PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL ACTIVITIES OF THE LEAVES OF *KALANCHOE PINNATA*.

BY

TESFAYE YADETA EJETA

A THESIS SUBMITTED TO

PROGRAM OF APPLIED CHEMISTRY

SCHOOL OF APPLIED NATURAL SCIENCE

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

OFFICE OF GRADUATE STUDIES

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DECLARATION

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

Name: Tesfaye Yadeta Ejeta

Signature_____

This MSc thesis has been submitted for examination with my approval as thesis advisor/ Supervisor.

Name: Hailemichael Tesso (PhD)

Signature _____

Date of submission_____

ADVISORS APPROVAL SHEET

To Chemistry department

Subject: Thesis Submission

This is to certify that the thesis entitled "isolation and structural elucidation of natural products from the leaves of *Kalanchoe pinnata* and evaluation of their antibacterial activities." submitted in partial fulfillment of the requirements for the degree of Master's in chemistry, the graduate program of department of chemistry, and has been carried out by Tesfaye Yadeta Ejeta with Id. No GSS/0228/05, under my/our supervision. Therefore, I/we recommend that the student has fulfilled the requirements and hence hereby he/she can submit the thesis to the department.

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SCHOOL OF APPLIED NATURAL SCIENCE

PROGRAM OF APPLIED CHEMISTRY

This was to certify that the thesis prepared by Tesfaye Yadeta Ejeta entitled: isolation and structural elucidation of natural products from the leaves of *Kalanchoe pinnata* has been submitted in partial fulfillment of the requirements for the Degree of Master of Science in chemistry complies with the regulations of the university and meets the accepted standards with respect to the originality and quality and signed by the examining committee:

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Internal examiner Legesse Adane (PhD)		25 Sep, 2017
External examiner		

ACKNOWLEDGMENTS

First of all, I would like to give my special thanks to God who allow me to alive and helped me to do this research. The success and completion of this thesis would not been possible without the contribution of many different people in their different ways.

I would like to express my sincere gratitude to my major advisor, Dr. Hailemichael Tesso for his timely guidance and exceptional support on structure elucidation.

My special thanks go to my co-advisor Dr.Yadesa Melaku, for his guidance through difficulties during laboratory work and constructive criticism at all stages of my research which could not be expected ever and Dr. Milkyas Endale for his friendly approach during lab Work.

I would also like to thank Addis Abeba University Chemistry Department in general for the NMR, FTIR and UV analyses.

My appreciation also goes to Oromia public health research laboratory center, Adama, for conducting the antibacterial assays, which adds valuable contribution to the thesis.

I am deeply grateful to Solomon Girmay, Head of Applied Chemistry Program in ASTU, and lab technicians for their kindly support.

My special thanks go to miss. Yadeshi Gemeda for giving me the strength that I needed throughout my work thanks for all your help, your advices and for your patience.

At last but not least my sincere gratitude goes to Adama Science and Technology University for providing the financial support for this research work.

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LISTS OF ACRONYMS AND ABBREVIATIONS

ASTU	Adama Science and Technology University
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
d	doublet
dd	double doublet
DEPT	Distortion less Enhancement by Polarization Transfer
dt	doublet triplet
FTIR	Fourier Transform Infrared Spectroscopy
^{1}H-NMR	Proton-Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
MHA	Muller Hinton Agar
m	Multiplet
s	Singlet
TMS	Tetramethylsilane
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

The plant K.Pinnata is mostly found on plains, tropical and temperate regions of Africa, Australia and America and has a wide ethno medicinal use for the treatment of various diseases. These include its use for healing bacterial infections on cuts and wounds and applied externally to heal ulcers and reduce body temperature, vomiting, earache, smallpox, cough, and headache. This study was directed towards organic solvents (n-hexane, EtOAc and methanol) extraction, isolation, and structural elucidation of compounds from the leaves of a medicinal plant Kalanchoe pinnata using chromatographic and spectroscopic techniques. Moreover, antibacterial activity determination of the crude extracts and isolated compounds were carried out against some selected bacterial strains. The antibacterial test was performed using disc diffusion method against selected gram-positive and gram-negative bacterial strains by observing the zone of inhibition. The ethyl acetate extract showed colored spots on TLC and hence subjected to fractionation over silica gel column chromatography which led to the isolation of three compounds ***KP-1*** *(**tetra decanoic acid**),* ***KP-2 (Z)-hexadec-9-enoic acid*** *and the third compound* ***KP-3*** *is a diterpene derivative. These compounds were not reported before from this plant leave. The ethyl acetate crude plant extract and the isolated compounds (**KP-1** and **KP-2)** were tested for their antibacterial activity against S. aurens, E. coli, P. mirabilis and K. pneumonia compared with Gentamycin as standard drug. Compound* ***KP-2*** *showed remarkable activity with acceptable selectivity against p. mirabilis bacteria with 16mm inhibition diameter compared with standard drug Gentamycin inhibition diameter of 15mm. However, the ethyl acetate extract and* ***KP-1*** *has moderate anti bacterial activities of 11mm maximum inhibition diametr against the tested bacterial strains compared with the standard drug (Gentamycin). The obtained results rationalize the folkloric use of the plant and can be further investigated to isolate the active compounds responsible for the biological activities.*

Key words*;- K. pinnata, extraction, phytochemical screening and antimicrobial tests .*

1. INTRODUCTION

1.1. Background of the Study

The universal role of plants in the treatment of disease is exemplified by their employment in all the major systems of medicine irrespective of the underlying philosophical premise. The use of plants as medicine in ancient times include their use in Mesopotamia, Egypt, the Unani (Islamic) and Ayurvedic (Hindu) systems centered in western Asia and the Indian subcontinent and those of the Orient (China, Japan, Tibet, etc.). How and when such medicinal plants were first used is, in many cases, lost in pre-history, indeed animals, other than man; appear to have their own material medical. Following the oral transmission of medical information came to the use of writing (e.g. the Egyptian *Papyrus Ebersc.dated over 1600 B.C*), baked clay tablets(documented over some *660 cuneiform tablets dated about 650 B.C* records from Ashurbanipal's library at Nineveh, now in the British Museum, refer to drugs well-known today), parchments and manuscript herbals, printed herbals (*invention of printing 1440 ad*), pharmacopoeias and other works of reference (first *London Pharmacopoeia*, *1618;* first *British Pharmacopoeia*, *1864*), and most recently electronic storage of data. Similar records exist for Chinese medicinal plants (*texts from the 4th century B.C)* [1].

In addition to the above recorded information there is a great wealth of knowledge concerning the medicinal, narcotic and other properties of plants that is still transmitted orally from generation to generation by tribal societies, particularly those of tropical Africa, North and South America and the Pacific countries. These are areas containing the world's greatest number of plant species, not found elsewhere, and with the westernization of so many of the peoples of these zones there is a pressing need to record local knowledge before it is lost forever.

In addition, with the extermination /destroy/ of plant species progressing at an alarming rate in certain regions, even before plants have been botanically recorded, much less studied chemically and pharmacologically, the need arises for increased efforts directed towards the conservation of gene pools. Therefore, natural products, such as plants extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity [2]. According to the world health organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary health care needs [3]. In recent times, there have

been increases in antibiotic resistant strains of clinically important pathogens, which have led to the emergence of new bacterial strains that are multi-drug resistant [4,5]. The non-availability and high cost of new generation antibiotics with limited effective span have resulted in increase in morbidity and mortality [6].

Therefore, there is a need to look for substances from other sources with proven antibacterial activity. Consequently, this has led to the search for more effective antimicrobial agents among materials of plant origin, with the aim of discovering potentially useful ingredients that can serve as source and template for the synthesis of new antimicrobial and antioxidant drugs [7,]. Even though pharmacological industries have produced a number of new antibiotics, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [8]. Such a fact is cause for concern, because of the number of patients in hospitals who have suppressed immunity, and due to new bacterial strains, which are multi-drug resistant. Consequently, new infections can occur in hospitals resulting in high mortality around all over the world [9].

The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem to control the use of antibiotic and develop research to understand the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies [10].

The use of herbal medicines in the world represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases [11]. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. According to World Health Organization medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed or developing countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to understand their properties, safety and efficiency

[12]. In the present study, the researcher deals with the isolation, phytochemical screening and characterization of chemical constituents from the crude leaf extracts of the plant and further the isolated constituents was evaluated for their antibacterial potential.

1.2. Justification of the Study

This thesis has practical and experimental result of a scientific relevance and therefore it is very important. In this research we would like to make additional information to the work in the future on *K. pinnata* plant, because the plant leaves contain many chemical constituents that are not isolated by this work and the plant leaves are highly effective traditional medicine to destroy mice from garden and prevents bacterial infections on human body traditionally in rural areas currently. Scientific literature regarding this plant species is especially scarce on the isolation of all chemical constituents from the plant leaves that are traditionally used in our country. To promote this traditional medicine to modern drug and for documenting of knowledge for drug industry; this particular thesis tries to fill the gap.

1.3. Statement of the Problem

There are many plant species that are traditionally used for the treatment of different diseases like inflammation, asthma and infectious microorganisms. The genus '*Kalanchoe*' consists of about 125 species of tropical, succulent flowering plants belonging to the family Crassulaceae. In most part of the world, *Kalanchoe* species are primarily ornamentals and houseplants, but some have escaped cultivation and can be found in the wild habitat and popularly known as 'Christmas tree plant'. It is an erect stout perennial shrub which grows up to a height of 1 to 1.5 m. These plants are cultivated as ornamental plants [13], [15]. In the past, the genus was divided into three genera: *Kalanchoe, Bryophyllum, and Kitchingia*. But today, most botanists recognize it as one genus [14]. Although the plant has medically used as a medicinal plant in both developed and developing countries in traditional cultures, users are required to have a great deal of experience to use safely and carefully. Most researchers conduct their research on different parts of the plants and distinguished the phytochemical constituents of the plant extract which are poisons and harmful in traditional method of using the plant extract as medicinal value. But still there is a gap in the use of the leaves of the plant extract as medicinal use [16]. Therefore, this study was designed to fill the gap regarding lack of scientific backing of the traditional use of the plant

leave extract and to add experience and knowledge of the plant to use the plant leave extract carefully. Moreover, the study adds knowledge of using the plant extract traditionally and the risks of the traditional use of the plant on human beings were recommended to fill the gap [17].

(a) **(b)**

Figure 1. Photo of *Kalanchoe pinnata* (Taken by Tesfaye Yadeta from Sago kebele, Jardaga Jarte District, Horro Guduru Wollega Zone, Oromia regional State (a) and Pulverized powder of the plant leaves (b)

1.4. Significance of the Study

Medicinal plants have emerged as some of the most widely studied plants and significant interests have been shown in their chemistry because of their potential applications in medicine. The constituents present in medicinal plants play a significant role in the identification of crude drugs. The treatment of infectious diseases and others still remains an important and challenging problem because of a combination of factors including emerging infectious diseases and the increasing number of multi-drug resistant microbial pathogens.

The study on the extracts of the leaves of *K. pinnata* might help to search for new natural products that could display significant antibacterial activities. This might lead to further investigations to arrive at new antimicrobial drugs. In this study, extraction, phytochemical screening of *K. pinnata* leaves and isolation of compounds from the extracts were carried out. Hence, once a chemical constituent of the plant was isolated and its structure was determined, it

could serve as a starting point in a search for new biologically active compounds that can be used as therapeutic agents.

1.5. Objectives of the Study

1.5.1. General Objective:

- To extract, isolate and characterize the chemical constituents from the leaves of *Kalanchoe pinnata* plant and evaluate their antibacterial activity.

1.5.2. Specific Objectives

- To extract leaves of *K. pinnata* sequentially using n-hexane, ethyl acetate and methanol.
- To carry out phytochemical screening on the plant crude extract.
- To isolate compounds from the crude plant extracts and characterizes the isolated compounds.
- To study the antibacterial activities of the crude extracts and isolated compounds against *Staphylococcus aurens, Escherichia coli, Protus mirabilis* and *Klebsella pneumonia*.

2. REVIEW OF RELATED LITERATURE

2.1. General Description of Medicinal Plants

The study of natural products has been played a major role in the development of organic and medicinal chemistry, including fundamental aspects of stereochemistry, bio-synthesis and mechanisms of biological actions as well as providing medically useful compounds. Plants are the best known source of primary and secondary metabolism in which they are classified by their chemical structure and/or physical properties in to one or more of the following groups: alkaloids, terpenoids, steroids, saponins, peptides, lipids, steroidal glycosides, ethereal oils, resins and balsams. Diseases are on the rise since the advent of life on earth. Procuring a defensive mechanism is a challenge for researchers. Plants are a boon to mankind. They are explored, researched and exploited to combat various dreadful diseases. Plants that are taken as a cure to diseases are considered medicinal. Their therapeutic nature also prevents occurrences of certain conditions. Such plant-based compounds are less toxic and show minimal or nil side effects.

2.2. *Kalanchoe Pinnata* Description

Kalanchoe is a genus that has many species most of which are used as agents to treat various ailments. Plants belonging to this genus have been traditionally known for their pharmaceutical value and have been studied by scientists for a very long time. *K. pinnata* (synonym: *Bryophyllum pinnate*) belongs to the Crassulaceae family and it could be cut off from the parent and cultivated separately on pots or barren lands [20]. This plant is a water-storing perennial herb that grows about 1 to 1.5 m tall. The leaves of this plant are thick green, fleshy, distinctively scalloped. The stems are tall and hollow bearing pendulous bell-like flowers [21].

This plant is mostly found on plains, tropical and temperate regions of Africa, Australia and America.

2.3. Ethnobotanical Uses of the Plant Leaves

The leaves of this plant are medicinally important and many reports explained the use of the plant to cure several diseases in traditional system of medicine particularly in folklore.

Traditionally, the crushed leaves are used to make counter irritant remedies all over Asia. In most part of the world the powdered leaves are used for coughs and colds, heal boils and wounds

and used in lotions for small pox [16]. The crushed leaves are also used to cure headache and are applied externally to reduce body temperature and to heal ulcers, leave extracts are also applied to wounds, to soothe inflammation and taken orally to cure diabetes. The juice from the leaves is drunk to treat bilious diarrhea and dysentery [18, 19]. It is one of the ethno medically used medicinal plants by the folklore of Asia [22]. In Africa, *K. pinnata* is used to facilitate childbirth, treat ulcers, skin diseases and rheumatism. The plant has high wound healing properties. It is used to treat stones in the gall bladder. In vitro experimental studies showed the antihistaminic activity of this plant and studies also demonstrated improvement of sleep quality in pregnant women, and tocolytic effects similar to the beta-agonist but with fewer adverse effects [23-27] .

A research study suggested that the juice extracted from the plant leaves were better tolerated than beta-agonists and used in vitro relaxant effect in human myometrium. They reported that the plant extract effectively reduced the spontaneous contractions and oxytocin-induced contractions in humans, thus exhibiting change in uterine contractility pattern. The juice from fresh leaves have been used to treat vomiting, earache, smallpox, cough, asthma, bronchial disorders, diarrhoea, blood dysentery, jaundice, gout, headache, convulsion and general debility .A study was conducted to investigate the mutagenic as well as antimutagenic activity of the juice extract of the plant. Phytochemicals such as phenols, flavonoids, saponins, tannins, triterpenoids, glycosides, carbohydrates, sterols and amino acids are found in this plant leaf extracts [28]. These chemical components are responsible for exhibiting the above mentioned pharmacological effects. In this review, an effort has been made to discuss the various fore-mentioned activities of *K. pinnata* and to gain knowledge about its therapeutic and ethno medical values.

2.4. General Pharmacological Effects of the Plant

2.4.1. Wound healing activity

The ethanolic leave extract of *K. pinnata* showed significant wound-healing activity by decreasing the size of the affected area. A study reported that this might be due to the presence of steroidal glycosides and phenolic antioxidants. Water, petroleum ether and alcoholic extracts of the plant have been reported the potential to heal wounds and water extract showed more activity than the other two extracts [29].

2.4.2. Antioxidant activity

Antioxidative agents in K.pinnata plant leave protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite. Antioxidants possess reducing properties generally associated with the presence of reductones, which have shown to exert antioxidant action by breaking the free radical chain either by donating a hydrogen atom or an electron. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Potential antioxidant activity has good correlations in the treatment of cardiovascular disorders and found that scavenging capacity increased with increase in concentration [30].

The leaves were reported to show maximum scavenging effects than stems and the ethanolic extract showed more total phenolic and flavonoidal content than other extracts. The high amount of phenols and flavonoids in the extracts may be the reason for their high antioxidative activity. The phenolic constituents have the ability to interact with the transition metals even in lipid phase and chelate them by filling their aqua-coordination sites and generating metal-coordinated insoluble complexes. Inhibition of lipid auto-oxidation could be attributed to the ability of phenolics to stabilize the radicals through generation of stabilised phenoxyl radicals by directly scavenging peroxyl radicals and ethanolic extract of *K. pinnata* has strong protective potential than standard antioxidants against oxidative stress in both aqueous and lipid phases [30].

The metal-chelating ability of the extract was found to be dependent on its ability to reduce metal induced peroxidative stress. Study identified the antimicrobial and antioxidant activities of ethyl acetate and methanolic extracts of the plant and isolated seven derivatives from ethyl acetate extract and tested for their antioxidant ability. It was reported that methanolic extract showed highest activity by scavenging of free radicals along with inhibition of microorganisms by using hexane, chloroform, ethyl acetate, acetone and ethanol extracts of the plant [31].

2.4.3. Antitumour activity

A study conducted on mice by inducing tumour formation in the peritoneal region of the body. Methanolic and aqueous plant leave extracts of the plant were administered as drugs in specific dosages. These extracts decreased the ascetic fluid volume and arrested the tumour growth, acting as a tumour suppressing agent. Thus, the extracts were reported to possess antitumor activity. The extract exhibited apoptosis-inducing property, growth-inhibitory activity, on

cervical cancer cells due to the presence of certain phytocompounds. The study also discussed on the antiviral antineoplastic activity properties of the plant [32]**.** In the study, the intake of the aqueous extract for diethyl nitrosamine induced hepato carcinogenesis in rats decreased the hepatic damage. The protective effect may be due to the antioxidant and antiperoxidative effects coupled with an ability to correct the abnormalities in lipid and lipoprotein metabolism through an increase in the activities of few lipid metabolizing enzymes. Diethyl nitrosamine has the tendency to generate free radicals as its metabolism takes place in the liver, disturbing the antioxidant status, ultimately leading to oxidative stress and carcinogenesis. That is, it is considered as a major environmental hepatocarcinogen [22].

2.4.4. Antiviral activity

Human papillomavirus (HPV) is one of the sexually transmitted viruses, acting as a major threat to humans. Cervical cancer which is on the rise is caused by HPV. The plant leave extract fractions when subjected to cancer cell lines, suppressed the expression of viral proteins thus inhibiting viral as well as tumour growth. Epstein Barr virus is a herpes virus that affects the B-lymphocytes of humans, leading to the formation of tumors [33]**.** A research conducted on the activity of compounds isolated from methanolic extract of the plant reported that the bioactive compounds in the plant inhibited the activation of the virus and suppressed tumour promotion [34]**.**

2.4.5. Antimicrobial activity

Researchers isolated two bufadienolides, two flavonoids and an alkaloid from the methanolic extract of *K. pinnata* and found to exhibit strong antimicrobial activity of the plant [21]. These phytocompounds inhibited growth of some commonly found gram-negative and positive bacteria and fungi. It was also reported that the antimicrobial ability of aqueous and methanolic extracts of the plant stem are quite significant. Bacteria that are found on skin can cause skin infections, and when they enter the body, they cause respiratory diseases, food poisoning, wound infections, abscesses, osteomyelitis, endocarditis, pneumonia and other complications. The reported data can be used to prepare antibacterial and antifungal cream for commercial use. Research confirmed the traditional use of the plant for curing respiratory tract infections including pneumonia. The petroleum ether and aqueous extracts of leaves and stems of *K. pinnata* were reported to show almost the same effect as that of the antifungal agent used as a standard [30].

2.4.6. Antileishmanial activity

The protozoans of the genus Leishmania cause the disease Leishmaniasis. The aqueous extract of *K. pinnata* was given orally to mice infected with Leishmania amazonensis. After the trial, few observations were made such as the decrease in size of the lesions and the parasitical load at the infected area. Continuous treatment with the extract not only controlled the growth but also prevented from further occurrence of the infections. It was suggested that this method could be applied for visceral leishmaniasis. The antileishmanial activity may be attributed to the presence of flavonoid glycosides in the extract of the plant [35].

2.4.7. Anthelmintic activity

Different literature review, show that there are a comparative study done on the activities *of extracts* of different solvents against the commonly found earthworms and roundworms. The results of their study revealed that chloroform, methanolic and aqueous extract of roots of *K. pinnata* possess anthelmintic activity, while petroleum ether extract did not show any activity against the worms [36]. The methanolic extract was found to be the most effective when compared with the root extract of the plant not only demonstrated paralysis, but also caused death of worms especially at higher concentration in shorter time span. The reason may be attributed to the presence of tannins, as they can bind to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite and cause death [37].

2.4.8. Insecticidal activity

Bufadienolidses Isolated from *K. pinnata* plant leaves were reported to exhibit strong insecticidal activity against third instar larvae of the silkworm and the reason was associated with the presence of 1, 3, 5-orthoacetate moiety of the bufadienolides [38].

The effect of anti-allergic activity of *K. pinnata* was studied on mast cells. These cells play a pivotal role for the development of allergic asthma. The study showed that aqueous extract of the plant effectively inhibited mast cell degranulation, thus preventing the development of allergic airway diseases. The reported data suggested that the extract can also act as an immunosuppressive agent [39]. Allergic anaphylaxis is another life-threatening immune reaction that causes death under extreme situations. Continuous administration of aqueous extract of *K. pinnata* has been found to prevented acute events related to allergen-induced anaphylaxis. The potential antiallergic activity may be due to the presence of flavonoids [40].

2.4.9. Antinociceptive activity

Methanolic leave extract of *K. pinnata* has been reported to show significant antinociceptive effect on mice when compared with standard drug aspirin. It reduced the number of acetic acid-induced writings in a dose-dependent manner [41]. Aqueous extract of leaves of the plant showed antinociceptive effects against thermally and chemically induced pain in mice. The aqueous extract relieved pain and protected the mice. The aqueous extract may probably have exerted its antinociceptive effects by inhibiting the release, synthesis and/or production of inflammatory cytokines and mediators, including prostaglandins, histamine, polypeptide kinins and so on [42].

2.4.10. Anti-inflammatory activity

An experiment to determine the anti-inflammatory activity of the plant was performed by petroleum ether, chloroform, acetone and methanol fractions from leaves were administered to experimental models with formaldehyde-induced oedema. Out of which, methanolic fraction was reported to exhibit more significant inhibition of paw oedema than other extracts [42]. Formaldehyde induces inflammation from cell damage, which initiates the production of extract could be due to the presence of bufadienolides and other water-soluble constituents in the extract. Endogenous mediators such as, histamine, serotonin, prostaglandins and bradykinin. It is also known that inhibition of oedema induced by formalin in rats is one of the activities against anti-inflammatory [43].

2.4.11. Antidiabetic activity

Diabetes is a major risk for cardiovascular diseases such as stroke; heart attack which affects majority of the global population reported that the ethanolic extract of *K. pinnata* decreased the blood glucose level of rats affected by diabetes. Thus, decreasing the serum glucose level and increasing glucose tolerance [44]. The plant extract also increased the pancreatic secretion of insulin and researchers investigated the antidiabetic activity of ethanolic and aqueous extracts of the dried stem of *K. pinnata* against alloxan-induced diabetes in rats. They reported that both the extracts exhibited hypoglycemic activity, that is, significant antihyperglycemic activity [45].The activities of both the extracts were compared and it was observed that the ethanolic extract showed more inhibitory activity of α- amylase enzyme than the aqueous extract, while the aqueous extract showed more hypoglycemic activity than the ethanolic extract research report determined the hypoglycaemic activity of aqueous extract of the plant by inducing diabetes in

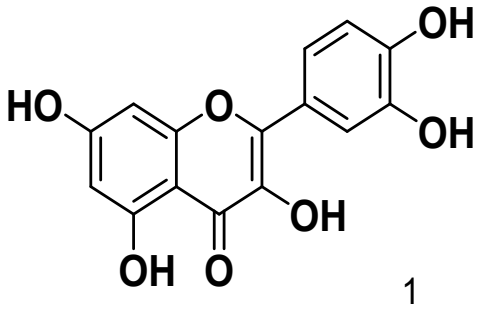
rats through streptozotocin treatment [42]. Once the aqueous extract was orally administered, the reduction in blood glucose level was observed. And, after 24-hour continuous observation, the glucose levels subsided to normal baseline levels indicating the antidiabetic potential of the plant.

2.4.12. Immunomodulatory activity

The aqueous extract of *K. pinnata* significantly depressed the delayed- hypersensitivity reactions to mice having ovalbumin-induced allergy thus proving to be an immunosuppressive agent. The aqueous extract prevented antigen-induced mast cell degranulation and histamine release, due to the presence of quercitrin, a flavonoid, which prevented fatal anaphylaxis through down-modulation of pro-anaphylactic-induced immune responses and modulation of acute events related to shock which leads to death (extreme allergic reaction) [40]. This immune response caused is highly critical for the resistance phenotype prophylactic therapy for hypersensitive people under the risk of anaphylactic shock.

2.5. Phytochemistry of *Kalanchoe pinnata*.

Some compounds isolated from the plant leaves include kaemferol (**1**), cholesterol, (**2**), Rutin (**3**), Sigmasterol (**4**), Bryophollenone (**5**), Quercitine (**6**), Bufalin (**7**) β-amyrin-3-acetate (**8**), and glut-5(6)-en-3-one (**9**).



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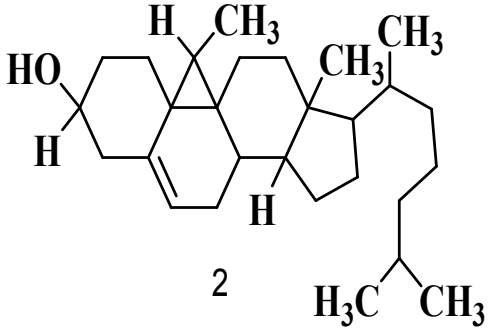

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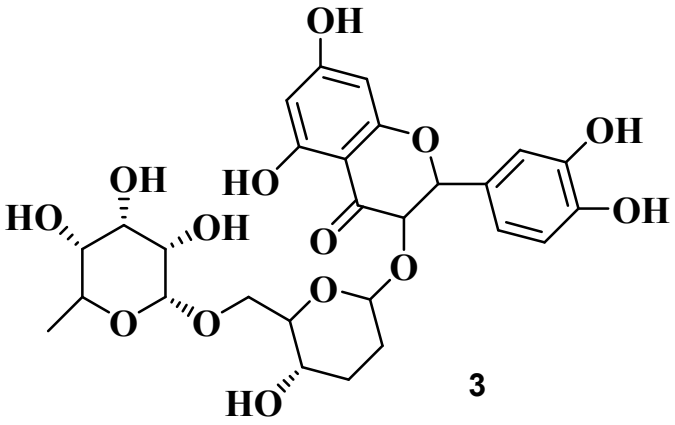

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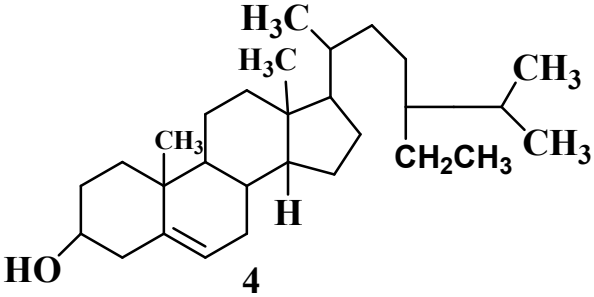

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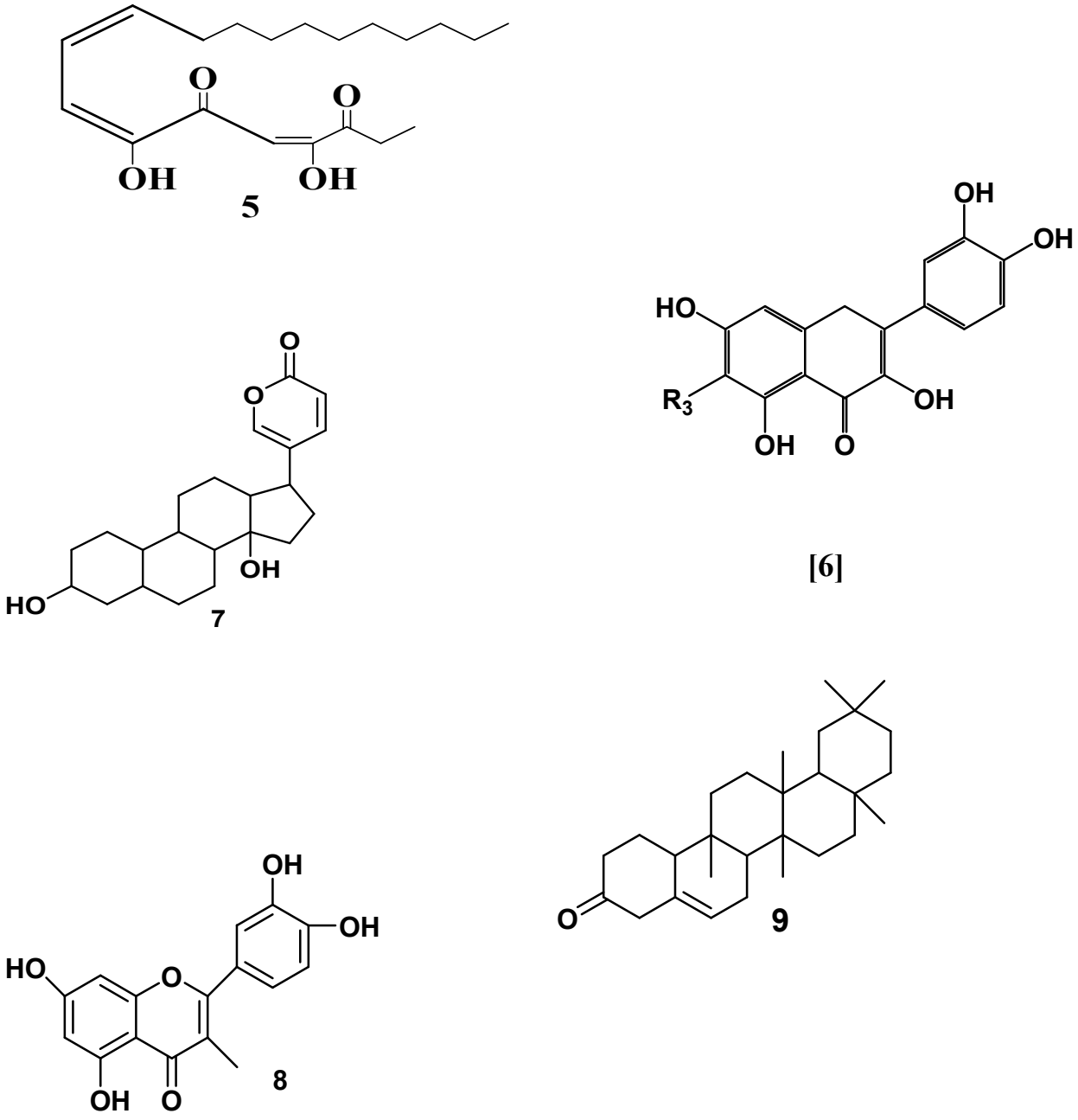


Figure 2. Some compounds reported from *K. pinnata* plant leave.

3. MATERIALS AND METHODS

3.1. Experimental

Solvent extraction and chromatographic separation, was done at Adama Science and Technology University whereas, NMR, FTIR and UV-Vis analysis was done in Addis Ababa University Department of Chemistry research laboratory. Antibacterial assay tests were done at Oromia public health research, capacity building and quality assurance laboratory center, Adama.

3.2. Apparatus and Instruments

The apparatus and instruments which were used in this study include column, separatory funnel, oven, TLC plate, Whatman No.1 filter paper, analytical mills (mortar and pestle), UV lamp, UV light cabinet, electronic balance, capillary tube, aluminum foil, vials, rotary evaporator, chromatographic chamber, and refrigerator. ^{1}H-NMR spectrum was measured on a Bruker Avance 400 MHz and ^{13}C-NMR spectrum was measured with the same machine operating at 100 MHz. The ultraviolet and visible (UV-Vis) spectrum was taken on electronic UV-Vis scanning spectrometer. Infrared (IR) absorptions were measured on a Perkin Elmer Spectrum 65 FT-IR spectrometer as KBr pellets in the range of 4000-400cm^{-1}.

3.3. Chemicals, Reagents and Media

The chemicals and reagents that were used in the study includes: silica gel, solvents n-hexane, chloroform, dichloromethane, ethanol, methanol, ethyl acetate, lead acetate, conc. sulfuric acid, vanillin, hydrochloric acid, NaOH solution, $CuSO_4$ solution, acetic acid, $FeCl_3$, Dragondorff's reagent, Mayer's reagent, iodine and distilled water.

3.4. Experimental Methods

3.4.1. Collection and identification of the plant material

Fresh and fully grown plant leaves were collected in month of September 2016 from Sego kebele, Jardega Jarte Distirict, Horo Guduru Wollega Zone, in the particular area (Adi Wolde) is 390 kms far from Addis Ababa. The plant was identified by prof. Legese Negash, Botanist in Addis Abeba University), and specimen was deposited at the National Herbarium in the Department of Biology with voucher no K-001/2016.

3.4.2. Extraction

The collected plant material was cut in to smaller pieces to facilitate drying, and was dried under room temperature. Air dried plant leaves were grinded using mortar and pestle until a uniform size powder was obtained. Finally, 300g of the pulverized powder was obtained which then was packed in polyethylene bags to avoid entrance of air or any other mixing of surrounding material. The powdered leaves of the plant were macerated with 1500 mL of *n*-hexane for three days i.e. for 72 hrs. After three days of maceration the n-hexane extract was filtered with Whatman no 1 filter paper by changing the filter paper periodically and collected in different beakers and the n-hexane filtrate was concentrated using rotary vapor at 40ºC and stored under refrigerator at 4°C until further analyses.

The marc from n-hexane extraction was macerated with 1500mL ethyl acetate for three consecutive days with periodic shaking to obtain ethyl acetate crude extract at room temperature. The extract was filtered using Whatman no.1 filter paper and the filtrate was concentrated using rotary vapor at 40^{o}C and stored under refrigerator at 4^{0}C for further analyses. The marc from ethyl acetate extraction was again macerated with 1500mL methanol for three consecutive days with periodic shaking to obtain methanol crude extract. After three days of maceration, the extract was filtered with filter paper by changing the filter paper and the extracts were concentrated on Rotary vapor under reduced pressure at 40^{0}C and stored under refrigerator at 4^{0}C.The mass of each crude extracts was finally measured on electronic balance.

3.5. Phytochemical Screening Tests

The n-hexane, ethyl acetate and methanol crude leave extracts of *K. pinnata* were subjected to preliminary phytochemical screening tests of various constituents in the plant leaves following standard methods reported in literature [46-47].

Test for Alkaloids (Mayer's test)

0.5 g of the each crude extract was mixed with 3 mL of n-hexane, ethyl acetate and methanol taken in separate test tubes, 1% dilute solution of hydrochloric acid and Wagner's reagent were added to each crude extract. Formation of yellowish buff colored precipitate was observed.

Test for Flavonoids

0.5 g of the each crude extract was mixed with 1 mL of n-hexane; ethyl acetate and methanol taken in separate test tubes and a few drops of dilute sodium hydroxide were added. An intense yellow color was observed in all extracts which become colorless on addition of a few drops of dilute sulfuric acid.

Test for Tannins (Lead acetate test)

0.5 g of the each crude extract was mixed with 5 mL of n-hexane, ethyl acetate and methanol in different test tubes and a few drops of 1% lead acetate were added and the formation of a yellow precipitate was observed.

Test for Saponins

Foam Test: Three test tubes each containing 0.5 g of crude extracts were dissolved with 1 mL of ethanol and 10 mL distilled water and shaken for 15 minutes and kept aside. The formation of a 1 cm layer of foam after standing for 30 minutes indicates the presence of saponins.

Lead Acetate Test:-0.5 g of each plant crude extract was mixed with 1 mL of n-hexane, ethyl acetate and methanol and treated with 1% lead acetate solution. The formation of white precipitate indicates that there are Saponins in the crude extracts.

Test for Terpenoids

On to three separate test tubes containing 0.5 g of each crude extracts 2 mL of n-hexane, ethyl acetate and methanol and 2 mL chloroform and 2mL conc. H_2SO_4 were added successively and the formation of a grayish color indicates the presence of terpenoids.

Test for Steroids

On to three separate test tubes containing 0.5 g of the three crude extracts, 2 mL of n-hexane, ethyl acetate and methanol were added respectively, followed by addition of 2 mL chloroform and 2 mL conc. H_2SO_4 and the formation of a red color on the lower layer of chloroform indicates the presence of steroids.

Test for Phenolics

Three separate test tubes containing 0.5 g of n-hexane, ethyl acetate and methanol crude extracts were mixed with 5 mL of distilled water and 5 % $FeCl_3$ successively. The formation of dark green color indicates the presence of phenolic compounds.

Test for glycosides

Liebermann Burchared test

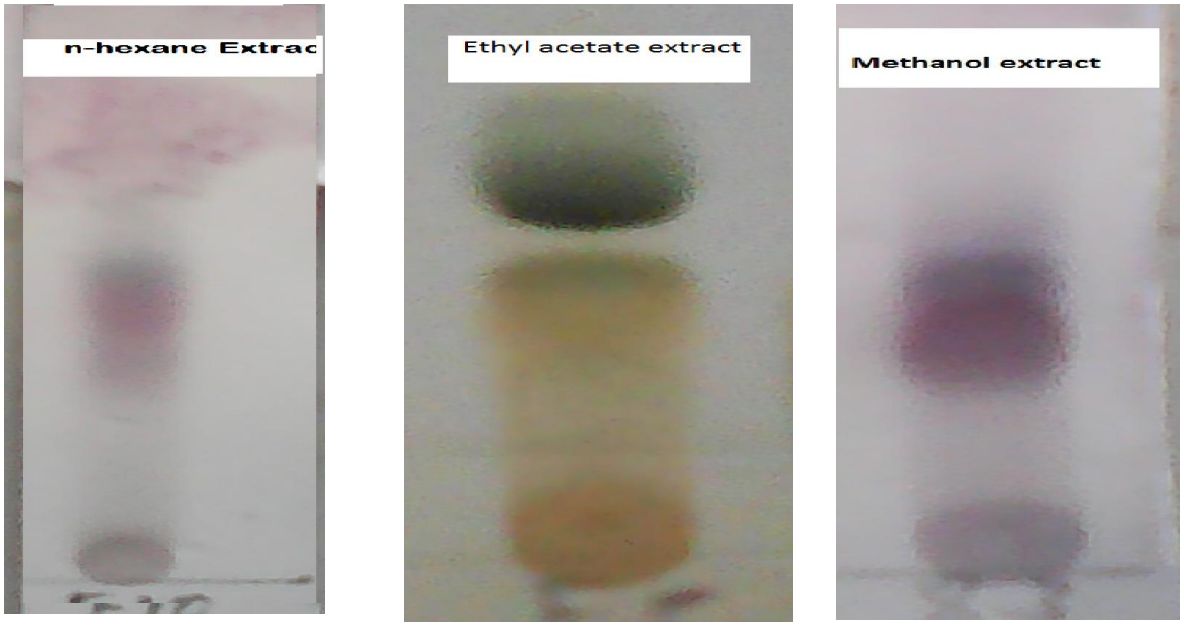
Three separate test tubes containing 0.5 g of each crude extract was mixed with 2 mL of n-hexane, ethyl acetate and methanol followed by addition of 2 mL chloroform and 2 mL of acetic acid respectively and the solutions were cooled in ice and H_2SO_4 was added carefully. A color change from Violet to blue to green indicates the presence of glycosides.

Salkowiski's test

In the same manner on to the three test tubes containing 0.5 g of the plant organic extracts 2 mL of chloroform and 2 mL of H_2SO_4 were added carefully and shaken gently. The formation of a reddish brown color indicates the presence of glycosides.

3.6. TLC Analysis of Crude Extracts

After concentrating of the three crude plant extracts, TLC profile of the three crude extracts were seen in order to select the extract for column elution for isolation of compounds. In this manner, 2 mL of each crude extract after concentrating was tested on TLC using n-hexane, ethyl acetate and methanol crude plant extracts by increasing the polarity of the solvents. Finally, TLC profile of the three crude extracts showed that the ethyl acetate extract to be selected candidate for column isolation of compounds



(a) **(b)** **(c)**

Figure.3 TLC profile of the three crude plant leave extracts n-hexane (a), ethyl acetate (b) and methanol (c)

3.7. Isolation of Compounds

Purification of compounds was carried out using column chromatography that was packed with silica gel (250-400) Merck as stationary phase. The mobile phase used in this study was a mixture of n-hexane and ethyl acetate with increasing gradient of ethyl acetate in n-hexane. The elution progress of each fraction and sub-fractions were monitored by TLC using n-hexane, ethyl acetate, dichloromethane, methanol and/or mixtures of them in different proportions.

Visualization of the chromatogram was achieved by spraying of appropriate reagents such as iodine, vanillin, ammonia solution and UV lamp at λ_{max} 254 nm and 366 nm. A total of 25 fractions were collected (table 1).

Table 1 Fractions collected from larger column of ethyl acetate crude extract.

No	Solvent system	Ratio (%)	Volume	Fractions code
1	n-hexane	100	100 mL	-
2	n-hexane/ethyl acetate	95:5	100 mL	-
3	n-hexane/ ethyl acetate	90:10	100 mL	F_1
4	n-hexane/ ethyl acetate	85:15	100 mL	F_2
5	n-hexane/ ethyl acetate	80:20	100 mL	F_3
6	n-hexane/ ethyl acetate	75:25	100 mL	F_4
7	n-hexane/ ethyl acetate	70:30	100 mL	F_5
8	n-hexane/ ethyl acetate	65:35	100 mL	F_6
9	n-hexane/ ethyl acetate	60:40	100 mL	F_7
10	n-hexane/ ethyl acetate	55:45	100 mL	F_8
11	n-hexane/ ethyl acetate	50:50	100 mL	F_9
12	n-hexane/ ethyl acetate	50:50	100 mL	F_{10}
13	Ethyl acetate/n-hexane	60:40	100 mL	F_{11}
14	Ethyl acetate/n-hexane	70:30	100 mL	F_{12}
15	Ethyl acetate/n-hexane	80:20	100 mL	F_{13}
16	Ethyl acetate/n-hexane	90:10	100 mL	$_{F14}$
17	Ethyl acetate	100	100 mL	F_{15}
18	Ethyl acetate/methanol	90:10	100 mL	F_{16}
19	Ethyl acetate/methanol	80:20	100 mL	F_{17}
20	Ethyl acetate/methanol	70:30	100 mL	F_{18}
21	Ethyl acetate/methanol	60:40	100 mL	F_{19}
22	Ethyl acetate/methanol	50:50	100 mL	F_{20}
23	Methanol/ethyl acetate	60:40	100 mL	F_{21}
24	Methanol/ethyl acetate	70:30	100 mL	F_{22}
25	Methanol/ethyl acetate	80:20	100 mL	F_{23}
26	Methanol/ethyl acetate	90:10	100 mL	F_{24}
27	Methanol	100	100ML	F_{25}

3.7.1. Isolation of Compounds from the Ethyl acetate Crude Extract

TLC profile of each fraction was observed and most of the fractions were colored. But F-3, F-4, F-10, F-14 and F-15 showed better resolved spots on TLC and were chosen for further study. Therefore; for further chromatography F_3 and F_4 were selected and subjected to smaller column chromatography. 126 mg of F_3 obtained from the larger column chromatography was purified with the smaller column in which silica gel packed with n-hexane and eluted with increasing gradient of ethyl acetate in n-hexane and a total of five (5) fractions were collected by monitoring the polarity of the selected fraction between 100% n-hexane to (50:50) n-hexane: ethyl acetate ratio based on the larger column elution progress (Table 2).

Table 2: Fractions collected from smaller column of F_3 using n-hexane/ethyl acetate system.

No	Solvent	Ratio %	Fraction code	Yield of each fraction	TLC profile
1	n-hexane	100	K_1	5 mg	Not good
2	n-hexane/ethyl acetate	90:10	K_2	8 mg	Not good
3	n-hexane/ethyl acetate	80:20	K_3	111 mg	two spots
4	n-hexane/ethyl acetate	70:30	K_4	12 mg	Three spots
5	n-hexane/ethyl acetate	60:40	K_5	6 mg	Not good
6	n-hexane/ethyl acetate	50:50	K_6	3 mg	Not good

Fractions K-3 eluted with n-hexane: ethyl acetate (80:20 %) was a colorful spot on TLC. In addition, it has larger yield compared to the other fractions, and therefore, it was selected for further separation using a smaller column chromatography to isolate the two colored mixed spots carefully by starting from n-hexane (100%), through n-hexane: ethyl acetate (90:10 %) and n-hexane: ethyl acetate (80:20 %) ratios according to the TLC profile of the spots on smaller column chromatography shown on (Table 3).

Table 3 Fractions collected from K-3 using n-hexane/ethyl acetate solvent systems.

No	Solvent	Ratio	Fraction code	Yield of each fraction	TLC profile
1	n-hexane/ethyl acetate	90:10	KP-1	37mg	Compound 1
2	n-hexane/ethyl acetate	80:20	KP-0	10mg	Less mass
3	n-hexane/ethyl acetate	70:30	KP-2	25mg	Compound 2

Two compounds were isolated from the smaller column of K-3 of the ethyl acetate extract, and were labeled as compound **KP-1** and compound **KP-2**.

Fraction no 4 or (F_4) that was isolated and selected as a very good spot in larger column was again purified in the same manner as fraction no three (F_3) and seven fractions were collected (Table 4).

Table 4 Fractions collected from F-4 using n-hexane/ethyl acetate solvent systems.

No	Solvent	Ratio (%)	Fraction code	Yield of each fraction	TLC profile
1	n-hexane/ethyl acetate	80:20	F_{41}	4.2mg	Smaller mass
2	n-hexane/ethyl acetate	70:30	F_{42}	8mg	Smaller mass
3	n-hexane/ethyl acetate	60:40	F_{43}	24mg	two spots
4	n-hexane/ethyl acetate	50:50	F_{44}	89mg	one spot with impurity
5	n-hexane/ethyl acetate	40:60	F_{45}	11mg	Not good spot
6	n-hexane/ethyl acetate	30:70	F_{46}	3mg	Not good spot
7	n-hexane/ethyl acetate	20:80	F_{47}	19mg	One spot

Fractions F_{44} that was obtained from n-hexane: ethyl acetate (50:50 %) was a very colorful spot on TLC plate. More Over, it had larger yield as compared to the other fractions, and therefore, subjected to column chromatography further for purification using isocratic elution method using n-hexane: ethyl acetate (50:50 %) solvent systems (Table 5).

Table 5 Fractions collected from purification of F-44 using n-hexane/ethyl acetate (50:50 %) isocratic solvent systems.

No	Solvent	Ratio (%)	Fraction code	fraction Yield	TLC profile
1	n-hexane/ethyl acetate	50:50	KP-3	15mg	Compound 3
2	n-hexane/ethyl acetate	50:50	KP-4	10mg	less mass
3	n-hexane/ethyl acetate	50:50	KP-5	9.3mg	less mass
4	n-hexane/ethyl acetate	50:50	KP6	3.7mg	Less mass

Finally, **KP-3** was the third compound that was isolated from the plant leave through isocratic purification of fraction F-44 on smaller column chromatography with a total mass of 15 mg.

3.8. Spectroscopic analysis

The compounds that were isolated from column chromatography through TLC analysis were characterized by using Infra Red, UV and Nuclear Magnetic Resonance (^{1}H-NMR ^{13}C-NMR, DEPT-135) and the spectra were recorded in deuterated chloroform [$CDCl_3$], with Tetramethylsilane (TMS) as internal standard. Complete structure determination was achieved by comparing the IR and NMR data obtained with that in literature.

3.9. Antibacterial assay

The antibacterial activity was only done on the ethyl acetate crude extract, compounds **KP-1** and **KP-2** isolated from the ethyl acetate extract. No bacterial assay was done on compound **KP-3** due to its smaller yield. Four bacterial cultures having gram positive and gram negative strains namely *Staphylococcus aurens, Escherichia coli, Protus mirabilis* and *Klebsella pneumonia* were tested using the disc diffusion method. Muller-Hinton agar (MHA) was used as bacterial medium. The filter paper discs (6 mm in diameter) were individually impregnated with 20 µg/mL of the stock solution of the extract and then placed onto the agar plates which had previously been inoculated with the tested microorganisms. The plates were inoculated with bacteria incubated at 37°C for 24 h. The diameters of inhibition zones were measured in millimeters. Gentamycin, 20µg/mL, was served as the positive control with ≥15mm inhibition diameter which was taken as a standard solution for bacterial test. Bacterial inhibition zone ≥7mm was said to be sensitive(S), but ≤ 6mm was taken as resistance (R) against the tested bacterial strains with the selected chemical substance [50].

4. RESULTS AND DISCUSSION

4.1. Extraction yield

300 g of the plant leave extracts were successively obtained using n-hexane, ethyl acetate and methanol solvents as mentioned in the methodology part and the mass of the pulverized plant leave extracts were weighed and the extraction yields (w/w) were calculated as:

$$Percentage\ Yield = \frac{weight\ of\ dry\ extract}{weight\ of\ dry\ material}\ x\ 100$$

The Percentage yields of the plant leave extracts isolated by using different solvent ratios are listed in (Table 6).

Table 6 Percent yields of the plant extracts after macerated and concentrated with solvents.

Wt.of the plant macerated before in (g)	Solvent used for Maceration	Mass of the crude extract collected after concentration	% yield
300g	n-hexane	6g	2%
294g	Ethyl acetate	12g	4%
282g	Methanol	25g	8.3%

4.2. Preliminary phytochemical analysis

As described in literature review (section 2.1) based on the physiological activities secondary metabolites in plants are classified as saponins, tannins, terpenes, alkaloids, flavonoids, steroids, glycosides and etc. The phytochemical screening tests for the analysis of the plant leave extracts of *K.pinnata* revealed the presence of phytochemicals such as flavonoids, terpenoids, tannins, saponnins, steroids and Phenols in both ethyl acetate and methanol crude extracts. The n-hexane crude extract however could only contain flavonoids, saponnins, phenols and steroid chemical compounds. But the ethyl acetate and methanol crude extracts contain most of the secondary metabolites except alkaloids and glycosides (Table 7).

Table 7 Phytochemical constituents of the leaves of *K.pinnata* in crude extracs.

Phytochemicals	n-hexane	Ethyl acetate	Methanol
Alkaloids	_	-	_
Flavonoids	+	+	+
Saponins	+	+	+
Tannins	_	+	+
Terpenoids	_	+	+
Steroids	+	+	+
Phenols	+	+	+
Glycosides	_	_	_

Key: + Presents; - Absent

The phytochemical screening analysis of n-hexane, ethyl acetate and methanol crude plant extracts revealed the presence of secondary metabolites such as flavonoids, saponins, steroids, phenolics in all extracts and tannins and terpenoids in ethyl acetate and methanol crude extracts only, which are used as medicinal use in traditional culture due to the functional groups on the extracts which help as therapeutic agents for the treatment of infectious and/or chronic diseases in traditional medicine. More Over, on careful extraction of these secondary metabolites from the plant, the continued traditional use of the plant was encouraged.

4. 3. Characterization of the compounds

4.3.1. Characterization of compound KP-1

Compound **KP-1** was obtained as a white crystalline solid substance isolated from ethyl acetate extract with Rf value of 0.65 with melting point of $(53\text{-}54)^0$C and the compound was characterized as follows.

In the UV spectrum at λ max in EtOH (Appendix 1) absorption maximum at 220 nm revealed the molecule has unsaturated carbonyl and C=O bond chromophores ($\pi \rightarrow \pi^*$) conjugation. In the IR (KBr disk) spectrum (Appendix 2) of the compound a broad absorption band at 3440 cm^{-1} was due to the presence of hydroxyl groups and medium absorption band at (2938 and 2858) cm^{-1} due to asymmetric and symmetric methylene C-H bond stretch and medium absorption band at

1633 cm^{-1} due to the presence of carbonyl carbon (C=O) in the structure and medium absorption peak at 1393 cm^{-1} due to C-H bending in the structure.

The ^{1}H-NMR (400 MHz, $CDCl_3$): spectrum (Appendix 3 and table 8) revealed the presence of proton signals at δ_H 2.36 (2H, St J=7.8,4.5 Hz), revealed the presence of a CH_2 proton attached to carbon of the carbonyl, signal at δ_H 1.62 (2H, m) revealed a CH_2 group proton on the structure, δ_H 1.27 (17H, m) indicates broad overlapping CH_2 proton on the structure, δ_H at 1.32 (2H, m), shows a CH_2 proton attached to a terminal carbon in along long carbon chain. A signal at δH at 0.89 (3H, t) 4Hz, indicates a terminal proton on aliphatic chain.

The ^{13}C-NMR spectrum (Appendix 4 and Table 8) revealed a total of fourteen carbon signals. Signals at δ_C 179.9, is signal of quaternary carbon, signal at δ_C 33.9 is attributed to a CH_2 carbon in along carbon chain attached to quaternary carbon, and signals at δ_C 31.9, 29.6, 29.5, 29.4, 29.3, 29.2, 29.0, 27.2, 24.6, 22.6, and 14.1 revealed due to long aliphatic carbon chain on the structure, respectively.

The multiplicity of each carbon atom was determined using DEPT-135(Appendix 5), revealed the presence of one methyl group, twelve CH_2 groups, one quaternary carbon with a total of fourteen carbon atoms in the structure. (Appendix 3 and Table 8) [48].

Table 8: ^{1}H-NMR, ^{13}C-NMR and DEPT-135 data of compound **KP-1**

Position	^{1}H-NMR δ (ppm) (J-Hz)	^{13}CNMR δ (ppm)	Literature δ (ppm)	DEPT-135 δ (ppm)
1	-	179.2	179.2	-
2	(2.32, t)	33.9	34.0	CH_2
3	(1.60, m)	24.6	24.7	CH_2
4-11	(1.27, *br, s)*	(29.0-29.6)	(29.0-29.7)	$(CH_{2)}$ x8
12	(1.32, m)	31.9	31.9	CH_2
13	(1.27, m)	22.6	22.7	CH_2
14	(0.86, t)	14.1	14.1	CH_3

Based on the above ^{1}H, ^{13}C and DEPT-135 (Appendix 5) data the proposed structure of compound **KP-1** was proposed to tetradecanoic acid (Figure 3).

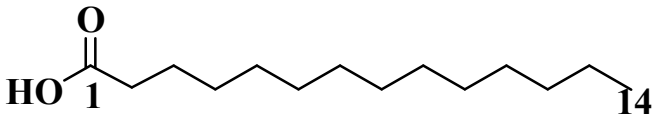


Figure 4.The proposed structure of compound **KP-1**

4.3.2. Characterization of compound KP-2

Compound **KP-2** was obtained as a white crystaline substance having Rf. value of 0.76 with melting point (1-2) °C and the compound were characterized as follows:-

In the UV spectrum at λ max in EtOH (Appendix 6) absorption maximum at 231 nm revealed the molecule has unsaturated carbonyl (C=O) bond chromophores ($\pi \rightarrow \pi^*$) conjugation.

In the IR (KBr disk) spectrum (Appendix 7) showed broad absorption band at 3443 cm^{-1} due to the presence of (OH) group and another strong absorption band at (2918 and 2838) cm^{-1} due to asymmetric and symmetric (C-H) bond stretching and medium absorption band at 1694 and 1653 cm^{-1} due to the presence of carbonyl carbon (C=O) and methylene group in the structure and weak absorption peak at(1500-1400)cm^{-1} due to C=C conjugation in the structure and medium absorption band at 1390 revealed the C-H bending stretch and at 749 cm^{-1} there indicates the presence of one (C-H) bending for the hydrogen on the carbon connected with the double bond.

The ^{1}H-NMR δ_H (400 MHz, $CDCl_3$) spectrum (Appendix 8 and Table 9) revealed the presence of proton signals at δ_H 2.14 (2H, st J=7.8,4.5 Hz), revealed the presence of a CH_2 group attached to carbon of the carbonyl group, δ_H 2.08 (2H, m), δ_H 1.50 (2H, m), signals at δ_H 1.13 (2H, m), δ_H at 1.10 (2H,m)(Table 9) revealed due to the CH_2 groups attached to the carbonyl group in the structure. Signal of δ_H at 3.43 (2H, dt), shows a CH_2 group in long chain fatty acid attached to carbon having a double bond in the structure, δ_H at 5.42-5.33 (2H,tt) is due to two CH groups attached to each other in the structure. Signals of δ_H at (2.77,dt), δ_H at 2.26 (2H, tt) δ_H at 1.50 (2H, tt), 1.13(2H, m), and 1.06(2H, m)(Table 9) are due to long chain fatty acids attached to carbon of the double bond in the structure. Finally, signal of δ_H 0.8 (3H, t) is due to a terminal carbon in along chain fatty acid.

The ^{13}C-NMR spectrum (Appendix 9 and Table 9) revealed a total of sixteen carbon signals. Signals at δ_C 179.4, is signal of quaternary carbonyl carbon attached to a long chain fatty acid group and signal at δ_C 33.9 is attributed to a CH_2 carbon in along chain fatty acid attached to a

carbonyl group in along chain fatty acid and signals at δ_C 31.9, 29.7, 29.6, 29.5, and 29.4 are due to straight chain fatty acid between the carbonyl group and the double bond in the chain. But carbon signals at δ_C, 129.8 having two carbon atoms attached to each other with a double bond. Finally, signals at δ_C 29.3, 29.2, 29.0, 27.2, 24.6, 22.6 and 14.0 (Table 9) are due to carbon of the long chain fatty acid attached to the carbon of the double bond to the terminal carbon in the structure respectively. The multiplicity of each carbon atom was determined using DEPT-135 experiment, which revealed the presence of one methyl group, the presence of twelve CH_2 groups, two CH attached to each other in a double bond and one quaternary carbon with a total of sixteen carbon atoms in the structure (Appendix 10 and Table 9) [49].

Table 9 ^{1}H-NMR, ^{13}C-NMR and DEPT-135 for compound **KP-2**

Position	^{1}H-NMR δ (ppm) (J-Hz)	^{13}CNMRδ (ppm)	Literature data	DEPT-135 δ (ppm)
1	-	179.4	180.0	Qc
2	(2.1, t) 7.8, 4.5 Hz	33.9	34.0	CH_2
3	(2.0, m)	24.6	24.6	CH_2
4	(1.5, m)	29.3	29.0	CH_2
5	(1.1, m)	29.4	29.4	CH_2
6	(1.1, m)	29.5	29.0	CH_2
7	(2.7,tt)	29.6	29.6	CH_2
8	(3.4, dt)	29.7	29.7	CH_2
9	(5.3, t)	129.8	129.8	CH
10	(5.4, tt)	129.8	128.7	CH
11	(2.2, dt) 4.5, 2.4 Hz	29.2	29.2	CH_2
12	(1.5, m)	29.0	29.0	CH_2
13	(1.1, m)	27.2	27.4	CH_2
14	(1.1, m)	31.92	31.7	CH_2
15	(1.0, m)	22.6	22.6	CH_2
16	(0.8, t) 3Hz	14.0	14.1	CH_3

Based on the above ^{1}H, ^{13}C and DEPT-135 data the proposed structure of compound **KP-2** would be the next structure.

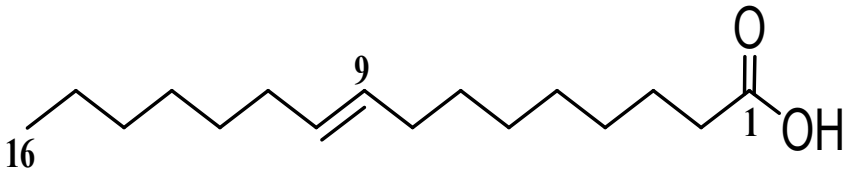


Figure 5. The proposed structure of compound **KP-2** /Palmitoleic acid/

4.3.4. Characterization of compound KP-3

Compound **KP-3** was obtained as a reddish brown crystalline substance having Rf. value of 0.73 isolated from ethyl acetate plant leave extract and the compound was characterized as follows.

In the UV spectrum at λ_{max} in EtOH (Appendix 11) absorption maximum at 475 nm revealed the molecule has homo and hetroannular double bonds, i.e., diene systems that require least energy or longer wave length for the absorption.

In the IR (KBr disk) spectrum (Appendix 12) should a broad absorption band at 3465 cm^{-1} due to the presence of hydrogen bonded (OH) groups bond stretching vibrations and another strong absorption band at 2935 and 2857 cm^{-1} revealed due to asymmetric and symmetric methylene C-H bond stretching and medium absorption band at 1632 cm^{-1} due to the presence of unsaturated carbon-carbon double bond which are asymmetrical due to polar substituent's in the structure and medium absorption peak intensity at 1390cm^{-1} due to bending vibrations of methylene and methyl groups in the structure and medium intensity peak at 1030 cm^{-1} arises due to C-O bond stretching.

The ^{1}H-NMR δ_H (400 MHz, $CDCl_3$): spectrum (appendix 13 and table 10) revealed the presence of proton signals at δ_H 1.98 (2H, d, J=10.9 Hz), indicates the presence of a CH_2 group, δ_H 4.0 (1H, m) a CH group on the structure, δ_H 1.62 (2H, dd, J=2.5, 2.34Hz) indicates a CH_2 proton signal on the structure, signals at δ_H 4.2 (1H, m) indicates a CH group, δ_H at 1.52 (1H,dd, J=10.9, 6.2Hz), revealed the presence of CH group on the carbon skeleton on the structure. Signal at δ_H 6.64, 6.25 on carbon chain indicates proton on conjugated double bond respectively. δ_H at 1.69 (1H, dd, J=10.9, 2.0Hz), δ_H at (1.49,m) shows a CH_2 group on the structure, δ_H 6.19 (1H,dd,J=15.0, 8.4Hz), 6.40 (1H,dd, J=15.0,6.3Hz), 6.18 (1H,dd, J=10.8,6.8), 6.28 (1H,dd, J=10.4, 5.8Hz) on the structure respectively a indicates a double bond protons on the structure. δ_H at 2.35 (1H, m) indicates a proton on, δ_H at 1.71 (2H,d,J=9.8Hz) indicates a CH_2 proton on the

structure, δ_H at 5.45 (1H,d,J=11.7, 2.0Hz) and δ_H at 5.56 (1H, m) indicates protons signal connected with a double bond, δ_H at 1.64 (3H, d, J=8.4Hz) and δ_H 1.76 (3H,d,J=6.3Hz) indicates a CH_3 proton on the structure respectively. δ_H at 0.86 (3H, s) indicates a CH_3 proton on the structure which was attached to a quaternary carbon atom on the structure.

The ^{13}C-NMR spectrum (Appendix 14 and Table 10) revealed a total of twenty one carbon signals. Signals of carbon at δ_C 48.4, 42.5, 44.5 and 30.2 are due to a methylene groups on the structure respectively. δ_C at 65.1 and 65.9 revealed due to a hetro atom attached to methylene group respectively. δ_C at 54.9 revealed due to carbon signal attached to three carbon atoms containing a hetro atom, a double bond and another quaternary carbon. δ_C at 131.3 and 132.5 indicates (CH) carbon atoms that are connected with a double bond. δ_C at 29.5 revealed due to (CH) carbon atom connected to a CH_2 and CH carbon chains. δ_C at 24.2 revealed the presence of quaternary carbon atom attached to a methyl and another three carbon chains in the structure.

δ_C at 137.5, 130.8, 125.5, 137.7 indicate presence of a conjugated double bonds on carbon chains connected to each other respectively. δ_C at 34.0 is due to a (CH) carbon, δ_C at 130.0 and 124.9 revealed due to carbon atoms connected to each other respectively. δ_C 12.8, 21.6 and 20.1 indicates a CH_3 groups on different carbon positions on the structure. The multiplicity of each carbon atom was determined using DEPT-135 experiment, which revealed the presence of three methyl groups, four CH_2 groups, thirteen CH groups and one quaternary carbon with a total of twenty one carbon atoms which are indicated on (Appendix 15 and Table 10).

Table 10 ^{1}H-NMR ^{13}C-NMR and DEPT-135 for compound **KP-3.**

Position	1H-NMR δ (ppm) (J-Hz)	13CNMRδ (ppm)	DEPT-135 δ (ppm)
1	1.9 (dd,J=10.9,6.1Hz)	48.4	CH_2
2	4.0, m	65.1	CH
3	1.6(dd, J=2.5,2.34Hz)	42.5	CH_2
4	4.2, m	65.9	CH
5	1.5 (dd, J=10.9, 6.2Hz)	54.9	CH
6	6.6(dd,J=8.5, 4.3Hz)	131.3	CH
7	6.2(dd,J=8.6, 3.3Hz)	132.5	CH
8	1.6(dd,J=1.9, 2.07)	29.5	CH
9	1.4(d, J=2.5Hz)	44.5	CH_2
10	-	24.2	Qc
11	6.3(dd, J=15.0, 8.4Hz)	137.5	CH
12	6.4(dd, J=15.0 ,6.3Hz)	130.8	CH
13	6.1(dd, J=10.8, 6.4Hz)	125.5	CH
14	6.2(dd, J=10.4, 5.8Hz)	137.7	CH
15	2.3, m	34.0	CH
16	1.7(d, J= 9.8Hz)	30.2	CH_2
17	5.4(dt,J=11.7,4.351Hz)	130.0	CH
18	5. 5, m	124.9	CH
19	1.6 (d,8.4Hz)	12.8	CH_3
20	1.7(d, J=1.6Hz)	21.6	CH_3
21	0.8(d, J=1.6Hz)	20.1	CH_3

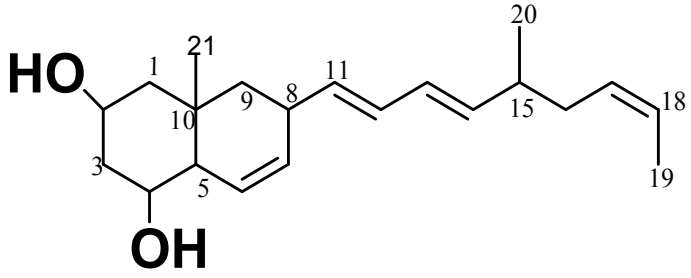


Figure 6.The proposed structure of compound of **KP-3.**

4.4. Antibacterial assay

The antibacterial test of the plant leave extract was tested against four different bacterial species having gram positive and negative strains. The bacterial activity was given in terms of inhibition zone (in mm) (Table 11).

Table 11 Antimicrobial activities of compound **KP-1**, **KP-2** and ethyl acetate crude extract of *Kalanchoe pinnata*.

Samples selected for bacterial test	Conc.in µg/mL	Type of bacteria with mean inhibition diameter (in mm)			
		S. aurens (+)	*E. coli* (-)	*P. mirabilis* (-)	*K.pneumonia* (-)
Ethyl acetate crude extract	20	11	7	8	8
KP. 1	20	11	11	-	7
KP.2	20	11	14	16	11
Gentamycin	20	16	18	15	18

In the present study, the antibacterial test was performed using the Agar disc diffusion method reported in the literature [50]. The antibacterial activities of the ethyl acetate and the two isolated compounds (**KP-1** and **KP-2**) were tested against selected gram positive and gram negative bacterial strains. The results indicate that the ethyl acetate crude extract and the two compounds showed significant sensitivity to the different strains of bacteria used in the study (Table 11). The experimental result of the bacterial test indicated that compound **KP-2** inhibited the growth of *P. mirabilis* (16 mm) bacteria more than the other extracts compared to the standard drug Gentamycin inhibition zone of (15 mm). Compound **KP-2** has also more efficient antibacterial activity towards *E. coli* bacteria with (14 mm) inhibition diameter compared to standard drug with (18 mm) inhibition diameter. Compound **KP-1** has moderate inhibiting activity towards *S. aurens and E. coli* the same inhibition diameter (11 mm) compared with Gentamycin (16 and 18 mm) showing that the compounds have moderate antibacterial inhibition effect. Compound KP-3 was not tested on bacterial assay due to its smaller mass. The experiment was only conducted with certain species of bacteria and no fungus strains were tested due to absence of fungi cultures. Therefore, further research is needed to evaluate the sensitivity of the plant extract and the isolated compounds against other species of bacteria, fungi and virus.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

For decades, traditional medicine has been used and continues to be an alternative approach on treatment for various diseases. The phytochemical screening tests for the major secondary metabolites were performed in this research work. From the study, the plant leave extracts were found to contain saponins, tannins, terpenoids, phenols, steroids and flavanoid compounds. The presence of these important phytochemicals in the plant is a scientific justification of the plant use in the traditional medicine for the treatment of various diseases affecting humans and animals and enhances their pharmaceutical and therapeutic potentials. After repeated successive solvent extraction and column chromatography different chemical compounds were isolated from the plant leaves. The structures of the compounds were characterized on the basis of spectral data (UV, IR, ^{1}H-NMR, ^{13}C-NMR, and DEPT-135) by comparison with the literature data. Among the isolated compounds, three compounds coded as **KP-1**, **KP-2** and **KP-3** were characterized as **KP-1** is **tetra decanoic acid** (Myristic acid), **KP-2** is **(Z)-hexadec-9-enoic acid** (Palmitoleic acid) and the third compound was a diterpene derivative isolated from ethyl acetate plant leave extract. To the best of our knowledge three compounds are reported here from the plant leaves for the first time. The obtained results rationalize the folkloric use of the plant and can be further investigated to isolate the active compounds responsible for the biological activities. Based on TLC analysis, the plant contains several polar chemical constituents which were not isolated in this study because of financial and time constraints.

Therefore, any researcher interested to study on this plant leaves, could be better to concentrate on the methanol extract which have polar compounds rather than non-polar extracts.

5.2. Recommendations

In this study the presence of some of the chemical compounds that were identified from the plant leaves confirmed the presence of bioactive chemicals that are useful as a traditional drug. The continued traditional medicinal use of these plants is therefore encouraged while it is suggested that further work should be carried out to isolate, purify and possibly characterize more bioactive constituents form the plant.

To improve the yields of high polarity crude extracts, the availability of solvent for the extraction should be maximized to isolate more chemical compounds from the plant leaves. Therefore, it

would be better if further study is carried out on identifying high extracting solvents, to elucidate the complete and correct structure of pure compounds and additional experimental data should be carried out to explore the potential of this plant extracts in the treatment of infectious diseases. In addition to this the following recommendations were made.

- Further bioassay guided isolation and characterization work should be done to fully characterize more bioactive constituents of the plant leave extracts to identify all active compounds against a variety of bacteria and fungi.
- It is recommended that further similar studies should be conducted on other parts of the plant such as root, stem, and bark to isolate more bioactive compounds.
- Toxicity studies of the plant should also be done to determine the safety indices of the extracts.
- Advanced chromatographic techniques such as GC-MS and HPLC techniques were recommended to isolate and characterize pure compounds from the plant body.

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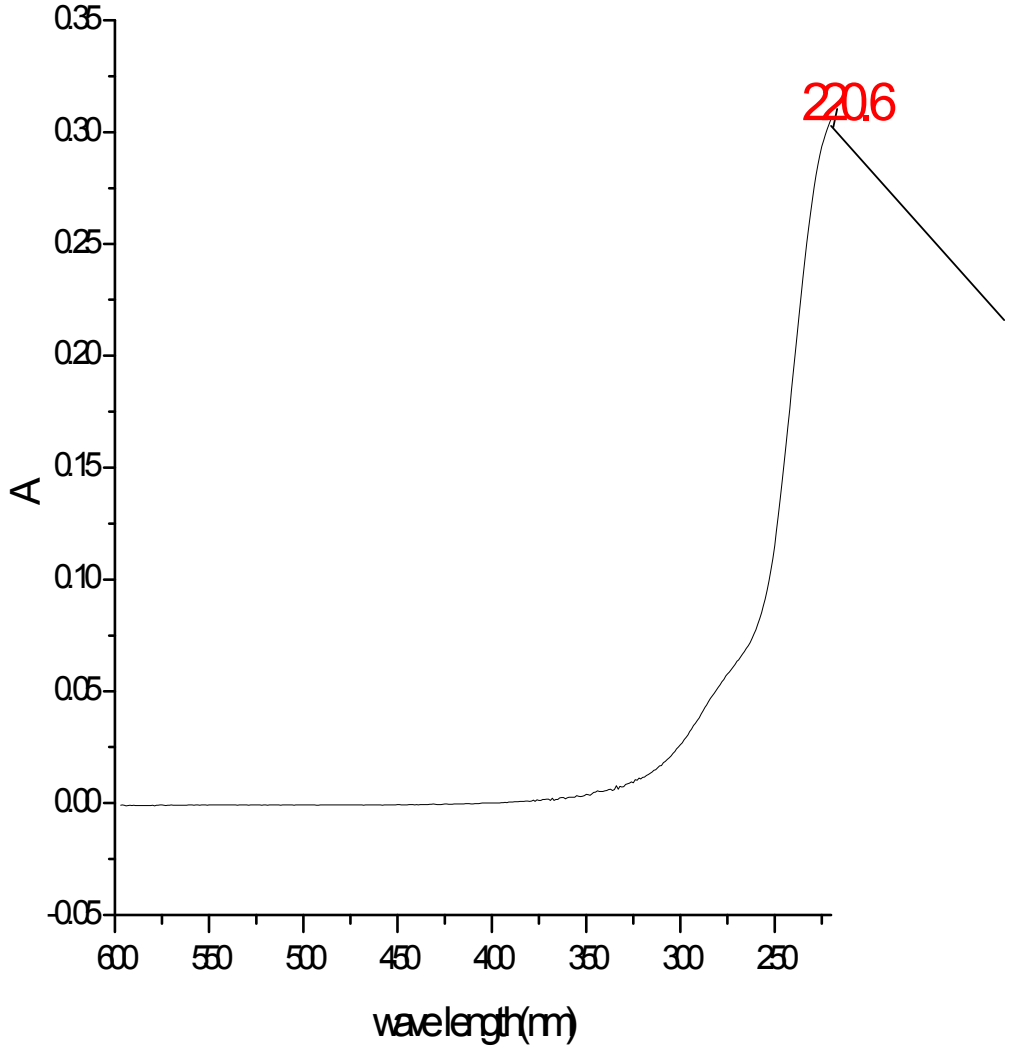
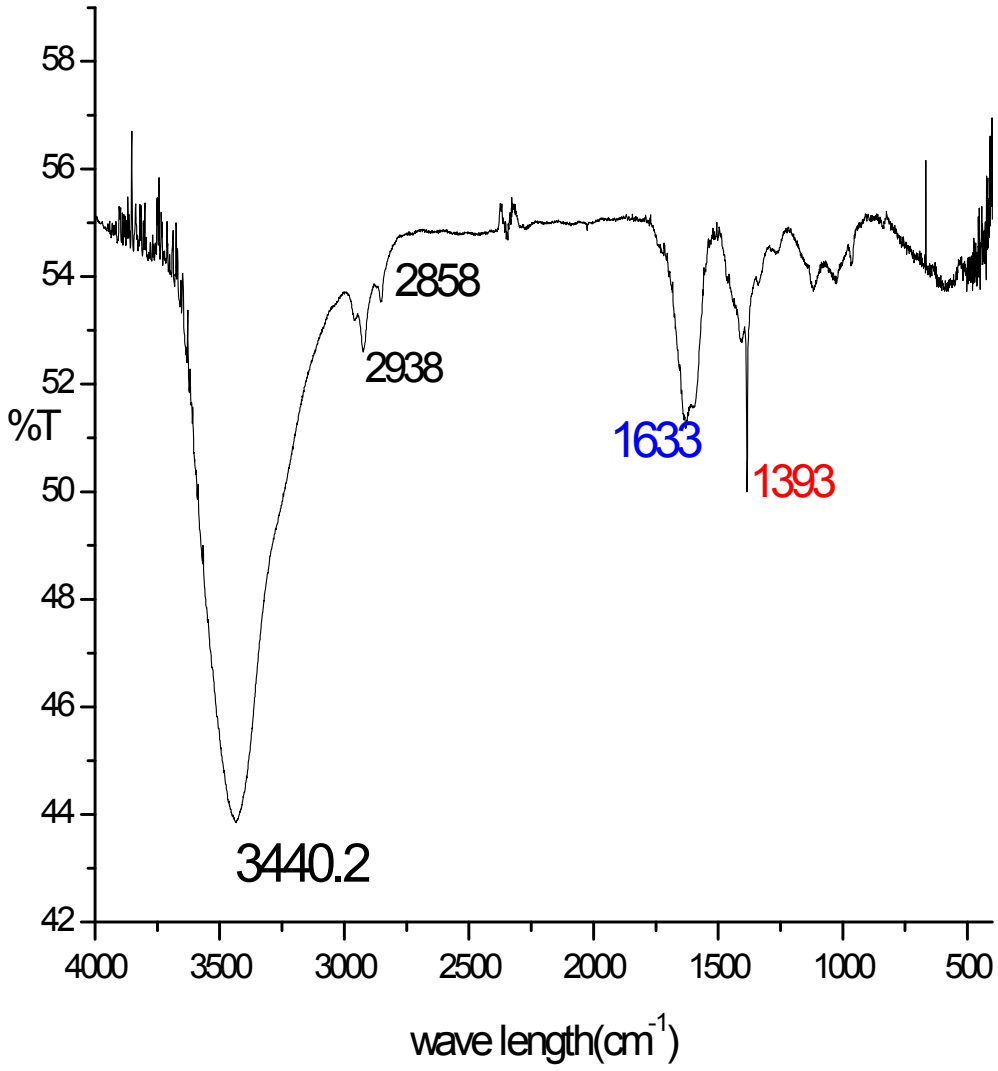
APPENDICES

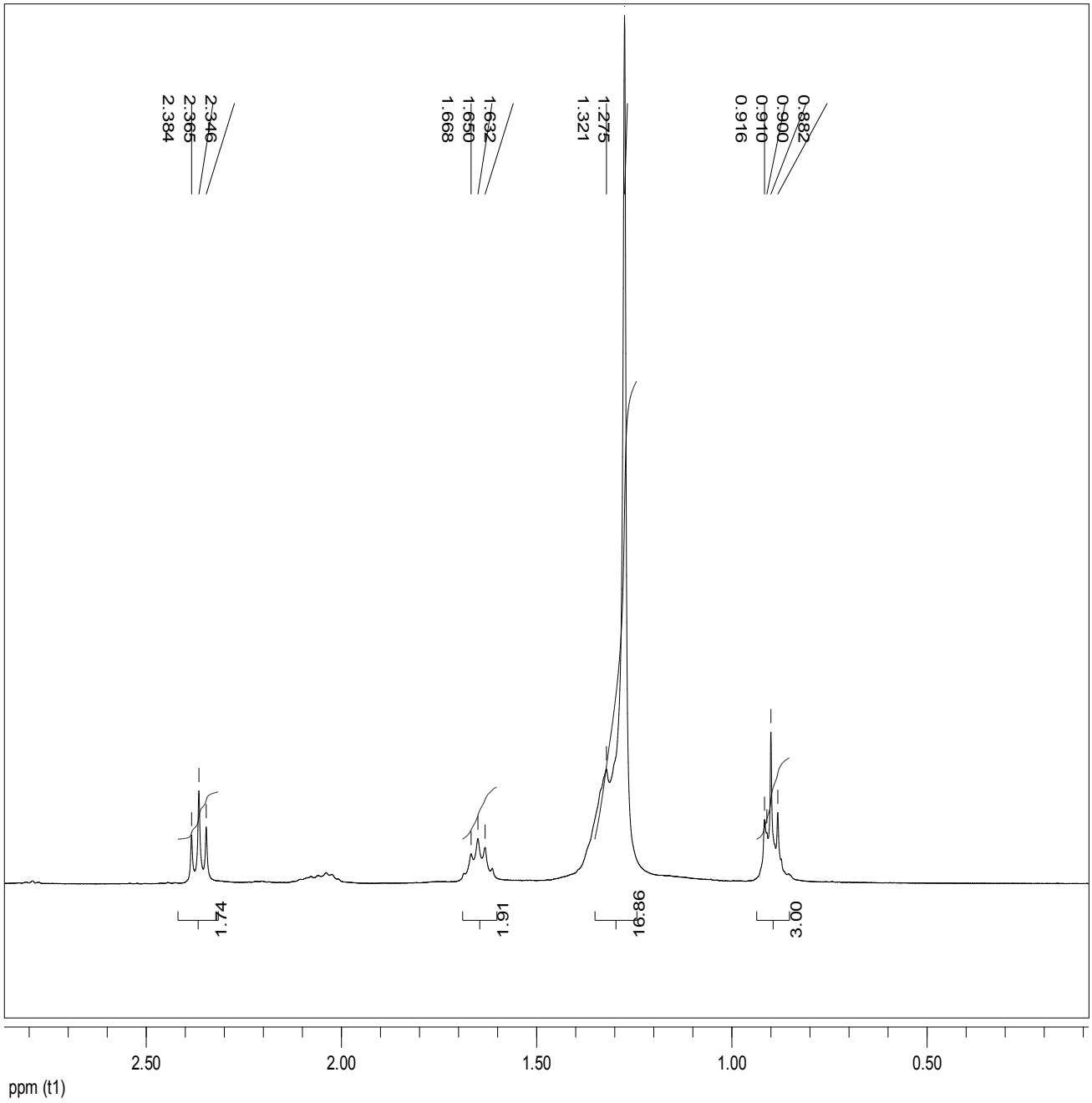
Appendix 1: UV-spectrum of compound **KP-1.**



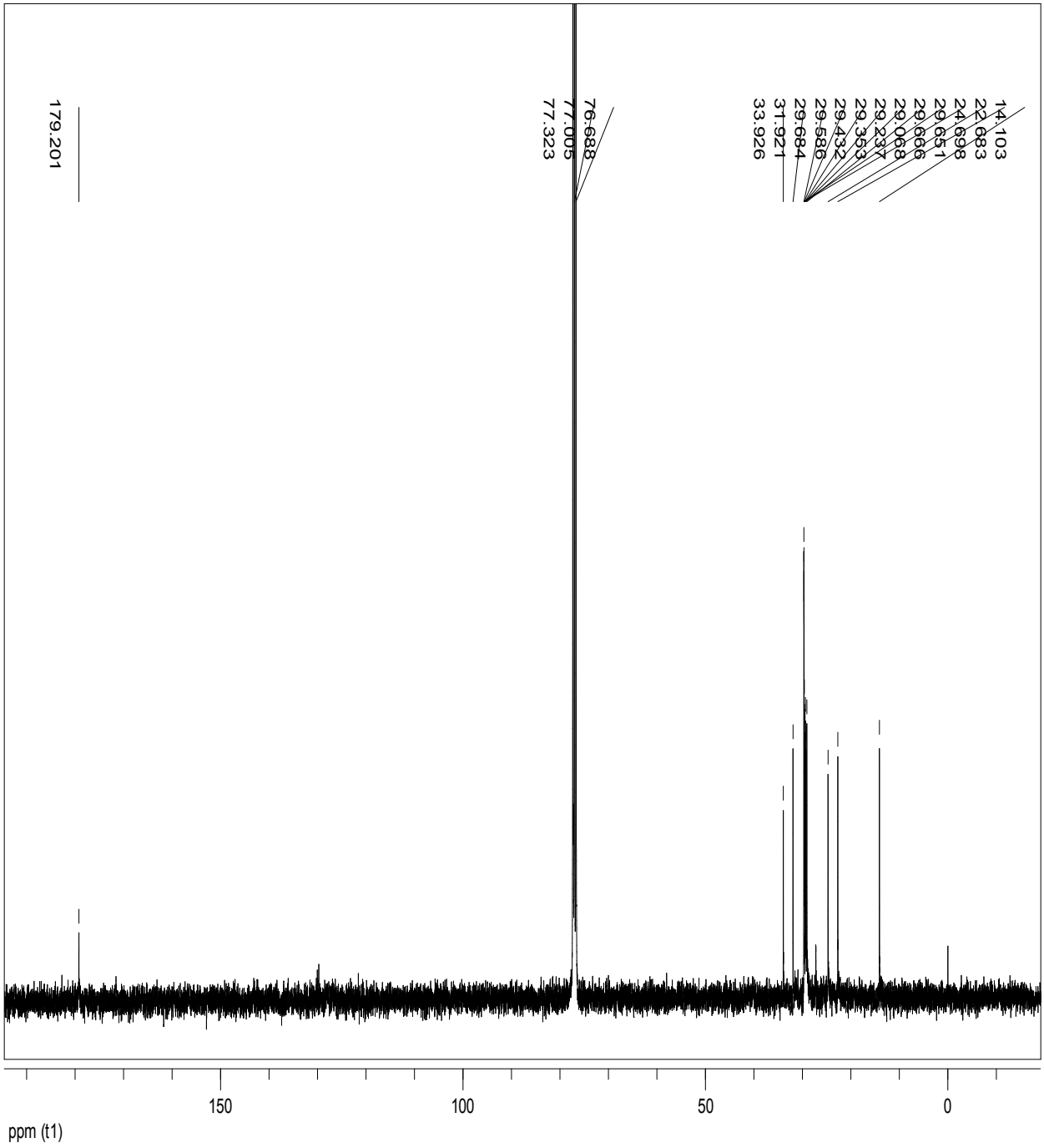
Appendixes 2: IR spectrum of compound **KP-1**



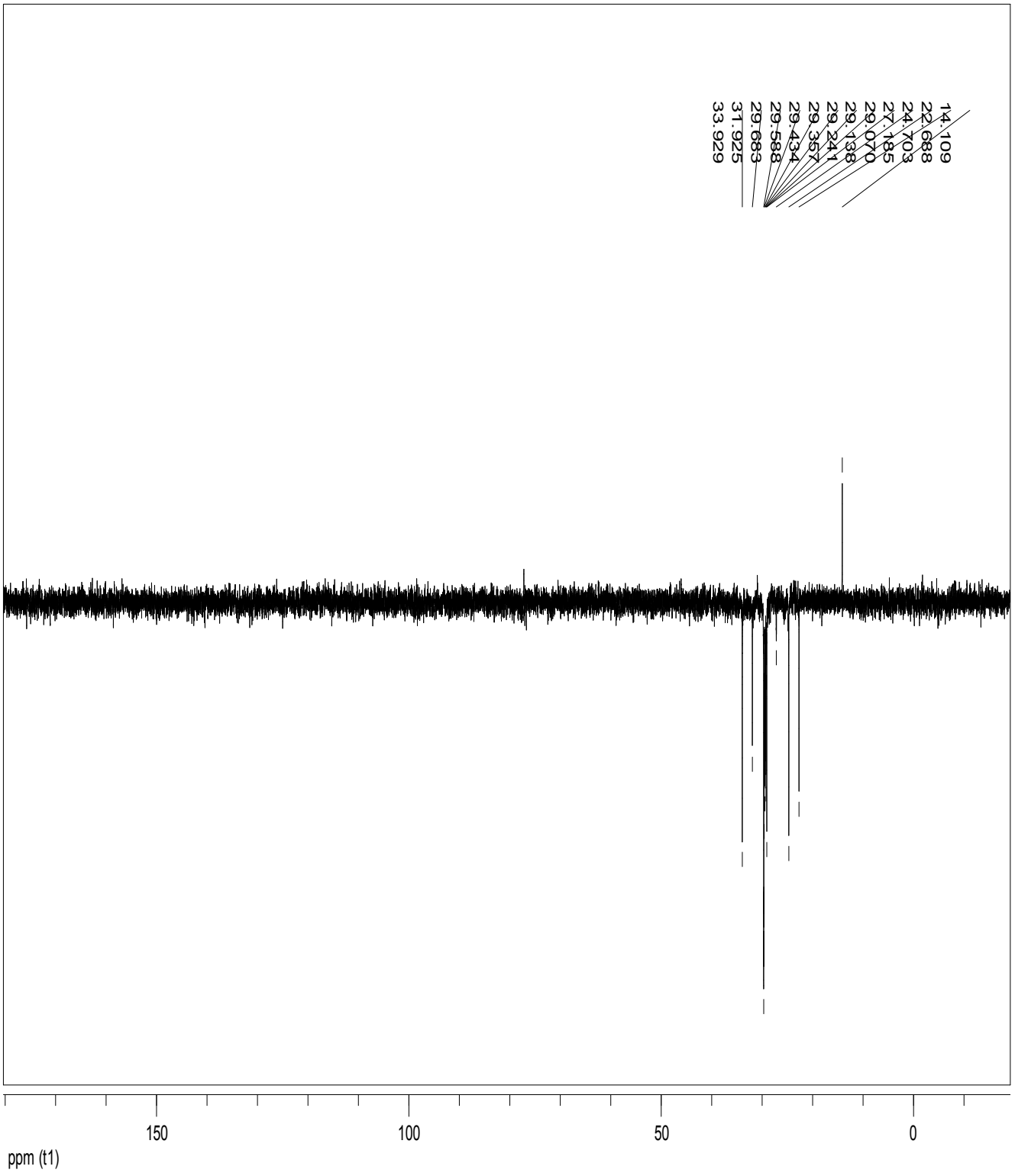
Appendix 3: ^{1}H-NMRSpectrum of compound **KP-1**



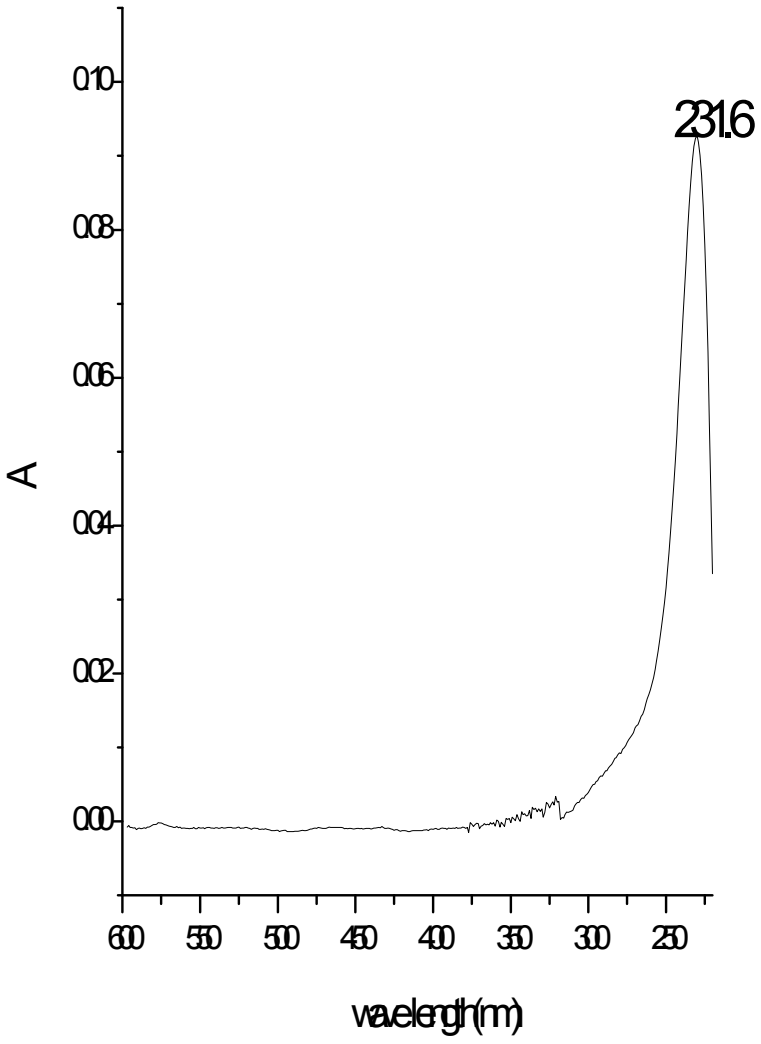
Appendix 4: ^{13}C-NMR spectrum of compound **KP-1**



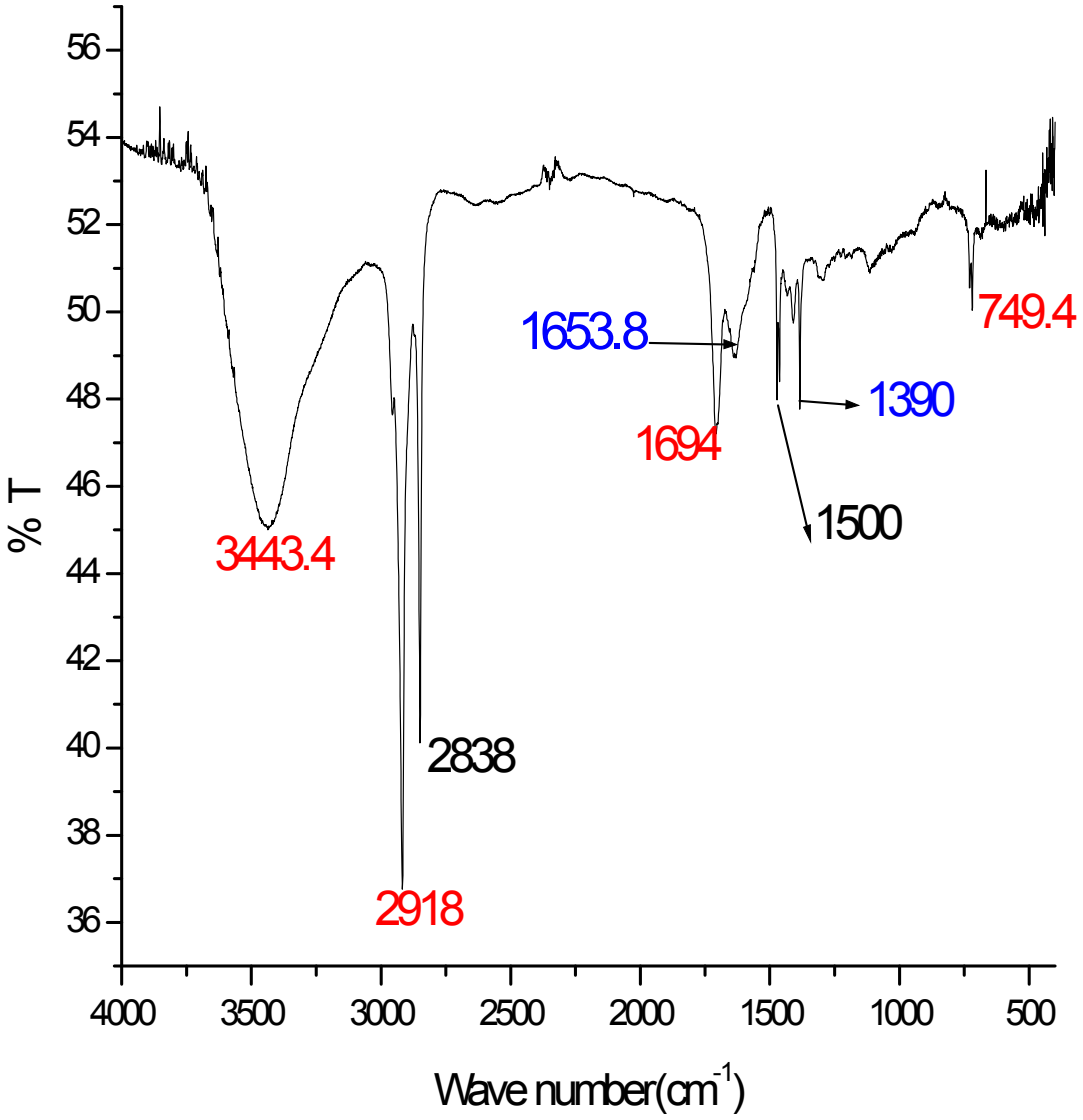
Appendix **5:** DEPT -135 spectrum of compound **KP-1**.



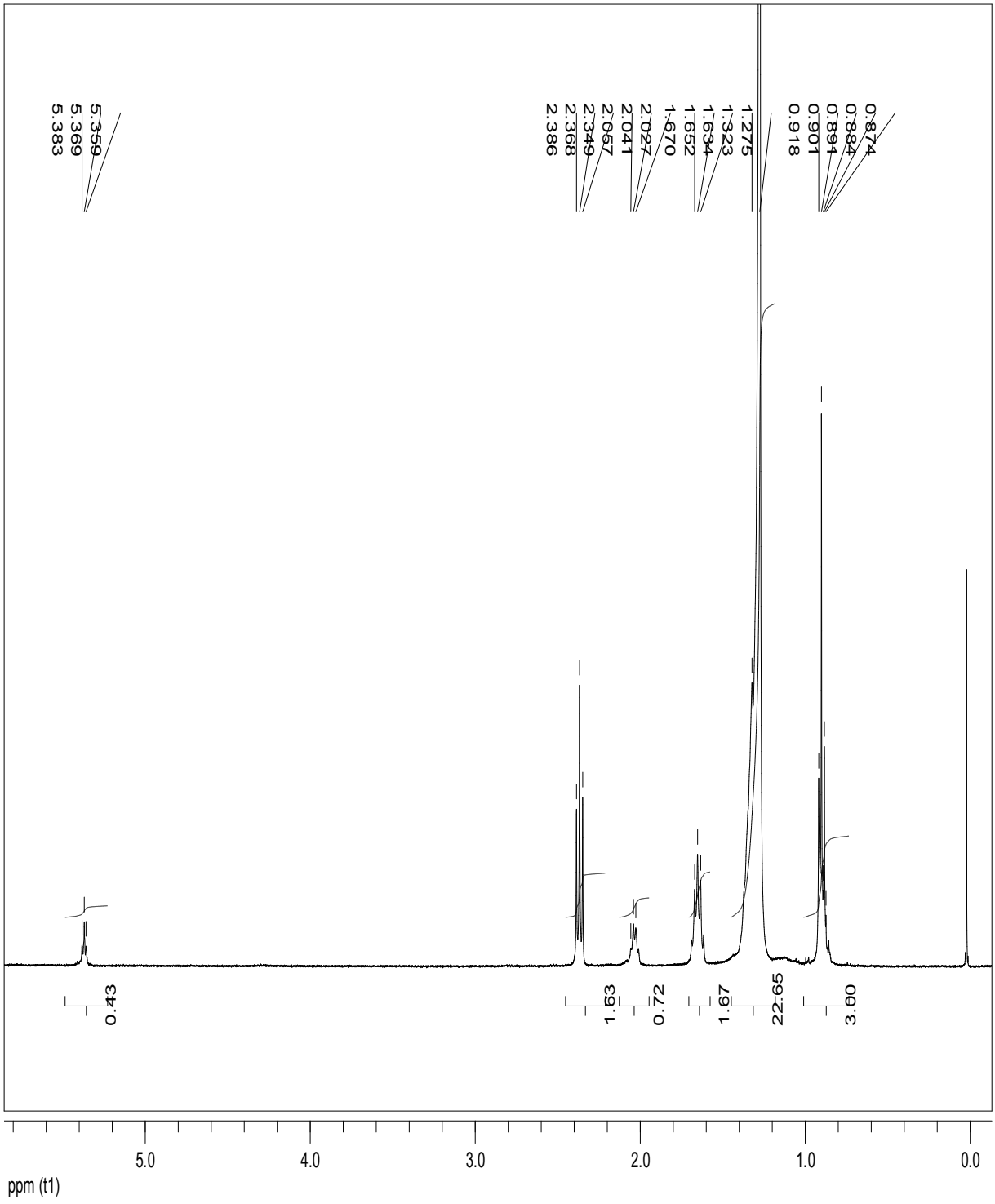
Appendix 6: UV spectrum of compound **KP-2**.



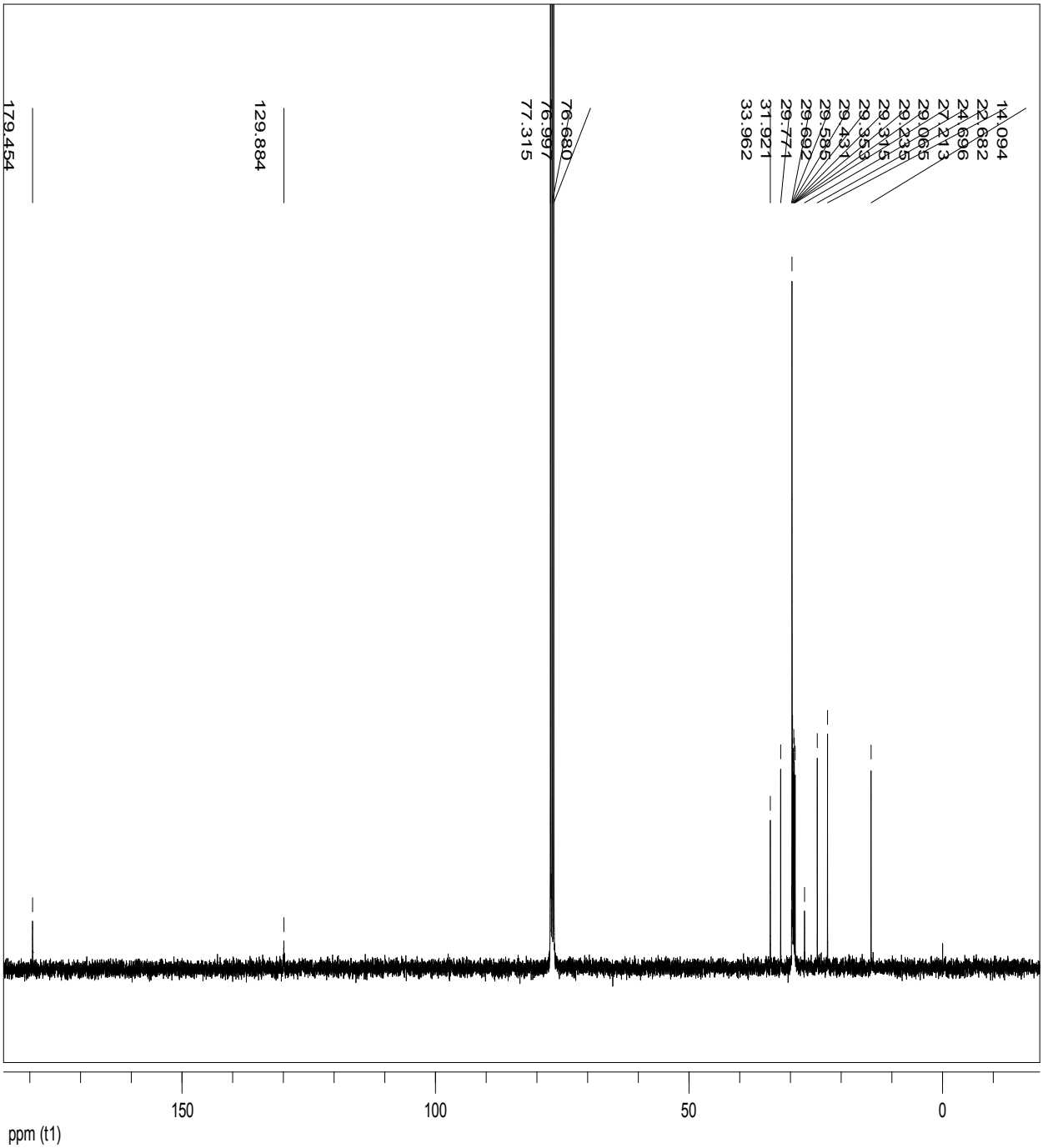
Appendix 7: IR spectrum of compound **KP-2.**



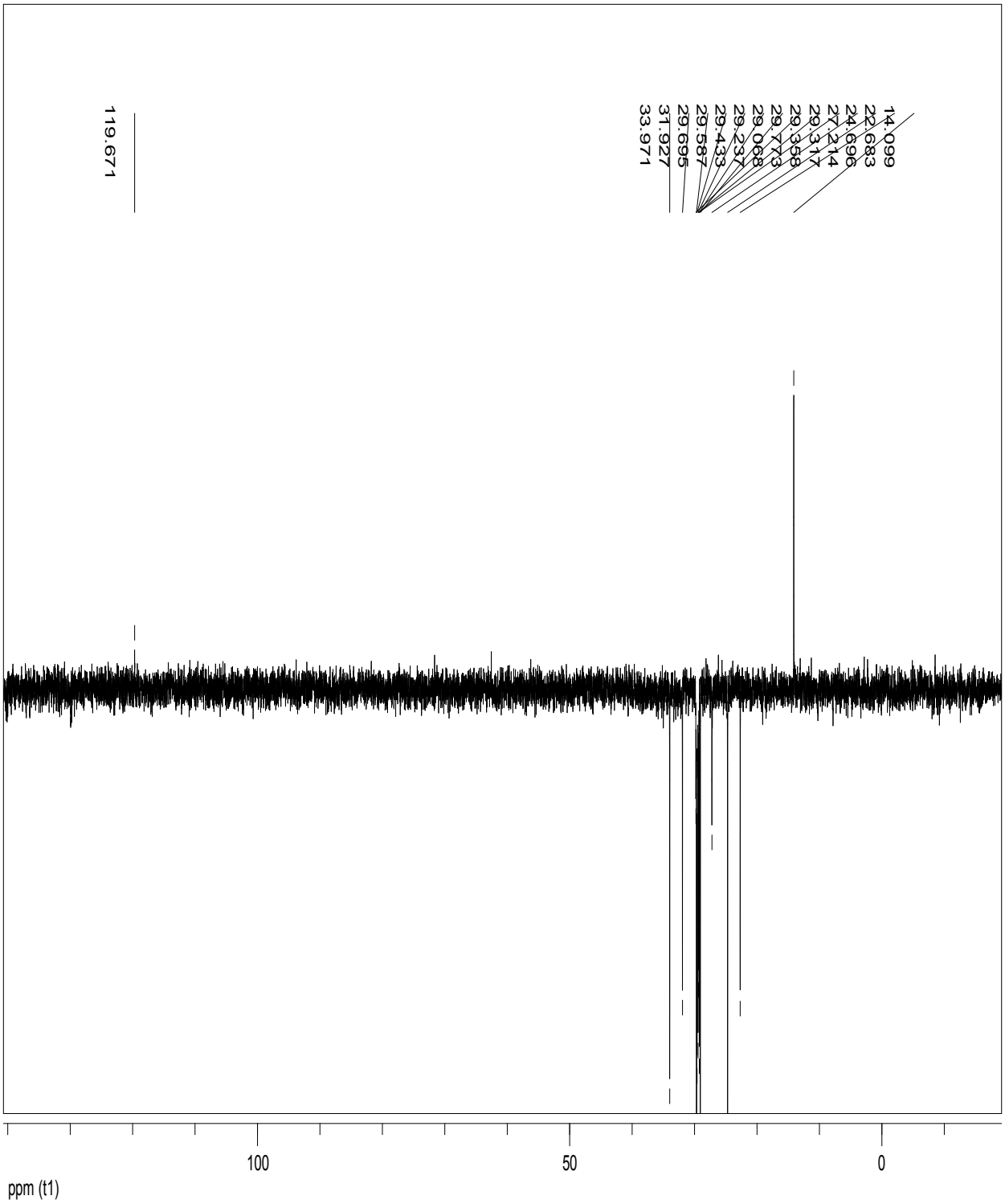
Appendix 8: ^{1}H-NMR spectrum of compound **KP-2.**



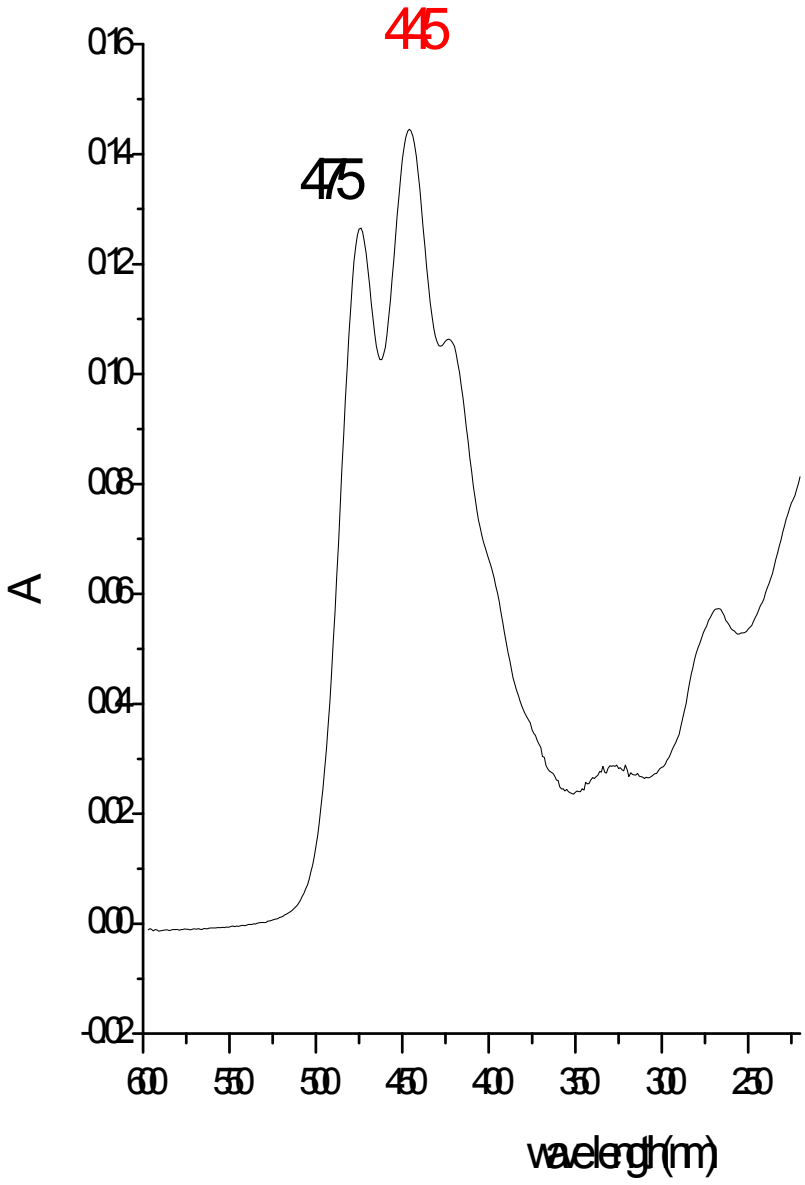
Appendix 9: ^{13}C-NMR spectrum of compound **KP-2**.



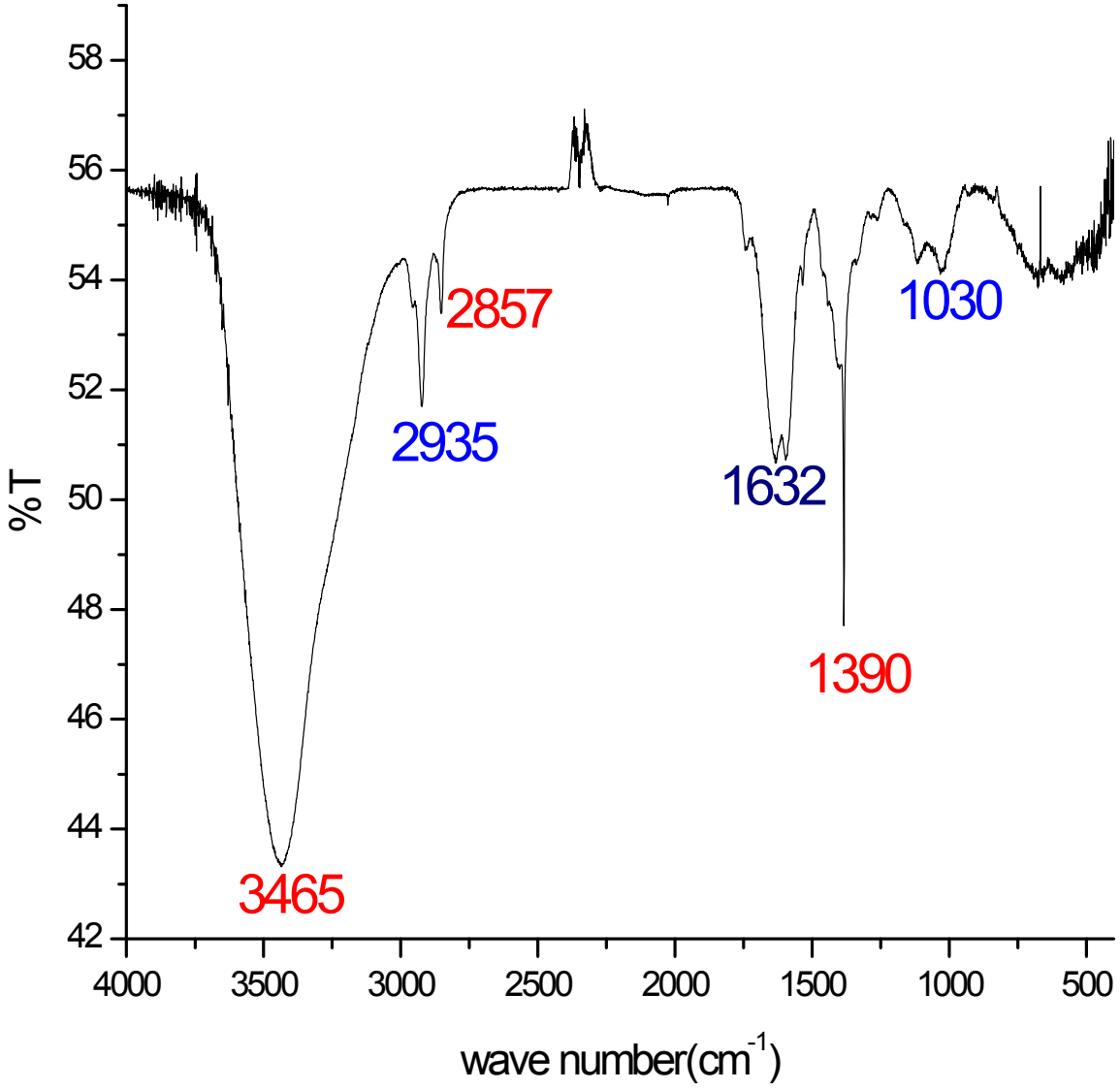
Appendix-10: DEPT -135 spectrum of compound **KP-2.**



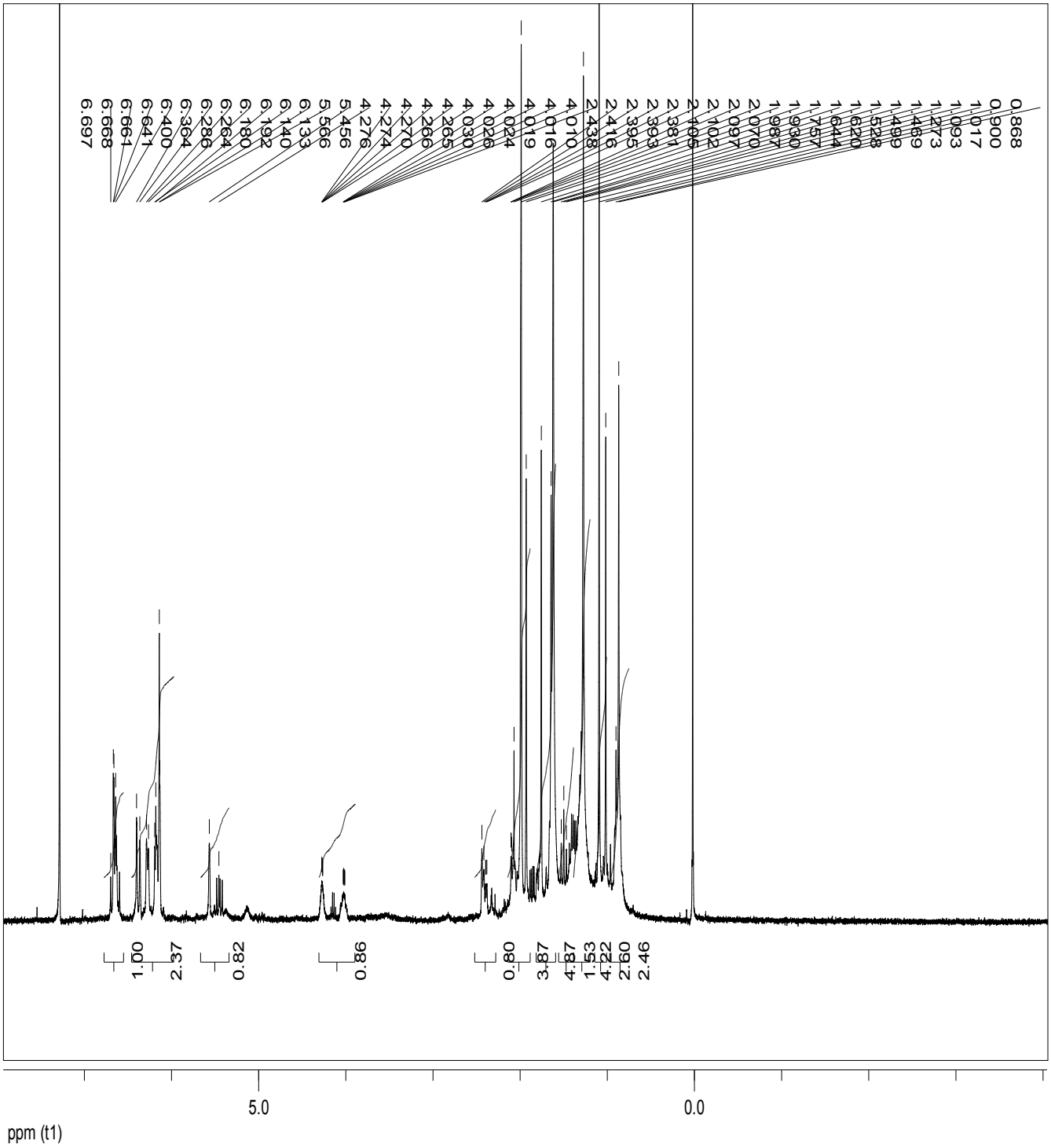
Appendix 11: UV spectrum of compound **KP-3.**



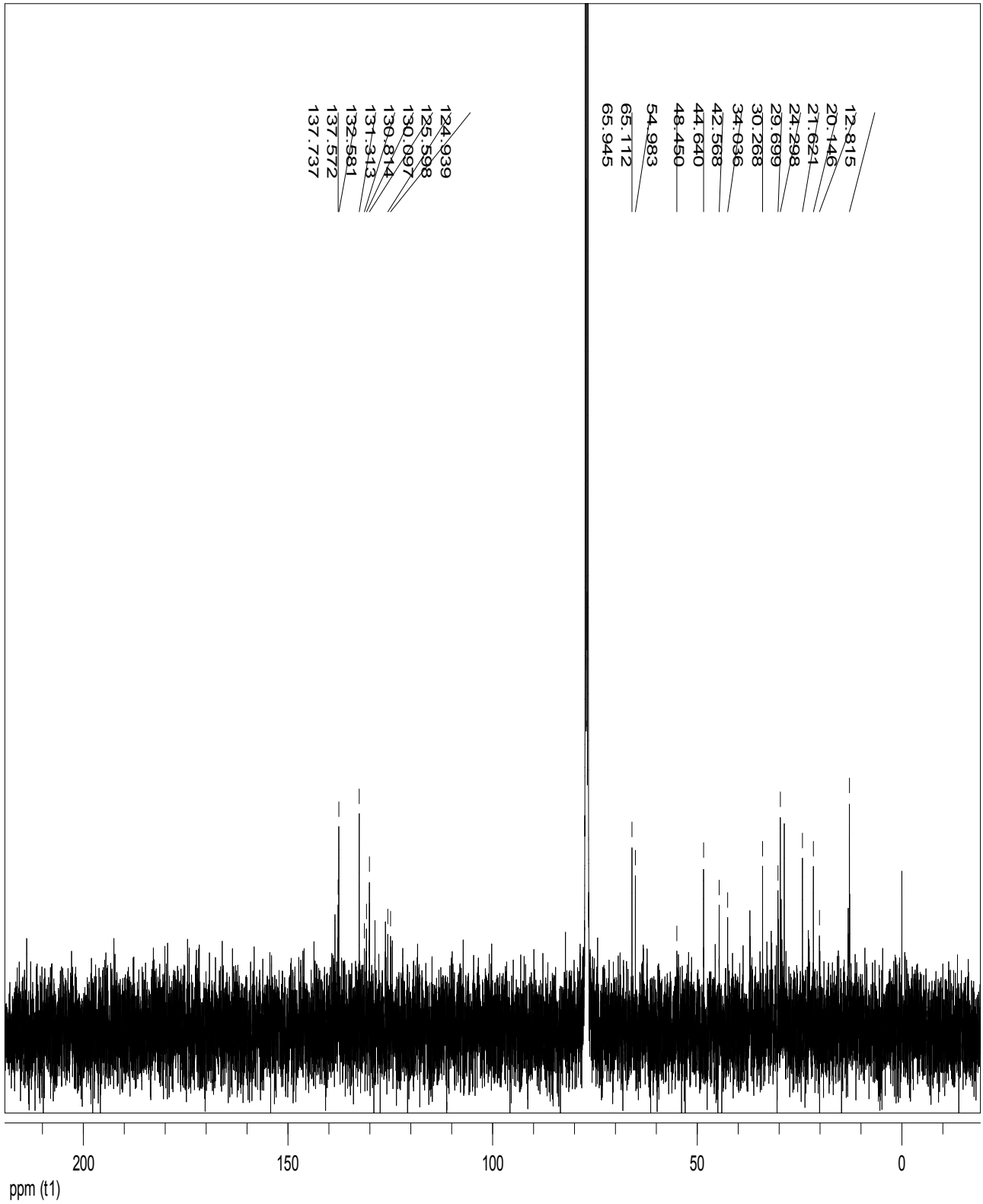
Appendix 12: FTIR of compound **KP-3.**



Appendix 13: ^{1}H-NMR spectrum of compound **KP-3.**



Appendix 14: ^{13}C-NMR spectrum of compound **KP-3**.



Appendix 15: DEPT -135 spectrum of compound **KP-3**.