Phytochemical Investigation and Antibacterial Evaluation of Roots of *Kniphofia schimperiana*

Adama Science and Technology University,

School of Applied Natural Science,

Department of Applied Chemistry

By

Kebede Shenkute

Thesis Submitted to Office of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science in Applied Chemistry (Medicinal Chemistry)

July, 2018

Adama, Ethiopia.

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Major Advisor: Milkyas Endale, PhD

CO-Advisor: Rajalakshmanan Eswaramoorthy, PhD

July, 2018

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DECLARATION

I, hereby declare that this thesis is my original work and has not been presented for a degree or diploma in any other University. All sources or materials used for this thesis have been duly acknowledged.

KEBEDE SHENKUTE

Signature ----------

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

ADVISORS

Major Advisor: Milkyas Endale (PhD)

Signature ----------

Co-advisor: Rajalakshmanan Eswaramoorthy, (PhD)

Signature ----------

Date of submission ----------

APPROVAL SHEET

This is to certify that the thesis entitled "phytochemical investigation and Antibacterial Evaluation of Roots of *Kniphofia Schimperiana*" submitted in partial fulfillment of the requirements for the degree of Master of Science in Applied Chemistry with specialization in Medicinal Chemistry of MSc program of Department of Applied Chemistry, Adama Science and Technology University and is a record of original research carried out by **KEBEDE SHENKUTE** (ID. N^{o} GSR/0181/09), under our supervision, and no part of the thesis has been submitted for any other degree or diploma in any other University.

Submitted by

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Approved by

Major Advisor	Signature	Date
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Examiner-2	Signature	Date
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Head of Program	Signature	Date
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School Dean	Signature	Date
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PG Dean	Signature	Date

DEDICATION

THIS THESIS IS DEDICATED TO MY WIFE MESERET AWULACHEW FOR HER ADVICE, LOVE AND SUPPORT SHE MADE ME WHO I AM TODAY YOU WILL ALWAYS BE IN MY HEART.

KEBEDE

Acknowledgment

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Acronyms

Å	Angstrom
ANOVA	Analysis Of Variance
CFU	Colony Forming Unit
DMSO	Dimethyl sulfoxide
EMB	Eosin Methylene Blue
IC_{50}	The half maximal inhibitory concentration
IR	Infrared
Km	Kilo meter
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MRSA	Methecilin Resistance Staphylococcus Aureus
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
Nm	Nano meter
PTLC	Preparative thin layer chromatography
PDB	Protein Data Bank
R_f	Retention factor
SPSS	Statistical Package for the Social Science
TLC	Thin layer chromatography
TSB	Tripticase soy broth
UV-Vis	Ultraviolet visible

Abstract

*Medicinal plants have been used throughout human history for treating various diseases. The genus Kniphofia (Asphodelaceae) one of the medicinal plants used to treat various diseases in Ethiopia. The genus comprises 71 species, commonly known as red hot pokers, is a rich source of monomeric and dimeric anthraquinones, anthrones, phenylanthraquinones and oxanthrones. The phytochemical screening test of CH_2Cl_2:CH_3OH (1:1) and 100% CH_3OH root extract of Kniphofia schimperiana revealed that the presence of glycosides, saponins, sterols, tannins, phenols and flavonoids and absence of alkaloids. Silica gel column chromatographic separation of CH_2Cl_2:CH_3OH (1:1) and 100% CH_3OH root extract of Kniphofia schimperiana of yielded three compounds of which one of them is a Knipholone derivative (**21**). Complete characterizations of isolated compounds were done with the help of spectroscopic techniques ^{1}H NMR, ^{13}C NMR and DEPT-135 and comparison with literature reports. Anti bacterial activities test of the crude extract and isolated compounds were done by the disk diffusion method compound 20 showed better activity than the other on S.aurouse with zone of inhibition of 11mm. of the four isolated compounds two of them (compound 21 and compound 7) were didn't show any antibacterial activities against both gram positive and gram negative bacterial strains.*

Key words: *Antibacterial, Kniphofia schimperiana, Phytochemical screening, anthraquinones*

1. INTRODUCTION

1.1. General background

Since ancient times, mankind has used plants to treat common diseases and some of these traditional medicines are still included as part of the habitual treatments of various maladies [1]. Plants are prospective source of antimicrobial agents in different countries. Literature report showed that about 60 to 90% of populations in the developing countries use plant-derived medicine [2]. Folk medicine, mainly based on plants, enjoys a respectable position today, especially in the developing countries, where the availability of modern health services is limited. However, in the absence of a scientific base, such practices may generate serious adverse effects. The analyses of the pharmacological activities of plant extracts may therefore make possible the design of less expensive therapies to be used in economically unprivileged regions [3].

Mainstream medicine is increasingly receptive to the use of antimicrobials and other drugs derived from plants, as traditional antibiotics become ineffective and as new, particularly viral, diseases remain intractable to this type of drug. Another driving factor for the renewed interest in plant antimicrobials in the past 20 years has been the rapid rate of plant species extraction [4].However, the full acceptance of phytopharmaceuticals and the integration of phytotherapy into the concept of classical medicine can be achieved only if they meet the same criteria of quality as synthetic pharmaceuticals. Moreover, the ideal procedures for standardization of the phytopharmaceuticals are based on knowledge of the major pharmacological and toxicological assays [5]. Plants are rich in a variety of phytochemicals including tannins, terpenoids, alkaloids, and flavonoids which have been found *in vitro* to have a wide range of antimicrobial properties [6].

African traditional medicine is the oldest, and perhaps the most assorted, of all therapeutic systems. Africa is considered to be the cradle of mankind with a rich biological and cultural diversity marked by regional differences in healing practices [7]. The extensive use of traditional medicine in Africa, composed mainly of medicinal plants, has been argued to be linked to cultural and economic reasons. This is why the WHO encourages African member states to

promote and integrate traditional medical practices in their health system [8]. In many parts of Africa, medicinal plants are the most easily accessible health resource available to the community. In addition, they are most often the preferred option for the patients. For most of these people, traditional healers offer information, counseling, treatment to patients and their families in a personal manner as well as having an understanding of their patient's environment [7, 9]. Africa is blessed with enormous biodiversity resources and it is estimated to contain between 40 and 45,000 species of plant with a potential for development and out of which 5,000 species are used medicinally. This is not surprising since Africa is located within the tropical and subtropical climate and it is a known fact that plants accumulate important secondary metabolites through evolution as a natural means of surviving in a hostile environment [10].

Ethiopia and Eritrea are two countries with varied topography and climate from hot semi-desert temperature and an altitude of 115 m below sea level in the Afar depression to cooler climates at 4620 m altitude at the top of the Semen Mountains. These contrasting temperatures and altitudes coupled with geology have resulted in niches that include about 6000 species of higher plants and of which about 10–12% are endemic. Of these species, about 1322 species are monocots (22%), with grasses accounting to 612 species (*c.*10%) and the remaining monocots including orchids cover about 710 species (*c.*12% of the flora) [11]. Despite, the wide spread use of Western medicine becoming more widespread in Ethiopia, people in the country tend to rely more on traditional medicine. Moreover Conventional medical services remain concentrated in urban areas and have failed to keep pace with the growing population, keeping health care access out of reach for most Ethiopians living in rural Ethiopia. Because traditional medicine is culturally entrenched, accessible, and affordable, up to 80% of the Ethiopian population relies on traditional remedies as a primary source of health care [12].

The genus *Kniphofia* moench (Asphodelaceae) ,local name of the plant is "lella" (in affaan oromiffaa) comprises 71 species, commonly known as "red hot pokers" because of the charismatic and conspicuous inflorescences of many species. It is traditionally used to treat wide ranges of ailments including menstrual pains, infertility, abdominal cramps, wounds, malaria, chest complaint and hepatitis B. *Kniphofia* is almost entirely African plant with two species from

Madagascar and one from Yemen, and has a disjunct Afromontane "island" distribution as it prefers temperate mountainous grassland and moist habitats. In southern Africa (SA) 48 species are currently recognized and the center of diversity and endemism is the Drakensberg region. Fifteen *Kniphofia* species have been recorded in Eastern Africa, of which seven occur in Ethiopia, of which five are endemic and two of them are wide spread including *Kniphofia foliosa* Hochst, *K. isoetifolia, K. schimperi, K. hildebrandtii K. insignis*. *K. thomsonii Baker and K. pumila.* [29].

1.2. Statement of problem

Global prevalence of infectious diseases caused by bacteria is a major public health problem [13]. The bacterial agents including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Proteus vulgaris* cause several human infections [14]. Recent emergence of antibiotic resistance and related toxicity issues limit the use of currently available antimicrobial agents [15] and are prompting a revival in research of the antimicrobial role of plants against resistant strains due to comparable safety and efficacy [2]. Therefore, investigation of medicinal plants for potential antibacterial agent has to continue in search of new, potent and safe antibacterial drug templates.

1.3. Significance of the research

The studies on antimicrobial activities of Ethiopian medicinal plants are still scarce, and there is need for further investigations and evaluate the effectiveness of these plants phytochemical constituents and crude extracts against disease causing agents and their ultimate utilization in drug development. Clinical observations on traditional remedies are possible and useful. Some herbal remedies may be safe and effective for the treatment of microbial infection. Nevertheless, better evidence from randomized clinical trials and cytoxicity assay is required before herbal remedies can be recommended on a large scale. In order to prioritize remedies, there is need for preliminary clinical observational studies based on existing data from the ethnobotanical and ethnopharmacological study of Traditional medicine of Ethiopian plants. This research can provide an evidence base for the traditional use of the plants by identifying their chemical

constituents and validate through biological screening. This research is expected to contribute in the identification of lead compound(s) with antibacterial activities which could lead to cheap and readily available alternative drugs. Moreover, this research work is expected to provide scientific information for researchers working in similar area.

1.4. Objectives

1.4.1. General objective

The main objective of this research is phytochemical investigation and evaluation of antibacterial activity of secondary metabolites from the roots of *Kniphofia schimperiana.*

1.4.2. Specific objectives

- To extract the root of *Kniphofia schimperiana* with CH_2Cl_2:CH_3OH (1:1) and CH_3OH successively.
- Isolation of compounds from the extract by chromatographic method.
- To elucidate the structures of the isolated compounds using spectroscopic methods.
- To analyze the antibacterial activities of the crude extracts and isolated compounds using disk diffusion method against three gram negative (*E.coli, P.aeroginasa* and *K,neumonia*) and one gram positive (*S.cabrous*) bacteria.

2. LITERATURE REVIEW

2.1. General concept

The term antibiotics literally means “against life” in this case, against microbes. There are many types of antibiotics such as antibacterial, antiviral, antifungal, and antiparasitics. Some drugs are effective against many organisms. These drugs are called broad-spectrum antibiotics. Others are effective against just a few organisms and are called narrow spectrum antibiotics [17]. Infectious diseases are known to cause a serious health and economic problem to human being throughout the world, ultimately causing millions of mortalities, many of which are a premature death of children under age of five [18]. The use of plants and plant-based products for healing human diseases is an old experience and practice [19]. In addition to the traditional application, plants and their products have also laid a great foundation for the modern drug, For example, the majorities of anticancer drugs and drugs for the treatment of infectious diseases have been derived from plants and other nature-based sources and, still continue to serve as potential sources [20, 21]

2.2. Botanical Information

2.2.1. The family *Asphodelaceae*

The genus *Kniphofia* belongs to the family *Asphodelaceae* which is comprised of 17 genera (10 of which occur in South Africa) and about 750 species. The family *Asphodelaceae* is divided into two subfamilies; the *Asphodeloideae* and the *Alooideae.* Accordingly the genera *Asphodeline, Asphodelus, Bulbine, Bulbinella, Eremurus, Hemiphlacus, Jodrellia, Kniphofia, Paradisea, Simethis,* and *Trachandra* are placed in the subfamily Asphodeloideae while *Aloe, Gasteria, Haworthia, Lomatophylum,* and *Poellnitzia* are placed in the *Alooideae* [27].

On the other hand some authors consider the above two subfamilies as distinct families i.e. the *Asphodelaceae* and the *Aloaceae.* The generations of chemical information on species belonging to these two groups are believed to reveal the relationships among the various taxa and to assist in establishing taxonomic classifications at various levels [28].

2.2.2 Genus *Kniphofia*

The genus *Kniphofia* Moench (*Asphodelaceae*) comprises 71 species, commonly known as "red hot pokers" because of the charismatic and conspicuous inflorescences of many species. It is traditionally used to treat wide ranges of ailments including menstrual pains, infertility, abdominal cramps, wounds, malaria, chest complaint and hepatitis B. *Kniphofia* is almost entirely African plant with two species from Madagascar and one from Yemen, and has a disjunct Afromontane "island" distribution as it prefers temperate mountainous grassland and moist habitats.

Figure 1 *Kniphofia schimperiana* picture taken in November 2017, by Kebede Shenkute From Fasil Angaso, Bale, Oromia, Ethiopia.

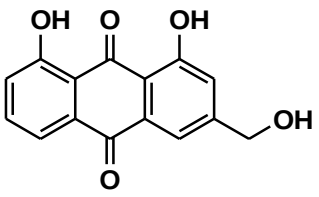
2.2.3. Chemical constituents of the genus *Kniphofia*

The genus *Kniphofia* is a rich source of monomeric and dimeric anthraquinones, anthrones, phenylanthraquinones and oxanthrones. The sources of these compounds are summarized in Table 1 below.

Table 1 List of compounds isolated from the genus *Kniphofia*

Compounds	Species (parts)	Reference
Monomeric anthraquinones		
Aloe-Emodin **(1)**	*K. foliosa (leaves, flowers, fruits)*	[30]
	K. insignis (flowers)	
	K. isoetifolia (flowers)	
	K. schimperi (Flowers)	
	K. thomsonii (root)	[31]
Aloe-Emodin acetate **(2)**	*K. foliosa (leaves)*	[30]
	K. foliosa (leaves, flowers, fruits)	
	K. isoetifolia (flowers)	
	K. thomsonii (root)	[31]
Chrysopanic acid **(3)**	*K. foliosa* (Leaves)	[32] [30]
	K. isoetifolia (Flowers, Leaves, Rhizomes)	[30]
Dimeric anthraquinones		
Asphodelin **(4)**	*K. albescens* (Root)	[33]
Chrysalodin **(5)**	*K.folosa* (Leaves)	
Knipholone **(6)**	*K. insignis* (Rhizomes) *K. isoetifolia* (Rhizomes) *K. pumila* (Rhizomes) *K. schimperi* (Rhizomes) *K foliosa* (Rhizomes)	[35]
10-Hydroxy-10-(chrysophanol-7'-yl)-chrysophanol anthrone **(7)**	*K.foliosa* (Root) *K. thomsonii* (Root)	[24] [31]
10-Bichrysophanolanthrone **(8)**	*K. thomsonii* (Root)	[31]
10-Hydroxy-10-(chrysophanol-7'- yl)-aloe-emodin anthrone (**9**)	*K.Thomsonii* (Root)	
10-hydroxy-10-(islandicin-7'-yl)-aloe-emodin anthrone **(10)**	*K. thomsonii* (Root)	[31]
Phenyl anthraquinones and anthrones		
Isoknipholone **(11)**	*K.foliosa* (Stem)	[36]
Isoknipholone anthrone **(12)**	*K.foliosa* (Stem)	[36]
Knipholone anthrone **(13)**	K. foliosa (Stem)	[37] [36]

Oxanthrones		
Foliosone **(14)**	*K.foliosa* (Stem)	[36]
Isofoliosone **(15)**		



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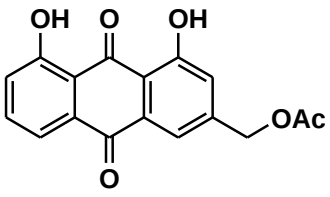

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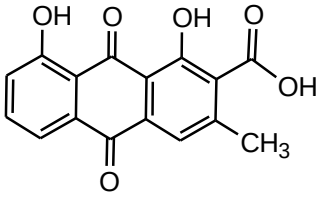

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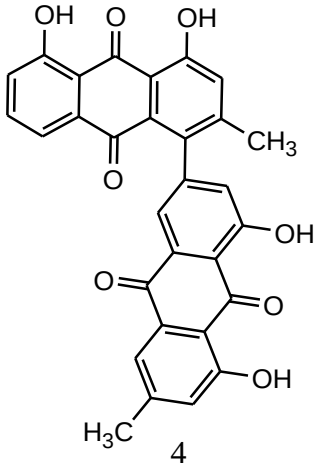

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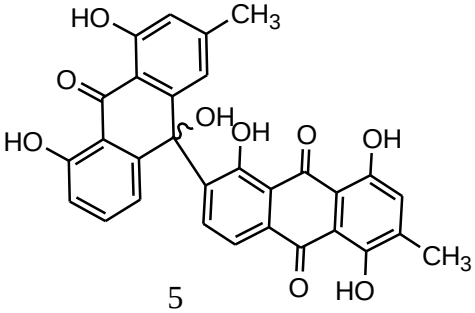

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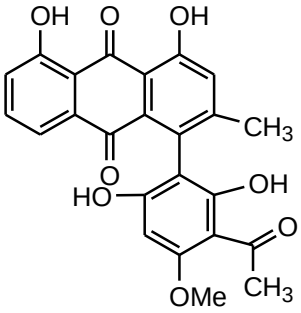

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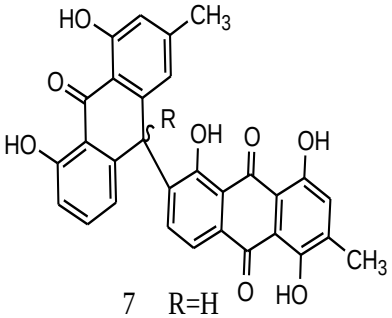
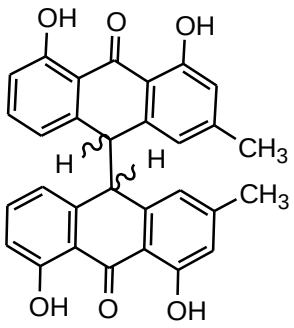


7 R=H
8 R=OH



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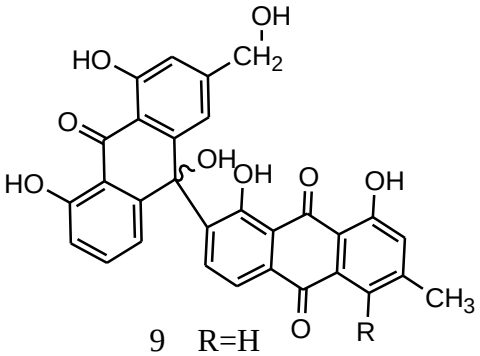
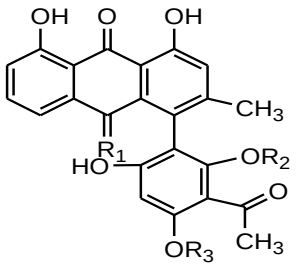
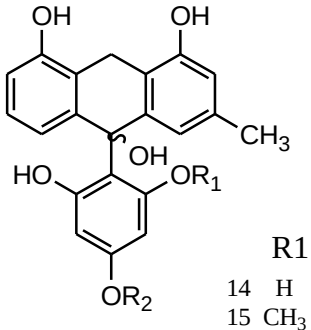


9 R=H
10 R=OH

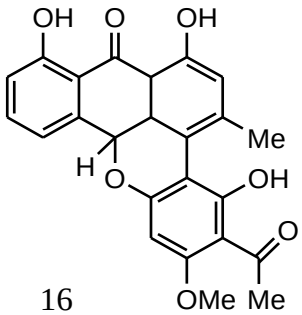


	R1	R2	R3
11	O	CH_3	H
12	H,H	CH_3	H
13	H,H	H	CH_3



	R1	R2
14	H	CH_3
15	CH_3	H

Extracts of the rhizomes of *Kniphofia foliosa* exhibited antiplasmodial activities against the chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *Plasmodium falciparum* with IC_{50} values of 3-5 µg/ml. A phenyloxanthrone, named 10-acetonylknipholone cyclooxanthrone (**16**) is isolated from the rhizomes, along with known quinones, including the rare phenylanthraquinone dimers, joziknipholones A and B [38].



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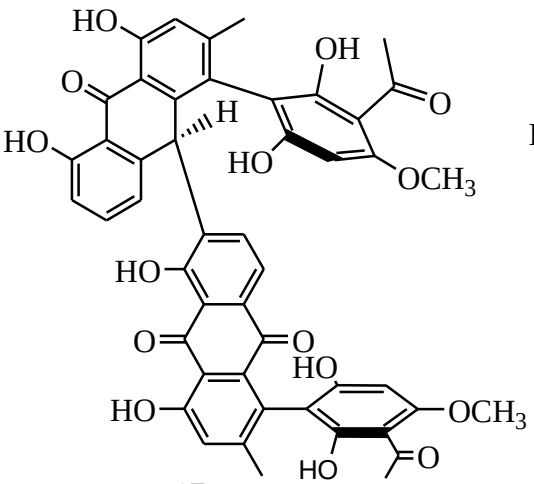

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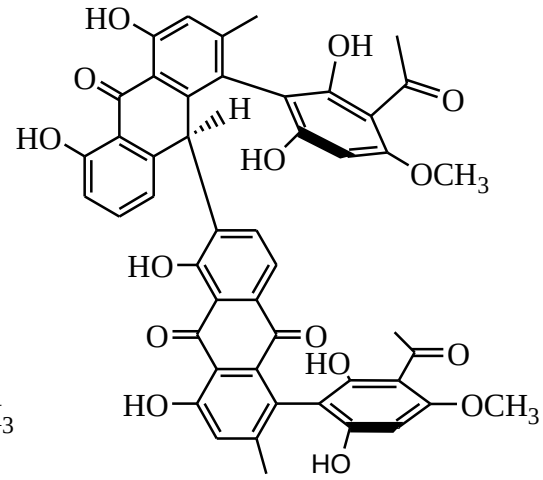

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2.2.4. Ethno botanical uses of the genus *Kniphofia*

Although the genus is widely recognized for its ornamental value owing to their colorful flowers, the use of the genus *Kniphofia* in traditional medicine is limited to few species which is summarized in Table 2.

Table 2 Summary of the Ethnobotanical uses of the genus *Kniphofia*

Species	Plant part	Used for/as	References
K.buchananii	Plant infusion	Snake deterrents, Chest ailments	[22,23]
K.caulescens	Part not specified	Charm against lightening	[22]
K.foliosa	Roots	Abdominal cramp and ache Wound healing	[24, 23]
K.isoetifolia	Roots	Gonorea, hepatitis B	[25]
K.laxiflora	Plant infusion	Snake deterrents, Chest ailments	[23]
K.linearifolia	Roots	To treat infertility	[26]
K.parvifolia	Plant infusion	Snake deterrents, Chest ailments	[23, 22]
K.ritualis	Part not specified	Shoulder pains	[22]
K.rooperi	Plant infusion	Chest ailments and snake deterrent	[3, 22]

2.2.5. Biological activity of the genus *Kniphofia*

The use of plants and plant-based products for healing human diseases is an old experience and practice [19]. In addition to the traditional application, plants and their products have also laid a great foundation for the modern drug, for example, the majority of anticancer drugs and infectious diseases has been derived from plants and other nature-based sources and, still continue to serve as a potential source [20, 21]. Some of the compounds isolated from *Kniphofia foliosa* have shown promising antimalarial activity. The diverse biological activity of the compounds isolated is summarized in Table 3.

Table 3. Biological Activity of the genus *Kniphofia*

Biological Activity	**Compound**	**References**
Antioxidant activity	Knipholone anthrone (**13**)	[39] [40]
Antiprotozoal Activity and radical Scavenging activity	Knipholone anthrone (**13**)	[39]
Antitumor Activity	Knipholone (**6**) and Isoknipholone (**11)**	[40]
Antimalarial	Isoknipholone(**11**),Knipholone anthrone (**13**)	
Inhibition of leukotriene (Treatment of Asthema and Inflamatory Diseases)	Knipholone (**6**)	[41]

3. MATERIAL AND METHODS

3.1. Chemicals, Apparatus and Instruments

3.1.1. Materials required

Apparatus and instruments used in this study were electrical grinder, drying oven, measuring cylinders, bottles, shaker, ruler, whatman No **1** filter papers, funnels, round bottom flasks, rotary evaporator, vials, TLC Chamber, TLC plates, capillary tubes, UV-lamp, column chromatography and NMR- Spectrometer.

3.1.2. Chemicals

The chemicals used in the experiment were *n*-hexane, ethyl acetate, dichloromethane, ethanol, methanol, acetone, oxalic acid, silica Gel (230-400 mesh size), Wagner's reagent, Keller's reagent, Keller Kiliani reagent, Salkowski reagent, distilled water, sulfuric acid, ferric chloride, potassium hydroxide, lead acetate and sodium hydroxide.

3.2. Plant material

The roots of *Kniphofia schimperiana* were collected from Oromia region, Bale Zone around Goba town specifically Fasil Angaso kebele which is 412km from Addis Ababa. The plant was identified by botanist Mr. Shambel Alemu in National Herbarium of Ethiopia, Department of Biology, and Addis Ababa University, Ethiopia. The plant material was dried under shade at Chemistry laboratory of Adama Science and Technology University. The dried plant material was pulverised using an electric grinder for further analysis.

3.3. Extraction and isolation of compounds

The air-dried and grounded powder of roots of *Kniphofia schimperiana* (500 g) was soaked for 72 hrs in 1:1 ratio of dichloromethane/methanol the extract was evaporated by using rotary evaporator to yield 40.52 g (8.104%) crude extract. The marc left after dichloromethane/methanol (1:1) extraction was soaked in 100% methanol for 72 hrs then filtered. The extract was evaporated under reduced pressure by using Rotary evaporator and yield

46.87 g (9.374%) of crude extract. The TLC profile of the crude extract obtained from the extraction was monitored by TLC and visualized using UV lamp at (254 nm and 365 nm).
The dichloromethane/methanol (12 g) was subjected to silica gel column chromatography on oxalic acid impregnated silica gel (150 g), (250 g silica gel Merck 60 H 230-400 Mesh) deactivated with 0.75 g of oxalic acid in one liter distilled water, and eluting with increasing gradient from *n*-hexane, ethyl acetate, dichloromethane and methanol. A total of 136 fractions (100 mL each) were collected. The constituent profile of each fraction was monitored by TLC (30% ethyl acetate in *n*-hexane) and visualized under UV lamp (254 and 366nm). Fractions 32-33 were combined based on their TLC profile and was further purified by column chromatography (eluent; isocratic ethyl acetate in *n*-hexane) then to give compound **19** (16mg). Fractions 99-100 (one major spot) were combined, dried and washed successively with *n*-hexane to give compound **20** (15 mg).

The crude extract of methanol (12 g) was subjected to silica gel column chromatography on oxalic acid impregnated silica gel (150 g), (250 g silica gel deactivated with 0.75 g of oxalic acid in one liter distilled water, Merck 60 H 230-400 Mesh) and eluting with increasing gradient from *n*-hexane, ethyl acetate, dichloromethane and methanol. A total of 106 fractions (100 mL each) were collected. The constituent profile of each fraction was monitored by TLC (30% ethyl acetate in *n*-hexane) and visualized under UV-Vis light (λ_{max} 254 and 366 nm). Fractions 64-69 were combined base on their TLC profile and was further purified. A total of 15 fractions (15mL each) were collected from which fractions 7-9 which showed similar R_f were combined to afford compound **21** (18 mg).

3.4. Phytochemical screening of crude extract

Phytochemical screening is important in giving information about the class of chemical compounds existing in the plant material and that would help the investigator to decide which extract to be further isolated. The phytochemical screening of the plant extracts were carried out using the standard procedures reported in the literature [42]. The phytochemical screening of the root of *Kniphofia schimperiana* was revealed that the presence of glycosides, saponins, sterols,

tannins, phenols and flavonoids. The following table showed the qualitative test of secondary metabolite of the crude extract of root of *Kniphofia schimperiana.*

3.5. Structure elucidation

The isolated compounds were fully characterized with the help of spectroscopic techniques such as 1D NMR (^{1}H NMR and ^{13}C NMR).

3.6. Antibacterial Activity test

Four microorganisms were selected for the antimicrobial activity test of the crude extract and isolated compounds of root of *Kniphofia shimperiana*. One Gram-positive, *Staphylococcus aureus*, and three-Gram negative bacteria, *K.neumonia, Escherichia coli* and *Pseudomonas aeruginosa* were used for *in vitro* evaluation of the anti-bacterial activity using the disk diffusion method. The bacteria stock cultures were maintained on the nutrient agar slants, which were stored at 40°C. The test solutions were prepared by dissolving 50mg of the test samples to achieve final stock concentrations of 50 mg/mL in DMSO. The freshly grown liquid culture of the test pathogens solution of having similar turbidity with 0.5 McFarland were seeded over the Müeller-Hinton Agar medium with sterile swab. Sterile Whatman No **1** filter paper discs were soaked with 30µL of the above stock solution concentration of the samples and air dried to evaporate the solvent and then applied over the seeded plates at equidistance. The plates were then inverted and incubated at 37°C for 24 hrs. After the incubation period, the plates were observed for a clearance zone around the disks. The clear zones formed around each disks were measured in millimeter. Each experiment was carried out in triplicates. The mean of the inhibition zone of each test sample was taken for evaluating the antibacterial activity.

4. RESULT AND DISCUSION

4.1. Phytochemical screening

Phytochemical screening is important in giving information about the class of chemical compounds existing in the plant material and that would help the investigator to decide which extract to be further isolated. The phytochemical screening of the root of *Kniphofia schimperiana* revealed the presence of glycosides, saponins, sterols, tannins, phenols, flavonoids and absence of alkaloids (Table 4).

Table 4. Phytochemical components of crude extract of root of *Kniphofia schimperiana.*

Phytochemical compounds	MeOH Extract	DCM: MeOH Extract
Alkaloid	-	-
Glycoside	+	+
Saponin	+	+
Sterol	+	+
Tannins	+	+
Flavonoid	+	+
Phenolics	+	+

MeOH=Methanol, DM=dichloromethane, (-) absent, (+) = present

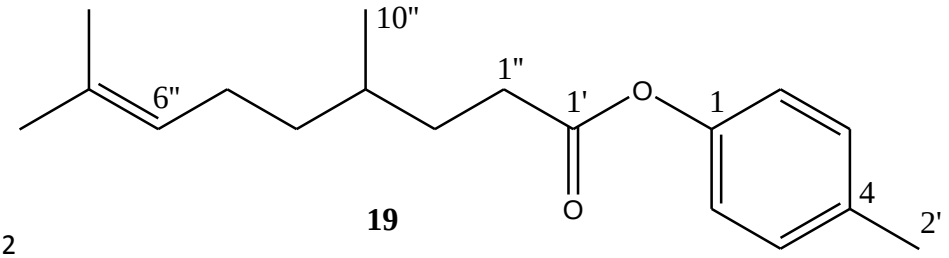
4.2 Characterization of compounds isolated from root of *Kniphofia shimperiana*

The extract was then subjected to silica gel column chromatography on oxalic acid impregnated silica gel which led to the isolation of three compounds. The characterization of these compounds is presented below.

4.2.1 Characterization of compound 19

Compound **19** was isolated as a yellow powder with R_f value of (7.1) in (1:1 ethyl acetate: n-hexane). The ^{1}H NMR spectrum revealed the existence of AA'XX' spin system suggesting a 1, 4 di-substituted aromatic ring (δ 6.87 (2H, *dd, J= 8, 2 Hz*) and 7.03 (2H, *dd, J= 8,2 Hz*)). The peaks at δ 4.35(3H, s) suggest the presence of a methyl group attached to phenyl ring (C-1''). The presence of an olefinic proton at δ5.2 (1H, t, H-6'') coupled with two symmetrical methyls groups at δ1.71 (3H, s, H-8'', 9''), four methylenes (C-1'', 2'', 4'', 5'') suggest a geranyl moiety.

The ^{13}C NMR spectrum revealed a total of 18 carbon peaks of these the presence of ester moiety at δ176, a 1, 4-disubstitued phenyl ring (δ130.4, 130.9, 128.8 and 161.0) are all clearly evident suggesting that the methyl is located at C-4 position of phenyl ring whereas the ester geranyl moiety is located at Para position to the methyl (C-1). Moreover, the 1D NMR spectral pattern is a good agreement that the geranyl moiety is directly connected to the ester. Thus, based on the above spectral data the compound **19** was proposed as shown below.



19

Figure 2

Table 5: NMR data of compound 19 (^{1}H NMR 400MHz, ^{13}C NMR 100 MHz, $CDCl_3$)

Position	^{1}H NMR (δ in ppm)	^{13}C NMR (δ in ppm)	DEPT-135 (δ in ppm)
1	-	129.1	-
2	7.03(2H, dd)	132.4	-
2’	2.0 (3H, s)	13.9	
3	6.87(2H,dd)	130.9	-
4	-	155.0	-
5	6.87(2H, dd)	130.9	-
6	7. 03(2H,d*d)*	132.4	-
1’	-	167.0	-
1’’	2.71 (2H,*t,)*	65.6	65.6
2’’	1.24(2H)	30.6	30.6
3’’	1.69(1H)	31.9	-
4’’	1.70(2H)	29.7	29.7
5’’	1.79(2H)	38.0	38.0
6’’	5.2(2H)	128.7	-
7’’	-	132.5	-
8’’	1.55(3H, s)	22.5	-
9’’	-	27.3	-
10’’	1.06(3H,nd)	19.7	-

4.2.2. Characterization of compound 21

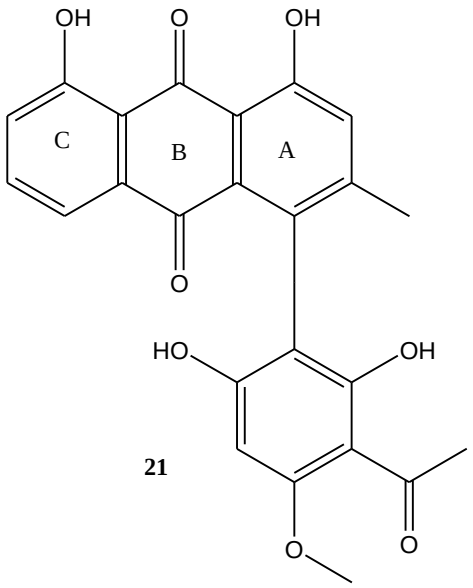
Compound **21** was isolated as yellow amorphous powdered with R_f value of 0.62.The ^{1}H NMR spectrum displayed the presence of three adjacent and mutually coupled aromatic protons at δ_H 7.20(*dd, J* = 7.8, 1.1 Hz, H-5), 7.56(t) and 7.77 (*dd*, J = 7.8, 1.1 Hz, H-2) suggesting ABX spin system for ring C. The presence of peak at δ12.98 (s, 1H, 1-OH) suggest that there is a peri hydroxyl group at C-8 position. The ^{1}H and ^{13}C NMR chemical shift pattern showing three aromatic protons having an ABX spin system, for ring C, coupled with a singlet at δ 7.25 (H-2) and a methyl peak δ 2.10 (C-3, δ_C 19.0), as expected biogenetically, are all in good agreement with the knipholoneanthrone derivatives. Signals of one chelated hydroxyl groups (C-1), a methyl group (C-3) as well as two isolated aromatic proton peaks (H-2 and H-5') coupled with three ABC aromatic protons peaks (H-5, 6, 7) indicate the chrysophanolanthrone part of the molecule is substituted at C-4 of ring A. The presence of a methoxy group at C-4' was evident from the peaks at δ_H3.1 (3H, s). The acetonyl group in acetylphloroglucinol moiety at C-3' is from a methyl at δ_H 2, 0 (s, 3H).

The ^{13}C NMR spectrum revealed a total of 26 peaks in good agreement with the knipholone skeleton. Of these, the two carbonyls are evident at δ192.96 (C-9) and 182.79 (C-10). The difference of more than 5ppm, between the two peaks suggests that one of the carbonyl (C-9) is peri to the hydroxyl group (C-8).

The proposed structure is in agreement with literature, the chemical shift value of methoxyl group (C-4') is up field, in those knipholone derivatives having an acetonyl group at C-3' with value of δ_H 3.35 and δc 59.6 which might be due to anisotropy effect of the acetonyl moiety at C-3'. Moreover, the upfeild chemical shift of methyl at C-3' appeared at δ_C31.3 in agreement with literature (37,38).Thus, the above spectral data suggest that the difference between compound **21** and that of knipholone is the absence of methoxy group at C-6' of the acetylphloroglucinol moiety. Consequently, based on the above spectral data the compound was proposed as shown below in (Table 6).

Table 6: NMR data of compound 21 (^{1}H NMR 400MHz, ^{13}C NMR 100 MHz, $CDCl_3$) (δ in ppm)

Position	**. Compound 21**			**6,8-O-dimethylknipholone (**Abdissa **et al., 2014**) (45)	
	^{1}H NMR	**^{13}C NMR**	**DEPT-135**	**^{1}H NMR**	**^{13}C NMR**
1	-	131.6	-	-	162.3
1**a**	-	117.48	-	-	116.2
2	7.79(s, 1H,H-7)	125.03	-	7.25(s,1H, H-2	125.3
3	-	147.77	-	-	149.4
3-CH_3	1.4 (s,3H,3-CH_3)	21.17	-	2.10(s,3H,3-CH3)	20.7
4	-	127.55	-	-	126.6
4a	-	130.34	-	-	130.9
5a	-	138.72	-	-	136.9
5	7.20 (dd,1H,*H-5*)	124.77	-	7.74 (dd,1H, H-5)	120
6	7.56(t,3H, H-6)	135.80	-	7.64(t,1H, H-6)	135.3
7	7.77(dd,1H,H-2)	118.95	-	7.29(dd,1H,H-2)	117.2
8	12.98(s,1H, 1-OH)	162.7	-	12.07(s,1H,1-OH)	160.2
8a	-	121.38	-	-	120.6
9	-	192.96	-		189
10	-	182.79	-	-	183.5
1’	-	108.5	-		109.3
2’	-	163.31	-	-	162.4
3’	-	104.0	-		106.2
3’C=O	-	203.33	-	-	203.5
4’	-	157.83	-	-	162.9
4’O**$\underline{CH_3}$**	3.19 (s,3H, 4-OCH_3)	53.16			55.3
5’	-	114		6.14 (s,1H, H-5’)	86.2
6’	-	162.5	-	-	162.6



21

Figure 3

4.2.3 Characterization of compound 20

Compound **20** was obtained as colorless precipitate with R_f value of 0.35 in ethyl acetate:*n*-haxane (1:1). The ^{1}H NMR spectrum ($CDCl_3$,400MHz) revealed a deshielded singlet proton at δ 14.10 (1H,*s*) due to the proton of the hydroxyl group at C-9 and two singlet aromatic protons at δ 6.90(1H, s, H-10) and δ 6.95 (1H, *s*, H-5). It also showed that at δ 4.25 (oxygenated methane at C-3), a singlet at δ 2.51(methyl group at C-8) and another singlet at δ3.85 (methyl ester and peaks at δ 2.70 (1H, *dd*, H-2), δ 2.80 (1H, *dd*, H-2); and δ 3.15 (1H *dd*, H-4), δ 2.88 (1H, *dd*, H-4) indicate the protons of methylenes.

The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) showed a total of sixteen different carbons; oxygenated quaternary carbons at δ 156.2(C-6) two carbonyl carbons at δ 204.1(C-1) and methyl at δ 21.1, two methylenes at 38.1 (C-4) and 46.9 (C-2). Moreover, the DEPT-135 spectrum displayed five upward peaks at δ 21.1, 52.6, 64.9 and 117.1 and two downward peaks at δ 38.1 and δ 46.9 attributed to two methylenes. Based on spectroscopic evidence compound **20** in good agreement with compound reported in literature as 3,6,9-trimethyl-1oxo-5,6,7,8-tetrahydroanthracene-carboxyic acid methyl ester known by trivial name aloesaponol I (figure 3) where the only difference observed between compound **20** and that of Aloesaponol is the acetyl

group at C-7 position is in reduced form (-CH_3). Aloesaponol was previously reported from *Aloe saponaria*, *Aloe turkanersis* and *Aloe secondiflora* and *Aloe Elegan* (45, 37, 38).

Table 7. NMR data of compound 20 (^{1}H NMR 400MHz, ^{13}C NMR 100 MHz, $CDCl_3$)

Position	Compound **20**			Literature data (44)	
	δ_H	δ_C	DEPT-135	δ_H	δ_C
1		202.4			203.7
2	2.70 (*dd* 5.5 Hz) 2.80 (*dd*,17.3Hz)	46.9		2.70 (*dd*, 5.4 Hz) 2.96 (*dd*,15.6)	46.6
3	4.25 (*m*)	64.9	CH (64.9)	4.24 (*m*)	64.4
4	2.88 (*dd*, 15.4 Hz) 3.15 (*dd*, 15.5Hz)	38.1	CH_2(38.1)	2.90 (*dd*, 15.6 Hz) 3.14 (*dd*, 15.8 Hz)	37.5
5	6.95 (*s)*	117.1	CH (117.1)	6.95(*s*)	116.6
6		156.2			155.1
7		141.4			140.8
8		137.7			137.2
9		166.5			165.9
10	6.90 (*s*)	108.1	CH (108.1)	6.92(*s*)	107.5
11		126.1			125.4
12		115.8			
13		137.1			136.6
14		110.8			110.2
8-CH_3	2.51(*s*)	21.1	CH_3 (21.1)	2.70 (*s*)	20.8
9-OH	14.10(*s*)			15.27(*s*)	
7-CH_3	2.2 9S)	19	CH_3 (19)	-	-

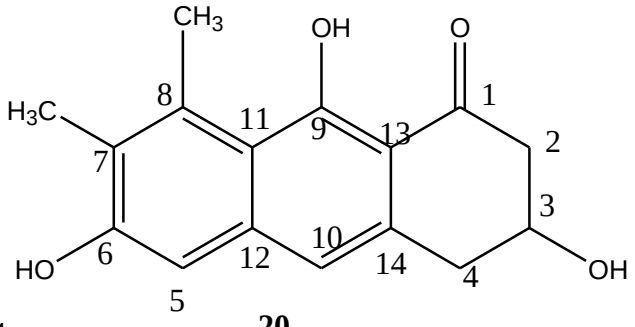


Figure 4 **20**

4. 3. Antibacterial Activity test

The two crude extracts (dichloromethane/methanol (1:1) ratio and 100% methanol) and four isolated compounds (compound 19, ksk-18 and compound 21) were evaluated for their *in vitro* antibacterial activity using disk diffusion method which showed varying degrees of responses against the bacterial strains(Table 8),. The two crude extracts showed low activity on Gram-positive (*S .aurous*) with zone of inhibition of (7 and 8 mm) table 8. Both Grams negative and gram positive bacterial strains were not showed activity with zone of inhibition 6 mm with no different activity was observed between the two crude extracts. The isolated compounds also showed a little activity in the *E. coli* ksk-18 with zone inhibition of 7mm and compound 20 showed better activity than the other on *S.aurouse* with zone of inhibition of 11mm. of the four isolated compounds two of them (compound 21 and compound 7) were didn't show any antibacterial activities against both gram positive and gram negative bacterial strains.

Table 8. Diameter of zone of growth inhibition (in mm) of crude extracts and isolated compounds from the roots of *K.schimperiana.*

St. no	**Sample code**	***S. aurous* in (mm)**	***P.aeroginosa* in (mm)**	***E.coli* in (mm)**	***K.pneumonia* in (mm)**
1	KSC-DM:ME	8	6	6	6
2	KSC-ME	7	6	6	6
3	compound 19	6	6	6	6
4	compound 21	6	6	6	6
5	KSK-18	6	6	7	6
6	Compound 20	11	6	6	6
7	Ceftraxone (PC)	20	22	15	17
8	DMSO(NC)	6	6	6	6

ME=methanol extract, PC=positive control, NC=negative control, DMSO=dimethyl sulfoxide

5. Conclusion and Recommendation

The work attempted to analyze the chemical constituents of medium polar and polar extracts of root of *Kniphofia schimperiana* which is endemic to Ethiopia. Phytochemical screening tests of the crude CH_2Cl_2/CH_3OH (1:1) and CH_3OH (100%) root extract of *Kniphofia schimperiana* revealed that the presence of glycosides, saponins, sterols, tannins, phenols and flavonoids. Silica gel column chromatographic separation of CH_2Cl_2/CH_3OH (1:1) root extract of *kniphofia schimperiana* yielded compounds **19** and **20** whereas CH_3OH (100%) root extract of *Kniphofia schimperiana* yielded compound **21**. In agreement with the previous study, the wide traditional use of the plant may be attributed to its rich anthraquinones and phenolic compounds constituents. Thus, further work is recommended on this endemic plant so as to validate the traditional use and identify more bioactive secondary metabolites in support of its traditional use.

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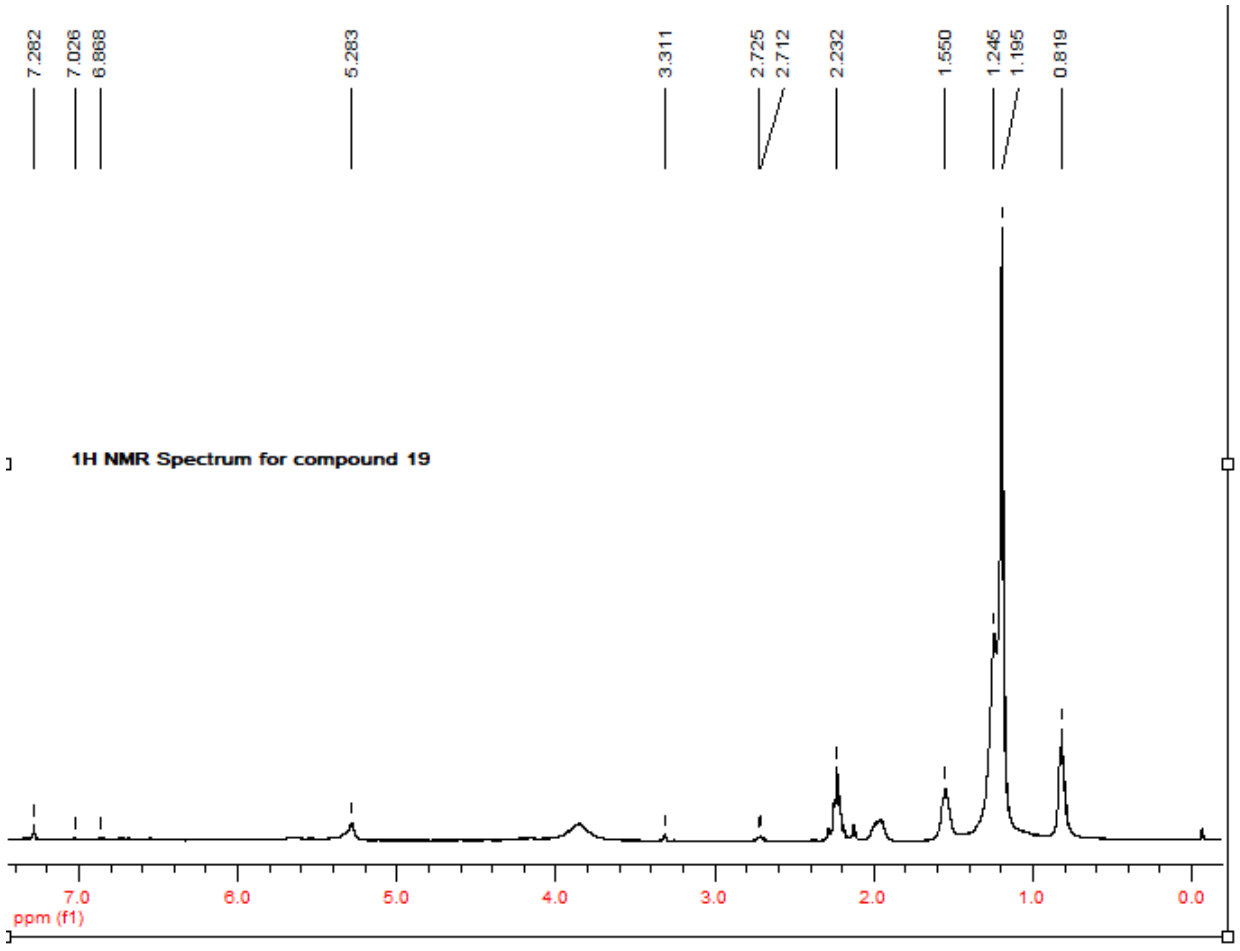
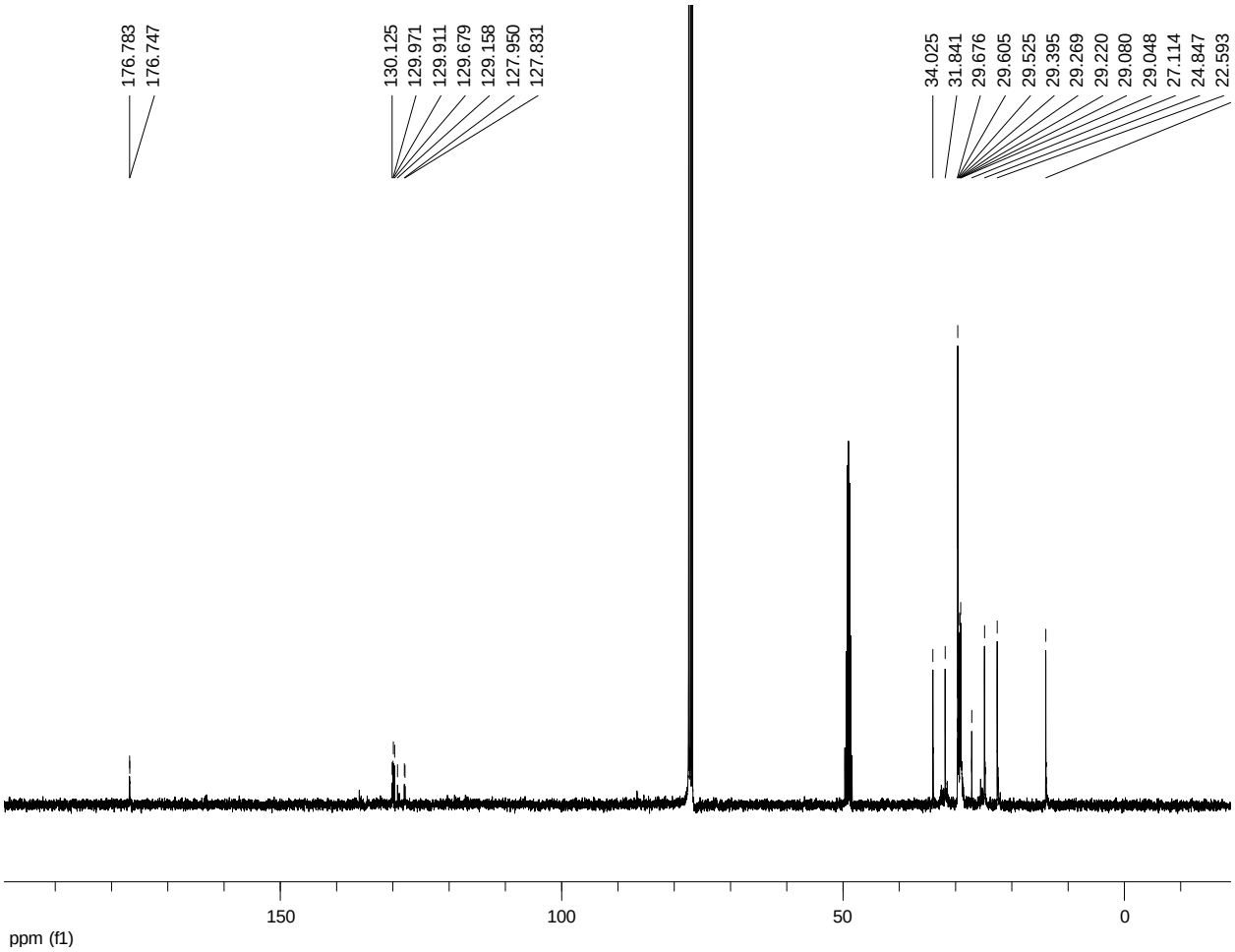
7. ANNEX

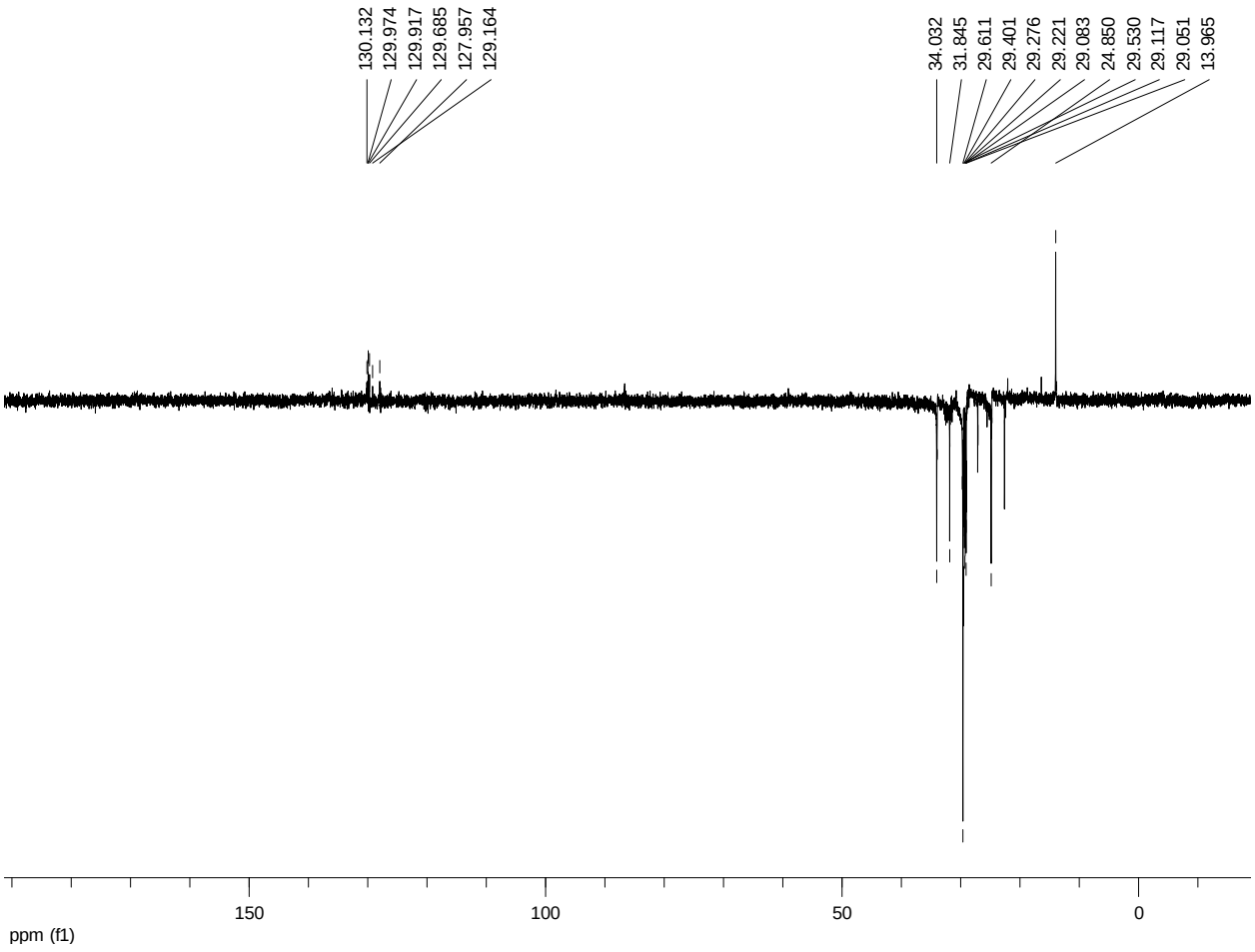
[1]HNMR spectrum for compound 19



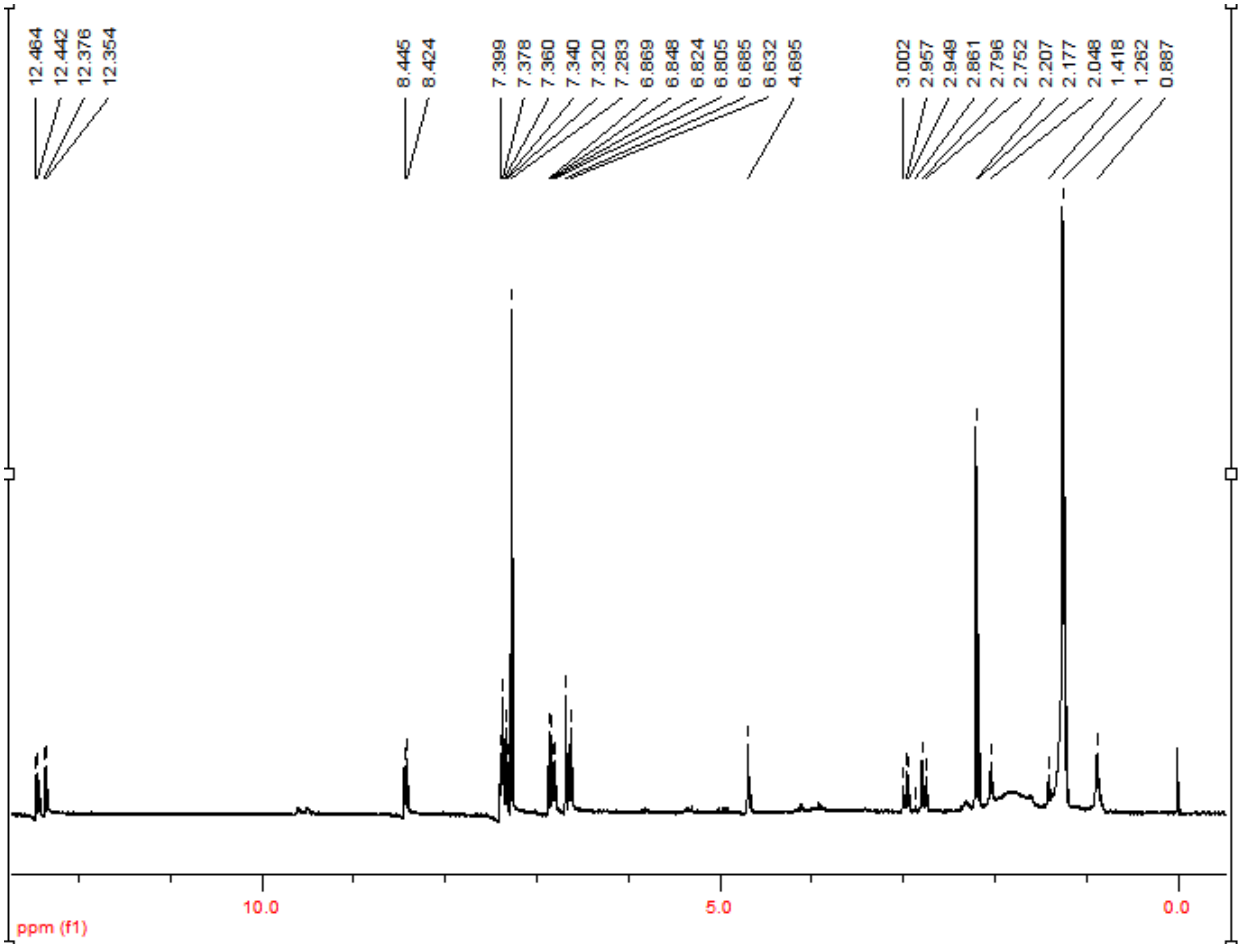
^{13}C NMR spectrum for compound 19



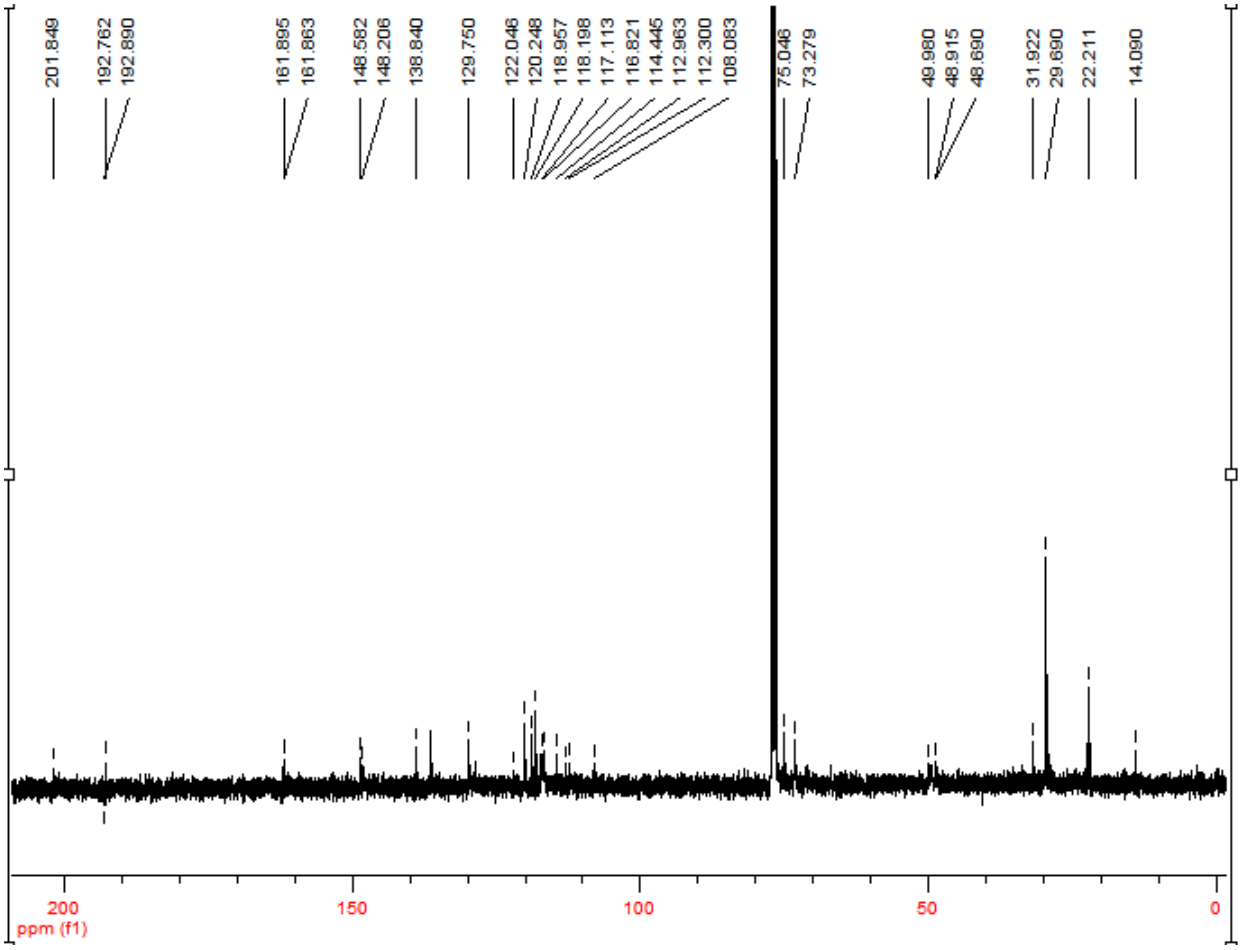
DEPT-135 spectrum for compound **19**



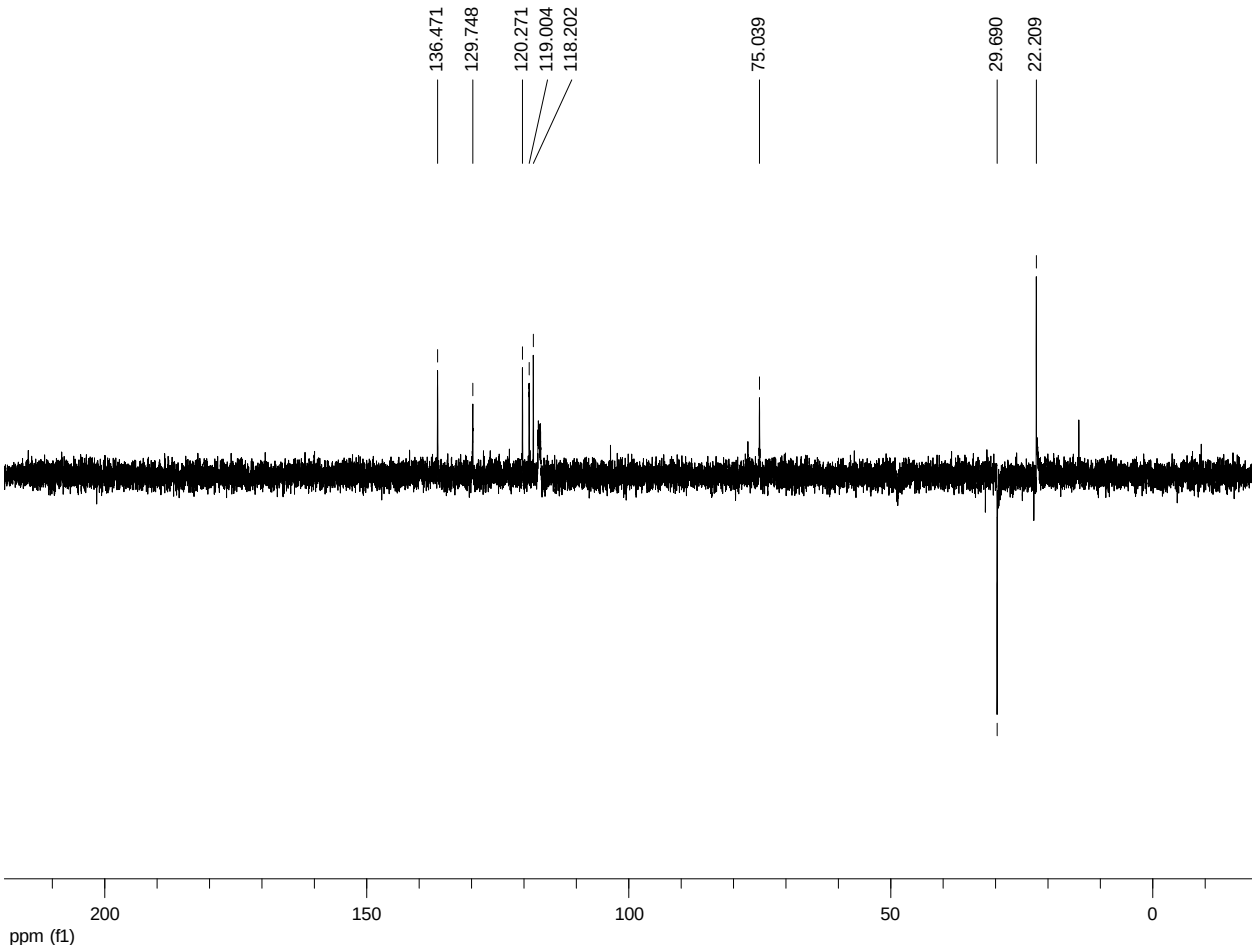
^{1}H NMR spectrum for compound **20**



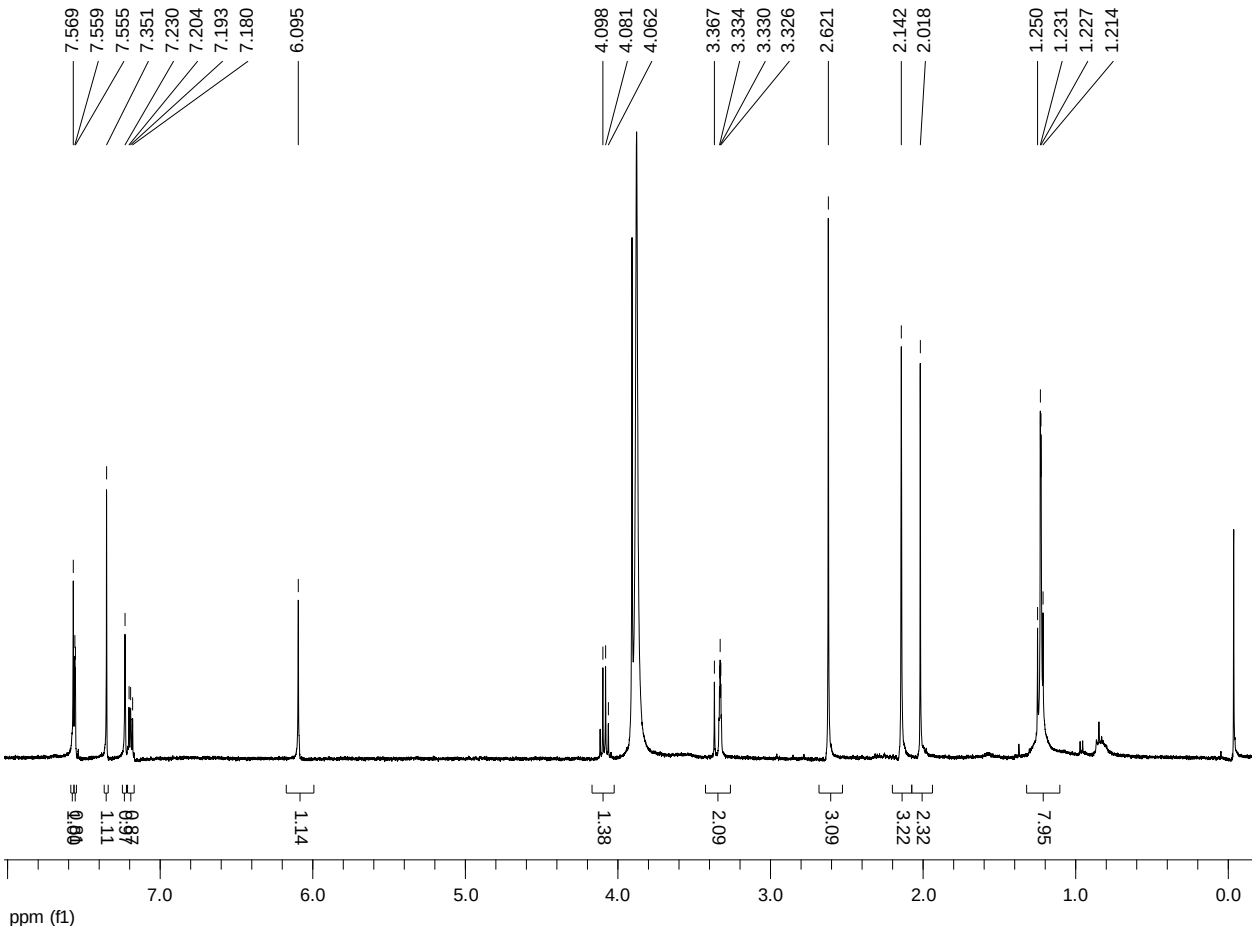
^{13}C NMR spectrum for compound **20**



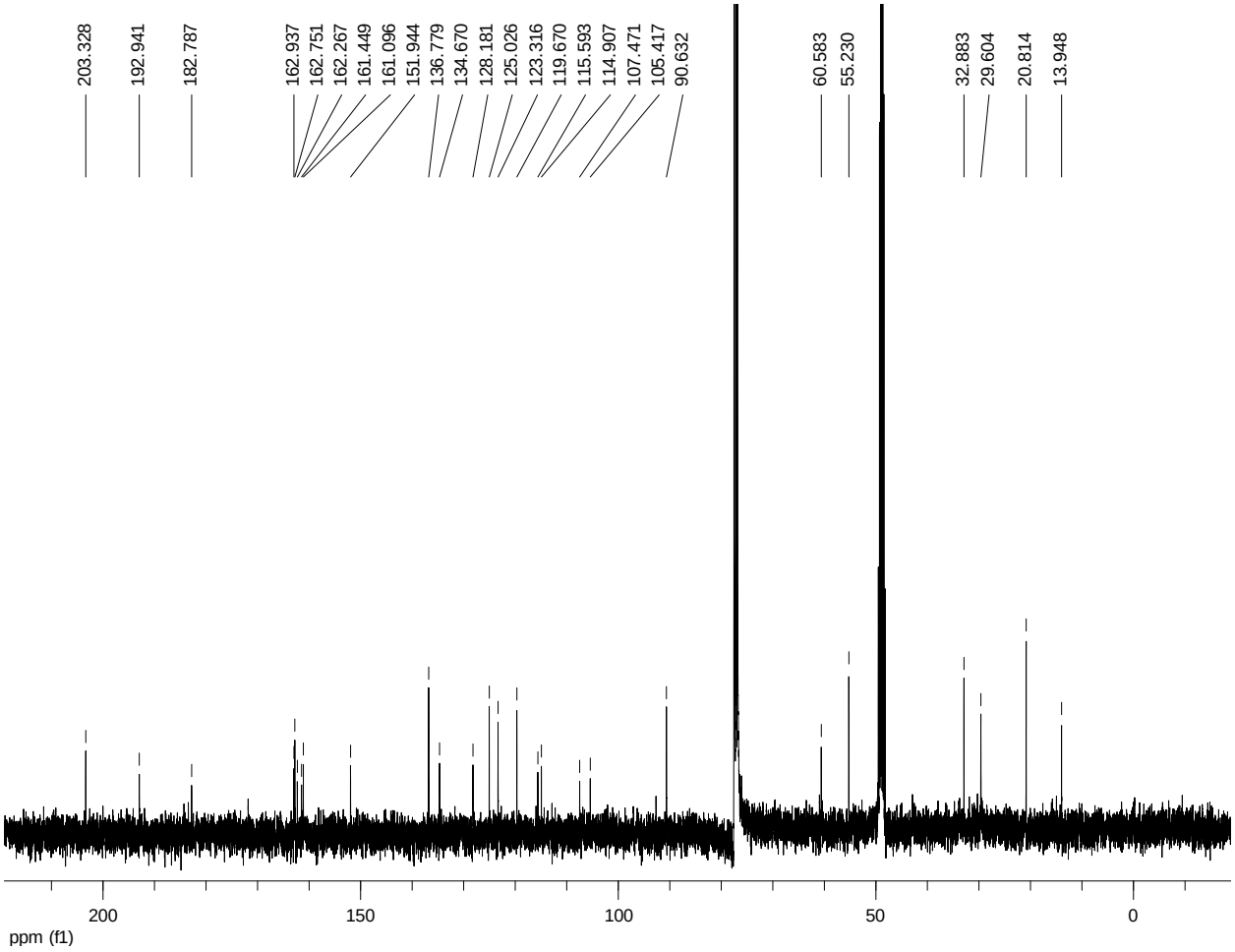
DEPT-135 spectrum for compound **20**



^{1}H NMR spectrum for compound **21**



^{13}C NMR spectrum for compound **21**



DEPT-135 spectrum for compound **21**