ISOLATION, CHARACTERIZATION, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF THE ROOTS EXTRACT OF *CYPHOSTEMMA NIVEUM*

BY

BELAY GEMECHU GUDU

A THESIS SUBMITTED TO

DEPARTMENT OF CHEMISTRY

SCHOOL O F APPLIED NATURAL SCIENCE.

PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MSC IN APPLIED CHEMISTRY

OFFICE OF GRADUATE STUDIES

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

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ADVISOR:- YADESA MELAKU (PhD)

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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: Belay Gemechu Gudu

Signature: _____

This MSC thesis has been submitted for examination with my approval as thesis advisor.

Name: Yadessa Melaku (PhD)

Signature: _____

Date of submission _____

SUPERVISORS' APPROVAL SHEET

To: Chemistry department

Subject: Thesis Submission

This is to certify that the thesis entitled "Isolation, characterization, antibacterial and antioxidant activities of the roots extract of *Cyphostemma niveum"* submitted in partial fulfillment of the requirements for the degree of Master's in applied chemistry, the graduate program of the department of chemistry, and has been carried out by Belay Gemechu Gudu Id. No GSS/0210/05, under my supervision. Therefore, I recommend that the student has fulfilled the requirements and hence hereby he can submit the thesis to the department.

Yadessa Melaku (PhD)	_____	_____
Name of major Advisor	Signature	Date
Milkyas Endale (PhD)	_____	_____
Name of co-Advisor	Signature	Date

APPROVAL SHEET

This is to certify that the thesis prepared by Belay Gemechu Gudu entitled 'isolation, characterization, antibacterial and antioxidant activities of the roots extract of *Cyphostemma niveum*' and submitted in fulfillment of the requirement for Master of Science in Chemistry complies with the regulation of the university and meets the accepted standards with respect to originality and quality.

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ABBREVIATIONS AND ACRONYMS

AA	Antioxidant activity
DPP	2, 2-diphenyl -1-picryl hydrazyl
IC50	half-maximal inhibitory concentration
IR	Infrared
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
NMR	nuclear magnetic resonance
TLC	thin layer chromatography
UV	Ultraviolet
UV-Vis	Ultraviolet visible
WHO	World health organization

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ABSTRACT

Cyphostemma niveum (*Vitaceae*) is *a plant traditionally used for the treatment of various ailments including wound healing and killing pest. The air dried roots powder (300g) of Cyphostemma niveum were extracted successively with hexane, ethyl acetate and methanol to furnish 1g (0.33%), 5.1g (1.7%) and 20g (6.7%) respectively. The EtOAc extract after silica gel column chromatography has led to the isolation of two compounds coded as CN02 and CN06. The structure elucidations of these compounds were undertaken using spectroscopic techniques (UV, IR and NMR). The methanol, EtOAc extract and isolated compounds were assessed for their antibacterial activity against S. aureus, E. coil, P. mirabilis and K. pneumonia. Results showed that CN02 displayed activity against all bacterial pathogens tested in this study while the methanol extract was found active only against S. aureus. Furthermore, the antioxidant activities of the extracts were evaluated using DPPH. The MeOH extract displayed modest DPPH radical scavenging activity compared with ascorbic acid*.

Key words*: Cyphostemma niveum, Antibacterial, Antioxidant, β-sitosterol.*

1.INTRODUCTION

1.1. Background

The wide spread use of traditional medicine could be attributed to cultural acceptability, economic affordability and efficacy against certain type of diseases as compared to modern medicines. Thus, different local communities in countries across the world have indigenous experience in various medicinal plants where they use their perceptions and experience to categorize plants and plant parts to be used when dealing with different ailments [1]. Plants have played a central part in combating many ailments in human and livestock in many indigenous communities including Africa [2]. According to World Health Organization more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Traditional healers, particularly medicinal plant herbalists have a detailed knowledge-base of traditional medicine in Africa. But, it is transferred orally from one generation to the next through professional healers, knowledgeable elders and/or ordinary people [3].

Similarly in Ethiopia, traditional medicine has played a significant role in treating both livestock and humans health problems [4]. The plant-based health care persists and remains as the main alternative treatment for different ailments in the country. This is largely due to shortage of pharmaceutical products, prohibitive distance of the health service stations, unaffordable prices by small holder farmers and pastoralists for conventional drugs, emergence and reemergence of certain diseases and appearance of drug resistant microbes and/or helminthes [5]. In medicinal chemistry, the chemist attempts to design and synthesize a drug or a pharmaceutical agent, which will benefit humanity [6]. In recent times, focus on plant research has increased all over the world, and a large body of evidence has been collected to show the immense potential of medicinal plants used in traditional systems [7]. The term medicinal plants include a various types of plants used in herbalism and some of these plants have a medicinal activities. These medicinal plants are considered as rich resources of ingredients which can be used in drug development and synthesis. Besides that these plants play a critical role in the development of human cultures around the whole world [8, 9].

Since time immemorial, medicinal plants have been used to prevent and treat various health problems. Plants are still an indispensable source of medicaments in the contemporary

health care delivery system. Globally, over 100 pure chemical substances extracted from higher plants are used in the modern medicine and most of these compounds have the same or related medicinal use with the original traditional therapeutic claims. Traditionally medicinal plants also serve as a source of starting material for the development of more complex synthetic compounds. The majority of the population in the developing countries still relies on herbal preparations to help enhance health. This is also true of Ethiopia where the majority of the population depends on plants for their healing action in various health problems. Among the 7000 higher plant species that are known exist in Ethiopia, about 800 of them are employed in the traditional health care delivery system to prevent and treat nearly 300 physical and mental disorders [10].

Since 1978, there have been some ethnobotanical studies conducted in different parts of the country to document medicinally important plants. Compared with the huge knowledge of medicinal plants available in the country, very little information is so far collected and documented [11]. Furthermore, little work is so far undertaken to identify the phytochemicals present in these medicinal plants [12]. *Cyphostemma niveum* is among medicinally significant plant in Ethiopia traditionally used for the treatments of wound. Despite the use of this plant by the traditional people, little has been done on the phytochemistry and biological activity of its roots.

1.2. Justification of the Study

Cyphostemma niveum is a plant used in Ethiopia against wound healing and killing pest. Scientific literature regarding the isolation of chemical constituents, antibacterial and antioxidant activities of the roots of this plant is scarce. Therefore, this research attempts to isolate and characterize secondary metabolites from the roots extracts of Cyphostemma niveum. Furthermore, the antibacterial and antioxidant activities of the extracts of the roots were also studied herein.

1.3. Statement of the Problem

Antibacterial resistance has become a global problem. It is therefore necessary to find new antibacterial compound which can effectively inhibit bacteria. One of the major strategies to improve the current situation includes research in finding new and innovative antibacterial agents from natural sources. This is supported by a report that a substantial number of new

antibiotics introduced on the market are obtained from natural or semi-synthetic resources. In developing countries like Ethiopia, a large proportion of the population utilizes medicinal plants for the treatment of infectious diseases. Among these medicinal plants, *Cyphostemma niveum* is used against wound healing and killing pest. Therefore, this research attempts to explore the chemical constituents and antibacterial activities of the roots extracts of *Cyphostemma niveum.* In view of the incidence of cancer as a result of the use synthetic antioxidants in food additives, it is necessary to find out antioxidant compounds from natural sources. Hence the roots extracts of *C. niveum* was also examined for the presence of antioxidant compounds.

1.4. Significance of The Study

The study serves as a springboard for researchers who were interested to conduct further studies in related areas. The study on the root of *Cyphostemma niveum* may help to arrive at new natural products. The extracts and compounds to be obtained from the plant may exhibit pronounceable antibacterial activities. Furthermore the biological activities displayed may validate the traditional uses of this plant.

1.5. Objectives

1.5.1, General Objective

To investigate the chemical constituents, antibacterial and antioxidant activities of the roots extracts of *Cyphostemma niveum*.

1.5.2, Specific Objectives

The specific objectives of the study are to:

1. successively extract the roots of *C. niveum* using hexane, ethyl acetate and methanol
2. isolate compounds from extracts of the roots of *C. niveum* using chromatographic techniques
3. Characterize the isolated compounds using spectroscopic methods UV, IR and NMR data.
4. evaluate the antibacterial activities of the extract and constituents of the root of *C. niveum*
5. assess the radical scavenging activities of the extract and isolated compounds of the roots extracts of *C. niveum*

2. LITERATURE REVIEW

2.1. The Family Vitaceae

Vitaceae (the grape family) dicotyledonous flowering plant consists of about 14 genera and 950 species primarily distributed in tropical and subtropical regions. Vitaceae is the grape family of flowering plants, in buckthorn order (Rhamnales), 14 genera of wood plants, most of them tendril-bearing vines. However, fruit of some genera of Vitaceae grown as an ornamental contains oxalate which is poisonous to mammals [13]

2.2. Genus *Cyphostemma*

The genus *Cyphostemma* belongs to the family of vitacea which consists of a wide range of creeping plants with broadly ovate leaves and stem. They have greenish tinge and are produced as berries of varying sizes. They are originated from Africa and Madagascar, even though various species now occur in different parts of the world. While *Cyphostemma quandragularis* is found in India, *Cyphostemma adnata* and *Cyphostemma pallida* are found in china. *Cyphostemma glaucophilla* and *Cyphostemma populnea* are found in parts of Nigeria in West Africa. These species serve many medicinal purposes [14].

The genus *Cyphostemma* includes around 300 species from the World, of which only a few are succulent, taking the form of caudiciform shrubs, trees and scrambling lianas. They are native to Southern Africa, Madagascar, and Arabia. The racemes of small flowers are followed by small poisonous berries with high tannin content and usually with a single relatively-large seed. The pachycaul species in this genus enhance any collection of succulent plants. Although large plants may withstand a touch of frost, they are best kept in frost-free conditions with a very free-draining potting mixture [13]. The genus *Cyphostemma* has tropical and subtropical plants which are chiefly woody vines of grape family Vitaceae and the genus was split according to some details of the flowers and the large caudiform species were moved to the genus *Cyphostemma*. These species include *C. bainesil, C. cirrhosum, C. Juhas, C. Crotalariode, C. elephantophus, C. haza and C. curorii*. *Cyphostemma* contains some of the most desirable and attractive of all caudiforms in their native habitat; these extra ordinary members of the Vitaceae are rare lived plants [15]

2.2.1.Traditional uses and Reported biological activities

Cyphostemma glaucophilla is a useful perennial herb locally used in the treatment of kwashiorkor and marasmus in children. Also, the ground leaf paste is used by local orthopaedics in setting of fractured and dislocated bones. The seed extract is also used as a local dye. Yorubas use it traditionally in treating female infertility, stomach upset and inflammation. The blended leaves are also used with honey in the treatment of cough. However, the leaves, flowers and stem use as an internal cleanser for new born by feeding the child with the water extract in Nigeria. The young shoots are also used as vegetables **[**14**]**.

There are many medicinal uses recorded for *Cyphostemma adenocaule*. The leaf sap is used to cure ophthalmia (DR Congo and Tanzania) and it is applied to cuts (Tanzania). Leaves are chewed to remedy a sore throat (Tanzania) and macerated leaves are mixed with honey as a treatment (Tanzania). Leaves heated over a fire are applied as a compress to reduce swelling (East Africa). Leaves are applied to the chest to cure pneumonia (East Africa) and an infusion of the leaves is taken as a purgative and to treat swollen abdomen. The root has been used to treat malaria .A paste made of the root is applied topically to draw abscesses and reduce swellings in northern Ghana, Gabon and East Africa .water in which roots have been boiled is drunk to treat syphilis, abdominal pain (related to pregnancy or no) and to prevent abortion. Root and leaf are prescribed against diarrhea with blood. Field tests were carried out Uganda with *Cyphostemma adenocaule* as a trap crop for *Taylorilygus vosseleri*, an insect pest of cotton. When treated with insecticide to prevent the development of large populations of the pest, *Cyphostemma adenocaule* considerable protection to the cotton crop. Leaves crushed in water are used as an insecticide against it and people cut the stems to obtain drinking water. In Tanzania a fiber is obtained from the bark and string is out of it. Dried stems are used for hut building in Uganda [13]

Another species belonging to this genus include *Cyphostemma glaucophilla.* It is a perennial herb with prostate branches, rooting from the node [16]. *C. glaucophilla* is a well-known plant to traditional medicine practitioners in Kogi and Kwara States of Nigeria. Different parts of the plants: the leaf, root, stem, bark, fruits are used in diverse ways for various purposes. The leaf is widely used by the Yorubas in treating infertility in women and stomach ailments in children also, the ground leaf paste is useful in the relief of pains,

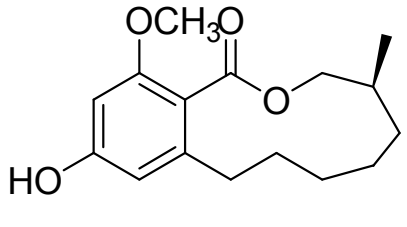
reduction of swelling and in accelerating the healing of fracture. The Ebiras of Kogi State, Nigeria use the stem and flowers with other plants to bath babies' immediately after delivery and also give the extract to new born babies to drink as internal cleansers. The concoction is also taken as cough remedy. The Ibajis in Igala speaking areas of Kogi State use the fruits as attractant to fish in hooks and bait [17].

It is an ancient medicinal house plant used in Indian folk medicine to heal fractures [18]. It is also used to treat gastrointestinal disorders and hemorrhoids [19, 20]. However, records that it has antioxidant, anti-microbial and anti-inflammatory actions, as well as the reduction of tissue infiltration by immune cells- a hallmark of tissue injury [21, 22]. *Cyphostemma quadrangularis* contains high concentrations of vitamin C and carotene as well as the phytochemical quercitin, an anti-inflammatory vasodilation flavonoid found in grapes, [23]. *Cyphostemma quadrangularis* contains beta sitosterol, a cholesterol based compound that also has anti-inflammatory as well as immune modulation effects (cortisol reducing effect) [24]. In the same vein, *Cyphostemma quadrangularis* has the ability to reduce glucocorticoid receptors expression [25]. *Cyphostemma quandragularis* is a medicinal plant of Indian origin. The use of this plant by the common folk for promoting fracture healing process is an old practice. It has been prescribed in ancient Ayurvedic test by Bhava Prakash and Chakra Dutta as a general tonic especially for the fractured bone. Since then it has been in extensive use by bone setters both for external application and as medicine to be taken with milk. The stem is also reported in Ayurveda as alternative anti-helminthic, dyspectic, digestive tonic and in the treatment of irregular menstruation and asthma. Scientific studies have also revealed that the leaf extract possesses cardio tonic and androgenic properties [26]

2.2.2. Reported Chemical Constituents

Studies carried out by Gupta and Verma showed that *Cyphostemma quandragularis* contains large quantities of vitamin C, carotene A, anabolic steroidal substances and calcium. This anabolic steroidal principle markedly influenced the rate of fracture healing by influencing early regeneration of all connective tissues of mesenchymal origin, namely the fibroblast, chondroblast and osteoblast involved in the healing and quicker mineralization of the callus. The stem extract causes less amount of tissue reaction in the fractured region leading to optimum decalcification in the early stage with minimum callus formation. Hence deposition of calcium is just enough to join the two broken segments of a

bone so that its remodeling phenomenon leads to early recovery of treated animals. It is also shown to cause early gain in the tensile strength of fractured bones of about ninety percent of its normal strength at the end of six weeks. The extract builds up the chemical composition of the fractured bone namely mucopolysaccharides, collagen, calcium, neutralized the anti-anabolic effect of steroids like cortisone in healing of fractures. This includes inhibition of tissue regeneration and repair, also retarding formation of the specific skeletal structures such that even if the cartilage is produced, its maturation and ultimate bone replacement do not take place in the normal pattern. Its main inhibitory action is on fibroblast and mast cells which produce mucopolysaccharides of connective tissues [27]. Some compounds isolated from the plant; the crude extract of *C. greveana* bark yielded the five compounds **1**–**5.** Compound **1** was identified as lasiodiplodin (1) by comparison of its spectroscopic data with those of the known compound. Compounds **2** and **3** were similarly identified as 12-hydroxy-15-oxo-selina-4,1l-diene (**2**) and 1β, 6α- dihydroxyeudesm-4(15)-ene (**3**) 16, 18-dihydroxykolavenic acid lactone (**4**), opposit-4(15)-ene-1β, 7-diol (**5**) respectively. [28]



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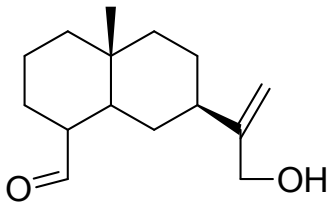

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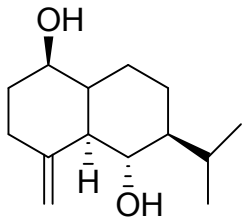

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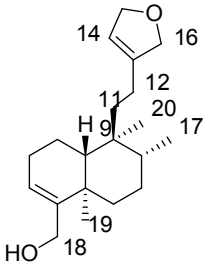

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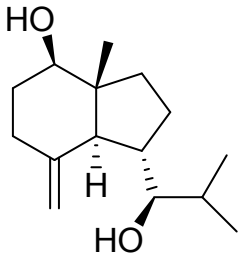

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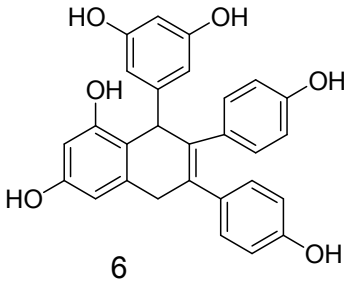
Compounds extracted from *Cyphostemma crotalarioides* identified as *cyphostemmin* A **(6),** *cyphostemmin* **B (7)**, resveratrol **(8),** parthenocissin A (**9)**, e-viniferin (**10)**, gnetin C (**11**), pallidol (**12)** , gnetin E (**13)** and ampelopsin D (**14) [**29**]**



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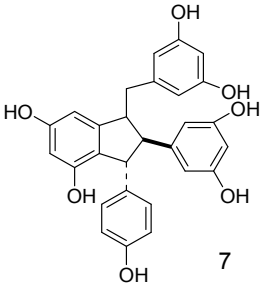

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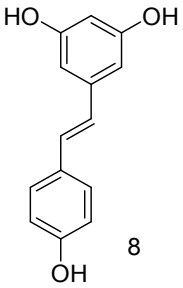

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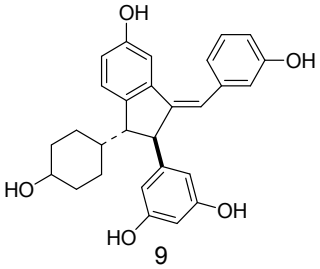

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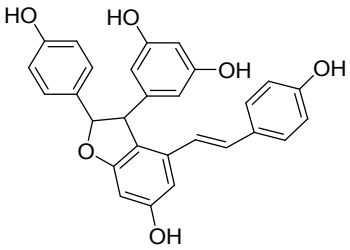

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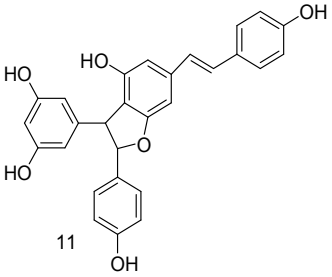

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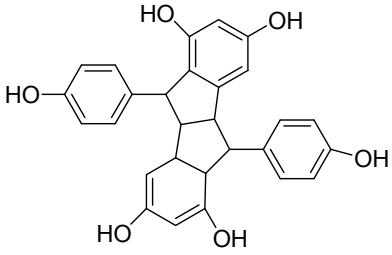

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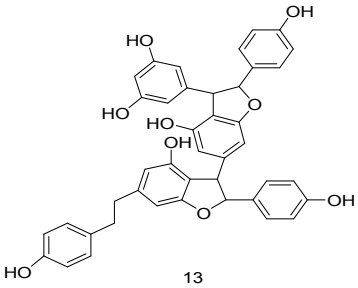

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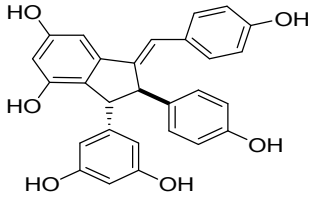

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3. MATERIALS AND METHODS

3.1. Chemicals

Chemicals used in the study include n-hexane (Ran chem PLC, Hind), ethyl acetate (EtOAc) (Carlo Erba, France), and methanol (MeOH) (Ranchem PLC, Hind), dichloromethane (Ranchem PLC, Hind). All these chemicals were of analytical grade and all of them were bought from Ran Chem General Trading PLC, Addis Ababa, Ethiopia, Silica gel, ammonia solution, HCl and Vanillin.

3.2. Instruments

Analytical thin layer chromatograms were run on a readymade 0.2mm thick layer of silica gel GF254 (Merck) coated on aluminium plate. Column chromatography was performed using silica gel (230-400 mesh) Merck. ^{1}H-, ^{13}C- and DEPT-135 NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz in $CDCl_3$. Infrared (IR) spectra were obtained on Perkin-Elmer 65FT IR ν_{max}KBr (4000-400) cm^{-1}) infrared spectrometer using KBr pellets.

3.3. Plant Material Collection and Identification

The root of *Cyphostemma niveum* were collected in March, 2017 from Leman town, Kersa Malima Woreda, South West Showa Zone, Oromia, Ethiopia. The plant material was authenticated by Professor Legesse Negash a botanist and voucher specimen (Voucher no-001) deposit in the National Herbarium of Addis Ababa University. The plant material was dried in the shade at the laboratory of Leman Preparatory School.

(a) (b) (c)

Figure1: Photo of *C. niveum* Leaves (a), Roots (b) and Roots Powder (c) (Picture taken by Belay Gemechu from Kersa Malima Woreda, South West Showa, Oromia).

3.4. Extraction and Isolation

Air dried ground roots (300 g) of *Cyphostemma niveum* was first soaked in n-hexane (1.5 L) for 72 h at room temperature, filtered and concentrated under reduced pressure at 40℃ using rotary evaporator to afford 1 g (0.33%) yellow crude extract. The marc was extracted with ethyl acetate (1.5 L) after soaking for 72 h at room temperature, filtered and concentrated using rotary evaporator to afford 5.1 g (1.7%) orange crude extract. Similar procedure as above was repeated this time with methanol to give 20g (6.67%) red orange crude.

a b c

Figure 2: TLC profile of the crude extract hexane (a), EtOAc (b) and MeOH (c) extract.

As shown in Figure 2 the EtOAc extract displayed well resolved spots and hence further analyzed for its chemical constituents using column chromatography

The EtOAc extract (5.1 g) was adsorbed and fractionated over silica gel (150 g) column chromatography using n-hexane: EtOAc: MeOH of increasing polarities as eluent to give 24 fractions each 100 mL. The whole procedure is depicted in Table 1

Table 1: Column chromatographic fractionation of the EtOAc extract

Eluent	Ratio	Fractions	Eluent	Ratio	Fractions
n-hexane	100%	F1	EtOAc/n-hexane	9:1	F13
n-hexane/EtOAc	9:1	F2	EtOAc	100%	F14
n-hexane/EtOAc	8:2	F3	EtOAc/Methanol	9:1	F15
n-hexane/EtOAc	7:3	F4	EtOAc/Methanol	8:2	F16
n-hexane/EtOAc	6:4	F5	EtOAc/Methanol	7:3	F17
EtOAc/n-hexane	5:5	F6	EtOAc/Methanol	6:4	F18
EtOAc/n-hexane	6:4	F7	EtOAc/Methanol	5:5	F19
EtOAc/n-hexane	6:4	F8	Methanol/EtOAc	6:4	F20
EtOAc/n-hexane	7:3	F9	Methanol/EtOAc	7:3	F21
EtOAc/ n-hexane	7:3	F10	Methanol/EtOAc	8:2	F22
EtOAc/n-hexane	8:2	F11	Methanol/EtOAc	9:1	F23
EtOAc/ n-hexane	8:2	F12	Methanol	100%	F24

Fraction 10 (90 mg) showed one spot on TLC developed using EtOAc/n-hexane (7:3) as a mobile phase and was labeled as CN02 and TLC showed one spot at Figure3.

Figure 3: TLC profile of compound CN02

Therefore, fraction F5, F6 and F7 are combined for further analyzed for its chemical constituents using column chromatography.

Figure 4: TLC profile of fraction F5, F6 and F7

As shown in Figure 4 the fraction F5, F6 and F7extract displayed well resolved spots and hence further analyzed for its chemical constituents.

Fractions $F_{5,}$ F6 and F_7 were combined and re-chromatographed over silica gel column chromatography using n-hexane: EtOAc of increasing polarities to furnish ten (10) fractions each 10mL. The whole procedure is depicted in Table 2.

Table2: Column chromatographic fractionation of combined F5, F6 and F7.

Eluent	Ratio	Fractions	Eluent	Ratio	Fractions
n-hexane	100%	Ff1	n-hexane/EtOAc	5:5	Ff6
n-hexane/EtOAc	9:1	Ff2	EtOAc/n-hexane	6:4	Ff7
n-hexane/EtOAc	8:2	Ff3	EtOAc/n-hexane	7:3	Ff8
n-hexane/EtOAc	7:3	Ff4	EtOAc/n-hexane	8:2	Ff9
n-hexane/EtOAc	6:4	Ff5	EtOAc/n-hexane	9:1	Ff10

Fraction Ff7 was found to have one spot on TLC and labeled as CN06 (30mg).

3.5. Structural Elucidation of Isolated Compounds

Structure elucidations of the isolated compounds were achieved using NMR (^{1}H-NMR, ^{13}CNMR, DEPT-135), UV-Vis and IR.

3.6. Studying Antioxidant Activities

The antioxidant activity of each extracts was carried out using DPPH. Antioxidant activity of different concentration of both methanol and ethyl acetate extracts were prepared in methanol 500, 250, 125 and 62.5µg/mL.

DPPH radical-scavenging activity was measured according to the method of

0.04% DPPH solution (4 mL) in methanol was added to four different vials each containing 1 mL of 500, 250,125 and 62.5 µg/mL of the methanol extract to furnish 100, 50, 25 and 12 µg/mL. The mixture was shaken vigorously and incubated at 37^{o}C for 30 min. The absorbance of the resultant solution was measured at 517 nm. Similar procedure was repeated for the EtOAc extract. The antioxidant activity was then calculated using the formula: $AA = (A_o-A_c)/A_0X100$, Where A_o = absorbance without extract A_c = absorbance with extract) [30]

3.7. Antibacterial Activities

The antibacterial activity of the MeOH extract and the two compounds isolated from ethyl acetate crude extract were tested against a gram positive bacteria (*Staphylococcus aureus*), a gram negative bacteria (*Escherichia coli, Proteus mirabilis*, *Klebsola pneumonia).* The bacteria strains were all obtained from the Oromia public health research capacity building and Quality assurance laboratory center at Adama. The agar-well diffusion methods were used to determine antibacterial activity. The agar was melted (50^{o}C) and the microorganism cultures were then added aseptically to the agar medium at 45^{o}C in plates. 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 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 and the solvent methanol as negative control. Bacterial inhibition zone >6mm was said to be sensitive (S), but >6mm was taken as resistance (R) against the tested chemical substance [31].

4. RESULTS AND DISCUSSION

4.1. Extraction yield

The roots of *Cyphostemma niveum* was successively extracted with n-hexane, ethyl acetate and methanol to afford 1 g (0.33%), 5.1 g (1.7%) and 20 g (6.67%) respectively. The dry extracts were weighed and the extraction yields (W/W) were calculated as:

$$\text{Percentage yield} = \frac{\text{weight of dry extract}}{\text{Weight of dry material}} \times 100\%$$

As seen above, the secondary metabolites were extracted better in methanol. This indicates that the root of *C. niveum* contains mainly polar compounds.

4.2. TLC Profile of the Extracts of the Roots of *C. niveum*

The n-hexane, EtOAc and Me OH extracts of the roots of *C.niveum* were analyzed with TLC and the profile is depicted in Figure 5.

(a) (b)

Figure 5: TLC profile of the EtOAc (a) and MeOH (b) extract.

As shown in Figure 5 the EtOAc extract displayed well resolved spots and hence further analyzed for its chemical constituents using column chromatography

4.3. Characterization of Isolated Compounds

In the course of this work two compounds, coded as CN02 and CN06, were isolated. The structure elucidations of these compounds were performed using NMR, IR and UV. The characterizations of these compounds were described as follows.

4.3.1. Characterization of CN06

CN06 was obtained as a white crystalline solid melting at 136-138^{o}C. The TLC showed spot at RF value 0.56 with EtOAc: n-hexane (6:4) as a mobile phase.

Figure 6:- TLC profile of compound CN06

The UV-Vis spectrum (EtOH) showed absorption maxima at 224 nm indicating the absence of conjugation in the structure of the compound. The IR spectrum showed absorption bands at δ 3480 cm^{-1} which is diagnostics for the presence of hydroxyl stretching. The band at δ 2906.9 cm^{-1} and 1626.7 cm^{-1} are evident for the presence of alkyl and carbon-carbon double bonds, respectively.

The ^{1}H-NMR spectrum ($CDCl_3$) of CN06 revealed the presence of six methyls at δ 0.7, 1.03, 0.95, 0.84, 0.86 and 0.88 (H-18, H-19, H-21, H-26, H-27 and H-29) respectively. The signal observed at δ 5.36 (1H, *t*, *J* = 3.2 Hz, H-6) is characteristics of olefinic proton in the structure of the compound. This clearly shows the presence of only one olefinic proton.

Also observed signal is at δ 3.52 (1H, *m*, *J*= 5.2 Hz, H-3) due to the presence of proton on oxygenated carbon and the doublet signal observed at δ 2.28 (1H, *d*, *J*= 2.4 Hz, H-4) .The ^{1}H-NMRspectrum also displayed other signals integrating for 30 hydrogens in the region between δ 1.45 to δ 1.67 (Table 3).

The proton decoupled ^{13}C-NMR and DEPT-135 spectra ($CDCl_3$) of CN06 (Table 3) revealed the presence of 29 well resolved carbon signals including six methyl, eleven methylene, nine methine, and three quaternary carbons. This is diagnostics for the presence

of sterol nucleus. The quaternary carbon signal observed at δ 140.7 (C-5) is due to olefinic carbon. The other olefinic carbon signal is evident at δ 121.4 (C-6). This clearly indicates that CN06 contains only one olefinic double bond. The carbon signal at δ 71.8 is assignable to C-3. The other carbon resonances were depicted in Table3. The data generated for characterization of CN06 agreed well β-sitosterol.

Table 3: ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C-NMR spectral data of CN06 and comparison with literature reported for β-sitosterol [32].

Carbon No	^{13}CNMR spectral data of CN06	^{13}C-NMR data of β -sitosterol [32]	^{1}H-NMR Spectral data of CN06	^{1}H-NMR data β-sitosterol [32]	DEPT-135
C-1	39.9	37.3	1.46	1.47	CH_2
C-2	29.4	31.9	1.56	1.56	CH_2
C-3	71.8	71.8	3.52	3.53	CH
C-4	42.3	42.4	2.28	2.28	CH_2
C-5	140.7	140.8	-	-	Cq
C-6	121.7	121.7	5.36	5.38	CH
C-7	31.6	31.7	2.2	2.03	CH_2
C-8	31.9	32.0	1.67	1.67	CH
C-9	50.1	50.2	1.45	1.48	CH
C-10	36.5	36.2	-	-	Cq
C-11	21.1	21.1	1.54	1.52	CH_2
C-12	37.3	39.8	1.51	1.49	CH_2
C-13	42.3	42.4	-	-	Cq
C-14	56.1	56.8	1.45	1.50	CH
C-15	24.3	24.3	1.56	1.60	CH_2
C-16	28.3	28.3	1.85	1.84	CH_2
C-17	56.8	56.1	1.46	1.49	CH
C-18	11.9	11.9	0.7	0.68	CH_3
C-19	19.82	19.41	1.03	1.02	CH_3
C-20	36.15	36.54	1.64	1.64	CH
C-21	18.78	19.07	0.95	0.94	CH_3
C-22	33.95	34.00	0.88	0.88	CH_2
C-23	26.08	26.16	1.1	1.04	CH_2
C-24	45.84	45.89	1.46	1.50	CH
C-25	29.70	29.23	1.66	1.65	CH
C-26	19.40	19.83	0.84	0.83	CH_3
C-27	19.04	18.81	0.86	0.85	CH_3
C-28	23.07	23.12	1.13	1.04	CH_2
C-29	11.88	12.01	0.88	0.8	CH_3

The ^{13}C-NMR spectral data of CN06 along with the literature reported for β-sitosterol are given in Table 3.

Therefore; the data generated led us to identify CN06 as *β*-sitosterol whose structure is shown in Figure 7

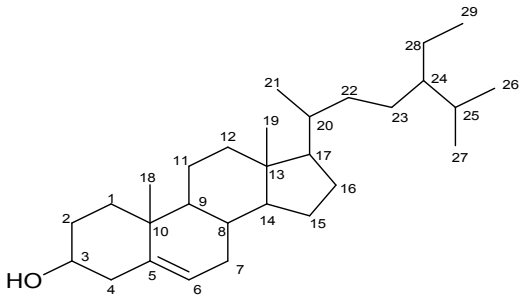


Figure 7: Chemical structure of *β*-sitosterol

β-Sitosterol reduces cancer of the colon. It shows anti-inflammatory, anti-pyretic, antiarthritic, anti-ulcer, insulin releasing and oestrogenic effects and inhibition of spermatogenesis. *β*- Sitosterol is mainly known and used for its cholesterol lowering property [32]. Therefore the presence of *β*-sitosterol adds one positive attributes to this plant.

4.3.2. Characterization of CN02

Compound CN02 was obtained as white crystalline solid from the EtOAc extract of the roots of *c .niveum.* Its TLC showed spot at an Rf value 0.7 with EtOAc: n-hexane (6:4) as a mobile phase.

The UV-Vis spectrum (EtOH) displayed an absorption band at λmax 270 nm indicating the presence of $\pi \rightarrow \pi^*$ in the structure of the compound. The IR spectrum showed absorptions bands at *δ* 3541 cm^{-1} due to hydroxyl stretching. The stretching frequencies of alkyl groups are evident at *δ* 2918 and 2858.1 cm^{-1}. The signal diagnostic of carbonyl group is evident at *δ* 1765.8 cm^{-1}. Furthermore the absorption bands at *δ* 1674 cm^{-1} and 1204 cm^{-1} are assignable to C=C and C-O stretching, respectively.

The ^{1}H-NMR spectrum of CN02 revealed the presence of four methyl protons at *δ* 0.78, 0.87, 0.94 and 0.97 all of which were turned out to be singlet. This indicates that all the four methyl groups are on quaternary carbons. The multiplets signal at *δ* 3.44 (1H) is ascribed to methine proton on an oxygenated carbon. The other signals observed at *δ* 4.05 (1H) and 4.37 (1H) are due to methylene protons on oxygenated carbons. The signal due to an olefinic

carbon β to a carbonyl was observed at δ 6.86. The other aliphatic proton signals integrating methyl hydrogen were detected in the region between0.78 to 0.97

The proton decoupled ^{13}C-NMR spectrum with the aid of DEPT-135 revealed the presence of 20 well resolved carbon resonances of which are five quaternary, five methine,

Six methylene and four methyls. The signal at δ 170.2, 136.3 and 126.9 are evident for the presence of α,β-unsaturated carbonyl group with the earlier signal accounted to the carbonyl carbon. The methine olefinic carbon was observed at δ 136.3. Signals due to oxygenated aliphatic methine carbon and methylene carbon are evident at δ 75.6 and 67.2, respectively. The other signals due to methylene carbons were observed at δ 25.0, 28.3, 24.1, 34.5 and 37.4 which are assignable to C1, C2, C6, C7 and C14 respectively. The other signals observed in the spectrum are depicted in Table 4.

Table 4: ^{1}H and ^{13}C-NMR spectral data of CN02

Carbon No	^{13}C NMR spectral data	^{1}HNMR spectral data	DEPT-135	Carbon No	^{13}C NMR spectral data	^{1}HNMR spectral data	DEPT-135
C1	25.0	1.67	CH_2	C11	136.3	6.9(J3.6HZ)	CH
C2	28.3	1.72	CH_2	C12	127.0	-	Cq
C3	75.7	3.44	CH	C13	37.0	2.4(J5.2)	CH
C4	49.3	-	Cq	C14	37.4	1.69	CH_2
C5	54.0	1.5	CH	C15	67.2	4.4(J9.2HZ)	CH_2
C6	24.1	1.61	CH_2	C16	170.2	-	Cq
C7	34.5	1.60	CH_2	C17	22.2	0.97	CH_3
C8	32.8	-	Cq	C18	17.9	0.95	CH_3
C9	51.1	1.49	CH	C19	14.1	0.87	CH_3
C10	40.6	-	Cq	C20	15.2	0.78	CH_3

The spectral data generated led us to identify compound CN02 whose structure is shown in Figure 8.

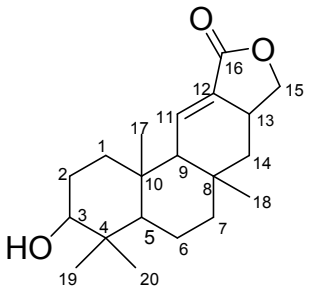


Figure 8: Proposed structure of compound CN02

4.4. Antibacterial test Results

The MeOH extract and EtOAc isolated compounds were evaluated for their antibacterial activities. All the tested extract and isolated compounds showed varying degrees of antibacterial activities against the pathogens. The MeOH extract were found active against *Staphylococcus aureus* which is the only gram positive bacteria assessed in this study but it was found inactive against all gram negative bacteria used in this study. As seen in Table5, CN06 was neither active in gram positive nor gram negative bacteria. Relatively an interesting result was obtained for compound CN02 which displayed activity against both gram positive and negative bacteria at concentration of 20µg/mL.

Table 5: Zone of bacterial growth inhibition (mm) for crude extract and isolated pure compounds from root of *C. niveum*

Sample code	*S. aureus*	*E. coli*	*P. mirabili*	*K. pneumonia*
Methanol extract	10	6	6	6
CN02	10	9	10	11
CN06	6	6	6	6
Gentamycin	15	15	15	15

The roots extracts of *C. niveum* contains phytochemicals having antibacterial activities. The activity displayed by the roots of this plant is likely due to the presence of CN02 which showed better activity compared with the extracts and CN06.

4.5. Antioxidant Activities

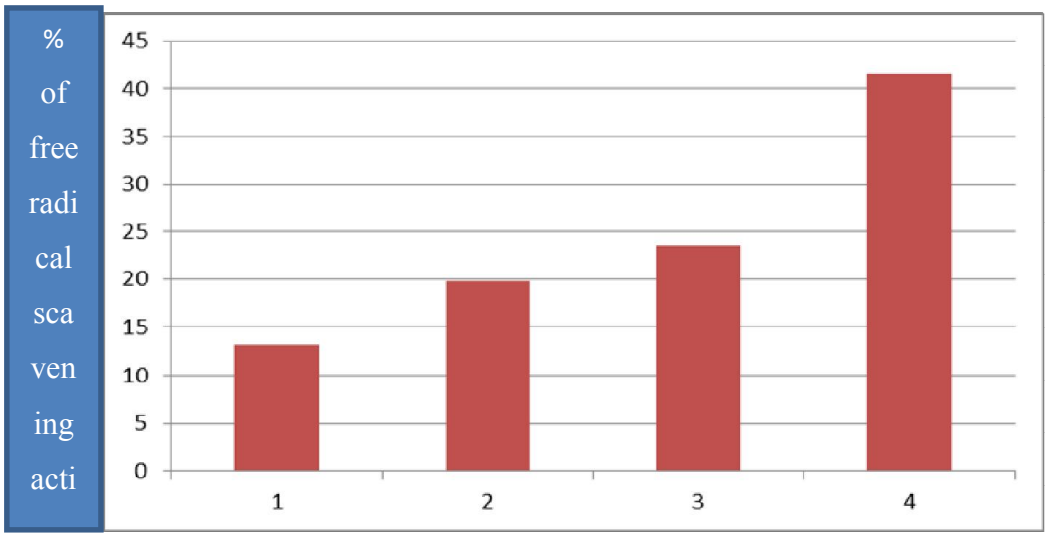
Antioxidant activity different concentration of both ethyl acetate and methanol extracts were prepared in methanol: 100, 50, 25 and 12 µg/mL. The antioxidant activity of extracts was carried out using DPPH.

Table 6: Antioxidant activity of EtOAc and MeOH root extracts of *Cyphostemma niveum*

EtOAc Extraction				MeOH extraction			
EtOAc concentration	Absorbance	% of free radical scavenging activity	IC50	MeOH concentration	Absorbance	% of free radical scavenging activity	IC50
12	0.92	13.2	40.0	12	0.84	20.8	29.0
25	0.85	19.8		25	0.72	32.1	
50	0.81	23.5		50	0.65	38.7	
100	0.62	41.5		100	0.52	50.9	

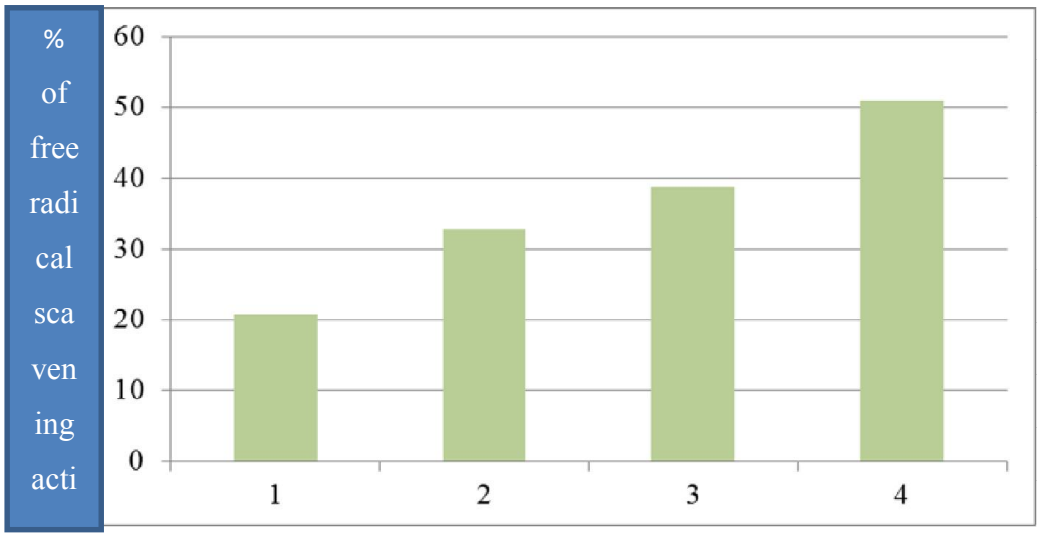
The EtOAc and MeOH extracts were evaluated for their antioxidant activity. As clearly seen from Table 6 the MeOH extract inhibited the DPPH radical by 50% at100 µg/mL which is much better than the EtOAc extract. The result was found to be modest compared with the standard drug, ascorbic acid (97%).

Chart 1: Antioxidant activity of EtOAc extract of *Cyphostemma niveum*



Concentration of EtOAc extraction in µg/mL (12, 25, 50 &100) of 1, 2, 3 &4 respectively

Chart 2: Antioxidant activity of MeOH extract of *Cyphostemma niveum*



Concentration of Methanol extraction in µg/mL (12, 25, 50 &100) of 1, 2, 3 &4 respectively

5. CONCLUSION AND RECOMMENDATIONS

5.1. CONCLUSION

The EtOAc extract after silica gel column chromatography has led to the isolation of two compounds, namely, *β*-sitosterol and labeled compound CN02. These compounds were characterized using spectroscopic methods such as NMR, IR and UV. The methanol extract showed antibacterial activity against *Staphylococcus* aureus gram positive bacteria while the EtOAc extract was found inactive in all bacterial pathogens tested in this study. CN02 showed activity against both gram positive and gram negative bacterial pathogens tested indicating its broad spectrum activity. The EtOAc and MeOH extract was not effective free radical scavenger.

5.2. Recommendations

- Although, the compounds isolated are not the only compounds present in the plant material as evidenced from TLC analysis, future work should aim at isolating other compounds present in the extract and determine their antibacterial activity.
- There is need to extend phytochemical investigations of these plants to methanol extracts and all fractions that were not analyzed in these studies.
- Future research should be extended to cover other biological activities.
- It is recommended that further *in vivo* anti-bacterial efficacy tests with the bacteria used in mice so as to validate the *in vitro* results.
- It is recommended that further similar studies should be conducted on other parts of the plant such as root, stem, and bark

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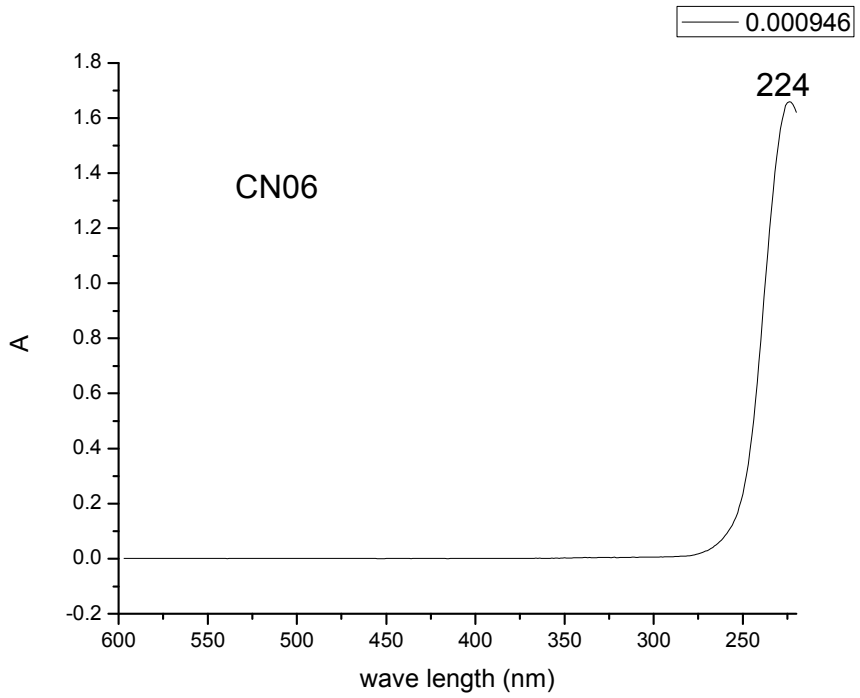
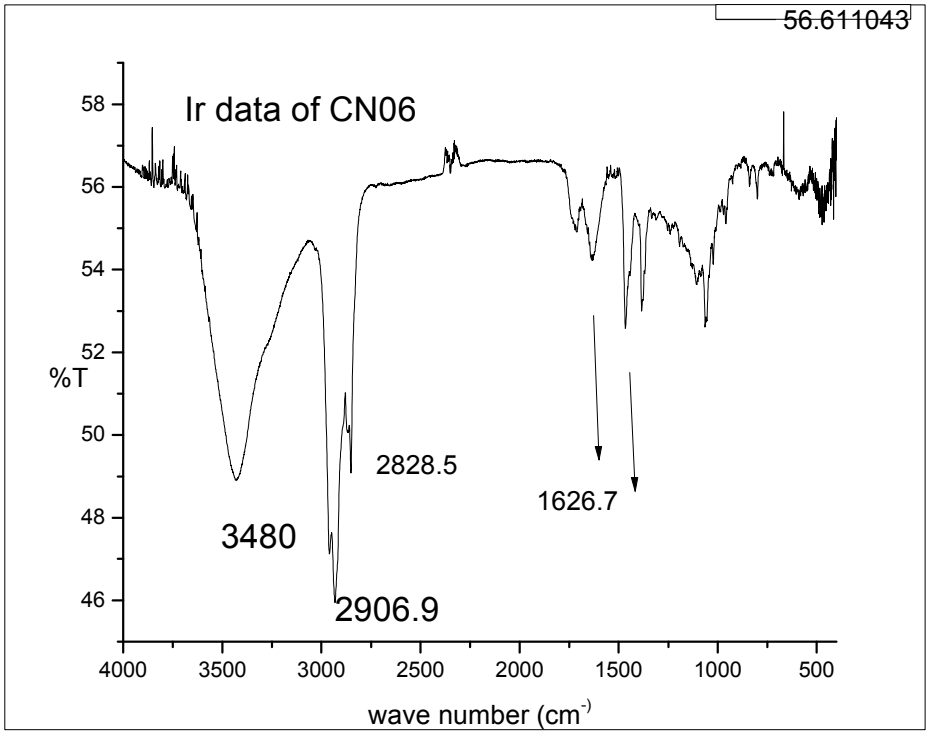
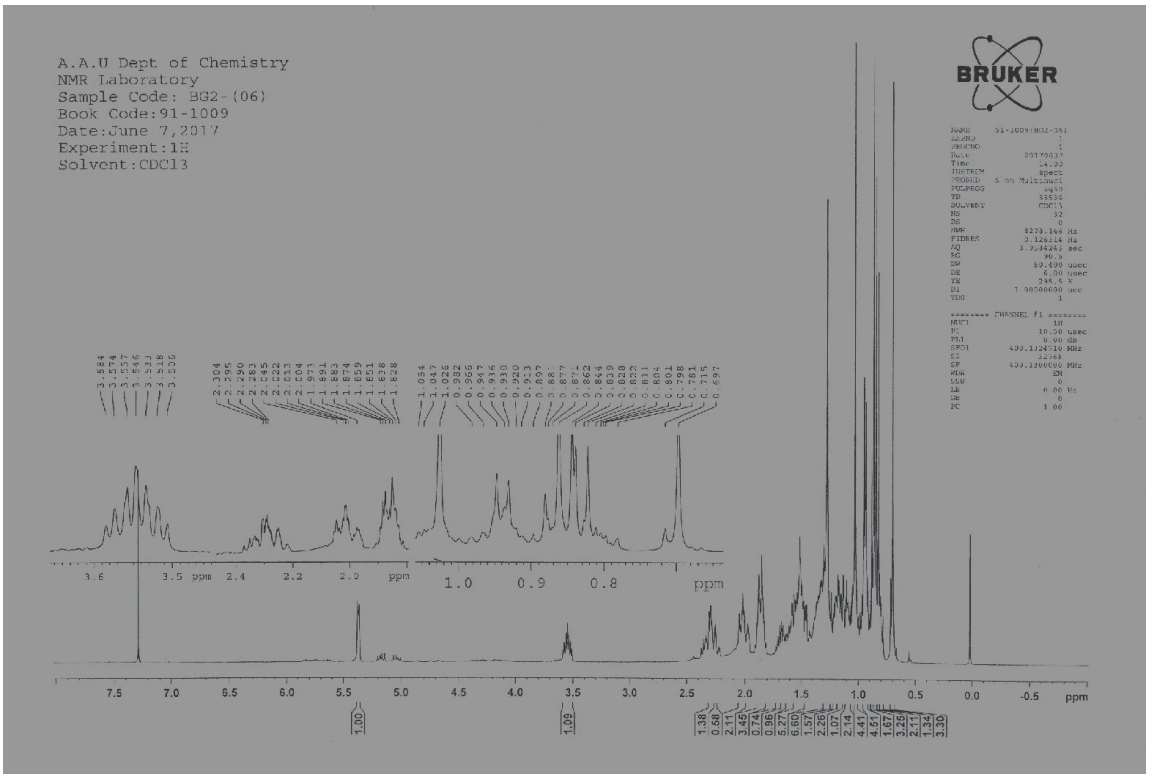
APPENDICES



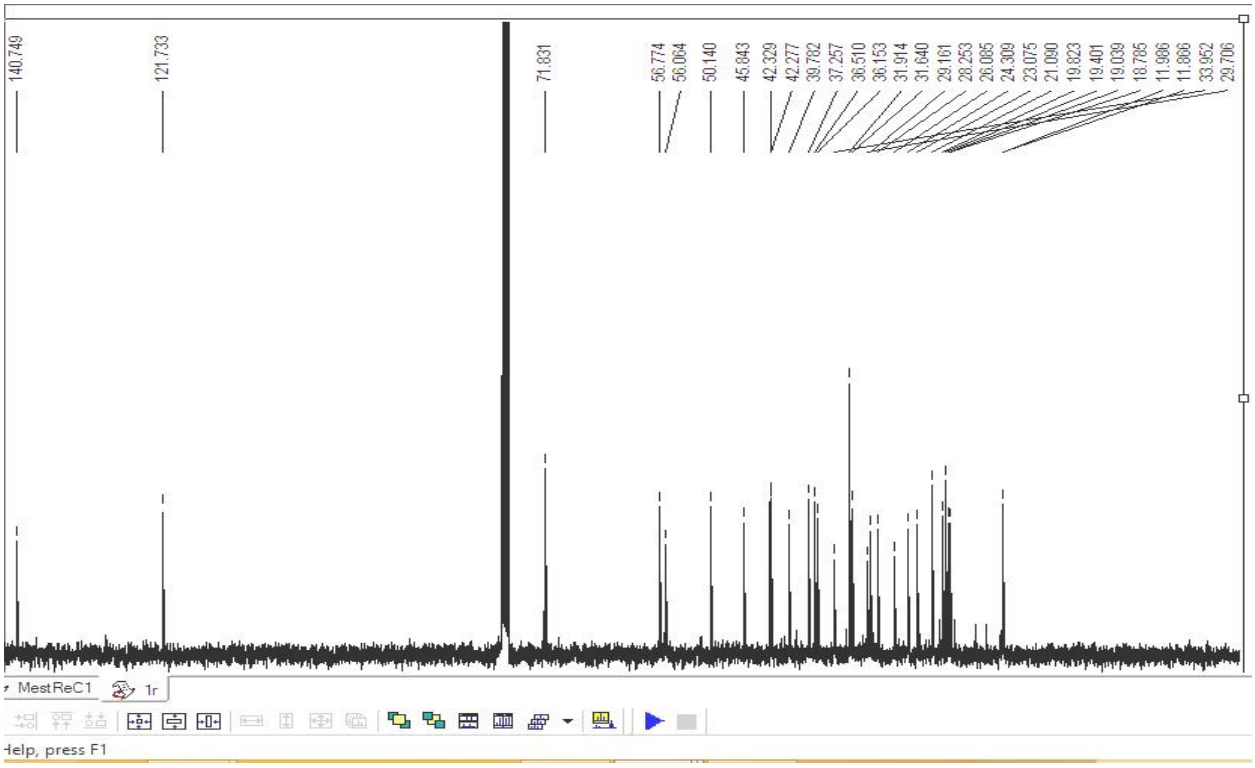
Appendix 1**:** UV data of CN06 Compound



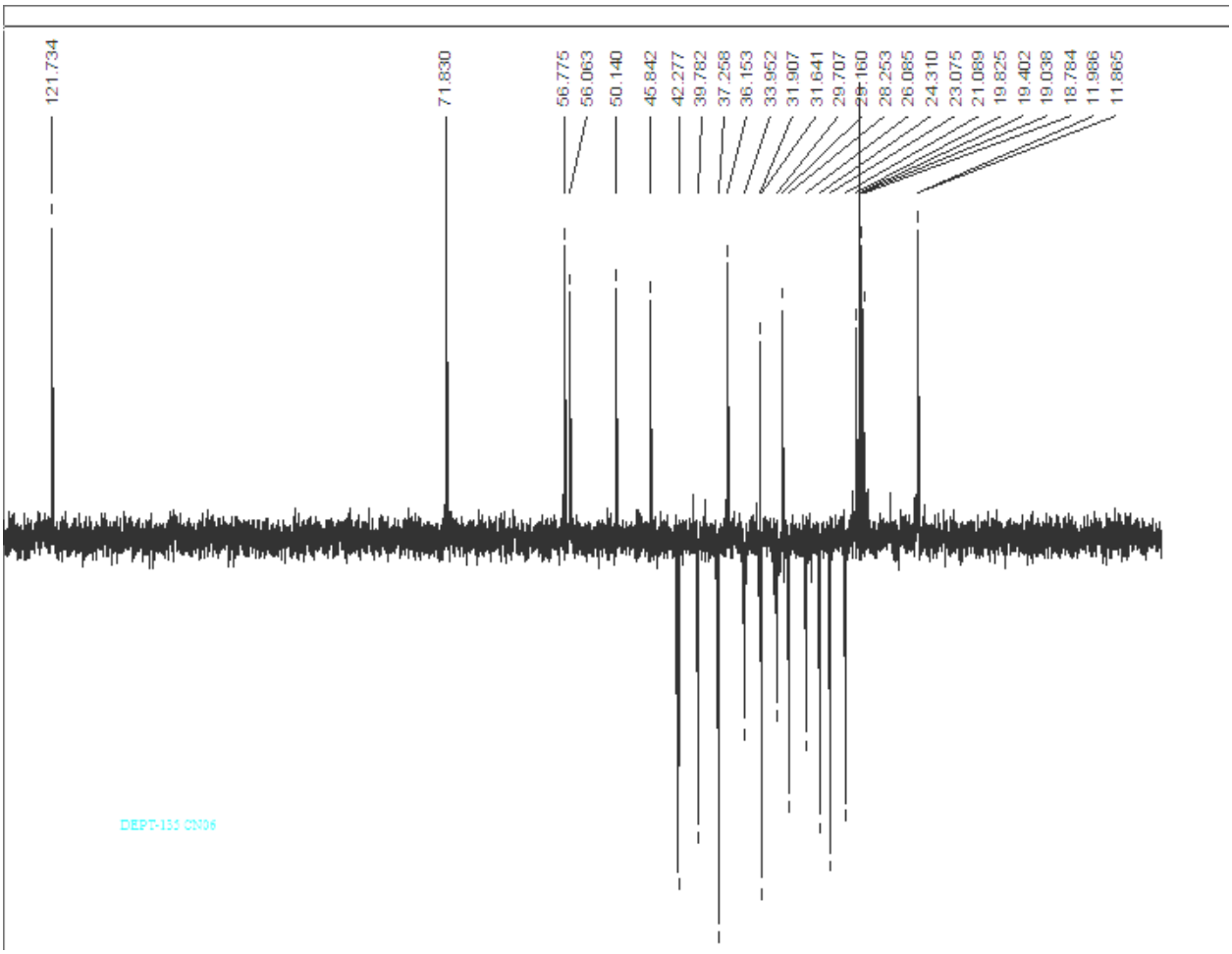
Appendix2: IR data of CN06 Compound



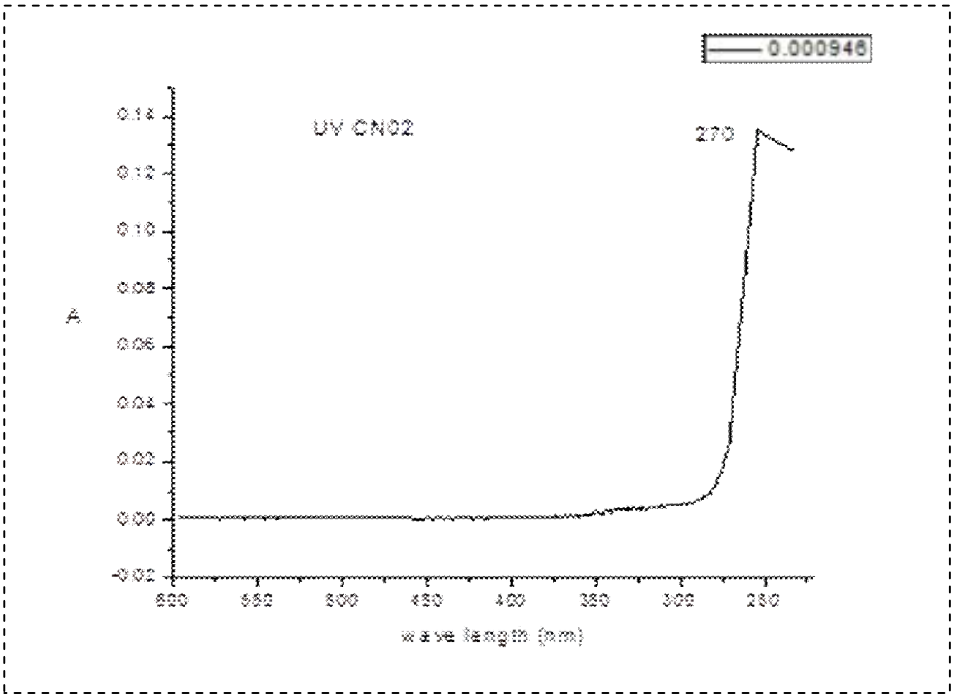
Appendix 3**:**[1]HNMR data of CN06 Compound



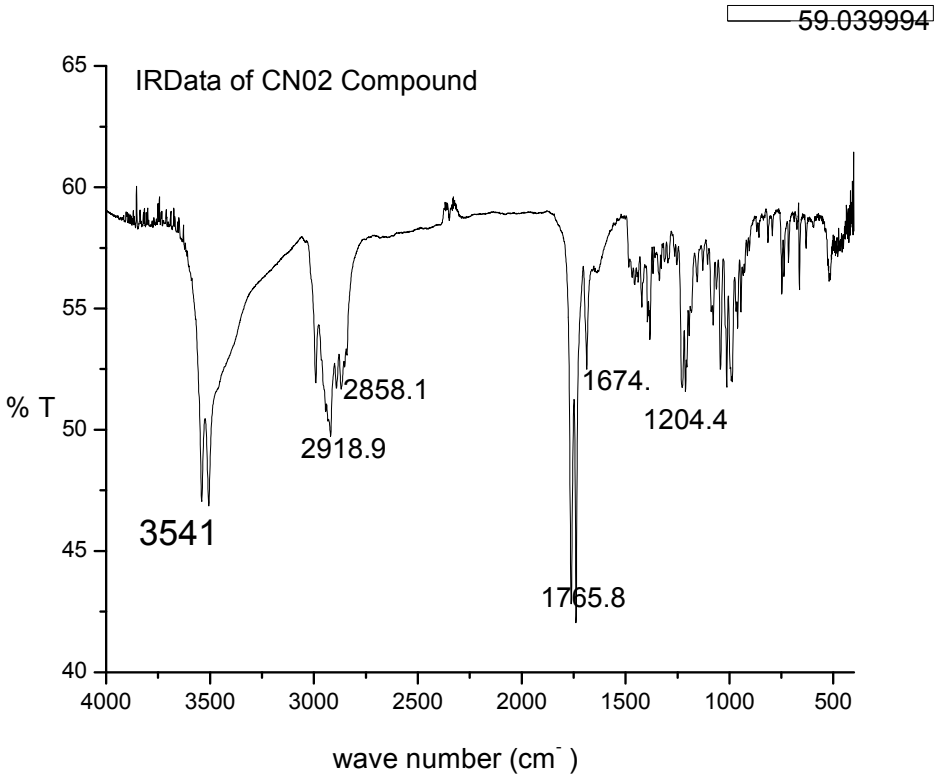
Appendix 4:[13]CNMR Data of CN06 compound



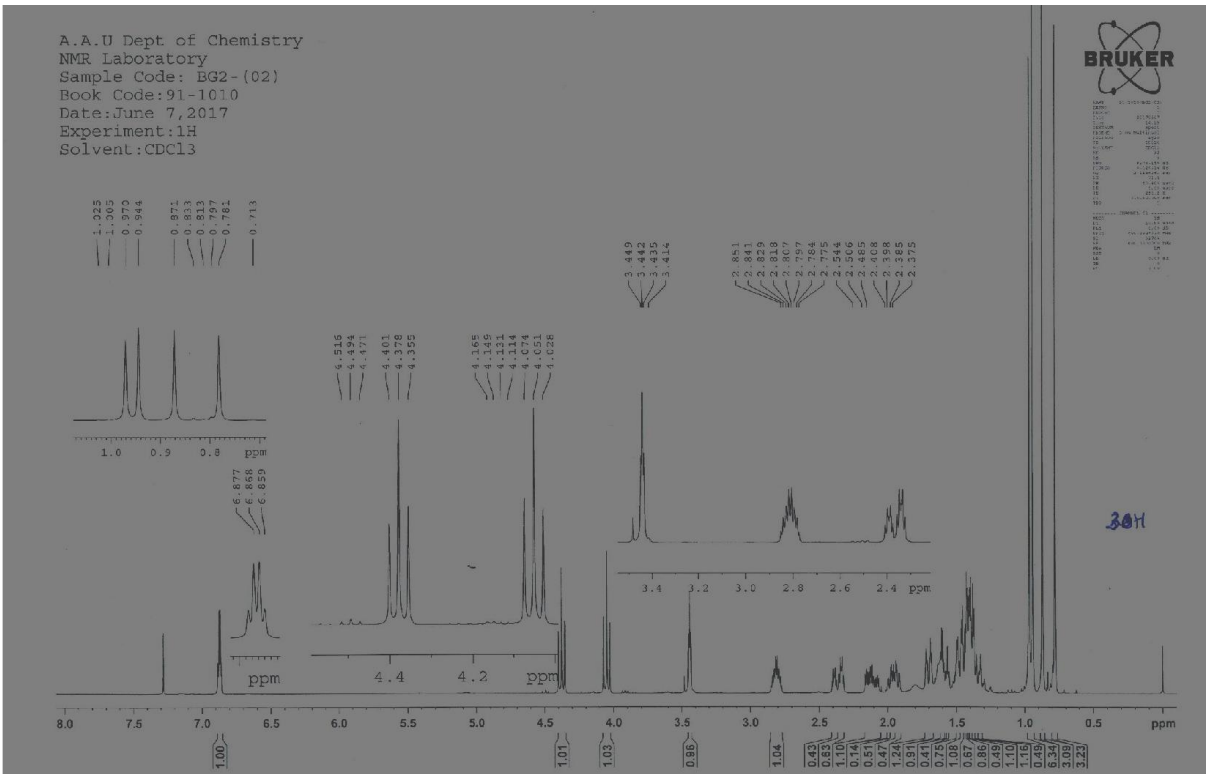
Appendix 5**:** DEPT-13 data of CN06 Compound



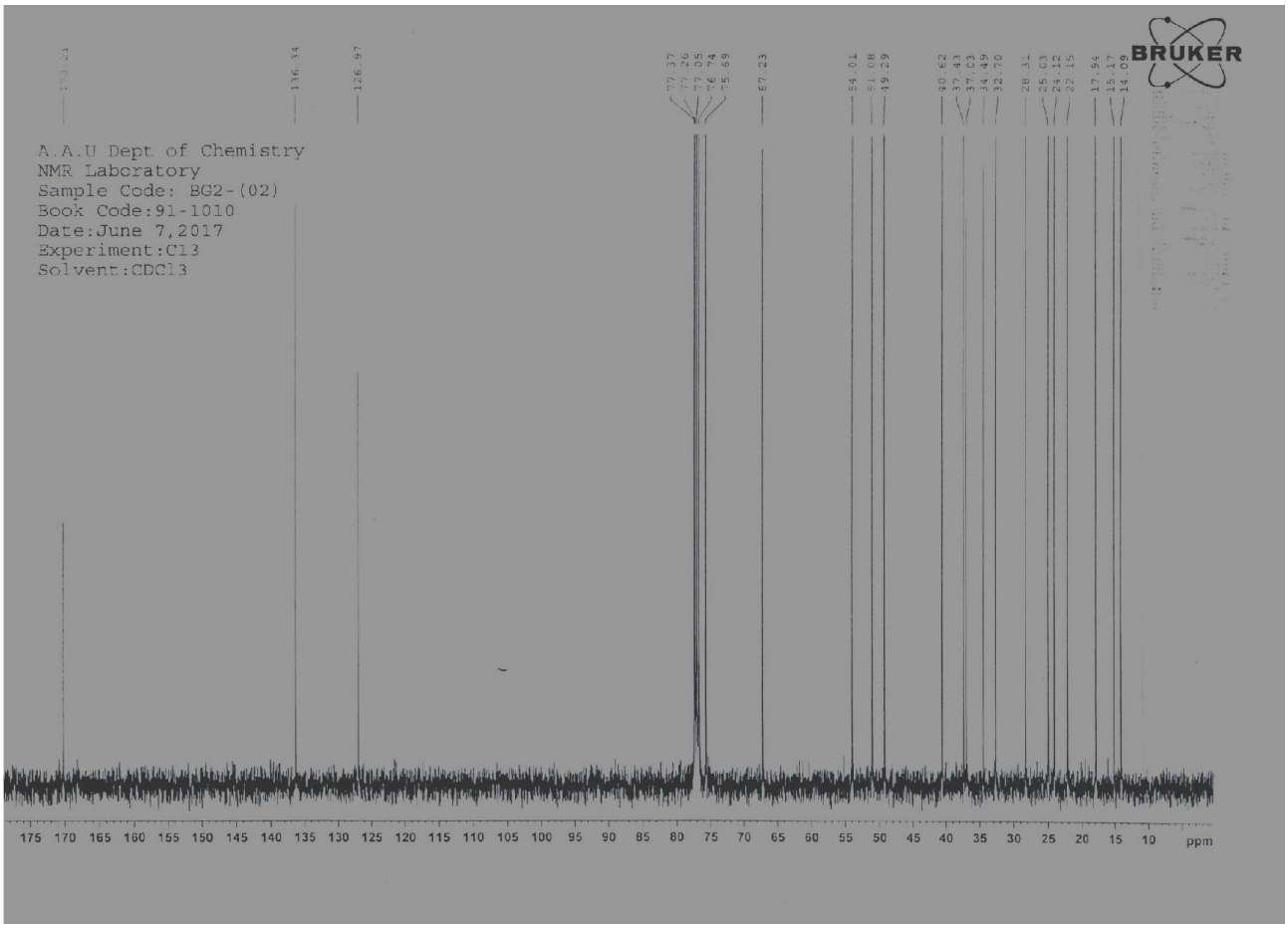
Appendix 6: UV-Vis data of CN02 Compound



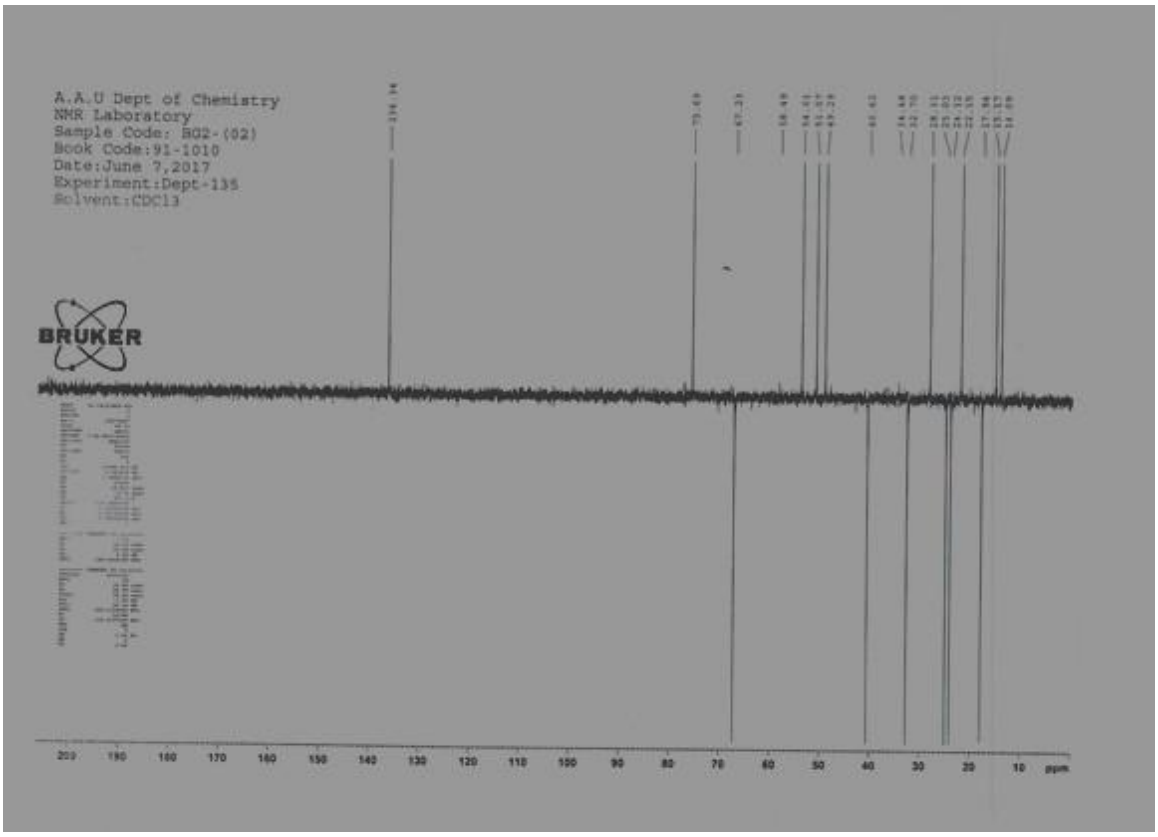
Appendix 7: IR data of CN02 Compound



Appendix 8:[1]HNMR data of CN02 Compound



Appendix 9: 13CNMR data of CN02 Compound



Appendix 10: DEPT-135 data of CN02 Compound