ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

SCHOOL OF APPLIED NATURAL SCIENCES

PROGRAM OF APPLIED CHEMISTRY

PHYTOCHEMICAL INVESTIGATION ON BERRIES OF *EMBELIA SCHIMPERI*

BY: TARIKU NEFO

ADVISOR: Dr. AMAN DEKEBO

SEPTEMBER, 2015

PHYTOCHEMICAL INVESTIGATION ON BERRIES OF *EMBELIA SCHIMPERI*

A THESIS SUBMITED TO THE SCHOOL OF APPLIED NATURAL SCIENCES

PROGRAM OF APPLIED CHEMISTRY

IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE IN CHEMISTRY

(Organic chemistry)

BY
TARIKU NEFO

ADVISOR: Dr. AMAN DEKEBO

SEPTEMBER, 2015

ACKNOWLEDGEMENTS

First my special and endless thanks go to my Almighty God in giving me success through my life. Second but the most I am very much indebted to my research advisor **Dr. Aman Dekebo** for his guidance, supervision from the beginning to the completion of this work and for giving me all the necessary materials required for the study.

I would like to thank **Nefo Duke, Lamesa Yadeta, Abebu Nefo, Dr. Belay Merera, Kibitu Lencho, Buzunesh Negasa, Zenebech Negasa, Dereje Ejigu, Abebe Terefe, Ebisa Nefo and Hinkosa Hordofa** who contributed by giving constructive guidance in one way or another while doing my study.

I am very much thankful to my brother **Engineer Alemayehu Nefo** and **Desalegn Nefo** for their unreserved continued encouragement and financial support of my study.

Finally, I express my deepest sense of gratitude to my lovely family teacher **Ayantu Merera** and **Robenit Tariku** for their continuous encouragement during the course of my study.

TABLE OF CONTENTS

Contents **Page**

ACKNOWLEDGEMENTS --I
Table of contents--II
List of figures--IV
List of tables ---V
List of scheme--VI
List of appendices ---VII
Glossary of abbreviations and symbols--VIII
ABSTRACT---IX
1. INTRODUCTION--1
1.2 Natural products in drug discovery---1
1.3 Statement of the problem---3
1.4 Significance of the study---3
1. 5 Objectives--4
1.5.1 General objective---4
1.5.2 Specific objective---4
2. LITERATURE REVIEW--5
2.1 Botanical descriptions of *Embelia schimperi* --5
2.2 Phytochemistry of the genus Embelia ---6
3. MATERIALS AND METHODS--11
3.1 Apparatus and equipments ---11
3.2 Chemicals ---11
3.3 Plant material collection and identification ---------------------------------------11
3.4 Method of extraction and isolation --12
3.5 Phytochemical screening tests--13
3.6 Isolation of F_4 and F_3--14
4. RESULTS AND DISCUSSION ---15
4.1 Phytochemical screening test --15
4.2 Characterization of compound F_3--17

4.3 Characterization of compound F_4--19

5. CONCLUSION AND RECOMMENDATION-----------------------------------22

6. REFERENCES ---23

7. APPENDIXES--25

List of figures **Page**

Fig. 1 *Embelia schimperi* locally called Enkoko in Amharic and
Hanku in Afan Oromo plant--5
Fig. 2 The structure of compound (F_3) --18
Fig. 3 The structure of compound (F_4) --21

List of table's **page**

Table 1. Compounds isolated from *Embelia genus* plants-----------------------------8

Table 2. Fractions of methanol extract--13

Table 3. Phytochemical screening test results--16

Table 4. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectral data of F_3 in DMSO-d_6----18

Table 5. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectral data of F_4 in DMSO-d_6----20

Scheme **Page**

Scheme 1. General procedures followed in the extraction of berries
of *Embelia schimperi*--12

List of appendixes **page**

Appendix 1: UV spectrum of F_3 --26
Appendix 2: ^{13}C-NMR spectrum of F_3 in DMSO-d_6--27
Appendix 3: DEPT-135 spectrum of F_3--28
Appendix 4: ^{1}H-NMR spectrum of F_3 in DMSO-d_6--29
Appendix 5: IR spectrum of F_3--30
Appendix 6: UV spectrum of F_4 --31
Appendix 7: ^{13}C-NMR spectrum of F_4 in DMSO-d_6--32
Appendix 8: DEPT-135 spectrum of F_4-- 33
Appendix 9: ^{1}H-NMR spectrum of F_4 in DMSO-d_6--34
Appendix 10: IR spectrum of F_4 --35

Glossary of abbreviations and symbols

^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
1D NMR	One-dimensional spectra
^{1}H-NMR	Proton-Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement by Polarization Transfer
RF	Radio frequency
WHO	World Health Organization
δ	Chemical shift
PTLC	Preparative Thin Layer Chromatography
DMSO-d_6	Deuterated dimethyl sulfoxide
IR	Infrared
MHz	Megahertz
R_f	Mobility relative to front (Retention factor)
TLC	Thin Layer Chromatography
UV	Ultraviolet
s	Singlet
d	Doublet
t	Triplet
dd	Double doublet
m	Multiplet
$CHCl_3$	Chloroform
EtOAc	Ethyl acetate
MeOH	Methanol
EtOH	Ethanol

ABSTRACT

Embelia is a genus of climbing shrubs in the family Myrsinaceae. *Embelia schimperi* is important in traditional medicine like the other species in the genus. The plant has been much known as local medicine for the treatment of tape worms. In this project berries of *Embelia schimperi* were subjected to exhaustive extraction with *n*-Hexane, ethyl acetate and methanol separately and yielded *n*-Hexane extract, ethyl acetate extract and methanol extracts. Based on TLC analysis and yield the methanol extract was selected for further isolation. Chromatographic purification (CC and PTLC) of the methanol extract gave two new compounds coded as F_3 and F_4. The compounds were identified to be [alkaloid 2-(pent-4-ol)-N-methyl cyclohexylamine (F_3) and alkaloid benzoate 2-methyl-4-(3-methyl butane)-6-(methyl benzoate)-N-methyl cyclohexylamine (F_4]. The structures of these compounds have been elucidated on the basis of spectroscopic evidences (^{13}C-NMR, DEPT, ^{1}H-NMR, UV and IR).

1. INTRODUCTION

The World Health Organization (WHO) defines traditional medicine as health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses and maintain well-being [1]. Plants have been used from ancient times to attempt cures for diseases, to relive physical suffering and also utilized as sources of spices, dyes, poisons, etc. Ancient peoples all had acquired some knowledge of medicinal plants. Oftentimes these primitive attempts at medicine were based on superstition and speculation. The chemical compounds responsible for these activities are often the secondary metabolites of plants or natural products as they are usually referred to [2].

The study of natural products offers challenging problems of stereo specific synthesis of compounds with a fascinating diversity of structures to synthetic organic chemists. These molecules of life have attracted the attention and efforts of the foremost organic chemists because of their economic importance in industry, their value in medicine and phytochemical studies of plants especially of medicinal plants are of great importance in developing drugs, study of chemotaxonomy and plant biodiversity as well as in documenting knowledge. Enhancing the knowledge of biological and pharmacological effects of plant constituent and determination of the structures of the active principles may help in sustaining the use of these products [2].

1.2 NATURAL PRODUCTS IN DRUG DISCOVERY

The contribution of natural products to the development of medicine could be demonstrated by the amount of plant derived drugs being used [3]. Medicinal agents used by humans since ancient times provided the starting point for the development of our current arsenal drugs. Higher plants have been the source of medicinal agents since earliest times, and today they continue to play dominant roles in the primary health care of about 80 % of the world's population due to cultural acceptability, it's attributed efficacy against certain types of diseases and economic affordability as compared to modern medicine. Research in to the chemical and biological properties of natural

products over the past two centuries has not only yielded drugs for the treatment of human elements, but has provided the stimulus for the development of modern synthetic organic chemistry, and the emergence of medicinal chemistry as a major route for the discovery of the novel and more effective therapeutic agents [4].

By 1882, more than 50 different herbs were commonly used to make medicines. The active ingredients of these plants were isolated from the herbs, berries, roots, and bark used in traditional medicine. Of the 119 plants derived drugs commonly in use, 74 % were discovered as a result of chemical studies directed at the isolation of the active constituents of plants used in traditional medicine [5].

Phytochemistry research is the backbone of herbal industry and directly or indirectly responsible for both failure and success of herbal drugs. For promoting the use of herbals in modern medicine, phytochemistry should be envisaged for: - isolation, purification and characterization of new phytoconstituents, use of newly isolated phytoconstituent as “lead” compound for the synthetic design of analogues with either improved therapeutic activity or reduced toxicity and conservation of lead phytoconstituents into medicinally important drugs. Scientists still search the world for plants, the oceans for flora and fauna that might yield new medicinal compounds.

A plant’s medicinal value is due to the presence in its tissues of some chemical substance or substances that produce a physiological action on the body. Worldwide there are several thousand plants that have been used and are still being used for medical purposes. Many of these are restricted in use by native people who have long resided in any given area [6].

The first official recognition of traditional medicine as important participant in primary health care was expressed in the World Health Organization’s Primary Health Care Declaration of Alma Ata in 1978. Traditional medicine according to the declaration is the sum total of all the knowledge and practices used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical

experience and observation handed down from generation to generation verbally or in writing [7].

Medicinal plant typically contains mixtures of different compounds that may individually or synergistically improve the health. The importance of medicinal plants lays not only on their chemotherapeutic effect but also in their role as a source of model compounds for drug development. In addition to plant constituents being used directly as therapeutic agents, they can be utilized as starting material or templates for drug synthesis [8]. In general it has been estimated that over 40 % of medicines have their origins in these active natural products [9].

1.3 STATEMENT OF THE PROBLEM

Previous report indicates that *Embelia* genus is the most known source of different class of compounds. Now this genus attracts the attention of researchers to study the bioactive compounds and to synthesize drugs in mass. *Embelia schimperi* is one of the medicinal plants which is present at various geographical locations and their presumptive folklore used to prescribe for parasitic helminthics, especially effective against tapeworm. Although chemical investigations on different part of the plant have been reported before, there is no report on the chemical constituents of solvent extract on berries of *Embelia schimperi*. Based on its medicinal value, this project was designed to investigate chemical constituents available on the berries of the plant

1.4 SIGNIFICANCE OF THE STUDY

This study addresses the *Embelia schemperi* berries of chemical constituents. In this study the pure compound isolated from solvent extracts of the plant berries might be used to expel intestinal parasitic tape worms in the support of traditional medicinal use of the plant by the local people. Hence, once a naturally occurring compound was isolated and its structure was determined, it can serve as a prototype in a search for other biologically active compounds in the plant or their synthesis.

1.5 OBJECTIVES

1.5.1 GENERAL OBJECTIVE

- To investigate chemical constituents from the berries of *Embelia schimperi* plant.

1.5.2 SPECIFIC OBJECTIVE

- To extract berries of the plant using methanol, ethyl acetate and n- hexane solvents.
- To fractionate the crude solvent extract using chromatographic techniques.
- To characterize isolated compounds using UV, NMR and IR techniques.

2. LITERATURE REVIEW

2.1 BOTANICAL DESCRIPTIONS OF *EMBELIA SCHIMPERI*

Embelia is a genus of climbing shrubs in the family Myrsinaceae; there are about 130 species which occur in tropical and subtropical areas across a wide range including Africa and from eastern Asia to the Pacific Islands as well as Australia [10]. The plant can grow to a maximum height of about 7 m [11]. The plant is a highland family of trees and/or shrubs that are established all over the world. In Kenya, the species are found in the West and South of Mt. Kenya, Ngong' Hills, Kakamega forests and Western slopes of Mau Ranges around Kericho District [12].

Embelia schimperi is scan dent or climbing shrub which reaches the height of 2-13 m [13]. The fruit, 5-8 mm in diameter, is orange yellow, reddish green to red in color when ripen. Each fruit often has one seed that has a diameter of 4.5-7 mm. It is brown in color with irregular orange markings when ripen [14].

Fig.1 *Embelia schimperi* locally called Enkoko in Amharic and Hanku in Afan Oromo plant Photo taken by researcher on November 2014.

2.2 PHYTOCHEMISTRY OF THE GENUS *EMBELIA*

The EtOH extract of the roots of *Embelia ribes* led to isolation of a new compound nitrogen-containing 3-alkyl-1,4-benzoquinone derivative, *N*-(3-carboxylpropyl)-5-amino-2-hydroxy-3-tridecyl-1,4-benzoquinone (1), and a gomphilactone derivative, 5,6-dihydroxy-7-tridecyl-3-[4-tridecyl-3-hydroxy-5-oxo-2(5*H*)-furylidene]-2-oxo-3(2*H*)-benzofuran (2), together with 14 known compounds, as well as the common plant metabolites sitosterol and daucosterol, were isolated from the plant. Their structures were elucidated by means of spectroscopic methods [17].

Chromatographic separation of an ethyl acetate extract of dried ground stem bark of *Embelia schimperi* led to the isolation of a new compound identified as 2,5-dihydroxy-3-methyl-1,4-benzoquinone (3) on the basis of spectroscopic and physical data. The plant's crude extract and pure compound 3 were assayed for *in vitro* antimicrobial activity against clinical strains of *Salmonella* spp., Proteusspp., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Cryptococcus neoformans*, *Shigella dysentriae* and *Staphylococcus aureus*. Disc diffusion method was used and zones of inhibition, after respective incubation periods, were used to quantify antimicrobial activity. Standard antibiotics namely: augmentin, cotrimoxazole, gentamycin, tetracycline and lyncomycin were used as controls. Compound (4) as exhibited by the zones of inhibition which ranged from 10-20 mm, the extracts were most active against *P. aeruginosa* as shown by the largest zone of inhibition of 20 mm, which was comparable to that of the control antibiotic gentamycin, with an inhibition diameter of 21 mm on the same microorganism [18].

Chromatographic analysis of air-dried berries of the plant from cold ethyl acetate extract led to the isolation of methyl vilangin (5) and Biembelin (6). Methyl vilangin was found to be lethal against 2nd instar larvae of *Aedes aegypti* (yellow fever vector) by first stopping the process of metamorphosis from the 2nd instar stage to the other stages and finally causing mortality to the larvae [19].

An investigation of MeOH extract of stem part of the plant led to the isolation of a new resorcinol derivative, 5-(7'Z-pentadecenyl) resorcinol (7), along with the known compounds lupeol (8) and β-sitosterol glucoside (9). Compound 5 exhibited moderate in vitro cytotoxic activity against human Hela cell line [20].

Fractionation of the methanolic extract of leaves of the plant has led to the isolation of novel flavonol glycosides (10). The compounds were characterized as isorhamnetin 3-O-β-galactoysyl (1→ 4)-β-galactoside and quercetin 3-O-[α-rhamnosyl (1→2)] [α-rhamnosyl (1→ 4)]-α-rhamnoside. Also reported from the same extracts were known compounds quercetin, myricetin, quercetin 3-O-α-rhamnoside, quercetin 3-O-β-glucoside, quercetin 3-O-rutinoside, myricetin 3-O-β-xyloside, isorhamentin 3-O-β-glucoside and myricetin 3-O-β-glucoside. Their structural elucidation was accomplished using spectral measurements and chemical methods [21].

Five oleanane-type pentacyclic triterpenoids were isolated by chromatographic separation of a chloroform extract of the stem bark of *Embelia schimperi.* Three of these compounds have a methyleneoxy bridge. Two compounds, embelinone and schimperinone, are reported here for the first time from a natural source (they have been synthesized previously during chemical transformations). Their structures were determined by spectroscopic techniques, among which 2D NMR was useful for complete characterization. Three of the triterpenoids exhibited mild antibacterial properties against the gram-positive bacteria strain *Rhodococcus* sp. [22]. (Table 1)

Table 1. Compounds isolated from *Embelia genus* plants

Compounds	Ref
1 2	17
3 4 2,5-dihydroxy-3-methyl-1,4-benzoquinone (1) and embelin (2)	18

Methyl vilangin (5)	19
Biembelin (6)	
7 8 9 5-(7’Z-pentadecenyl) resorcinol (5), lupeol (6) and β-sitosterol glucoside (7).	20

10. Isorhamnetin 3-O-β-galactoysyl (1→ 4)-β-galactoside and quercetin 3-O-[α-rhamnosyl (1→2)] [α-rhamnosyl (1→ 4)]-α-rhamnoside	21
Embelinone **(11)**	22

Embelia schimperi seeds found to possess antibiotic and anti-tubercular properties. A gum obtained from the plant is used as a warming remedy in the treatment of dysmenorrheal. Decoction of the leaves is used as a blood purifier [14]. Believed to eliminate adult *Taenia saginata*, the beef tape worm. This result indicates that the crushed seeds of *Embelia schimperi* taken orally by the Masai people indeed have an anthelmintic effect against human intestinal tapeworms [12]. This plant widely used in traditional medicine as an anthelmintic and anti-microbial, mostly known for anti-bacterial and anthelmintic properties [11]. The plant is reported to be effective against tapeworm but not against roundworm or hookworm [15]. The plant was screened for alkyl benzoquinones that the family is known to accumulate up to 5-16 %, w/w [16].

3. MATERIALS AND METHODS

3.1 APPARATUS AND EQUIPMENTS

^{1}H-NMR spectrum was measured on a Bruker Avance 400 MHz. ^{13}C-NMR spectrum was measured on a Bruker Avance 400 spectrometer at 100 MHz. The ultraviolet and visible (UV-Vis) spectrum was taken on Spectronic Genesys^{TM} 2PC UV-Vis scanning spectrometer. Infrared (IR) absorptions were measured on a Perkin Elmer System FT-IR spectrometer as KBr pellets in the range of 4000-400 cm^{-1}. Analytical thin layer chromatograms were run on ready made 0.25 mm thick layer of Merck silica gel 60 F_{254} coated on aluminum foil. The spots on the chromatograms were detected by observing in UV light and spraying with iodine. Silica gel with fluorescent indicator 254 nm on aluminum cards with layer thickness 0.1 mm used for TLC. Preparative thin layer chromatography were run on 1 mm thick layer Merck silica gel 60 PF_{254} coated on 20 x 20 glass plates. Column chromatography were conducted using column packed with Merck silica gel 60, particle size 0.063–0.200 mm (70-230 mesh ASTM), test tube, water bath, refrigerators, stand, beaker, round bottom flask, PTLC jar, gaze jar, spatula, stirrer, cotton, analytical balance, capillary, oven, ruler, pencil, pen, reagent bottle, pipette, Rota vapor, funnel, spatula, electronic balance, dropper, mortar, and pestle.

3.2 CHEMICALS

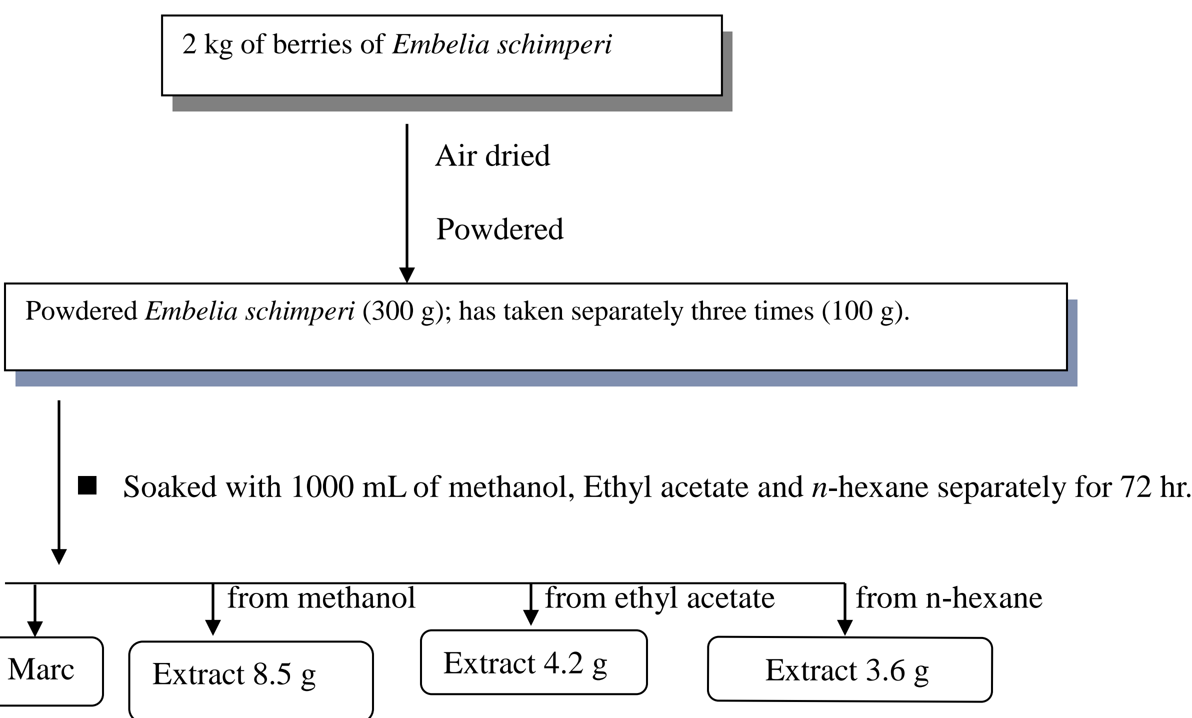
n-Hexane, ethyl acetate, iodine, chloroform, methanol, ethanol, distilled water, deuterated dimethyl sulfoxide–d_6 (DMSO-d_6), sulfuric acid, ferric chloride, KBr, TMS as an internal standard, silica gel, 2,4-dinitrophenylhydrazine, HCl, ammonia solution, Mayer's reagent, Dragendroff's reagent, ferrous sulphate, Fehling's solution, acetic acid anhydride, soap, tap water, sodium metal, $HgCl_2$, KI, sodium potassium tartarate, NaOH, $Bi(NO_3)_3$, and benzene.

3.3 PLANT MATERIAL COLLECTION AND IDENTIFICATION

The berries of *Embelia schimperi* were collected from Oromia region, Horo Guduru Wellaga Zone in Horo woreda, Loti-Ano kebele, which is 334 km west of Addis Ababa on December 13, 2015. The plant was identified by botanists in the Department of Biology, Addis Ababa University.

3.4 METHOD OF EXTRACTION AND ISOLATION

The berries of *Embelia schimperi* was dried with air and ground using mortar and pestle. 100 g of powdered berries of the plant were soaked with 100 mL of *n*-hexane, 100 mL of ethyl acetate and 100 mL of methanol for 72 hr separately, the filtrate was concentrated with Rota vapor to yield 3.6 g, 4.2 g and 8.5 g solid, respectively (Scheme 1). TLC analysis of crude extracts showed better TLC profile preferred for further work.



Scheme 1. General Procedures followed in the extraction of berries of *Embelia schimperi*.

By TLC monitoring 7.5 g of the crude extract of methanol was applied on to a column packed with n-hexane silica gel 210 g. The column was eluted using the following solvent system :- fractions 1-5 (100 mL x 5) by 100 % pure n-hexane, n-hexane/EtOAc (9:1) fractions 6-10 (100 mL x 5), n-hexane/EtOAc (8:2) fractions 11-16 (100 mL x 6), n-hexane/EtOAc (7:3) fractions 17-20 (100 mL x 4), n-hexane/EtOAc (6:4) fractions 21-23 (100 mL x 3), n-hexane/EtOAc (1:1) fractions 24-26 (100 mL x 3), pure chloroform fractions 27-28 (100 mL x 2), chloroform/methanol (9:1) fractions 29-30 (100 mL x 2), chloroform/methanol (8:2) fractions 31-32 (100 mL x 2),

chloroform/methanol (7:3) fractions 33-34 (100 mL x 2), chloroform/methanol (6:4) fractions 35-36 (100 mL x 2), chloroform/methanol (1:1) fractions 37-38 (100 mL x 2), pure methanol fractions 40-41 (100 mL x 2), 41 fractions were collected. (Table 2)

Table 2. Fractions of methanol extract

Solvent system	Ratio	Fractions Volume (mL)	Fractions	Combined	Code
n-hexane	100 %	500 mL	1-4	1-4	A1
n-hexane/EtOAc	9:1	500 mL	6-10	7-9	F
n-hexane/EtOAc	8:2	600 mL	11-16	11-16	B1
n-hexane/EtOAc	7:3	400 mL	17-20	17-20	B2
n-hexane/EtOAc	6:4	300 mL	21-23	21-23	B3
n-hexane/EtOAc	1:1	300 mL	24-26	24-26	B4
Chloroform	100 %	200 mL	27-28	27-28	C1
Chloroform/methanol	9:1	200 mL	29-30	29-30	C2
Chloroform/methanol	8:2	200 mL	31-32	31-32	C3
Chloroform/methanol	7:3	200 mL	33-34	33-34	C4
Chloroform/methanol	6:4	200 mL	35-36	35-36	C5
Chloroform/methanol	1:1	200 mL	37-38	37-38	C6
Methanol	100 %	200 mL	40-41	40-41	M

3.5 PHYTOCHEMICAL SCREENING TESTS

Test for Saponins: - 0.5 g of crude powder was shaken with distilled water in a test tube and it was warmed in water bath.

Test for Tannins: - 0.5 g of the crude powder was stirred with 10 mL of distilled water this was filtered and ferric chloride reagent was added to the filtrate.

Test for Terpenoids: - 0.5 g of crude powder was dissolved in 5 mL of methanol; 2 mL of the extract was treated with 1 mL of 2, 4-dinitrophenylhydrazine dissolved in 100 mL of 2 M HCl.

Test for Flavonoids: - 0.5 g of the crude powder was heated with 10 mL of ethyl acetate over a steam bath for 3 min, the mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution.

Test for Alkaloids: - 0.5 g of the crude powder was defatted with 5 % ethyl ether for 15 min, the defatted sample was extracted for 20 min with 5 mL of aqueous HCl on a boiling water bath, then the resulting mixture was centrifuged for 10 min. 1 mL of the filtrate was treated with few drops of Mayer's reagent and a second 1 mL with Dragendroff's reagent.

Test for Steroids: - 0.5 g of the crude powder was dissolved in 5 mL of methanol, 1 mL of the extract was treated with 0.5 mL of acetic acid anhydride and cooled in ice, this was mixed with 0.5 mL chloroform and 1 mL of concentrated H_2SO_4.

Test for Glycosides: - 0.5 g of the crude powder was dissolved in 5 mL of methanol, 10 mL of 50 % HCl was added to 2 mL of the extract and the mixture was heated in a boiling water bath for 30 min, 5 mL of Fehling's solution was added and the mixture was boiled for 5 min.

Test for Anthraquinones: - 0.5 g of the crude powder was shaken with 10 mL of benzene and was filtered, 0.5 mL of 10 % ammonia solution was added to the filtrate and the mixture was shaken.

3.6 ISOLATION OF F_4 AND F_3

TLC showed fractions (7, 8 and 9) analyzed with *n*-hexane/EtOAc (9:1) showed the same R_f (0.85) value and the same characteristic color on TLC were combined (0.66 g), then applied on PTLC using chloroform/methanol (7:3) and resulting in the isolation of four fractions the top band F_4 (41 mg) a single homogeneous brown spot R_f value 0.94 and next to the top band F_3 (22 mg) dark brown spot R_f value 0.82, F_2 (5 mg) reddish spot R_f value 0.176 and F_1 (2 mg) red spot R_f value 0.111 at bottom were collected.

4. RESULTS AND DISCUSSION

Ground berries part of *Embelia schimperi* (100 g) were subjected to exhaustive extraction with n-hexane, ethyl acetate and methanol separately and concentrated under reduced pressure using Rota vapor and yielded n-hexane extract (3.6 g), ethyl acetate extract (4.2 g) and a methanol extract (8.5 g). Based on TLC analysis and yield the methanol extract was selected for further isolation. Chromatographic purification of the methanol extract gave compounds coded as F_3 and F_4. The structures of these compounds have been elucidated on the basis of the following spectroscopic evidences.

4.1 PHYTOCHEMICAL SCREENING TEST

The following phytochemical tests were conducted from the methanol extract of berries of the plant using standard procedures. (Table 3)

Test for Saponins: - 0.5 g of crude powder was shaken with distilled water in a test tube and it was warmed in water bath no change is observed or no formation of froth this indicate the absence of saponins.

Test for Tannins: - 0.5 g of the crude powder was stirred with 10 mL of distilled water this was filtered and ferric chloride reagent was added to the filtrate, a blue-black ppt. was formed this confirms the presence of tannins in the extract sample.

Test for Terpenoids: - 0.5 g of crude powder was dissolved in 5 mL of methanol, 2 mL of the extract was treated with 1 mL of 2, 4-dinitrophenylhydrazine dissolved in 100 mL of 2 M HCl, yellow orange color is formed indicating the presence of terpenoids.

Test for Flavonoids: - 0.5 g of the crude powder was heated with 10 mL of ethyl acetate over a steam bath for 3 min, the mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution no change in color this confirm the absence of flavonoids.

Test for Alkaloids: - 0.5 g of the crude powder was defatted with 5 % ethyl ether for 15 min, the defatted sample was extracted for 20 min with 5 mL of aqueous HCl on a boiling water bath, then the resulting mixture was centrifuged for 10 min. 1 mL of the filtrate was treated with few drops of Mayer's reagent and a second 1 mL with Dragendroff's reagent and turbidity was observed indicating the presence of alkaloids.

Test for Steroids:- 0.5 g of the crude powder was dissolved in 5 mL of methanol, 1 mL of the extract was treated with 0.5 mL of acetic acid anhydride and cooled in ice, this was mixed with 0.5 mL chloroform and 1 mL of concentrated H_2SO_4 was added by pipette no change this indicate the absence of steroids.

Test for Glycosides:-0.5 g of the crude powder was dissolved in 5 mL of methanol, 10 mL of 50 % HCl was added to 2 mL of the extract was added to test tube and the mixture was heated in a boiling water bath for 30 min, 5 mL of Fehling's solution was added and the mixture was boiled for 5 min nothing is observed indicates the absence of glycosides.

Test for Anthraquinones:- 0.5 g of the crude powder was shaken with 10 mL of benzene and was filtered 0.5 mL of 10 % ammonia solution was added to the filtrate and the mixture was shaken well violet color in the layer phase was absorbed confirms the presence of anthraquinones.

Table 3. Phytochemical screening test results

Extract	Class of secondary metabolites	Present(+) and Absent (-)
Methanolic extract of *Embelia schimperi* berries	Saponins	-
	Terpenoids	+
	Anthraquinones	+
	Flavonoides	-
	Alkaloides	+
	Steroids	-
	Glycosides	-
	Tannins	+

4.2 CHARACTERIZATION OF COMPOUND F_3

Compound F_3 was obtained as an amorphous dark brown substance isolated from MeOH extract.

In the UV spectrum (appendix 1) revealed absorption maximum λ_{max} at 216 nm indicating that the molecule has lone pair electron chromophores.

In the IR (KBr) spectrum (appendix 5) the absorption band at 3435 cm^{-1} due to secondary amine (R_2NH) and hydroxyl group and strong absorption band at 2923 cm^{-1} and medium absorption band at 1465 cm^{-1} due to saturated C-H stretching.

^{1}H-NMR spectrum (appendix 4 and table 4) revealed signals at δ_H 2.72 (1H, m) is due to proton of methine attached to carbon bearing of secondary amine and δ_H 2.18 multiplet integrations for one proton of methine. Signals at δ_H 2.04-1.25 (14H, m) are due to methylene protons. Sharp singlet peak at δ_H 3.18 (3H) N-methyl proton and a signal at δ_H 0.88 (3H, d) is due to methyl protons.

^{13}C-NMR spectrum (appendix 2 and table 4) there were twelve carbon signals. Three tertiary carbons at δ_C 79.61, 49.05 and 29.34 and very intense signals at δ_C 79.61 which represents an sp^3 oxygenated methine bearing hydroxyl group, the signals at the δ_C 49.05 was assigned to the carbons that bearing amine part and δ_C 29.34 also carbons that links cyclohexane bearing hydroxyl group. The signals at δ_C 34.13, 31.74, 29.47, 27.06, 24.94 and 22.53 were assigned to the secondary carbons, two carbon signals at δ_C 29.47 are overlapped, and the carbon signal at the δ_C 40.21 is attributed to methyl linked to nitrogen and δ_C 14.39 stands for aliphatic methyl group.

The multiplicity of each carbon atom was determined using DEPT-135 experiment, which revealed the presence of two methyl groups (that is attached to nitrogen and the carbon bearing of alcohol), seven methylene and three methine carbons. (Appendix 3 and table 4)

Table 4. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectral data of F_3 in DMSO-d_6

Position	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	DEPT-135 (ppm)
1	2.72(1H, dt)	49.05	49.05
2	2.18(1H, m)	29.34	29.47
3	2.04(2H, dt)	31.73	31.74
4, 5	1.95(4H, m)	29.47	29.17
6	2.04(2H, dt)	34.12	34.13
N-CH_3	3.18(3H)	40.21	40.21
1`	1.34(2H, dt)	24.94	24.94
2`	1.25(2H, m)	22.53	22.53
3`	1.48(2H, dt)	27.06	27.06
4`	4.12(1H, m)	79.61	79.61
5`	0.88(3H, d)	14.39	14.39

Based on the NMR (1D), UV and IR spectra (appendix 1, 2, 3, 4, 5, and table 4) the tentative proposed structure for the compound F_3 was 2-(pent-4-ol)-N-methyl cyclohexylamine. (Fig 2)

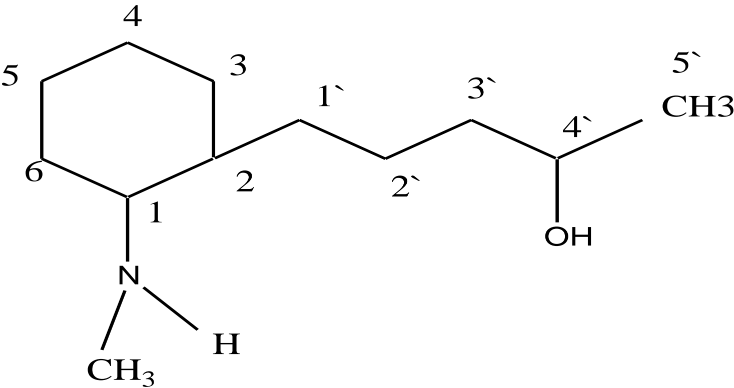


Fig. 2 The structure of compound (F_3)

4.3 CHARACTERIZATION OF COMPOUND F_4

Compound F_4 was obtained as an amorphous brown substance isolated from MeOH extract and the compound was characterized as follows.

In the UV spectrum at λ_{max} (in MeOH) (appendix 6) absorption maximum at 224nm revealed the molecule has unsaturated carbonyl chromophores ($\pi \rightarrow \pi^*$) conjugation.

In the IR (KBr disk) spectrum (appendix 10) showed absorption band at 3436 cm^{-1} due to the presence of secondary amine (R_2NH). Strong absorption band at 2925 cm^{-1} and medium absorption band at 1464 cm^{-1} due to saturated C-H stretching, strong absorption band at 1744 cm^{-1} due to ester group and absorption band at1255 cm^{-1} and 1112 cm^{-1} due to C-N stretching.

^{1}H-NMR δ_H (400 MHz, DMSO-d_6): spectrum (appendix 9 and table 5) revealed the presence of proton signals at δ_H 7.75 (2H, dd J=6.4 and 13.6 Hz), δ_H 7.575 (2H, dd J=4 and J=6 Hz) and δ_H 7.705 (1H, dd J=3.2 and 12.8 Hz) attributed to aromatic protons with a mono substituted phenyl ring, signals at δ_H 4.12 (2H, dd, J=4 Hz) due to oxygenated methylene group and signals at δ_H 1.28-1.62 (m, 8H) due to methylene protons. The signals at δ_H 2.75 (1H, dd J=4.2 Hz) showed proton of methine attached to carbon bearing of amine part. δ_H 2.25, 1.98, 1.68 and 1.5 with multiplet and integrated for four proton indicate methine. The presence of sharp singlet peak at δ_H 3.15 (3H) suggest N-methyl proton, two methyl group proton signal at δ_H 1.22 (6H, d) and one methyl group proton signal at δ_H 0.88 (3H, d, J=4 Hz).

^{13}C-NMR spectrum (appendix 7 and table 5) revealed a total of twenty-one carbon signals. Signals at δ_C 132.17, 130.05 and 129.05 attributed to mono substituted benzene ring, signals at δ_C 167.45 due to ester carbonyl group in the structure, and signal at δ_C 67.88 due to the presence of oxymethylene carbon. The signals at δ_C 30.26, 28.82, 23.71, and 22.85 were assigned to aliphatic carbons. The signal at δ_C 49.05 was assigned to the carbons that bearing amine part and signals at δ_C 38.55, 31.74, 29.44, and 27.06 were assigned for tertiary carbons of the compound. Signal δ_C 40.21 was assigned to the

methyl carbon attached to nitrogen. Signals at δ_C 14.33 and δ_C 11.22 were assigned for the methyl carbons.

The multiplicity of each carbon atom was determined using DEPT-135 experiment, which revealed the presence of four methyl groups (one is attached to nitrogen and three of them is attached to carbons), five methylene, five aromatic methane, five aliphatic methane, and two quaternary carbons. (Appendix 8 and table 5)

Table 5. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectral data of F_4 in DMSO-d_6

Position	^{1}H-NMR (ppm)	^{13}C-NMR(ppm)	DEPT-135
1	2.75(1H, dd)	49.05	CH
2	1.68(1H, dt)	29.44	CH
3	1.35(2H, dd)	28.82	CH_2
4	1.98(1H, m)	31.74	CH
5	1.62(2H, dd)	30.26	CH_2
6	2.25(1H, m)	38.55	CH
7	0.88 (3H, d, J=4 Hz)	14.33	CH_3
1``	1.28(2H, dt)	23.71	CH_2
2``	1.28(2H, dt)	22.85	CH_2
3``	1.5(1H, m)	27.06	CH
4``,5``	1.22(6H, d)	11.25	CH_3
N-CH_3	3.15(3H)	40.21	CH_3
1`	4.12 (2H, dd, J=4 Hz)	67.88	CH_2
2`	-	-	-
3`	-	167.45	Quaternary
4`	-	132.17	Quaternary
5`, 9`	7.75(2H,dd J=6.4 and 13.6 Hz)	130.05	CH
6`, 8`	7.575(2H,dd J=4 and J=6 Hz)	129.05	CH
7`	7.705(1H,dd J=3.2 and 12.8 Hz)	132.05	CH

Based on the NMR (1D), UV and IR spectra (appendix 6, 7, 8, 9, 10, and table 5) the tentative structure of compound F_4 was 2-methyl-4-(3-methyl butane)-6-(methyl benzoate)-N-methyl cyclohexylamine. (Fig 3)

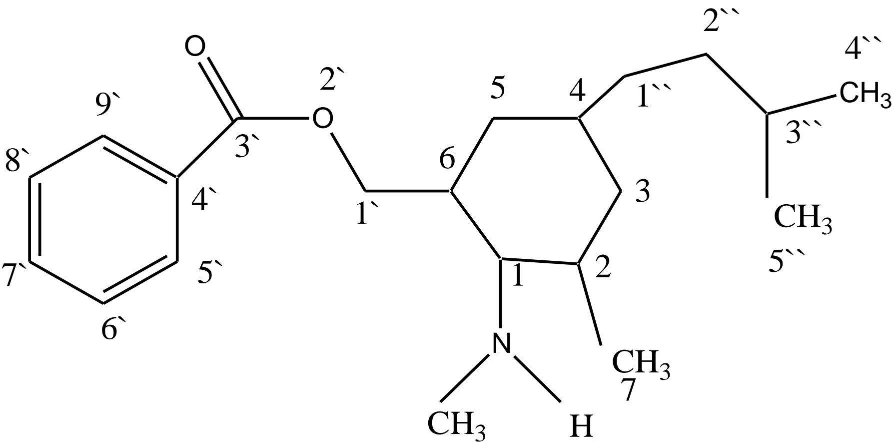


Fig. 3 The structure of compound (F_4)

5. CONCLUSION AND RECOMMENDATION

This work resulted in the isolation of two new alkaloid compounds [2-(pent-4-ol)-N-methyl cyclohexylamine (F_3) and 2-methyl-4-(3-methyl butane)-6-(methyl benzoate)-N-methyl cyclohexylamine (F_4)] isolated for first time, to the best of our knowledge, from the berries of *Embelia schimperi*. The structures of the compound were characterized on the basis of spectral data (UV, ^{1}H-NMR, ^{13}C-NMR, DEPT-135, and IR) as well as comparison with the literature data. Based on TLC analysis the plant contains several polar chemical constituents which were not isolated in this study because of financial and time constraints. It is possible to isolate more polar compounds using advanced chromatographic techniques such as MPLC and HPLC techniques with the help of reverse phase column. It is also important to evaluate the crude extracts, fractions and isolated pure compounds for antimicrobial activities to check their potential as lead compounds in the development of antimicrobial agents. Therefore, much more Phytochemical and biological study should be carried out on the plant in future.

6. REFERENCES

[1] WHO, Traditional medicines: global situation, issues and challenges, Geneva, 2011.

[2] LeQuesne, P.W.; Cooper-Driver, G.A.;Villani,M and Do,M.N. (1986) in new Trends in natural product chemistry, proceeding of the 2nd international symposium Pakistan U.S. Binational workshop, (eds. Atta-Ur-Rahman and Quesene, P.W.) Shamin printing press; Karachi, p.271.

[3] Hagos, M.; (1989) Ph.D. Dissertation ,Uppsala University, Uppsala, P.11.

[4] Bake, J.T.; Borris,R.P.; Curete.B.; Cragg.G.M.; Gupta,M.P.;lwu,M.M.; Madulid, *D.R.and Tyler,V.E.(1995*) in Natural products Drug Discovery and Development, New Perspectives on international Collaboration.*J. Nat. Prod*.; 58(9),.1325-1357.

[5] Karborne, J.B Grayer, R.J (1994) in the Flavonoids: Advance in research since 1986 (ed.Harborne,J.B.) Chapman and Hall, London, pp.589-618.

[6] Khurram, M. Studies on the isolation and characterization of secondary metabolites from Dodonaea viscosaand Quercus balootand their potential as antibacterial agents, PhD, thesis, Quaid-i-azam University, 2010; ,1-137.

[7] Shai, L.Characterization of compounds from Curtisia dentate (Cornaceae) active against Candida albicans, PhD, thesis, Pretoria University, South Africa, 2007; Pp1-177.

[8] Kamboj, A.and Kumar A.,isolation of stigmasterol and β-sitosterol from petroleum ether extract of aerial parts of ageratum conyzoides, *International Journal of Pharmacy and Pharmaceutical Sciences*, 2011;(3) 94-96.

[9] James, J.and Dubery I.Pentacyclic Triterpenoids from the Medicinal Herb, *Centella asiatica* (L.)*,* 2009;(14), 3922-3941.

[10] Muell, F.(2009), Genus *Embelia*". *PlantNET - New South Wales Flora Online*. Royal Botanic Gardens and Domain Trust, Sydney Australia. Retrieved 2009-08-11.

[11] Kokwaro, J.O.(1993), Medicinal Plants of East Africa, 2nd ed. E. A. Lit. Bureau, Nairobi, p 49 and 164.

[12] Bogh, H.O., Andreassen J., and Lemmic J. (1996), Anthelmintic usage of extracts of *Embelia schimperi* from Tanzania. *J Ethnopharmacol*. 50(1):35-42.

[13] Midiwo, J.O.Mwangi RW, Ghebremeskel Y (1995). Insect antifeedant, growth-inhibiting and larvicidal compounds from R.melanophloeos (Myrsinaceae). *Insect Sci. Appl*. 16(2): 163-166.

[14] Warrier, P.K. Nambiar VPK, Ganapati PM, Some Important Medicinal Plants of the Western Ghats, India: a Profile, Blackwell Publishers, New Delhi 2001, Pp 141-156.

[15] Zutshi, U. Johri RK, Atal CK (1989). Possible interaction of potassium embelate: A putative analgesic agent, with opiate receptors. *Ind Exp. Biol.*, 27: 656-657.

[16] Midiwo, J.O.; Arot, L.M.; Mbakaya, C.L. *Bull. Chem. Soc. Ethiop*. 1988, p 83.

[17] Pengcheng Lin, Shuai Li, Sujuan Wang, Yongchun Yang and Jiangong Shi *J. Nat. Prod.*, 2006, 69 (11), pp 1629–1632

[18] Ooko, S.A, Paul, C.K, Kipkemboi P.K, Festus Kaberia, and Andrew A.O. Naturforsch. 63 c, Pp 47-50 (2008).

[19] Kiprono, C.P., Midiwo J.O., Kipkemboi1, P.K. and Santino L, *Bull. Chem. Soc. Ethiop*. 2004, 18(1),Pp 45-49.

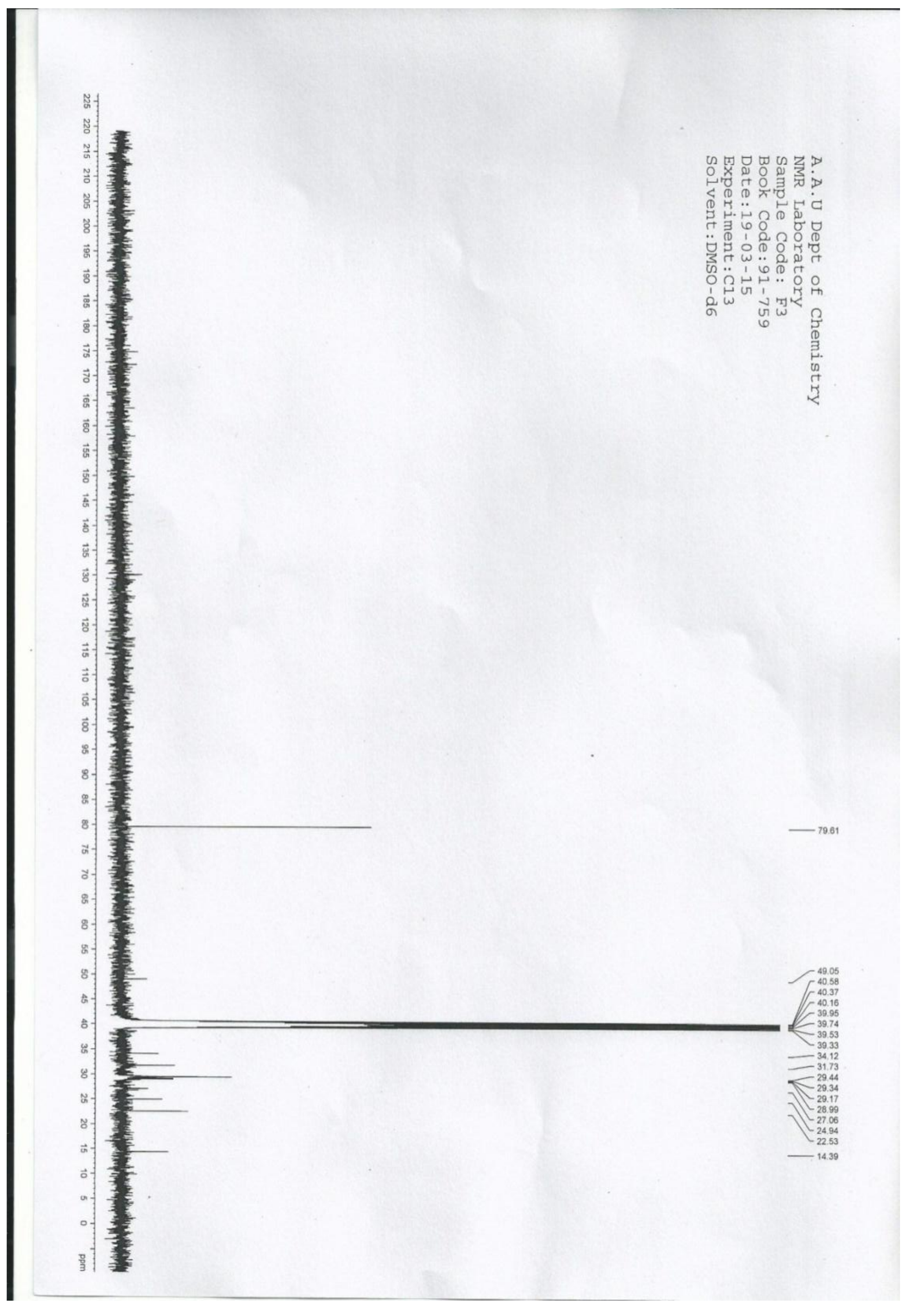
[20] Blanche, L.N, Faustine LMD, Michel F.T , Hippolyta. W, Guang-Zhi. Z, Ning-Hua. T and Pierre. T, *Rec. Nat. Prod*. 8:1 (2013) Pp 37- 40.

[21] Lawrence, O.A.M, Ivar. U and Peter. L, *Bull. Chem. Soc. Ethiop*. 2004, 18(1), 51-57.

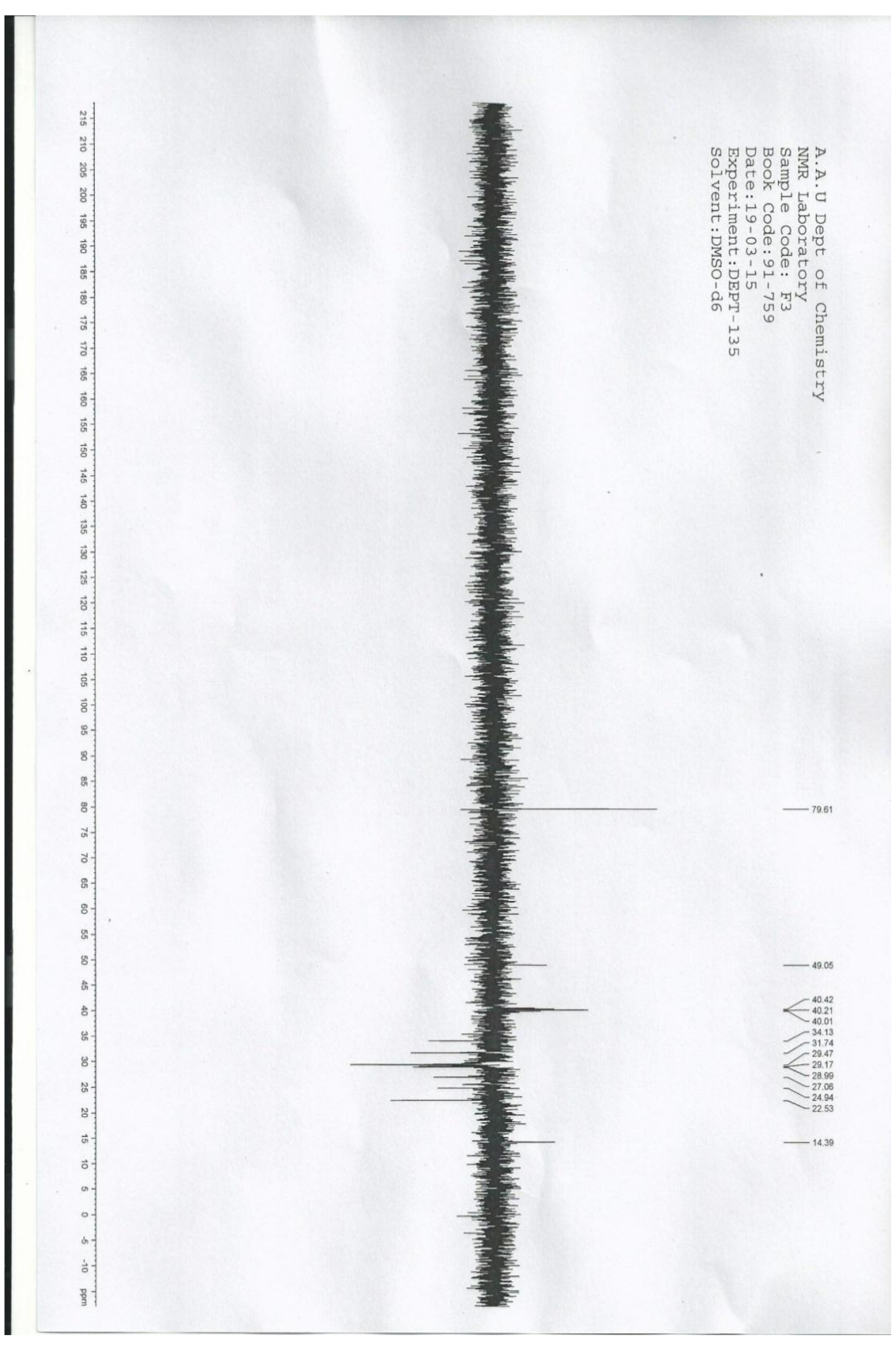
[22] Alex, K.M, Paul Kiprono, Sarina Grinberg and Shmuel Bittner isolate Pentacyclic triterpenoids from *Embelia schimperi* February 2003, Pages 573–577.

7. APPENDIXES

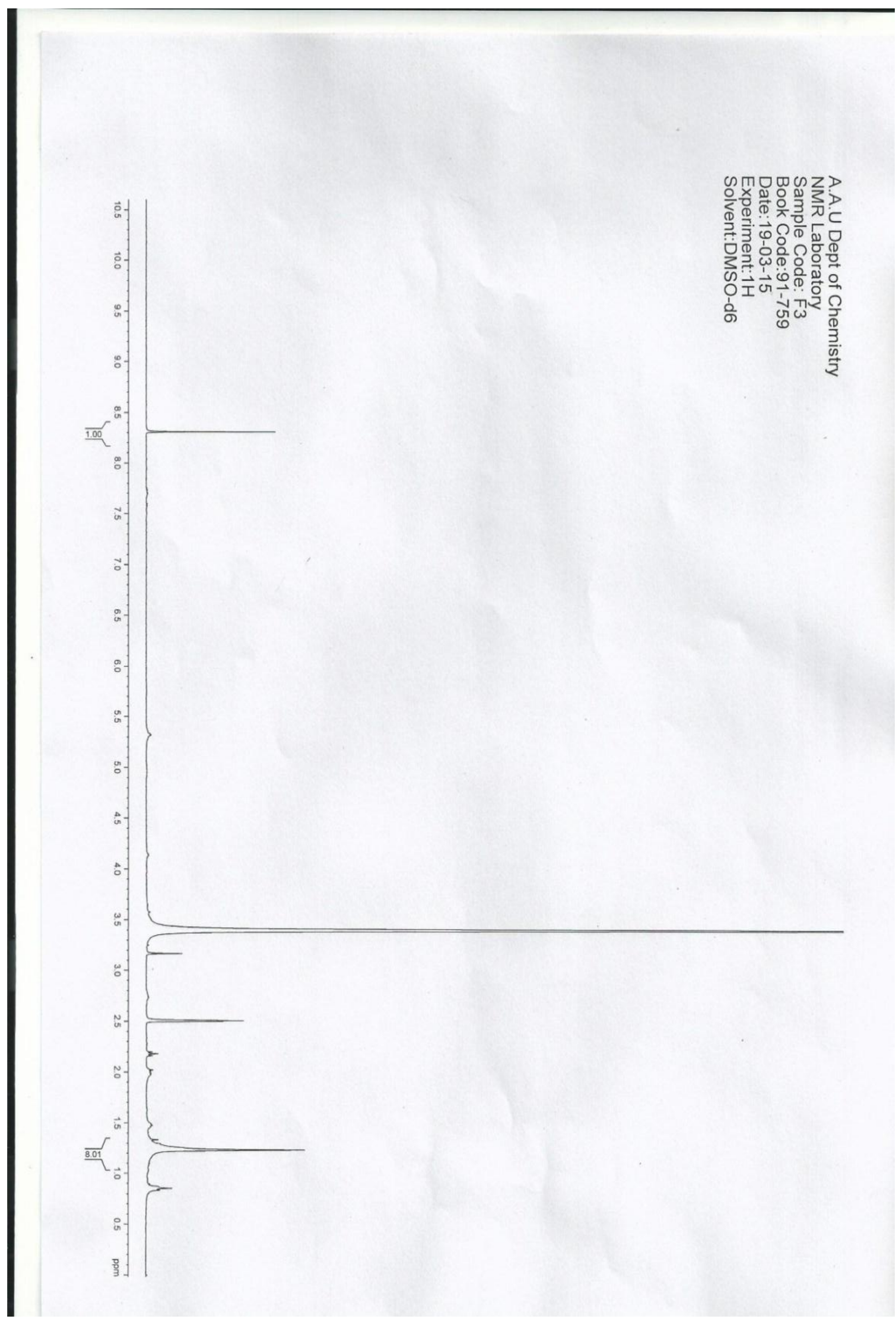
Appendix 1: UV spectrum of F_3



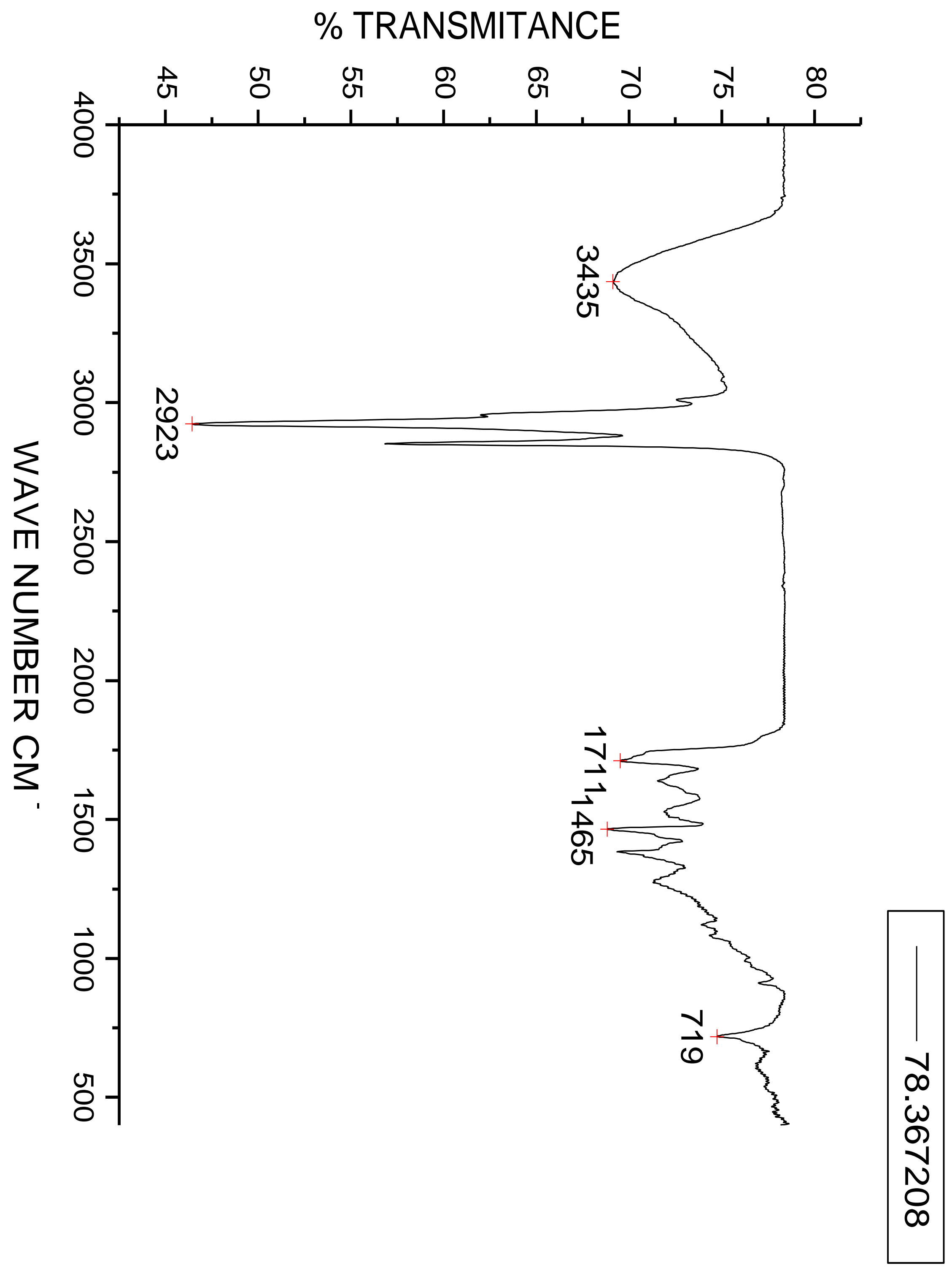
Appendix 2: ^{13}C-NMR spectrum of F_3 in DMSO-d_6



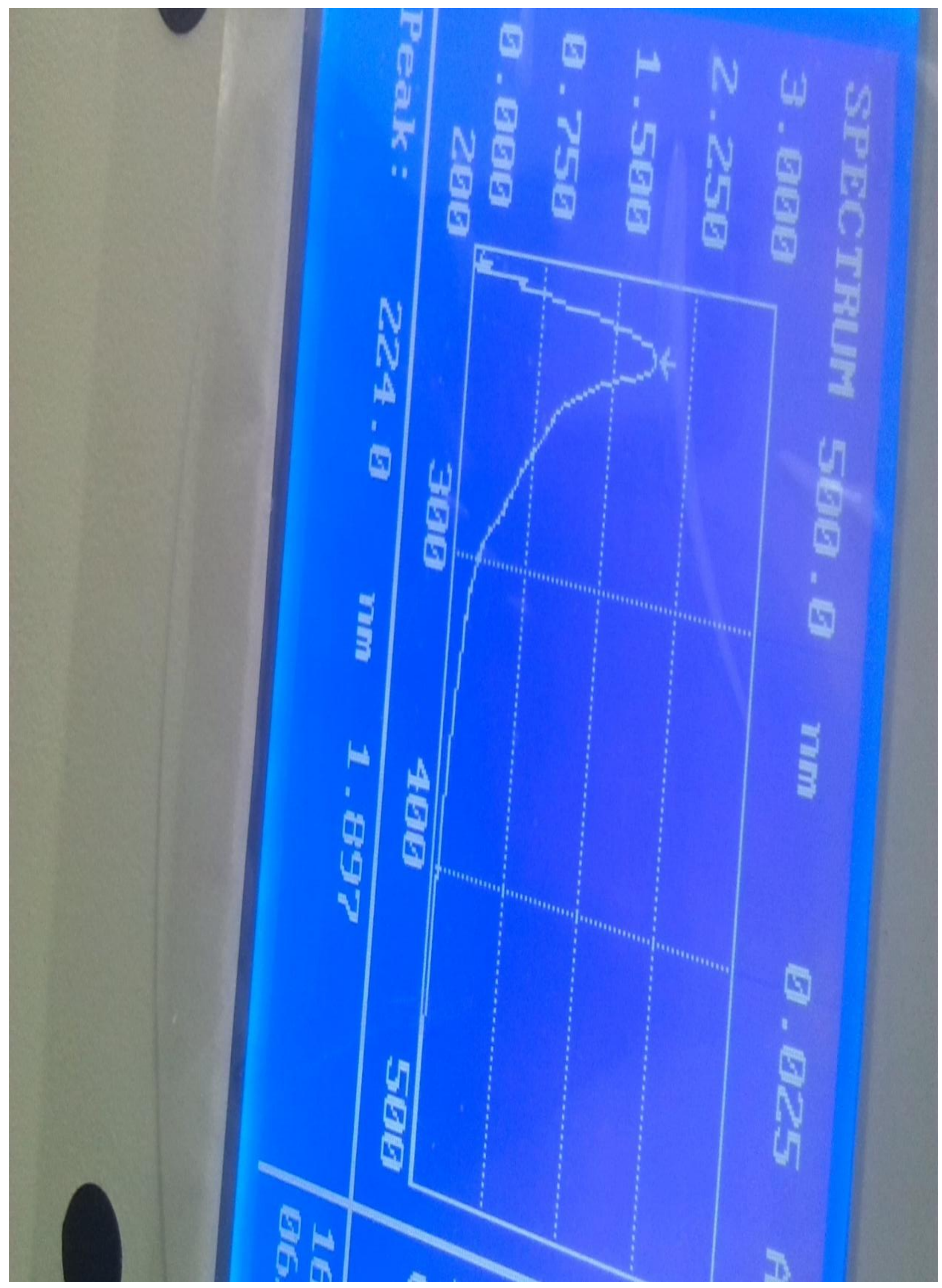
Appendix 3: DEPT-135 spectrum of F_3



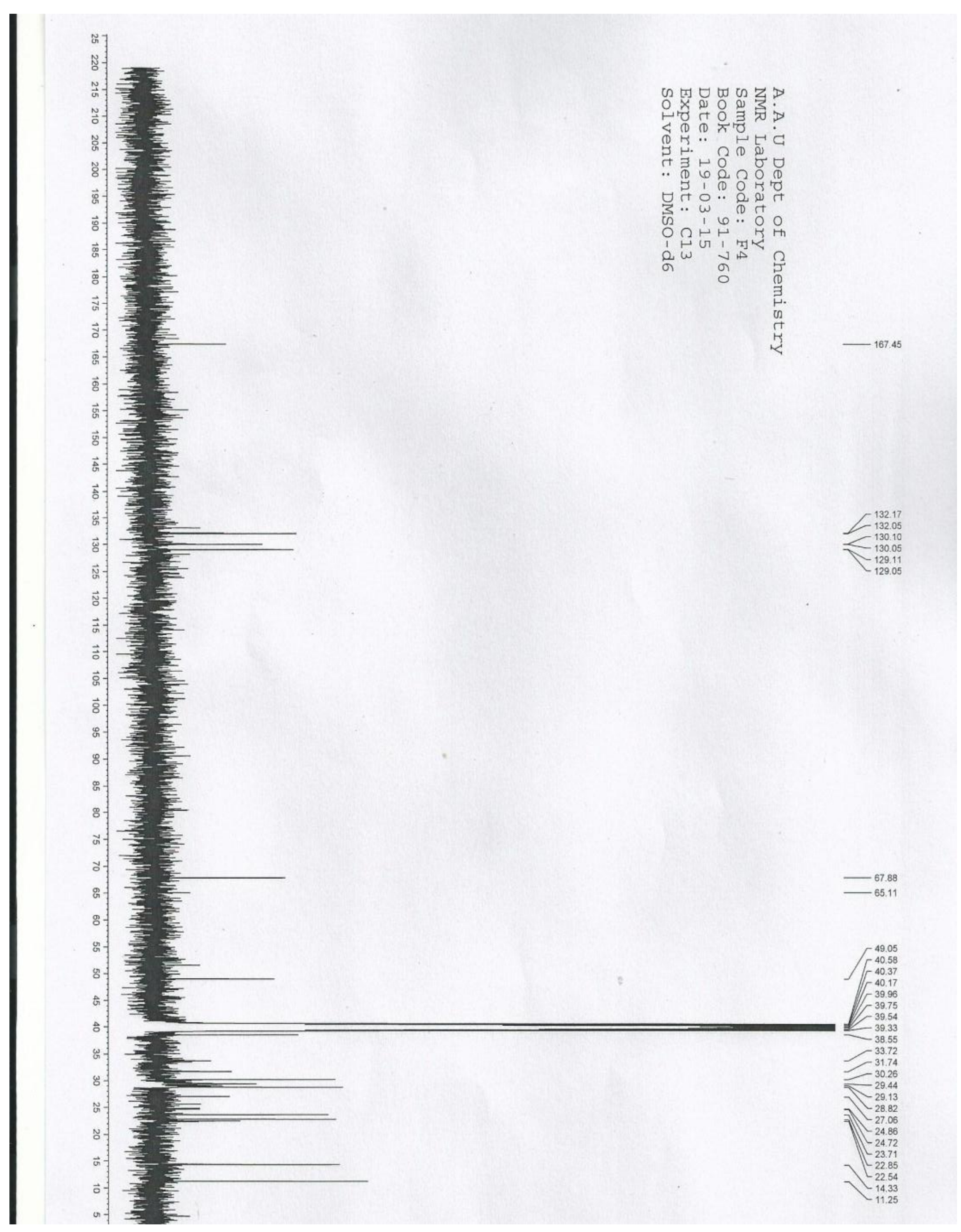
Appendix 4: ^{1}H-NMR spectrum of F_3 DMSO-d_6



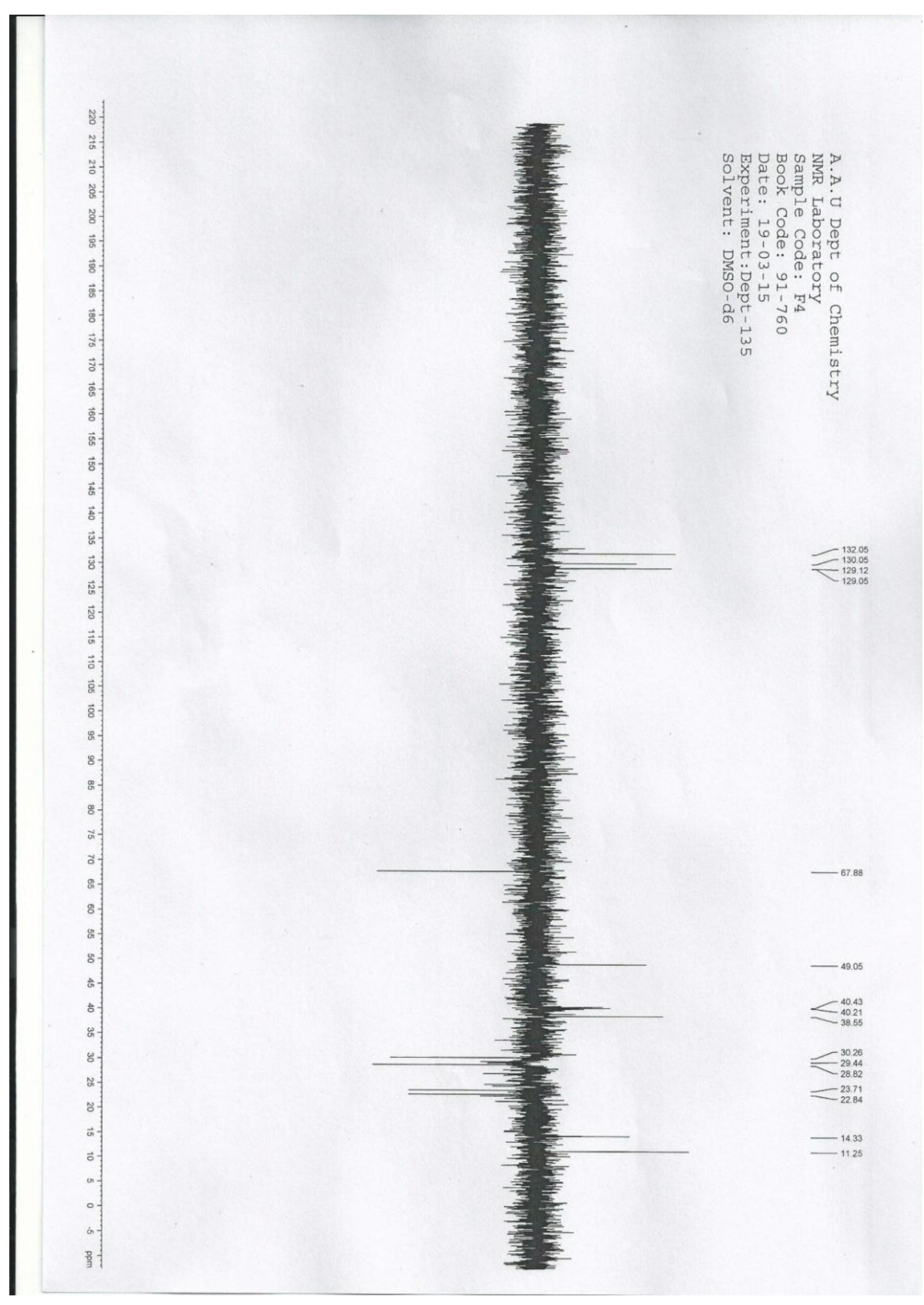
Appendix 5: IR spectrum of F_3



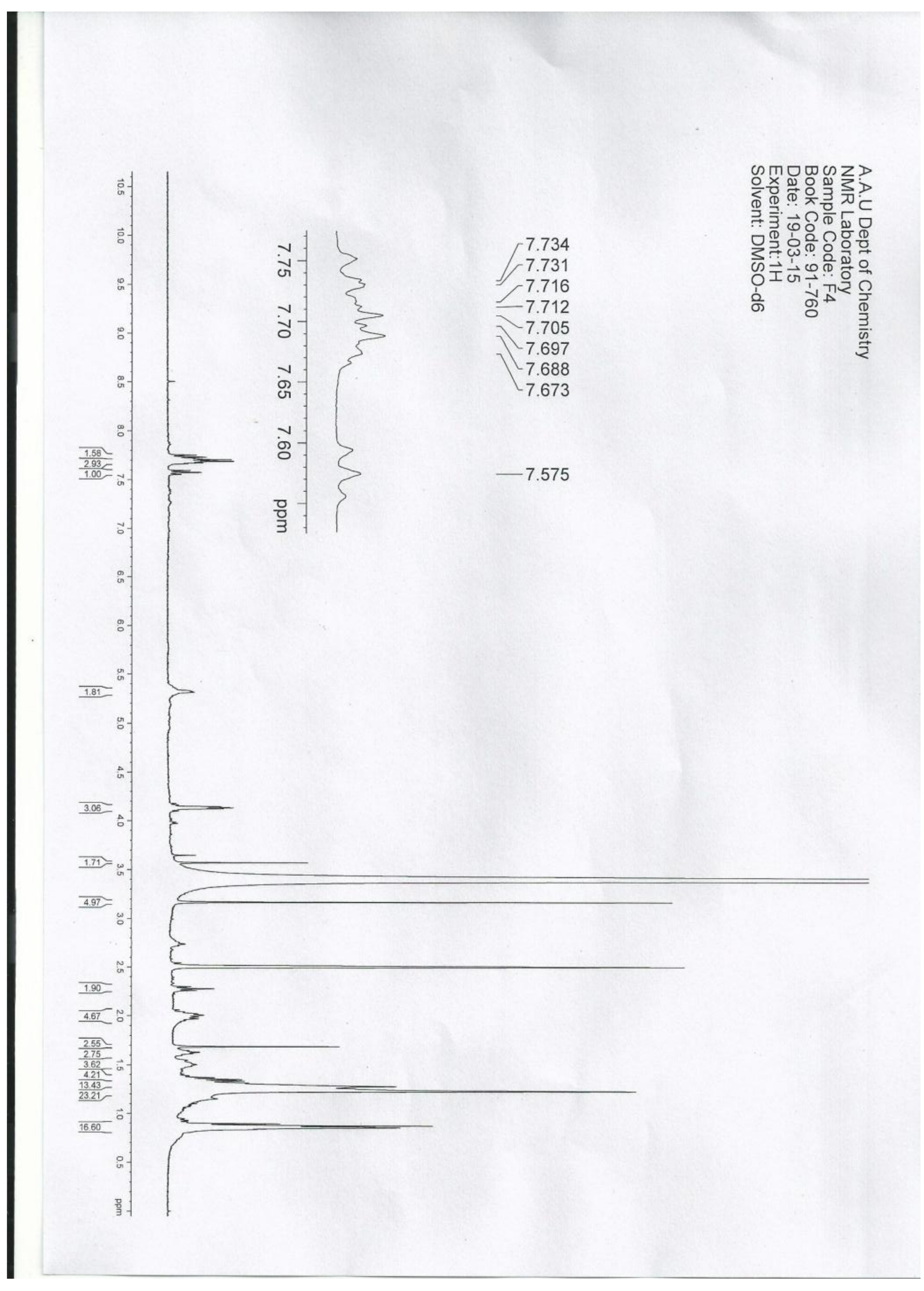
Appendix 6: UV spectrum of F_4



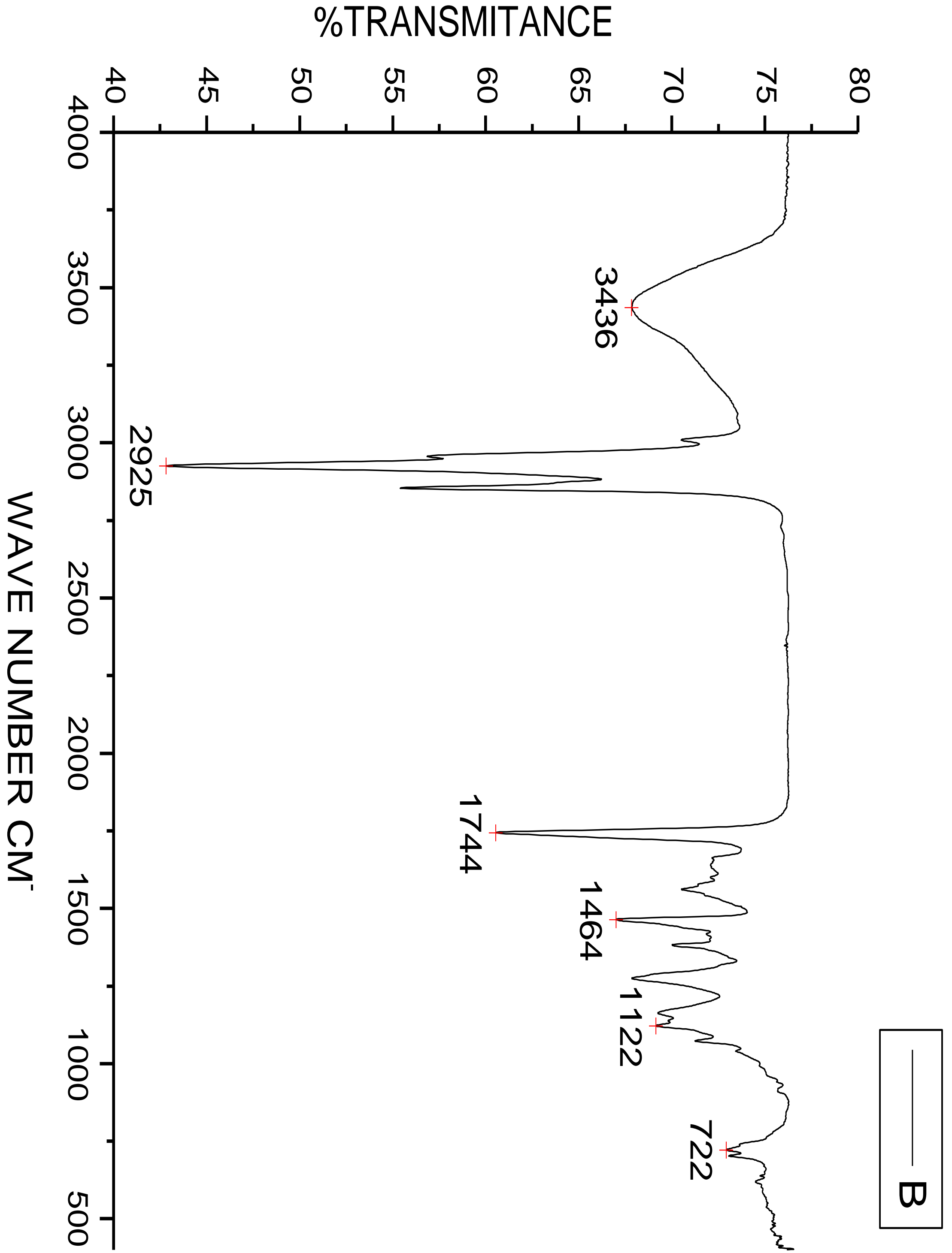
Appendix 7: ^{13}C-NMR spectrum of F_4 in DMSO-d_6



Appendix 8: DEPT-135 spectrum of F_4



Appendix 9: ^{1}H-NMR spectrum of F_4 in DMSO-d_6



Appendix 10: IR spectrum of F_4