PHYTOCHEMICAL, ANTIOXIDANT AND ANTISNAKE VENOM STUDIES OF THE METHANOL EXTRACT OF THE ROOT BARK OF *PHYLLANTHUS MUELLERIANUS* (KUNTZE) EXCELL

BY

EPHRAIM AYEGBA OKOLO

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY

FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY

ZARIA, NIGERIA

SEPTEMBER, 2014

PHYTOCHEMICAL, ANTIOXIDANT AND ANTISNAKE VENOM STUDIES OF THE METHANOL EXTRACT OF THE ROOT BARK OF *PHYLLANTHUS MUELLERIANUS* (KUNTZE) EXCELL

BY

Ephraim Ayegba OKOLO
B.SC. CHEMISTRY (KSU) 2008
M.SC./PHARM-SCI/9277/2011-2012

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES

AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE

IN PHARMACEUTICAL AND MEDICINAL CHEMISTRY

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,

FACVULTY OF PHARMACEUTICAL SCIENCE,

AHMADU BELLO UNIVERSITY,

ZARIA-NIGERIA

SEPTEMBER, 2014

DECLARATION

I declare that the work in this Thesis entitled **'PHYTOCHEMICAL, ANTIOXIDANT AND ANTISNAKE VENOM STUDIES OF THE METHANOL EXTRACT OF THE ROOT BARK OF *PHYLLANTHUS MUELLERIANUS* (KUNTZE) EXCELL'** has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Ephraim Ayegba OKOLO		
Name of Student	Signature	Date

CERTIFICATION

This thesis entitled **'PHYTOCHEMICAL, ANTIOXIDANT AND ANTISNAKE VENOM STUDIES OF THE METHANOL EXTRACT OF THE ROOT BARK OF *PHYLLANTHUS MUELLERIANUS* (KUNTZE) EXCELL** by Ephraim Ayegba OKOLO meets the regulations governing the award of the degree of Master of Science in Pharmaceutical and Medicinal Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literature presentation.

Dr. U.U. Pateh

Chairman, Supervisory Committee	Signature	Date

Prof. M.I. Sule

Member, Supervisory Committee	Signature	Date

Dr. U. U. Pateh

Head of Department	Signature	Date

Prof. Hassan Zoaka

Dean, School of Postgraduate Studies	Signature	Date

ACKNOWLEDGEMENT

First and foremost I am eternally grateful to God for making it possible for me to carry out this research work. He has been my anchor.

I also wish to express my sincere gratitude to my supervisors Dr. U.U. Pateh and Prof. M.I. Sule for their guide throughout the course of the work. Prof. M.S. Abubakar was helpful. He provided the venom for the pharmacological study. I am grateful.

My parents, Mr. and Mrs. Okolo F.O., and siblings have been of immense help and support. I cannot thank them enough. I also wish to use this opportunity to express my gratitude to Kogi State University, Anyigba for the rare opportunity of granting me a study leave to obtain a higher degree.

If I continue to mention names I will not finish. I want to express my sincere gratitude to all the staff and students of the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria. I really enjoyed a good learning and working relationship with you all.

God bless you.

DEDICATION

This research work is dedicated to God Almighty. Without Him I am nothing.

ABSTRACT

Phyllanthus muellerianus is distributed in secondary forests from Guinea-Bissau, Mali, West Cameroons and other parts of tropical Africa. It is a shrub or climber deciduous and occasionally arborescent. The root bark of this plant was extracted by maceration using methanol. The preliminary phytochemical screening of the methanol extract indicates the presence of terpenoids, flavonoids, tannins, saponins, alkaloids and carbohydrates. The methanol extract was then dissolved in water and partitioned with n-hexane, ethyl acetate and n-butanol. The partitioned n-butanol fraction was subjected to column chromatography, gel filtration chromatography and then preparative thin layer chromatography to yield a brown pentasaccharide PA - α-D-Glcp-(2→6)-[α-D-Fruf(1→2)]α-D-Fruf-(1→4)β-D-Glcp-(2→2)β-D-Fruf and sucrose PX - α-D-Glcp-(1→2)α-D-Fruf which is whitish in colour. The structures of the compounds were resolved using UV, IR, 1D- and 2D-NMR spectral results. n-Butanol was also added to the dry methanol extract. The n-butanol fraction of the dry crude extract was also subjected to gel filtration chromatography and preparative thin layer chromatography to give a brown amino alcohol glycoside CP1 – (2*S*, 3*S*, 4*S*, 5*R*)-2-((*E*)-3,10-diaminoicos-5-enyloxy)-tetrahydro-6-(hydroxymethyl)-2*H*-pyran-3,4,5-triol. The LD_{50} and LD_{99} in mice of the methanol extract and the venom of *Naja nigricollis* were determined to be 400mg/kg and 9.55mg/kg using Lorke's method and probit analysis respectively. Different doses of the methanol extract and the LD_{99} of the venom were used for *in vitro* and *in vivo* detoxification studies in mice. The extract exhibited 67% protection against lethality at an administered dose of 20mg/kg, 83% at 40mg/kg and 33% at 80mg/kg of the extract *in vitro* while there was 50% protection against lethality at an administered dose of 10mg/kg *in vivo*. The isolated pentasaccharide exhibited a free radical scavenging activity comparable to that of vitamin C at 95% confidence level using DPPH as the

standard free radical and methanol as solvent. Conclusively, the root bark of the plant is rich in saccharides and the ethnomedicinal claim as to the antivenin properties of the root bark of *Phyllanthus muellerianus* has been scientifically validated.

TABLE OF CONTENTS

Page

Cover Page……..……..…….i

Title Page…….……...……ii

Declaration…….…….iii

Certification…….…..……...iv

Acknowledgement……...v

Dedication……...vi

Abstract…….....vii

Table of Contents…….....ix

List of Tables…….……xvi

List of Figures……..……xviii

List of Plates…….....xx

List of Appendices……xxi

Abbreviations……...xxii

CHAPTER ONE

1.0 INTRODUCTION…….…1

1.1 General Introduction……1

1.2 Natural Products as Constituents of Herbal Medicine…….…….4

1.3 Problem Statement…….7

1.4 Justification of the Study……...8

1.5 Aim of the Study……8

1.5.1 Objectives……..……..……...8

1.6 Statement of Research Hypothesis……...9

CHAPTER TWO

2.0 LITERATURE REVIEW…….....10

2.1 The Plant *Phyllanthus muellerianus*…….....10

2.2 Habitat…….....10

2.3 Botanical Features…….....11

2.4 Ethnomedicinal Uses of *Phyllanthus muellerianus*…….....12

2.5 Pharmacological Actions of the Plants from the Genus *Phyllanthus*…...12

2.6 The Phytochemical Constituents of the *Phyllanthus* Genus and *Phyllanthus muellerianus* Species…….....13

2.7 Snake Bite Envenomation……....……..14

2.7.1 Classification and occurrence of snakes……..…..……14

2.7.2 Chemical constitution of snake venom…….……18

2.7.3 Biological actions of snake venom…….....…18

2.7.4 Antisnake venom plants……..……..…….19

2.7.5 Compounds from natural sources with antisnake venom activity……..19

2.8 Free Radicals, Antioxidants and Oxidative Stress……..……...25

CHAPTER THREE

3.0 MATERIALS AND METHODS……..…….27

3.1 Materials……..…….....27

3.1.1 Solvents/reagents and chromatographic materials……...27

3.1.2 Equipment…….……..……27

3.1.3 Experimental animals, and snake venom……...….28

3.1.4 Collection and identification of plant material……...…28

3.2 Methods……...……28

3.2.1 Extraction and partitioning…….…...……28

3.2.2 Preliminary phytochemical screening…….....28

3.2.2.1 Test for carbohydrates (Molisch's test)…….……...….29

3.2.2.2 Test for anthraquinones (Bontrager's test)…….....29

3.2.2.3 Test for saponins (Froth test)……..…29

3.2.2.4 Test for flavonoids (Shinoda test)……...…….…….29

3.2.2.5 Test for tannins (Lead sub-acetate and ferric chloride tests)……....29

3.2.2.6 Test for alkaloids (Dragendorf's, Meyer's and Wagner's tests)……...30

3.2.2.7 Test for terpenoids (Salkowski's test)…….…….30

3.3 Chromatographic Procedures…….....30

3.3.1 Thin layer chromatography of methanol extract and partitioned n-butanol fraction……..……...……30

3.3.2 Column chromatography of partitioned n-butanol fraction……..…….....31

3.3.3 Isolation of PA……...…….31

3.3.4 Isolation of PX……...…….32

3.3.5 n-Butanol fraction of the dry methanol extract of *Phyllanthus muellerianus*….32

3.3.6 Thin Layer chromatography of the n-butanol fraction of the dry methanol extract of *Phyllanthus muellerianus*……...33

3.3.7 Isolation of CP1……..……...33

3.4 Biological Studies…….....33

3.4.1 Acute toxicity studies……...33

3.4.2 In vitro snake venom detoxifying effect of the methanolic extract……..34

3.4.3 In vivo snake venom detoxifying effect of the methanolic extract…….34

3.4.4 In vitro antioxidant activity of PA…….……34

CHAPTER FOUR

4.0 RESULTS…….....36

4.1 Yield…….….....36

4.2 Result of Preliminary Phytochemical Screening……..……..36

4.3 Thin Layer Chromatogram of Methanol Extract (ME) and Partitioned n-Butanol Fraction (BF) Co-chromatographed on the Same Plate……38

4.4 Thin Layer Chromatograms of Column Fractions of the Partitioned n-Butanol Fraction (BF)…….....38

4.5 Thin Layer Chromatograms of Sephadex Fractions SE1-14...…….……40

4.6 Thin Layer Chromatograms of Sephadex Fractions S1-8…….....40

4.7 Thin Layer Chromatograms of Sephadex Fractions θ1-8, T1-8 and A1-10.41

4.8 Thin Layer Chromatograms of PA Developed in Different Solvent Systems and Determined Melting point……..…41

4.9 Thin Layer Chromatograms of PX Developed in Different Solvent Systems……41

4.10 Thin Layer Chromatogram of the n-Butanol Fraction of Dry Methanol Extract……..43

4.11 Thin Layer Chromatograms of SB1-16……..…….…..43

4.12 Thin Layer Chromatograms of CP1 Developed in Different Solvent System……....44

4.13 Spectral Analysis of PA ……..…….....45

4.14 1D- and 2D-NMR Spectra of PA…….....….54

4.15 Spectral Analysis of PX …….....57

4.16 1D- and 2D–NMR Spectra of PX……..…….....66

4.17 Spectral Analysis of CP1. …….....67

4.18 Summary of ^{1}H, ^{13}C, DEPT and HSQC NMR Spectral Data of CP1……..76

4.19 Summary of the COSY NMR Spectrum of CP1…….…...77

4.20 Summary of the HMBC NMR Spectrum of CP1…….….....78

4.21 Result of Acute Toxicity Studies……...…..79

4.22 *In vitro* and *In vivo* Antisnake Venom Activity of the Methanol Extract of the Root Bark of *Phyllanthus muellerianus*……..……...80

4.23 Free Radical Scavenging Activity of PA Compared to Vitamin C…….....81

CHAPTER FIVE

5.0 DISCUSSION...……..……..82

5.1 General Discussion……..82

5.2 Structure Elucidation of PA…….....83

5.3 Structure Elucidation of PX…….……...84

5.4 Structure Elucidation of CP1…….……86

5.5 Antivenin and *in vitro* Antioxidant Studies ……88

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS……...90

6.1 Summary……..…...90

6.2 Conclusion……..……90

6.3 Recommendations……..…….91

REFERENCES…….92

APPENDICES……..98

List of Tables

Table **Page**

1.1: Profile of plants used in Northern Nigeria for medicinal purposes……3

2.1: Classification of snakes according to their families……15

2.2: Venomous and non-venomous snakes and their percentage occurrence.. worldwide……...16

2.3: Occurrence of venomous species of snakes in the world……17

4.1: Results of preliminary phytochemical screening……37

4.2: ^{1}H, ^{13}C, DEPT and HSQC NMR data of α-D-glucopyranose, G1…….54

4.3: ^{1}H, ^{13}C, DEPT and HSQC NMR data of β-D-glucopyranose, G2…….54

4.4: ^{1}H, ^{13}C, DEPT and HSQC NMR data of β-D-fructofuranose, F1…….55

4.5: ^{1}H, ^{13}C, DEPT and HSQC NMR data of α-D-fructofuranose, F2…….55

4.6: ^{1}H, ^{13}C, DEPT and HSQC NMR data of α-D-fructofuranose, F3……....56

4.7: ^{1}H, ^{13}C, DEPT and HSQC NMR data of α-D-glucopyranose, G4……....66

4.8: ^{1}H, ^{13}C, DEPT and HSQC NMR data of α-D-fructofuranose, F4……..…..66

4.9: ^{1}H, ^{13}C, DEPT and HSQC NMR data of CP1……..……...76

4.10: Result of acute toxicity studies…….....79

4.11: Result of *in vitro* and *in vivo* antisnake venom studies…….....80

4.12: Free radical scavenging activity of PA compared to vitamin C…….81

5.1: Comparison of the ^{13}C NMR of PX and a synthesized L-sucrose……86

List of Figures

Figure **Page**

4.1: UV spectrum of PA……45

4.2: IR spectrum of PA……..46

4.3: Proton NMR spectrum of PA…….47

4.4: Carbon-13 NMR spectrum of PA……...48

4.5: DEPT NMR spectrum of PA……..49

4.6: HSQC NMR spectrum of PA……..…50

4.7: HMBC NMR spectrum of PA……...51

4.8: COSY NMR spectrum of PA…….……52

4.9: NOESY NMR spectrum of PA…….…….53

4.10: UV spectrum of PX…….…57

4.11: IR spectrum of PX……..…..58

4.12: Proton NMR spectrum of PX……..…….59

4.13: Carbon-13 NMR spectrum of PX……..…….60

4.14: DEPT NMR spectrum of PX…….…….61

4.15: HSQC NMR spectrum of PX……..……62

4.16: HMBC NMR spectrum of PX……63

4.17: COSY NMR spectrum of PX…….64

4.18: NOESY NMR spectrum of PX……..65

4.19: UV spectrum of CP1……..67

4.20: IR spectrum of CP1……68

4.21: Proton NMR spectrum of CP1……...69

4.22: Carbon-13 NMR spectrum of CP1……70

4.23: DEPT NMR spectrum of CP1……...71

4.24: HSQC NMR spectrum of CP1……..72

4.25: COSY NMR spectrum of CP1……..73

4.26: HMBC NMR spectrum of CP1……74

4.27: NOESY NMR spectrum of CP1……...75

List of Plates

Plate **Page**

I: *Phyllanthus muellerianus* in its natural habitat……10

II: TLC co-chromatogram of the methanol extract and partitioned n-butanol fraction…….38

III: TLC profile of column fractions 31-40……...…38

IV: TLC profile of column fractions 35-48……...……39

V: TLC profile of column fractions 49-60……...……39

VI: TLC profile of sephadex fractions SE1-14……...…..40

VII: TLC profile of sephadex fractions S1-8……...…..40

VIII: TLC profiles of sephadex fractions θ1-8, T1-8 and A1-10……...…….41

IX: TLC profiles of PA in different solvent systems……41

X: TLC profiles of PX in different solvent systems……42

XI: TLC profile of the n-butanol fraction of dry methanol extract…….43

XII: TLC profile of sephadex fractions SB1-16…….....43

XIII: TLC profiles of CP1 in different solvent systems……..44

List of Appendices

Appendix **Page**

I: A graph of administered dose versus percent survival (*in vitro* snake venom detoxifying test)……98

II: A graph of administered dose versus percent survival (*in vivo* snake venom detoxifying test)……..……98

III: A graph comparing the free radical scavenging activities of PA and vitamin C at various concentrations……..……99

ABBREVIATIONS

%	Percentage
^{0}C	Degree Celsius
^{13}C NMR	Carbon-13 nuclear magnetic resonance spectroscopy
1D-NMR	1-dimensional nuclear magnetic resonance spectroscopy
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
2D-NMR	2-dimensional nuclear magnetic resonance spectroscopy
A/NG	Australia and New Guinea
Approx	Approximate
BF	Partitioned n-butanol fraction
cm^{-1}	Per centimetre
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EME	Europe and Middle East
FTIR	Fourier transformed infrared spectroscopy
g	gramme
HMBC	Heteronuclear multiple bond coherence
HSQC	Heteronuclear single quantum coherence

kg	kilogramme
LA	Latin America
LD_{50}	Median lethal dose
LD_{99}	Minimum lethal dose
ME	Methanol extract
mg	milligramme
ml	Millilitre
NA	North America
nm	nanometre
No.	Number
NOESY	Nuclear overhauser effect spectroscopy
PAL	Phenylalanine ammonia lyase
POI	Pacific oceanic islands
ppm	Parts per million
S/No	Serial number
sp.	Species
TLC	Thin layer chromatography
UV	Ultraviolet
α	Alpha

β	Beta
δ_C	Carbon-13 chemical shift
δ_H	Proton chemical shift
μg	microgramme

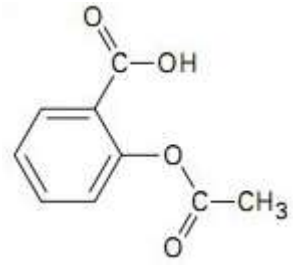
CHAPTER ONE

1.0 INTRODUCTION

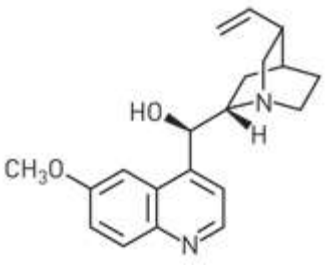
1.1 General Introduction

Traditional medicine has been defined as the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2013). For many years, it has proven to be effective in healthcare and disease prevention (Philipson, 2001). The World Health Organization has stated that in some Asian and African countries, 80% of the population primarily depend on traditional medicine for healthcare delivery. Herbal medicines, based on natural products, generate billions of dollars in revenue (WHO News, 2002). Its use is not limited to developing countries. Statistically, 70% of all medical doctors in France and Germany regularly prescribe herbal medicine (Murray and Pizzorno, 2000).

The medicinal properties of plants predate human history and are the basis of much of modern medicine. Many drugs originated from plant sources and about a century ago, most of the few effective drugs originated from plants. Examples are aspirin from willow bark, quinine from cinchona bark and digoxin from foxglove (Vickers and Zollman, 1999).



Aspirin



Quinine

The use of herbal medicine is now becoming increasingly popular (Miller, 1998). There are various reasons for this upsurge. One is the psychological comfort that it provides, especially, for patients of terminal diseases. Other reasons are safety, availability, ease of accessibility or convenience, and low cost as compared to conventional medicine (Winslow and Kroll, 1998).

Nevertheless herbal medicine can be harmful. They are heterogeneous in nature and impose challenges to quality control, assurance, and the regulatory process. Some of them contain heavy metals such as mercury, lead, arsenic, corticosteroids and poisonous organic compounds in harmful amount (Kew *et al*, 1993; De Smet, 1997; Sanjoy and Yogeshwer, 2003).

In China, Huperazine A, an extract of *Huperzia serrata* has been used for hundreds of years in the treatment of fever, swelling, blood disorders and Alzheimer's disease. It exhibits rapid absorption and penetration into the brain in animal studies. It is known to be a potent, reversible, and selective inhibitor of acetylcholine esterase (Ved *et al*, 1997).

The Gingko tree can be found worldwide but it is indigenous to Korea, Japan and China. Extracts from *Gingko biloba* have been used in the treatment of circulatory problems, asthma, vertigo, fatigue, and tinnitus. *Gingko biloba* was also used in the United States to treat age-related physical and cognitive disorders (Topic *et al*, 2002).

Since ancient times, the rhizome *Rhodiola rosea* has been used in East Asia as a tonic and an anti-aging agent. Evidences point to the fact that its extract possesses significant neuroprotective activity and antioxidative effects. Salidroside is one of the major compounds present in the water extract of the plant (Zhang *et al*, 2010).

A profile of plants used in Northern Nigeria for different purposes is given below. The plants were authenticated at the Herbarium Unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria (Aliyu *et al*, 2008).

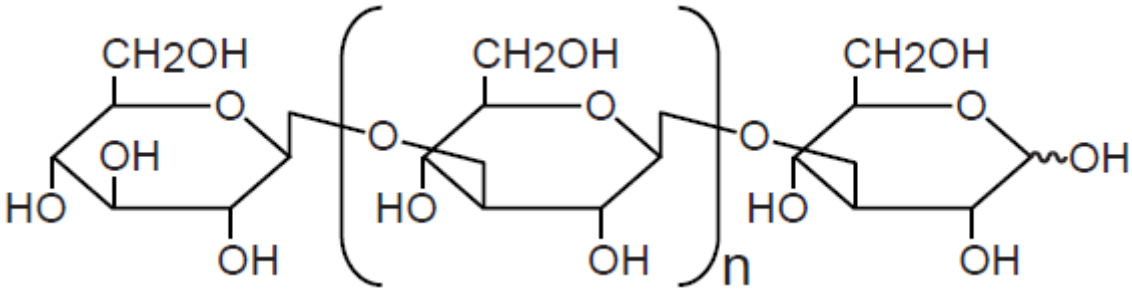
Table 1.1: Profile of plants used in Northern Nigeria for medicinal purposes

Botanical Name	Family	Local Name	Part used	Voucher number	Medicinal uses
Acacia albida Del.	Leguminosae	Gawo	Stem bark	900334	Skin infections
Anchomanes difformis Engl.	Araceae	Chakara	Roots	900283	Cough and respiratory diseases.
Boscia senegalensis (PERS) Lam.	Capparidaceae	Anza	Roots	6921	Cancer, ulcer and swellings
Bridelia ferruginea Benth.	Euphorbiaceae	Kirni	Stem bark	1750	Antibiotic, skin infection, diuretic, febrifuge and urethral discharge.
Ficus ingens (Miq.) Miq.	Moraceae	Kawuri	Stem bark	1504	Piles,diarrhea, laxative and diuretic.
Indigofera arrecta Hochst	Papilionoideae	Ba-ba	Aerial part	663	Infections, stomach pain, snake bite and epilepsy.
Indigofera pulchraI Willd	Papilionoideae	Bakin bunu	Aerial part	409	Infected wound,malaria, dysentery and fever.
Moringa oleifera Lam.	Moringacceae	Zogale	Leaf	571	Asthma, eye infection, migraine headache, febrifuge, abortifacient.
Mormodica basalmina Linn	Cucurbitaceae	Garahuni	Whole plant	3272	Laxative, antibiotic, antitumour, hypotensive, purgative.
Pavetta crassipes K. Schum.	Rubiaceae	Gadau	Leaf	3115	Respiratory diseases, hypertension.
Phyllanthus amarus Schumach & Thonn.	Euphorbiaceae	Geron tsuntsaye	Whole plant	3073	Fever, diarrhea, kidney ailments, ringworm, urinary tract infections, hemorrhoids and gonorrhea.
Vernonia blumeoides Hook. f.	Asteraceae	Bagashi	Aerial part	1784	Stomach or intestinal pain.

1.2 Natural Products as Constituents of Herbal Medicine

Natural products can be defined as organic compounds that are produced by living systems. They may be classified into three broad categories which are: the primary metabolites such as sugars, common amino acids and nucleic acids; the high-molecular-weight polymeric materials such as cellulose, lignins and proteins; and the secondary metabolites such as terpenoids, steroids, phenylpropanoids, alkaloids and others. Primary metabolites occur in all cells and are crucial to cell metabolism and reproduction while the high molecular weight polymeric materials form the cellular structures. Secondary metabolites are characteristic of a limited range of species. Their roles are not clear but they can function as defensive substances, antifeedants, attractants and pheromones (James, 2003). The three classes discussed above are known to have biological activity.

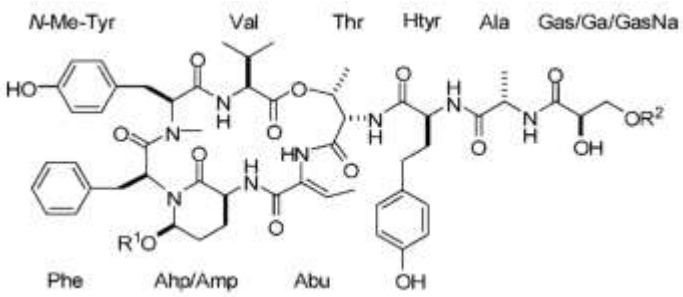
There is abundant literature concerning the biological activity of natural polysaccharides and oligosaccharides. ASLP is a polysaccharide isolated from a marine source that is known to affect the immune system. It does this by increasing the proliferation of splenocyte. Oligosaccharides with antioxidant, antitumor and immune-stimulation activities are known. They are obtained from fungi, land plants or algae containing only neutral sugars such as laminari-oligosaccharides corresponding to β-(1→3)-D-glucans (Josiane, 2009; Mayer *et al*, 2011). Fondaparinux the first synthetic pentasaccharide anticoagulant was derived from a natural polysaccharide, heparin.



Laminari-oligosaccharides

1. laminaribiose (n=0)
2. laminaritriose (n=1)
3. laminaritetraose (n=2)
4. laminaripentaose (n=3)
5. laminarihexaose (n=4)
6. laminariheptaose (n=5)

The biological activities of peptides and proteins are countless. Mirabamides A, C and D which are peptides isolated from sponge are known to have antiviral activity. They inhibit HIV-1 fusion. Lyngbyastatins 5-7 isolated from bacterium are also peptides that are known to possess anti-inflammatory properties through the inhibition of the elastase enzyme (Mayer *et al*, 2011).

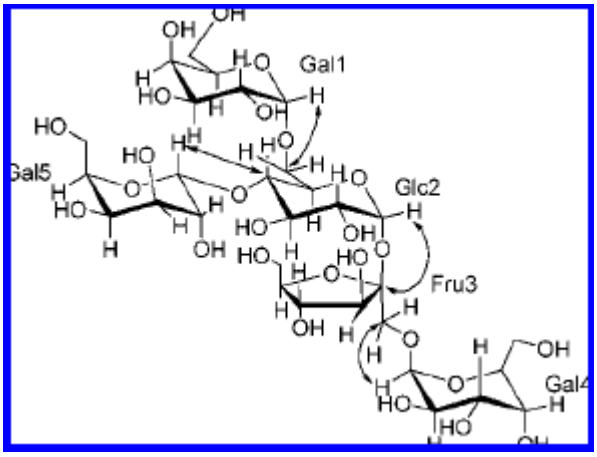


Lingbyastatin 5 $R^1 = H$ $R^2 = SO_3H$

Lingbyastatin 6 $R^1 = H$ $R^2 = H$

Apart from marine sources, oligosaccharides and peptides have been isolated from plants. Examples of peptides isolated from the seed of *Impatiens balsamina* are 1b-AMP1, 1b-AMP2, 1b-AMP3, and 1b-AMP4 with the following amino acid sequence respectively: GRRCCGWGPGRRYCVRWX, GRRCCNXGPGRRYCKRWC, RHRCCAWGPGRKYCKRWC and GRRCCGWGPGRRYCRRWC (Tailor *et al*, 1997).

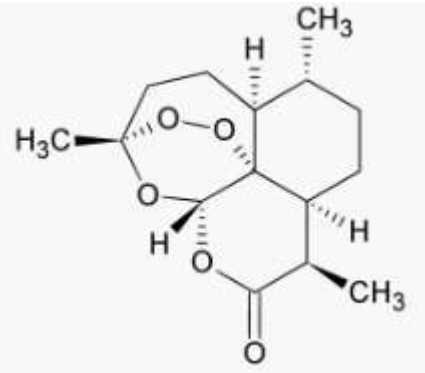
Examples of oligosaccharides isolated from plants are stellariose, a pentasaccharide from *Stellaria media* (Vanhaecke *et al*, 2008); a heptasaccharide and an octasaccharide isolated from *Paris polyphylla* (Zhou *et al*, 2003).



Stellariose with its HMBC connectivities (Vanhaecke *et al*, 2008)

Secondary metabolites, on the other hand, are widely known for their biological activity on other organisms and hence have attracted a lot of interest (Philipson, 2001). Examples are morphine and artemisinin which are used even today in orthodox

medicine. Morphine is an opium alkaloid obtained from *Papaver somniferum* while artemisinin is obtained from *Artemisia annua*.



Artemisinin

1.3 Problem Statement

(i) Envenomation from snake bites is an important yet neglected threat to public health (Kasturiratne, 2008).

(ii) At least 421,000 envenoming and 20,000 deaths occur worldwide yearly. It could be as high as 1.8 million envenoming and 94,000 deaths (Kasturiratne, 2008).

(iii) The highest rate of snake envenoming and death occur in South, South-east Asia and sub-Saharan Africa (Kasturiratne, 2008).

(iv) It has been reported that the incidence of bites in Nigeria is 497 per 100,000 population yearly, with a 12% natural mortality (Pugh and Theakston, 1980).

(v) In the northern savanna region, the incidence rate is 48 per 100,000 population with a mortality of 5.1% (Sasidharan *et al*, 2011).

(vi) Free radicals are implicated in aging, and other diseases such as cancer, cardiovascular diseases and so on (Sies *et al*, 1992).

1.4 Justification of the Study

(i) Snake bite is a major medical problem in the world and in Nigeria from the statistical known rates of envenoming and death.

(ii) Snake venom antisera are very expensive and beyond the reach of the common man in Nigeria and other African countries.

(iii) Nigeria being in the tropics is blessed with abundant flora. Therefore discovery of compounds having antisnake venom effect will be a great source of foreign exchange and economic growth.

(iv) The scientific investigation of the antisnake venom activity of the root bark extract of *Phyllanthus muellerianus* would confirm or otherwise disprove its traditional use.

(v) A range of diseases caused by free radicals can be prevented if a viable exogenous antioxidant can be isolated from the plant.

1.5 Aim of the Study

To characterize and evaluate the antioxidant property of isolated phytochemical(s) from the methanol extract of the root bark of *Phyllanthus muellerianus* and to verify the traditional claim as to its use in the treatment of snake bite.

1.5.1 Objectives

(i) To isolate and characterize compounds from the methanol extract of the root bark of *Phyllanthus muellerianus* using chromatographic techniques.

(ii) To determine the LD_{50} of the methanol extract of the root bark of *Phyllanthus muellerianus* in experimental mice.

(iii) To determine the *in vitro* and *in vivo* detoxifying effect of the methanol extract of the root bark of *Phyllanthus muellerianus* against the venom of *Naja nigricollis* in mice.

(iv) To determine the *in vitro* antioxidant activity of isolated phytochemical from the root bark of *Phyllanthus muellerianus* using DPPH as a standard free radical.

1.6 Statement of Research Hypothesis

The methanol root bark extract of *Phyllanthus muellerianus* possesses significant activity against the venom of *Naja nigricollis* at doses less than or equal to 30% of the LD_{50} and the isolated phytochemical possesses a significant *in vitro* antioxidant activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Plant *Phyllanthus muellerianus*

The plant *Phyllanthus muellerianus* belongs to the *Phyllanthus* genus which is in the Phyllanthaceae family. The species of the *Phyllanthus* genus range from 750-1200 with a well known diversity that includes annual and perennial herbs, shrubs, climbers, succulents, and floating aquatics. *Phyllanthus muellerianus* is a shrub or climber. Leaf flower is the most common name for all species belonging to this genus (Sheeja, 2012).

2.2 Habitat

Phyllanthus muellerianus is distributed in secondary forests from Guinea-Bissau, Mali, West Cameroons and other parts of tropical Africa.



Plate I: *Phyllanthus muellerianus* in its natural habitat

2.3 Botanical Features

It is a shrub or climber, deciduous and occasionally arborescent. It rarely develops a large stem. It is reported as rice weed in the Bendel region of Nigeria, and in Kenya it is known to be excellent firewood. Leaf flower is the most common name for all species belonging to this genus. Local names in Nigeria are kumci in Hausa, anya nunu in Igbo and arunjeran in Yoruba (Burkill, 1994; Sheeja, 2012).

It has about four to five sepals and stamens. The sepals are drying white in colour and are about 1.5mm long. The young fruits are conical, beaked or fibrous. The inflorescences are similar to the barren leafy shoots. The upper inflorescences are all male and sometimes with leaves while the lower ones are often bisexual. The leaves are about 2.5-7cm long and 2-4cm broad. The shape of the leaves are ovate, obtuse or slightly cuneate at base; acutely acuminate, and paler beneath (Hutchinson *et al*, 1958).

The genealogy of the plant is given as follows.

Kingdom: Plantae

Phylum: Tracheophyta

Order: Magnoliopsida

Class: Malpighiales

Family: Phyllanthaceae

Genus: Phyllanthus

Species: Muellerianus

2.4 Ethnomedicinal Uses of *Phyllanthus muellerianus*

The roots of *Phyllanthus muellerianus* are widely used for intestinal disturbances. In Ghana, the root is cooked with maize meal or other plant drugs for severe dysentery. In Congo (Brazzaville), the powdered root charred is taken with palm oil for stomach upsets and as an antiemetic. The aqueous extract of the powdered root is drunk for diarrhoea in Tanganyika. In Ivory Coast – Upper Volta, the powdered roots are used together with a bark decoction in draught and enema for stomach pains. The young root with young leafy twigs is given for jaundice in Nigeria. The powdered dried root and bark are sprinkled on wounds as a dressing in Tanganyika. The root is cut into small pieces with those of *Psychotria calva* (Rubiaceae), *Harrisonia abyssinica* (Simaroubaceae) in Ghana and drunk for cough. It is also recommended for whooping cough. The dried bark powder is taken in Congo (Brazzaville) for colds and sinusitis (Burkill, 1994).

In Ivory Coast, the leaves are believed to promote male fertility and a leaf decoction is taken for anaemia in Congo (Brazzaville). An infusion of young shoots is reputed to be remedy for chronic dysentery and chest complaints. Leaf sap is used as a wash for fevers and skin eruptions, and eye troubles. Leafy twigs with a pulp are topically applied on the body as cure for paralysis. In Ubangi, the leaf decoction is used as a mouthwash for toothache after the application of cooked leaves to the gums (Burkill, 1994).

2.5 Pharmacological Actions of the Plants from the Genus *Phyllanthus*

The pharmacological actions of *Phyllanthus sp.* are numerous. *P. corcovadensis, P. caroliniensis and P. flexuosus* are known to have analgesic activity. *P. Sellowianus, P. urinaria, P. niruri* among others are known to possess anti-inflammatory and analgesic

activity among other species demonstrating various kinds of pharmacological actions (Joao *et al*, 1998)

Additionally the antimicrobial properties of the extracts of different parts of *Phyllanthus* species are widely published. *Phyllanthus muellerianus* stem bark extracts using methanol and water as solvents have been found to have activity against *Clostridium sporogenes*, *Streptococcus mutans*, and *Streptococcus pyogenes*. The traditional use against wound infections and tetanus is validated by its activity against *Streptococcus pyogenes* and *Clostridium sporogenes* (Brussotti *et al*, 2011).

Ethnopharmacological claims list the following species: *P. amarus* (whole plant) [Walter *et al*, 2000]; *P.emblica* (fruit juice), *P. niruri* (flower paste), and *P. reticulates* (leaf infusion) [Inder and Devang, 2000], as having antisnake venom activity. The *P. muellerianus* species was not mentioned in the literatures as having antisnake venom activity. However, there is a folkloric claim in the eastern part of Kogi State, Nigeria, asserting the efficacy of the root bark of *Phyllanthus muellerianus* against snake venom.

2.6 The Phytochemical Constituents of the *Phyllanthus* Genus and *Phyllanthus muellerianus* Species

A great number of *Phyllanthus sp*. has been phytochemically investigated and many compounds have been isolated and identified. These compounds include alkaloids, flavonoids, terpenoids, steroids, lipids and sugars (Joao *et al*, 1998)

Lupeol, β-amyrin, phyllanthol and phyllanthoside have been isolated from *P. acidus*. Corilagin, chebulagic acid, bergenin, campesterol and phyllanthusiin A-E have been isolated from *P. flexuosus*. Phyllanthymide, xanthoxyline, scopoletin, glochidiol, levulose and saccharose have been isolated from *P. sellowianus* (Joao *et al*, 1998)

Compounds that have been reported to have been isolated from the diisopropyl ether extract of the bark of *P. muellerianus* are β-sitosterol, friedel-1-ene-22-β-ol, and friedellin-1-β-22-β-diol (Joao *et al*, 1998; Adesida *et al*, 1972).

2.7 Snake Bite Envenomation

Envenomation as a result of snake bites is an important public health problem in many tropical and subtropical countries (Kasturiratne, 2008). These envenomations take place in rural areas where subsistence farming is predominant. About 600 snakes are venomous out of the 3000 species that exist worldwide. Snakebites are either for defence against threat or for attack against provocation. *Viripedae* (pit vipers) and *Elapidae* (kraits and cobras) are families of snakes whose bites are particularly harmful to man. The potentially fatal effects of snake envenoming range from widespread bleeding, muscle paralysis, tissue destruction (necrosis) around the site of bite, permanent disability and even death (Kasturiratne, 2008).

2.7.1 Classification and occurrence of snakes

Snakes belong to the class *Reptilia* and order *Squamata*. They can further be sub-classified into various families (Smith *et al*, 1977; Wright and Wright, 1957a; Wright and Wright 1957b) or according to whether they are venomous or not. They are cold-blooded animals (Reid and Theakston, 1983; Rusell, 1980). The classifications are tabulated below.

Table 2.1: Classification of snakes according to their families (Smith *et al*, 1977; Wright and Wright, 1957a; Wright and Wright 1957b)

Family Name	Common Name	No. of Genus	Approx no. of *sp*.
Anomalepidae	Dawn blind snakes	4	15
Typhlopidae	Blind snakes	6	225
Leptotyphlopidae	Thread snakes	2	90
Anomochilidae	Dwarf pipe snakes	1	2
Anilidae	Pipe snakes	1	1
Cylindophidae	Asian pipe snakes	1	10
Uropeltidae	Shield tail snakes	8	45
Loxocemidae	MBP, CBP etc.	1	1
Xenopeltidae	Sunbeam snakes	1	2
Pythonidae	Pythons	8	30
Boidae	Boas	9	35
Boyleriidae	Round Island boas	2	2
Tropidophidae	Dwarf Boas	4	25
Acrochordidae	File snakes	1	3
Colubridae	Colubrine snakes	296	1800
Atractaspididae	Stilleto snakes, MV	11	65

Viperidae	Vipers, rattle snakes	33	240
Elapidae	Cobras, coral snakes etc	49	250
Hydrophiidae	Sea snakes	18	60

Table 2.2: Venomous and non-venomous snakes and their percentage occurrence worldwide is given below (Reid and Theakston, 1983; Rusell 1980)

Type	Percentage occurrence worldwide
a. Non-venomous snakes	
Non-venomous colubrids	64%
Blindsnakes	12%
Boas and related species	5%
b. Venomous snakes	
Vipers	8%
Cobras and related species	10%
Venomous colubrids	1%

Table 2.3: Occurrence of venomous species of snakes in the world (Wright and Wright, 1957b; Tan and Gnanajothy, 1990; Pugh and Theakston, 1980)

Family or sub-family	Number of species occurring in various regions
Colubridae	Latin America (LA) – 1, Africa – 2, Asia – 1
Atractaspidinae	Europe and the middle east (EME) – 2, Africa – 16
Acanthophiinae	Australia and New Guinea (A/NG) – 94, Pacific oceanic Islands (POI) - 4
Elapinae	North America (NA) – 2, LA – 54, EME – 3, Asia – 34
Hydrophiinae	NA – 1, LA – 1, EME – 6, Africa – 1, Asia – 33, A/NG – 31, POI-4
Laticaudinae	Asia – 9, A/NG – 2, POI – 3
Azemiopinae	Asia – 1
Viripinae	EME – 16, Africa – 31, Asia – 6
Crotalinae	NA-17, LA-89, EME-1, Asia-44
Total	NA-20, LA-145, EME-28, Africa-75, Asia-128, A/NG-127, POI – 11

2.7.2 Chemical constitution of snake venom

Snake venom is a complex mixture of proteins which are about fifty to sixty components and each protein has its biological activity. The composition and activity of a snake venom depends on the species the snake belongs. The closer the phylogenetic relationship between snakes, the more similar the composition and properties of their venoms will be (Singh and Tu, 1996).

2.7.3 Biological actions of snake venom

Different types of toxic effects result from snake bite envenomations because snake venom is a mixture of different proteins containing both toxic and non-toxic components (Singh and Tu, 1996).

The toxic components exhibit toxicity to varying degrees. The neurotoxins are usually the most potent. Therefore envenomations by cobra, kraits and sea snakes are very toxic since they contain potent neurotoxins. Such envenomations may lead to muscle paralysis and possibly death if poisoning is severe (Singh and Tu, 1996).

On the other hand, most rattle snakes contain few neurotoxins but contain potent tissue damaging toxins such as haemorrhagic and myonecrotic toxins. Envenomation by most common rattle snakes may therefore lead to haemorrhage and muscle damage of tissues and organs with a possible dysfunction or complete loss of a body part such as the toes or fingers (Singh and Tu, 1996).

Apart from toxic effects, snake venoms are known to have medicinal uses. They have been found to have fibrinogenolytic and fibrinolytic activity, cardiotonic and antiarrythmic activity, antineoplastic activity, antiparalytic, and antiarthritic activity.

Snake venom toxins could serve as leads for drug design to fight cardiovascular disorders, neurological problems and neoplastic growth (Sanjoy *et al*, 2002).

2.7.4 Antisnake venom plants

The antisnake venom properties of various plants have been widely reported in the literature.

Six extracts (leaf) of *Pouzolzia indica* were evaluated for antisnake venom activity against the Russell viper venom. Of these, the ethanolic and water extracts possessed the most significant activity by decreasing the inhibition of platelet aggregation and also by inhibiting the PAL enzyme. However, of the two extracts, the alcoholic extract possessed higher activity (Anas *et al*, 2010).

The methanol extract of the root bark of *Annona senegalensis* was found to reduce the mortality and significantly inhibit the onset, and severity of neurotoxic signs induced by the venom *Naja nigricollis nigricollis* Wetch. Both inhibition of animal lethality and *in vitro* detoxification effects were determined (Adzu *et al*, 2005).

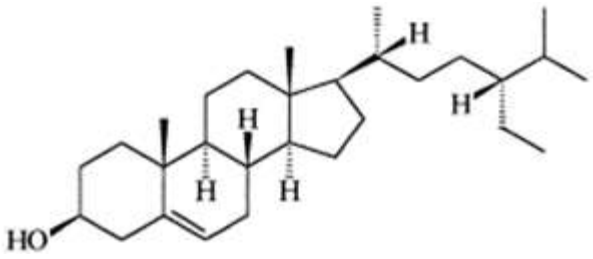
It was also found that the leaf extract of *Guiera senegalensis* using methanol as solvent possesses an in vitro activity against the venoms of *E. carinatus* and *N. nigricollis* (Abubakar *et al*, 2000).

Walter *et al*, 2000 has reported the antivenin effects of several plants, some of which are indigenous to Africa.

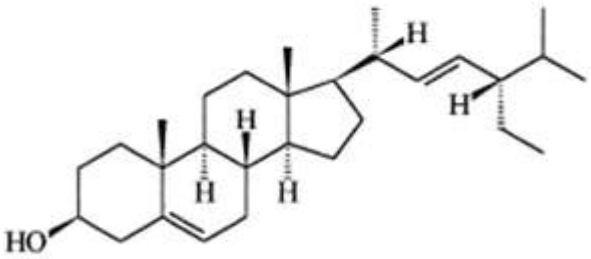
2.7.5 Compounds from natural sources with antisnake venom activity

Both primary and secondary metabolites present in plants are known to possess antisnake venom activity.

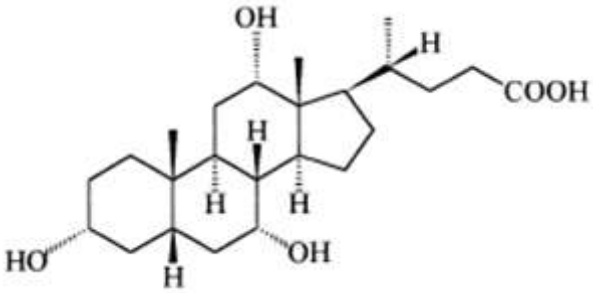
Steroids with such activity are β-sitosterol - 70% protection; stigmasterol, cholic acid, dehydrocholic acid - 80% protection; cholesterol - 60% protection; tigogenin - 40% protection and hecogenin - 20% protection (Walter *et al*, 2000).



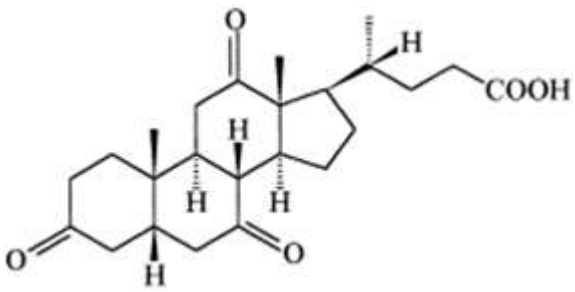
β-Sitosterol



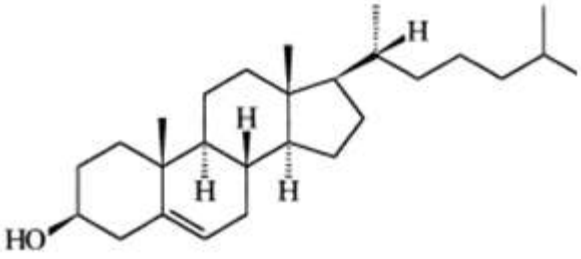
Stigmasterol



Cholic acid

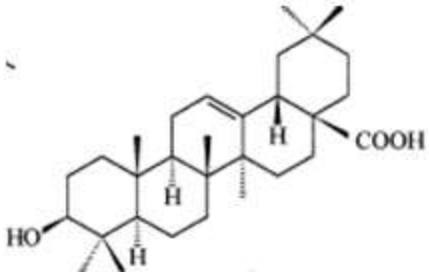


Dehydrocholic acid

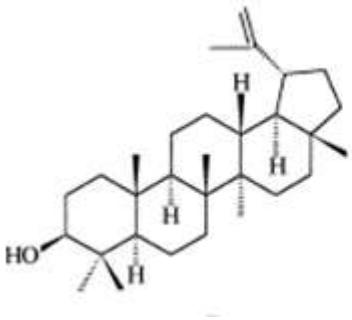


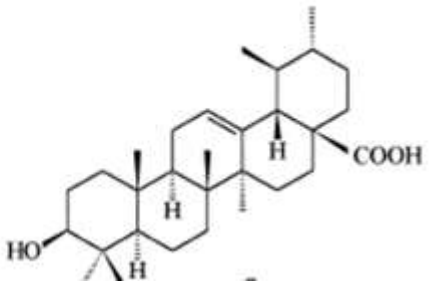
Cholesterol

Examples of triterpenoids with antisnake venom activity abound and they are: oleanolic acid, lupeol - 20% protection; ursolic acid, taraxerol, betulinol and betulinolic acid - 40% protection each, bredemeyeroside - 80% protection, euphol and others (Walter *et al*, 2000).



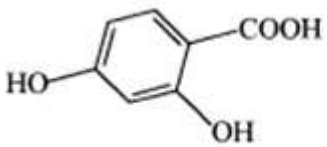
Oleanolic acid

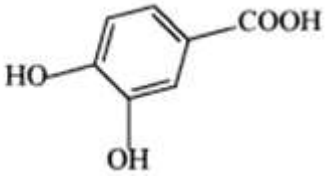




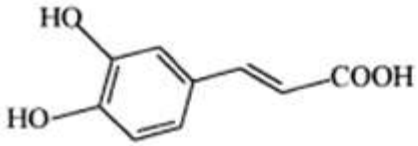
Lupeol Ursolic acid

Phenolic compounds with antisnake venom activity can be classified into hydroxybenzoic acids, cinnamic acid derivatives, coumarins, curcuminoids, flavonoids and vegetable tannins. Hydroxybenzoic acids active against snake venom are 2,4-dihydroxybenzoic acid - 83% protection; and 3,4-dihydroxybenzoic acid - 80% protection. Among the cinnamic derivatives, is caffeic acid, ferulic acid, rosmanic acid and chlorogenic acid - 60% protection. Similarly the curcuminoids are found to possess antisnake venom activity. Members include curcumin and artumerone. In addition the coumarins were observed to exhibit antisnake venom actions. Coumarin derivatives having this activity include coumarin itself - 40% protection; and umbelliferone while flavonoids having antisnake venom effects include primetin, quercetin - 40-80% protection, and tectoridin. Lastly the vegetable tannins having antisnake venom effects are typified by eufhorbins A and B (Walter *et al*, 2000).

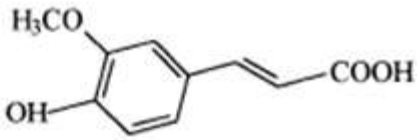




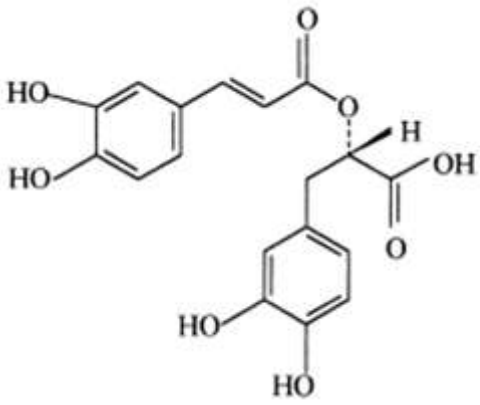
2,4-Dihydroxybenzoic acid 3,4-Dihydroxybenzoic acid



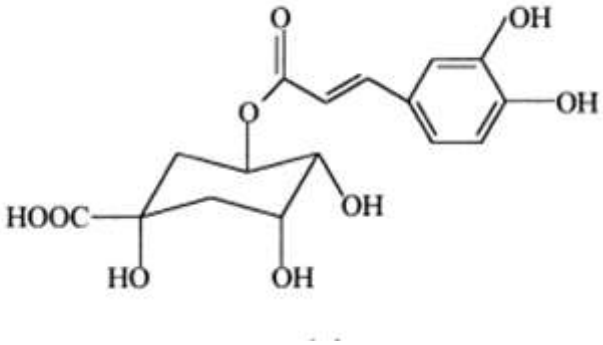
Caffeic acid



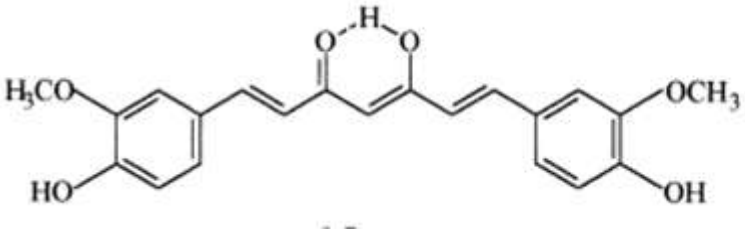
Ferulic acid



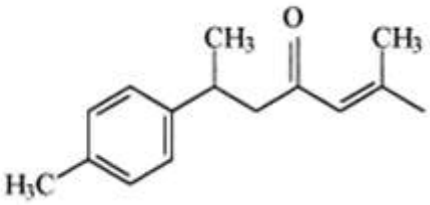
Rosmanic acid



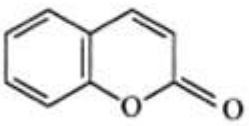
chlorogenic acid



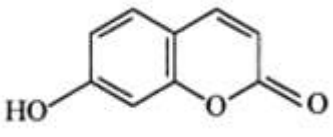
Curcumin



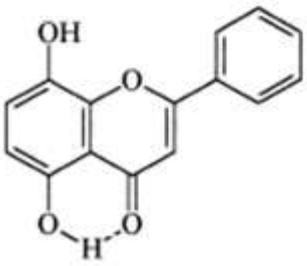
Artumerone



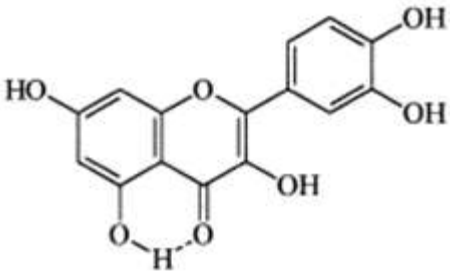
Coumarin



umbelliferone

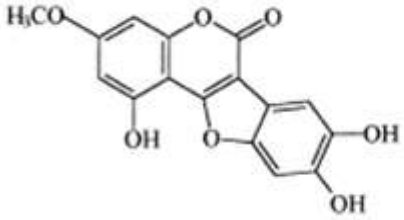


Primetin

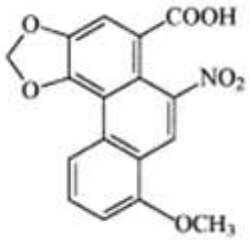


Quercetin

Pterocarpans known to possess activity against snake venom are cabenegrins A-I (22) and A-II (Nakagawa *et al*, 1982). Wedelolactone, 40% protection; is a coumestan. For the aristocholic acids, aristocholic acid and aristolactams in a mixture are known to possess antisnake venom activity.



Wedelolactone



Aristocholic acid

Polysaccharides are known to possess anti-inflammatory and immunomodulating properties and this can be extended to antisnake actions (Walter *et al*, 2000). The bark of *Casearia sylvestris* is a widely known antisnake venom remedy in Brazil. A mixture of polysaccharides separated from other phytochemicals have been proven to give 30% protection against snake venom (Barbi, 1992).

2.8 Free Radicals, Antioxidants and Oxidative Stress

Free radicals are chemical species that possess at least an unpaired electron in their outermost orbital(s). Generally they are very reactive and they cause chain reactions.

Free radicals were used to describe intermediate species in organic and inorganic chemistry but were later discovered to be important players in biological systems and responsible for damaging processes in the cell. Redox reactions are the bases of many biochemical pathways, cellular chemistry, biosynthesis and regulation. They would be needful in the proper understanding of biological oxidation, radicals and antioxidants. While the terms reducing and oxidizing agents are used chemically, biologically antioxidants and pro-oxidants are used respectively. An example of a type of pro-oxidants is the oxygen derived species commonly known as the reactive oxygen species (ROS). They attack biological targets such as lipids, proteins, DNA, and on the defending systems of the cell which are made up of enzymes and reducing equivalents, and antioxidants (Ron and Abraham, 2002).

It appears that the damage caused by free radicals is a major contributor to aging and other diseases such as cancer, cataracts, cardiovascular disease, immune system decline and brain dysfunction (Sies *et al*, 1992).

An antioxidant is a compound that significantly delays or prevents the oxidation of cell content when present in small concentrations (Vivek and Surendra, 2006). They are needed to maintain optimum health and vitality. They are the first line of defence against damages caused by free radicals in cells. They can stabilize or deactivate free radicals before they attack cells. The human system has a sophisticated antioxidant protective system (Mark, 1998).

Both the free radicals and antioxidants are present continuously in living systems and the balance between the two is tightly regulated for optimum cellular and biochemical functions. Oxidative stress is therefore defined as a change in this balance towards an increase in the pro-oxidant over the capacity of the antioxidant. The opposite is known as reductive stress. Both of these are capable of causing cellular damage (Ron and Abraham, 2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Solvents/reagents and chromatographic materials

Solvents used were of analytical grade and they include methanol, butanol, ethylacetate, chloroform and hexane. Reagents used were freshly prepared and include those for phytochemical screening such as Molisch's reagent, Salkowski's reagent, Shinoda's reagent, Dragendorf's reagent, Meyer's reagent, Wagner's reagent and Borntrager's reagent. Chromatographic materials used are TLC plates (aluminium), silica gel (60-120 mesh), sephadex LH 20, chromatographic tanks and columns.

3.1.2 Equipment

Thermoelectron UV machine at the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria was used for UV spectroscopy and antioxidant studies.

Shimadzu FTIR-8400S spectrophotometer at the National Research Institute for Chemical Technology, Zaria was used for infrared spectroscopy.

Bruker AVANCE III NMR spectrometer (600MHz) at the School of Chemistry and Physics, University of Kwa-Zulu Natal-Durban, South Africa was used for 1D- and 2D-NMR spectroscopy.

3.1.3 Experimental animals, and snake venom

Locally bred Swiss albino mice were obtained from National Veterinary Research Institute, VOM in Jos, Nigeria. They were kept in standard animal cages at the Animal House of the Department of Pharmacology and Therapeutics, at room temperature. They were fed with laboratory diet and water *ad libitum*. The venom of *Naja nigricollis* was obtained by the milking method of Markfarlane (1967) from 10 adult *Naja nigricollis* of both sexes. It was pooled, lyophilized and stored until required.

3.1.4 Collection and identification of plant material

The root of *Phyllanthus muellerianus* was collected in December, 2012 from Agojeju, Ejule in Kogi State. It was confirmed and authenticated at the Herbarium Section of the Department of Biological Sciences, Ahmadu Bello University, Zaria by comparing it with a standard specimen with voucher number 900351.

3.2 Methods

3.2.1 Extraction and partitioning

The root bark of the plant was dried, powdered and extracted with methanol using the maceration method. It was then taken in distilled water and partitioned with n-hexane, chloroform, ethyl acetate and n-butanol leaving the residual aqueous fraction.

3.2.2 Preliminary phytochemical screening

The preliminary phytochemical screening was carried out on the methanol extract according to standard procedures summarized below.

3.2.2.1 Test for carbohydrates (Molisch's test)

1ml of Molisch's reagent was added to 1ml of the extract and 1ml of concentrated sulphuric acid was then added. The formation of a reddish coloured ring at the interphase indicates the presence of carbohydrates (Silva *et al*, 1998).

3.2.2.2 Test for anthraquinones (Bontrager's test)

10ml of benzene was added to a solution of the extract and 5ml of 10% aqueous ammonia was then added. The formation of a pink red or violet colour indicates the presence of anthraquinones.

3.2.2.3 Test for saponins (Frothing test)

The extract was dissolved in water and then shaken. The formation of a persistent froth indicates the presence of saponins (Silva *et al*, 1998).

3.2.2.4 Test for flavonoids (Shinoda test)

Magnesium chip was added to a solution of the extract followed by five drops of concentrated hydrochloric acid. The formation of a red colouration indicates the presence of flavonoids (Silva *et al*, 1998).

3.2.2.5 Test for tannins (Lead sub-acetate and ferric chloride tests)

i. Three drops of lead sub-acetate was added to a solution of the extract. The formation of a coloured precipitate indicates the presence of tannins (Trease and Evans, 1996).

ii. In order to test for hydrolysable or condensed tannins, two drops of ferric chloride solution were added to a solution of the extract. The formation of a

green precipitate indicates the presence of hydrolysable tannins (Trease and Evans, 1996).

3.2.2.6 Test for alkaloids (Dragendorf's, Meyer's and Wagner's tests)

i. Few drops of Dragendorf's reagent were added to about 1ml of the extract solution. The formation of a red precipitate indicates the presence of alkaloids
ii. Few drops of Meyer's reagent were added to about 1ml of the extract solution. The formation of a cream precipitate indicates the presence of alkaloids
iii. Few drops of Wagner's reagent were added to about 1ml of the extract solution. The formation of a precipitate indicates the presence of alkaloids (Sofowora, 1982).

3.2.2.7 Test for terpenoids (Salkowski's test)

To 5ml of extract solution, 2ml of chloroform was added followed by 3ml of concentrated sulphuric acid (Salkowski's reagent). A reddish brown colouration at the interphase indicates the presence of terpenoids (Edeoga *et al*, 2005).

3.3 Chromatographic Procedures

3.3.1 Thin layer chromatography of methanol extract and partitioned n-butanol fraction

Pre-coated TLC plates were used to carry out thin layer chromatography by one way ascending technique. Capillary tubes were used to manually apply spots and the chromatogram was developed in an air-tight chromatographic tank at room temperature, employing ethyl acetate-methanol-water (10:5:1) as solvent system. The spots were visualized after been sprayed with 10% sulphuric acid followed by heating in an oven.

3.3.2 Column chromatography of partitioned n-butanol fraction

The column was packed using the wet slurry method. Cotton wool was placed at the bottom end of the column and solvent was added. The tap was then opened to be sure it runs. Silica gel was mixed with the solvent and stirred thoroughly after which it was packed into the column and allowed to settle. Cotton wool was also placed on the bed of the silica gel. The partitioned n-butanol fraction of the methanol extract was dissolved in methanol, and silica gel was added to form a powdered mixture which was allowed to dry. The dry mixture was then packed on the cotton wool on the silica gel bed.

As a result of the high polarity of the compounds present in the partitioned n-butanol fraction from TLC studies, the column was gradiently eluted starting from chloroform (100%), a gradient mixture of chloroform and ethyl acetate, through ethylacetate (100%) to ethyl acetate-methanol (85:15) before the collection of the target compounds in a less complicated mixture, as monitored by thin layer chromatography. Different column fractions collected were pooled together into six major combinations depending on the similarity of their TLC profiles. The six different combinations of the fractions are: 31-33, 34-35, 36-41, 42-44, 45-48, and 49-60.

3.3.3 Isolation of PA

Column fractions 36-41 was mounted on a small column packed with sephadex LH-20 and then eluted with methanol. This gave rise to sephadex fractions SE1-SE14 of which SE3-14 were pooled together. Column fractions 42-44 was also mounted on a small column packed with sephadex LH-20 and then eluted with methanol. This gave rise to sephadex fractions S1-S8.

The sephadex fractions SE3-14 and S8 were separately subjected to preparative thin layer chromatography using a pre-coated TLC plate. The plates were developed using

ethyl acetate-methanol-water (10:5:1) as solvent system. The bands containing the compounds of interest were scraped off and washed repeatedly with methanol to afford a brown compound. The respective compounds from the preparative TLC were subjected to co-TLC and their R_f values were found to be the same, hence, they were merged to afford compound PA. The spot of the pure compound was developed in different solvent systems to ascertain its purity. The melting point of PA was then taken using an electrothermal apparatus. The isolated compound was subjected to UV, FTIR, 1D- and 2D-NMR spectroscopy.

3.3.4 Isolation of PX

Column fractions 49-60 was mounted on a small column packed with sephadex LH-20 and then eluted with methanol. This gave rise to sephadex fractions θ1- θ8 which were pooled together. θ1- θ8 was remounted on sephadex to give T1-T8 which were pooled together. T1-T8 was remounted on sephadex to give A1-A10 which were pooled together.

The sephadex fractions A1-A10 were subjected to preparative thin layer chromatography using a pre-coated TLC plate. The plates were developed using ethyl acetate-methanol-water (10:5:1) as solvent system. The band containing the compound of interest was scraped off and washed repeatedly with methanol to afford a white compound PX. The spot of the pure compound was developed in different solvent systems to ascertain its purity. The melting point of PX was to be taken using an electrothermal apparatus but it charred in the process. The isolated compound was subjected to UV, FTIR, 1D- and 2D-NMR spectroscopy.

3.3.5 n-Butanol fraction of the dry methanol extract of *Phyllanthus muellerianus*

n-Butanol was added directly to powdered methanol extract and stirred thoroughly with a glass rod. It was then left to stand for about an hour after which it was decanted. The decantate was then dried and this afforded the n-butanol fraction of the dry methanol extract of *Phyllanthus muellerianus.*

3.3.6 Thin Layer chromatography of the n-butanol fraction of the dry methanol extract of *Phyllanthus muellerianus*

The TLC of the n-butanol fraction of the dry methanol extract was developed using chloroform- ethyl acetate-methanol (3:1:3) as the solvent system.

3.3.7 Isolation of CP1

The n-butanol fraction of the dry methanol extract was mounted on a small column packed with sephadex LH 20 and then eluted with methanol. This gave rise to sephadex fractions SB1-SB16. SB3 and SB4 were combined due to their similar TLC profile.

The sephadex fraction SB1, SB2 and SB3-SB4 were separately subjected to preparative thin layer chromatography to afford a brown compound CP1 which is present in the four fractions. The solvent system used is chloroform-ethylacetate-methanol 10:1:3. The isolated compound was then subjected to UV, FTIR, 1D- and 2D-NMR spectroscopy.

3.4 Biological Studies

3.4.1 Acute toxicity studies

The method described by Lorke (1983) was employed and the route of administration was intra-peritoneal. In the first phase, nine mice of either sex were divided into three groups containing three mice each. The first, second and third groups received

10mg/kg, 100mg/kg and 1000mg/kg respectively. In the second phase, four animals were used. Each of the four animals received different doses of the extract which are: 200mg/kg, 400mg/kg, 800mg/kg and 1600mg/kg. The second phase was repeated and the median lethal dose was calculated as recommended by Lorke.

3.4.2 *In vitro* snake venom detoxifying effect of the methanol extract

The method described by Abubakar *et al* (2000) was employed in this protocol. Twenty four mice were divided into four groups (n=6). Group 1 (control) received 10mg/kg of the venom only. Groups 2, 3 and 4 (treatment groups) received an equivalent of 10mg/kg of the venom containing 20, 40 and 80mg/kg of the extract. The venom and the extract were both incubated at 37^0C for 10 minutes prior to injection. The route of administration was intraperitoneal. Signs of toxicity such as inability to move and death were observed.

3.4.3 *In vivo* snake venom detoxifying effect of the methanol extract

The method described by Theakston and Reid, 1983 was employed. Twenty four mice were divided into four groups (n=6). Group 1 (control) received 10mg/kg of the venom only. Groups 2, 3 and 4 (test groups) received 5, 10 and 20mg/kg of the extract. The test groups were then injected with 10mg/kg of the venom 30 minutes after injecting the extract. The route of administration was intraperitoneal. Signs of toxicity such as inability to move and death were observed.

3.4.4 *In vitro* antioxidant activity of PA

The antioxidant activity of PA was evaluated using the DPPH method. Methanol served as the blank and absorbances were recorded at 518nm. Different concentrations of PA ranging from 1000, 500, 250, 125, 50, 25, 10 and 5µg/ml were prepared in methanol.

Similarly, 0.1mM of DPPH was prepared in methanol. 0.5ml of DPPH was then added to 2.5ml of each concentration of PA and allowed to react in the dark for 30 minutes after which absorbance readings were taken (Shirwakar *et al*, 2006). The same process was repeated for vitamin C as standard. The antioxidant activity was calculated as:

% Free radical scavenging activity = $(A_c - A_s) / A_c \times 100\%$

A_c = absorbance of control (DPPH)

A_s = absorbance of sample + DPPH

Values were taken in triplicates and the percentage free radical scavenging activity was taken as mean $\pm$ standard deviation.

CHAPTER FOUR

4.0 RESULTS

4.1 Yield

900g of the powdered bark of the root of the plant gave a 92.09g methanol extract. The n-butanol fraction (7.27g) was obtained after partitioning 72g of the methanol extract in water.

4.2 Result of Preliminary Phytochemical Screening

The preliminary phytochemical screening of the methanol extract revealed the presence of carbohydrates, saponins, flavonoids, tannins, alkaloids, and terpenoids. It is summarized in Table 4.1 below.

Table 4.1 Results of preliminary phytochemical screening

Constituents	Test	Observation	Inference
Carbohydrates	Molisch's test	A reddish coloured ring at the interphase	+
Anthraquinones	Bontrager's test	No change was observed	_
Saponins	Froth test	Persistent froth	+
Flavonoids	Shinoda test	Red colouration	+
Tannins	Lead sub-acetate test	Brown precipitate	+
Hydrolysable tannins	Ferric chloride test	Green precipitate	+
Alkaloids	a. Dragendorf's test	Red precipitate	+
	b. Meyer's test	Cream precipitate	+
	c. Wagner's test	Brown precipitate	+
Terpenoids	Salkowski's test	Reddish brown colour at interphase	+

Key + = Present – = Not present

4.3 Thin Layer Chromatogram of Methanol Extract (ME) and Partitioned n-Butanol Fraction (BF) Co-chromatographed on the Same Plate



ME-BF Ethyl acetate-Methanol-Water 10:5:1

Plate II: TLC co-chromatogram of the methanol extract and partitioned n-butanol fraction

4.4 Thin Layer Chromatograms of Column Fractions of the Partitioned n-Butanol Fraction (BF)



Plate III: TLC profile of column fractions 31-40



Plate IV: TLC profile of column fractions 35-48



Plate V: TLC profile of column fractions 49-60

4.5 Thin Layer Chromatograms of Sephadex Fractions SE1-14

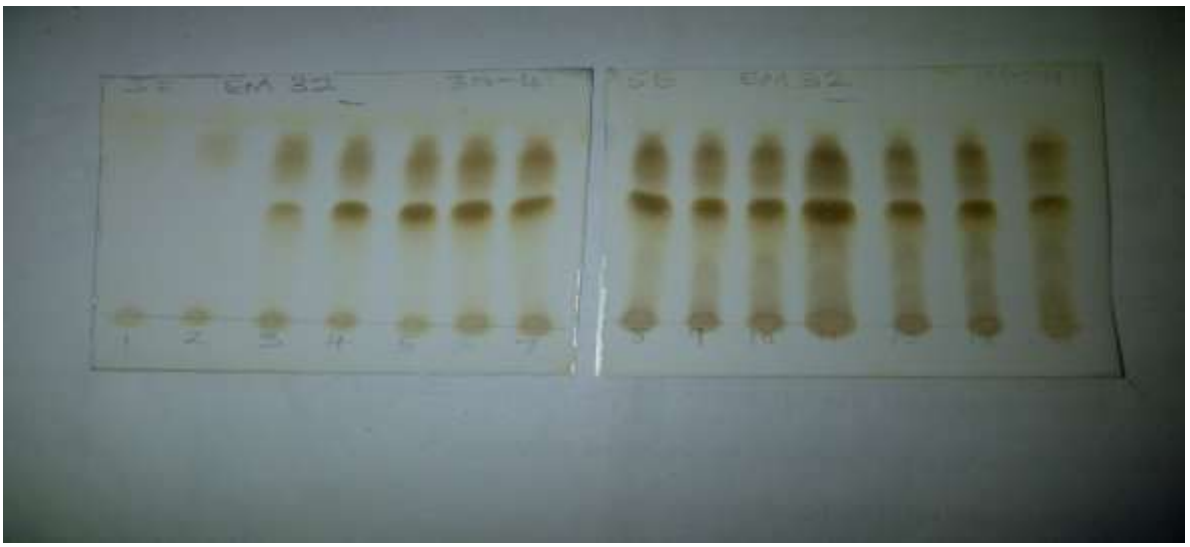


Plate VI: TLC profile of sephadex fractions SE1-14

4.6 Thin Layer Chromatograms of Sephadex Fractions S1-8

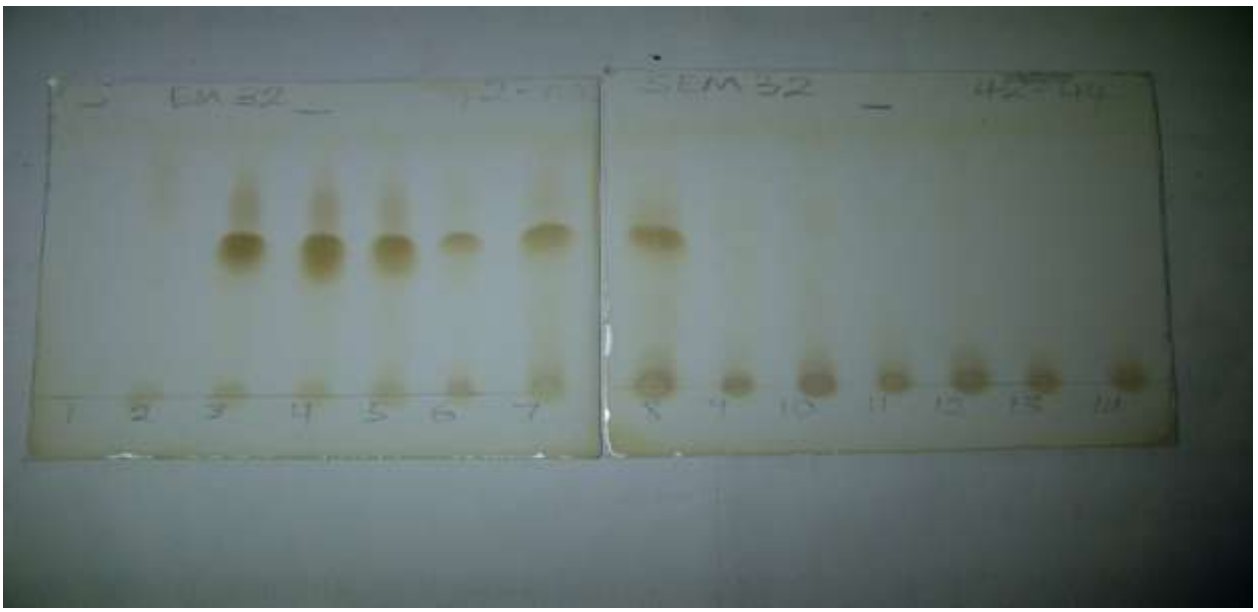


Plate VII: TLC profile of sephadex fractions S1-8

4.7 Thin Layer Chromatograms of Sephadex Fractions θ1-8, T1-8 and A1-10

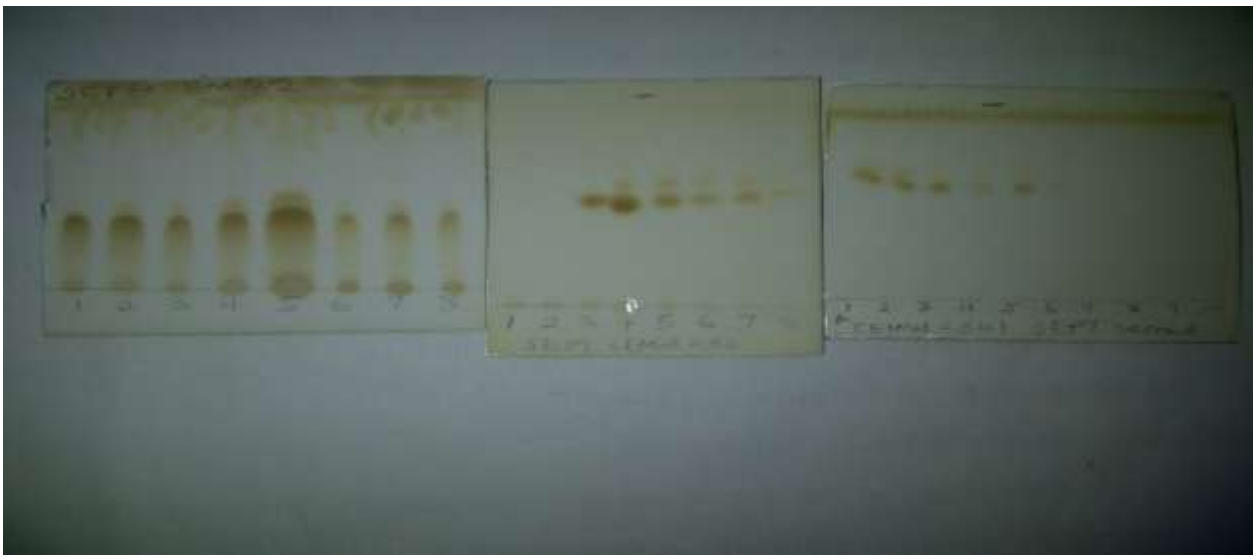


Plate VIII: TLC profiles of sephadex fractions θ1-8, T1-8 and A1-10

4.8 Thin Layer Chromatograms of PA Developed in Different Solvent Systems and Determined Melting point

The TLC chromatogram of PA was developed in ethyl acetate-methanol-water (EMW – 10:5:1) and the upper layer of n-butanol-acetic acid-water (BAW – 4:1:5). The melting point of PA was determined to be 158-160^{0}C.



PA (EMW – 10:5:1, R_f = 0.53) PA (BAW – 4:1:5, R_f = 0.30)

Plate IX: TLC profiles of PA in different solvent systems

4.9 Thin Layer Chromatograms of PX Developed in Different Solvent Systems

The TLC chromatogram of PX was developed in ethyl acetate-methanol-water (EMW – 10:5:1) and ethyl acetate-chloroform-methanol-water (ECMW – 10:2:5:1). PX charred in the process of determining its melting point.



PX (EMW - 10:5:1, R_f = 0.50) PX (ECMW – 10:2:5:1, R_f = 0.21)

Plate X: TLC profiles of PX in different solvent systems

4.10 Thin Layer Chromatogram of the n-Butanol Fraction of Dry Methanol Extract



Plate XI: TLC profile of the n-butanol fraction of dry methanol extract

4.11 Thin Layer Chromatograms of SB1-16

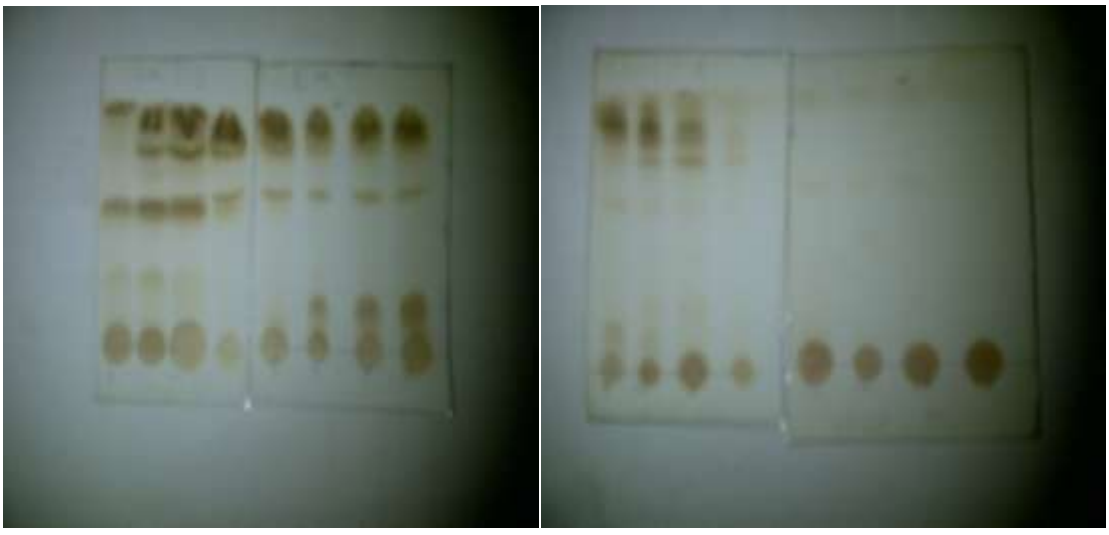


Plate XII: TLC profile of sephadex fractions SB1-16

4.12 Thin Layer Chromatograms of CP1 Developed in Different Solvent Systems

The TLC chromatogram of CP1 was developed in chloroform-ethyl acetate-methanol (CEM) 7:1:3 and 10:1:3.



CP1 CEM 7:1:3 (R_f = 0.63) CP1 CEM 10:1:3 (R_f = 0.38)

Plate XIII: TLC profiles of CP1 in different solvent systems

4.13 Spectral Analysis of PA

Figure 4.1 to 4.9 show the UV, IR, 1D- and 2D –NMR spectra of PA.

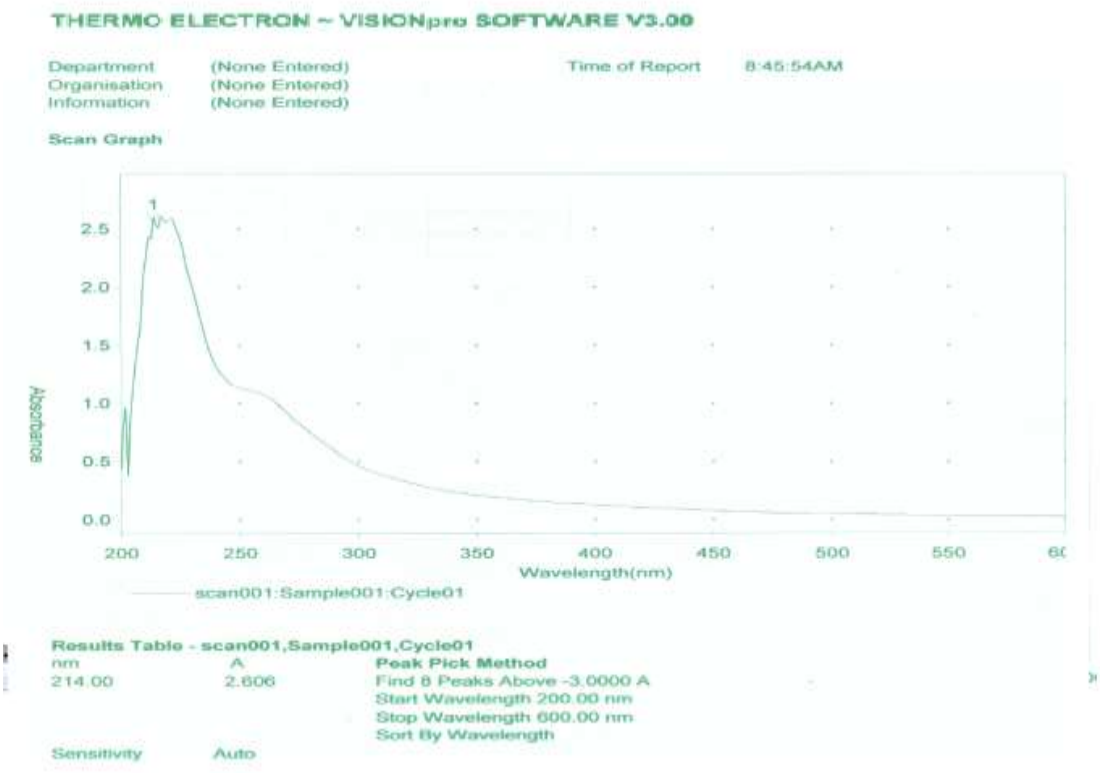
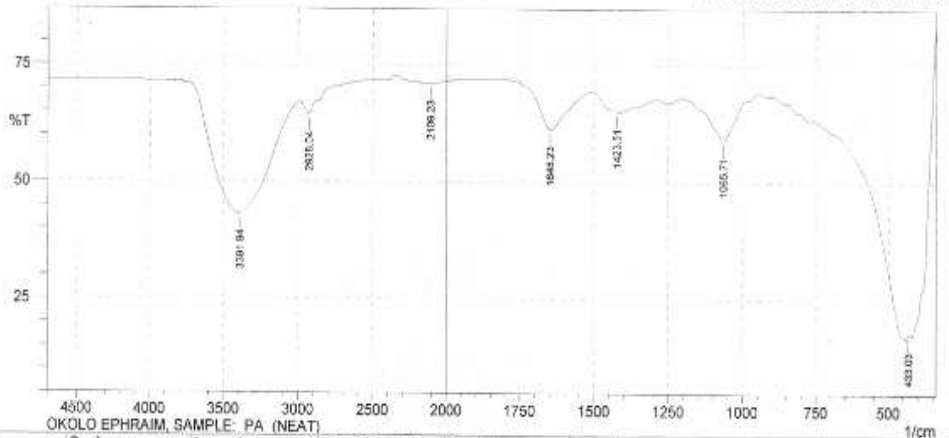


Figure 4.1 UV spectrum of PA

SHIMADZU

FTIR ANALYSIS RESULT NARICT,ZARIA

FTIR- 8400S FOURIER TRANSFORM INFRARED SPECTROPHOTOMETER



OKOLO EPHRAIM, SAMPLE: PA (NEAT)

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	433.03	17.83	0.09	434	431.1	2.16	0.01
2	1065.71	59.1	6.78	1206.51	986.62	42.25	5.34
3	1423.51	55.21	0.04	1439.91	1422.55	3.19	0
4	1648.23	61.39	0.13	1708.02	1647.26	11.4	0.12
5	2109.23	71.1	0	2110.19	2088.01	3.28	0
6	2928.04	64.26	2.97	2990.73	2874.03	21.16	1.03
7	3391.94	43.2	25.44	3693.81	2990.73	192.05	76.24

Comment;
OKOLO EPHRAIM, SAMPLE: PA (NEAT)

User; Administrator

No. of Scans;
Date/Time; 5/13/2013 12:00:12 PM
Resolution;

Figure 4.2 IR Spectrum of PA

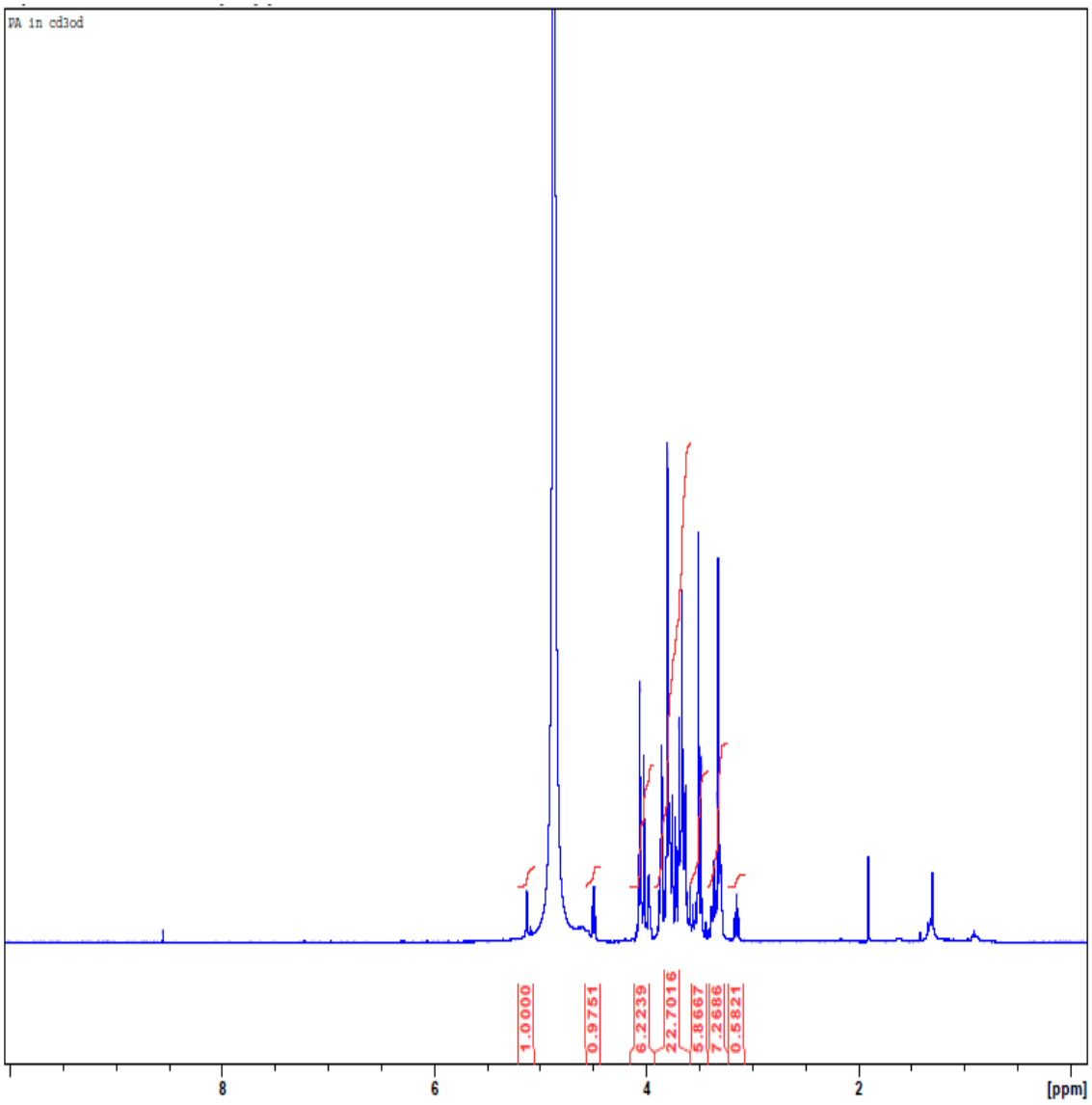


Figure 4.3 Proton NMR Spectrum of PA

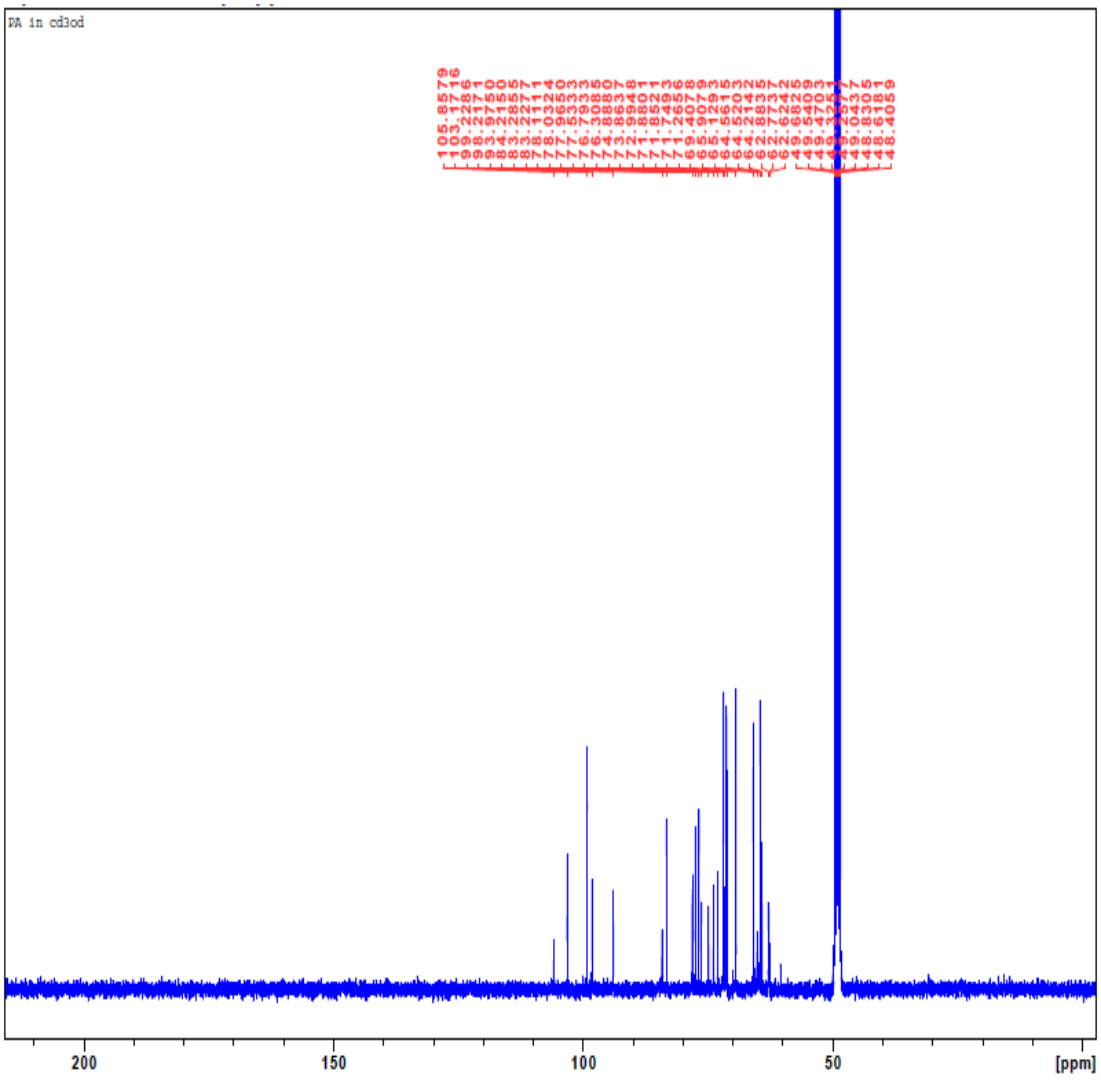


Figure 4.4 Carbon-13 NMR Spectrum of PA

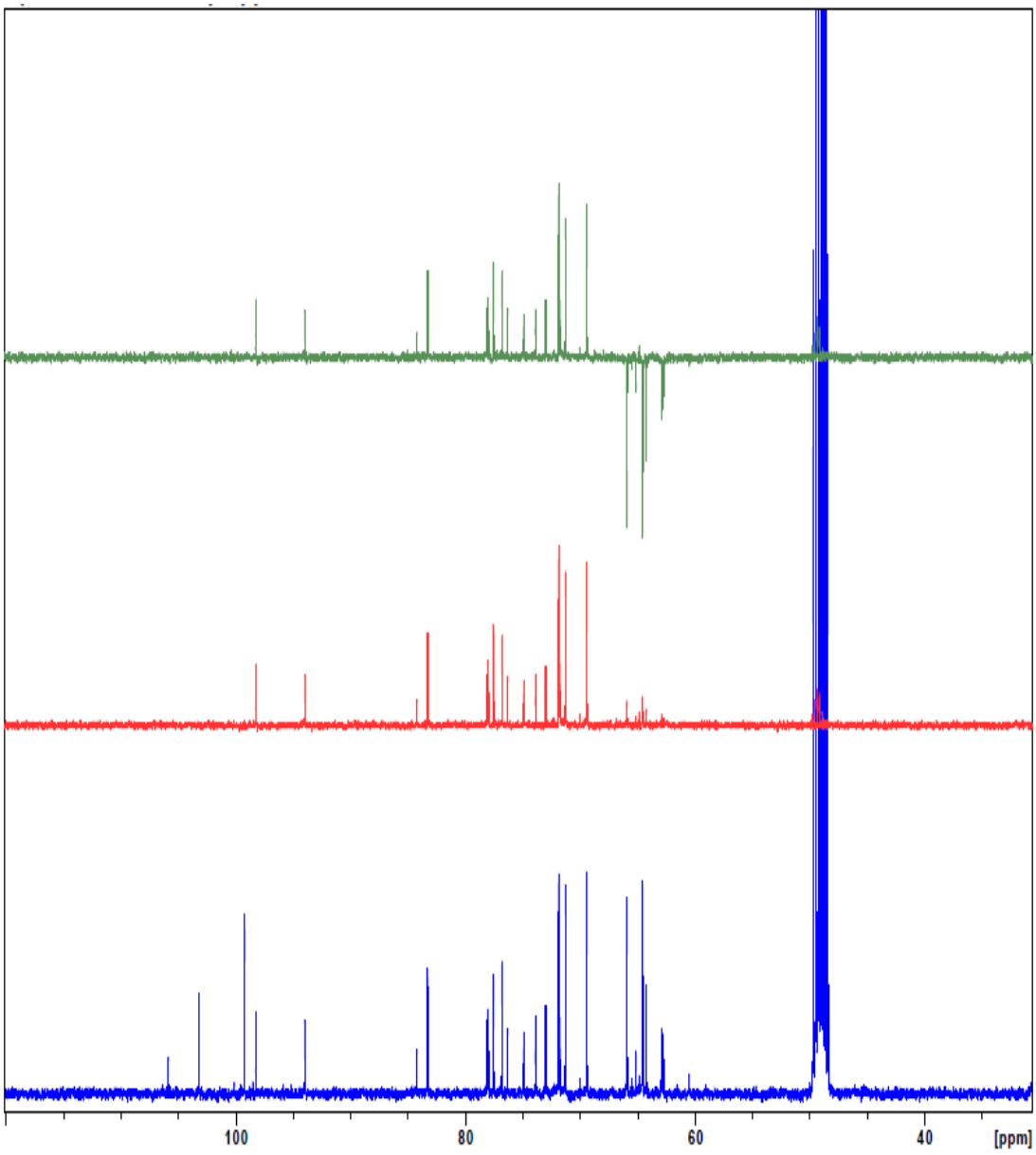


Figure 4.5 DEPT NMR Spectrum of PA

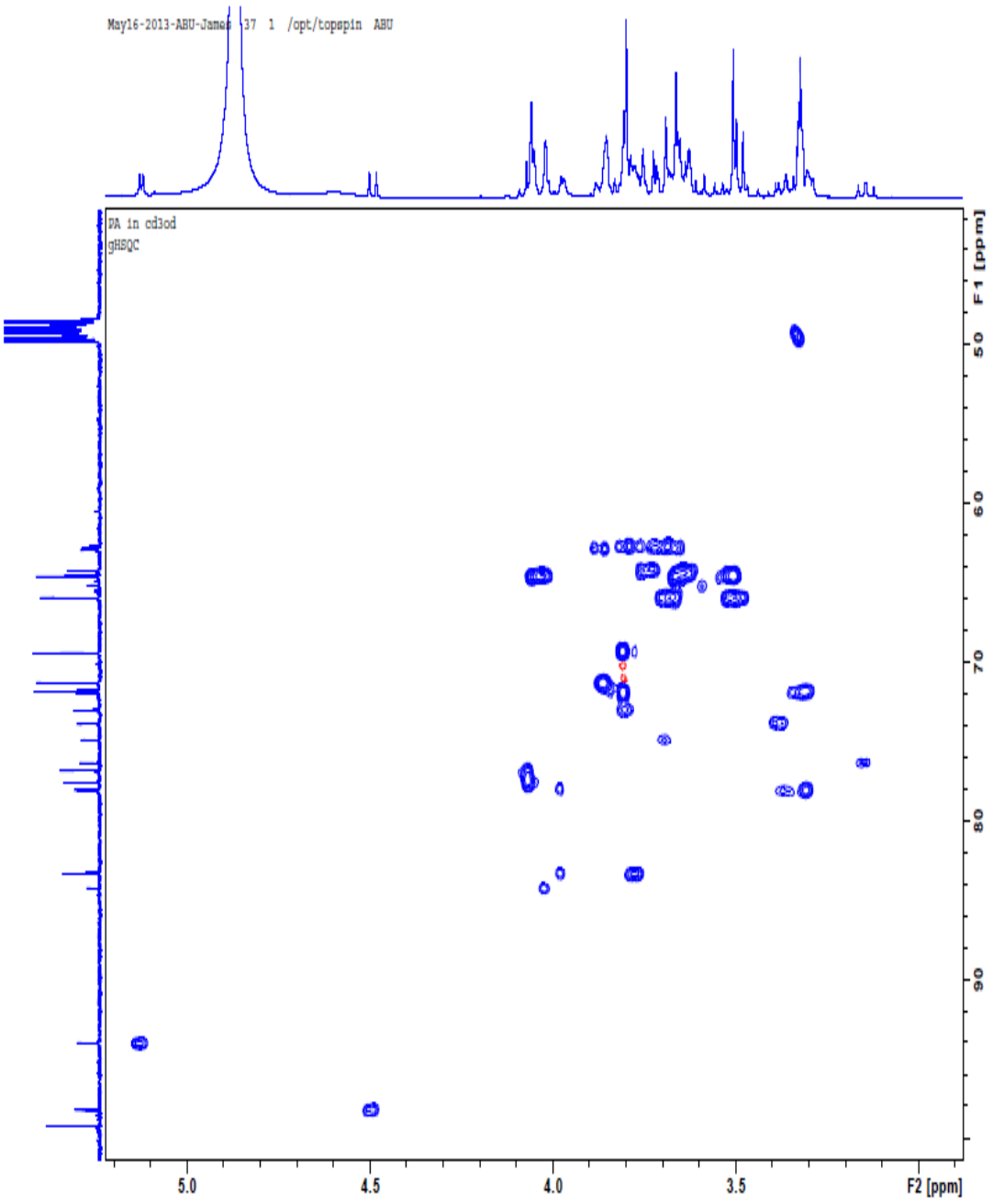


Figure 4.6 HSQC NMR Spectrum of PA

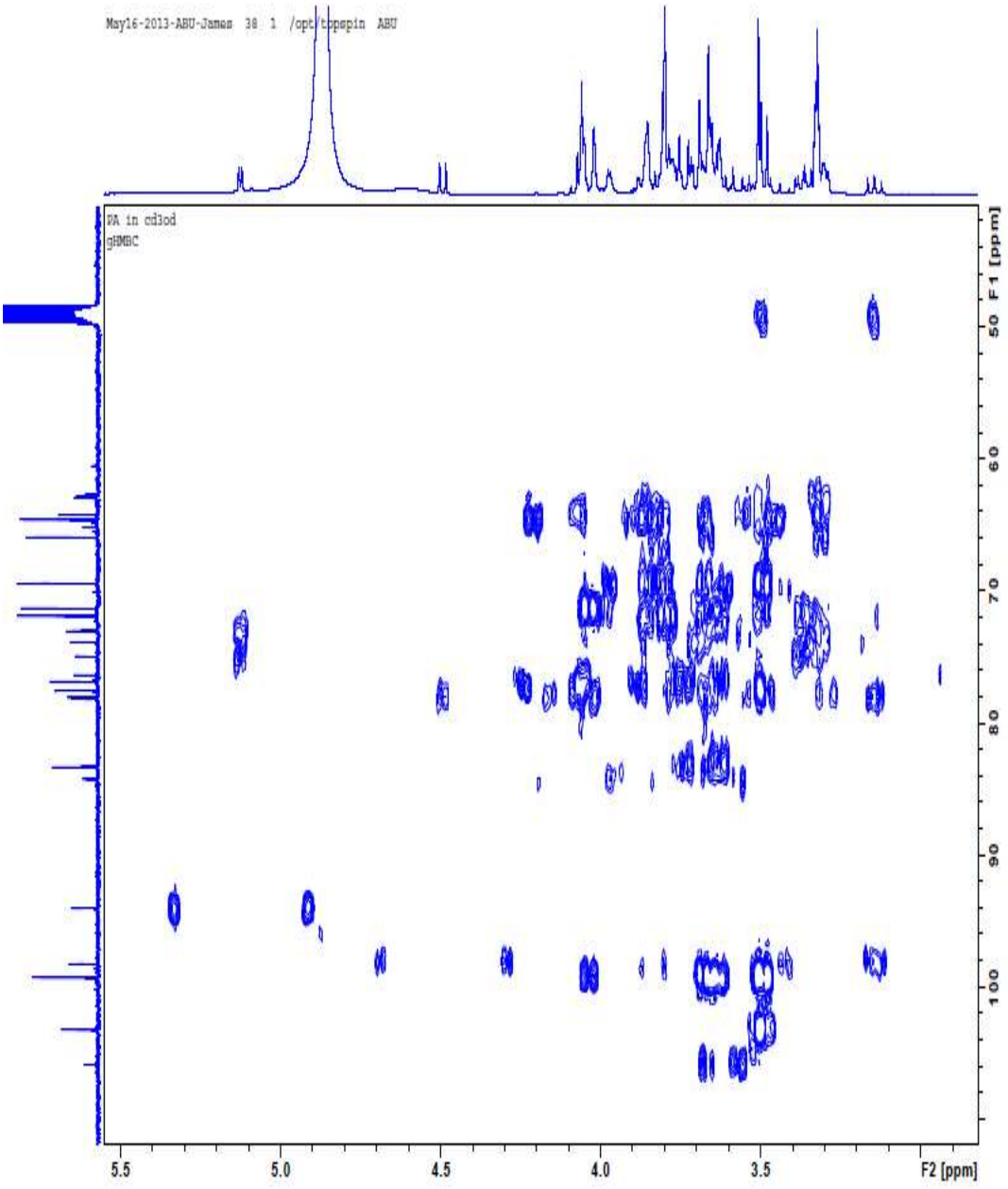


Figure 4.7 HMBC NMR Spectrum of PA

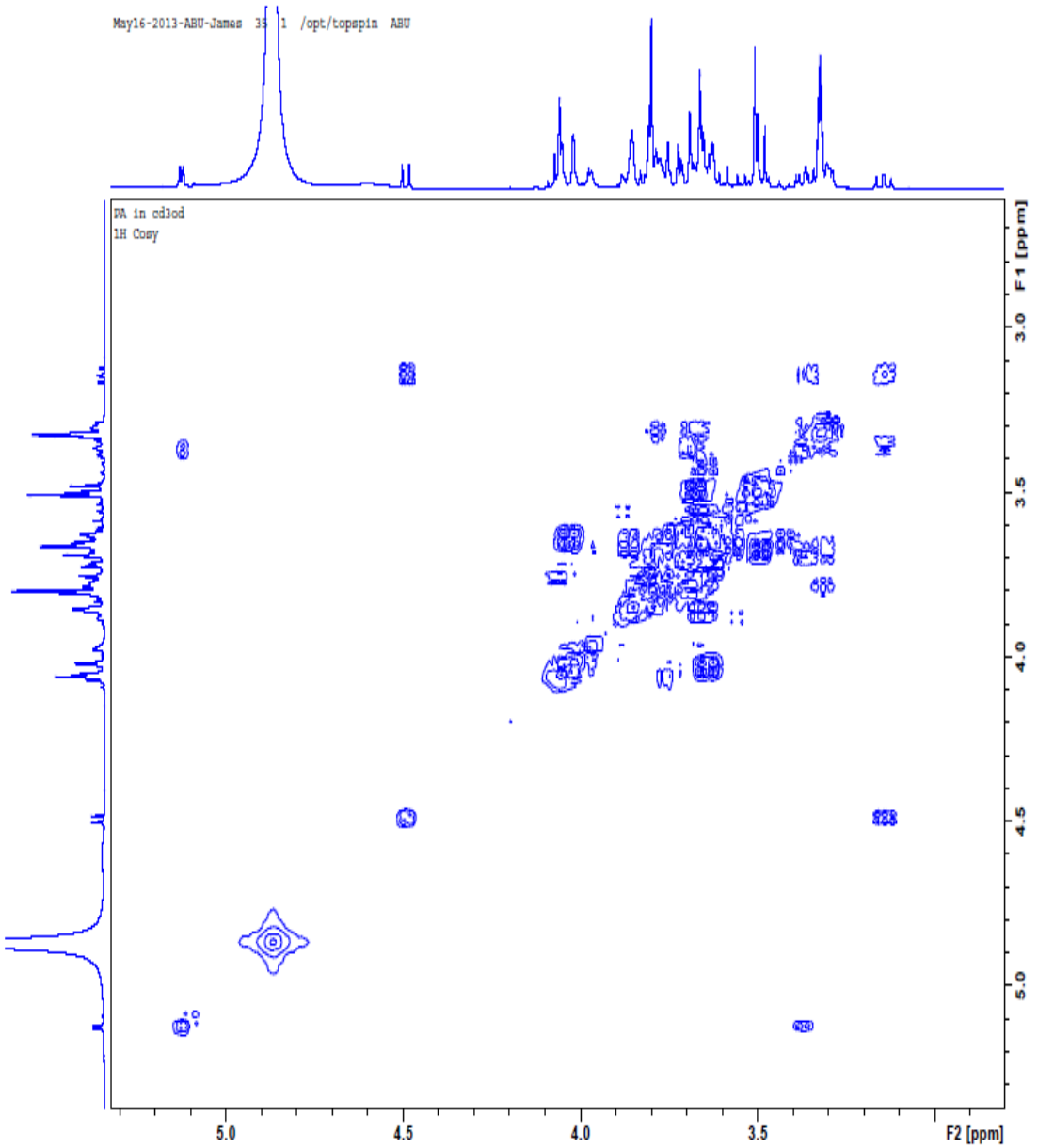


Figure 4.8 COSY NMR Spectrum of PA

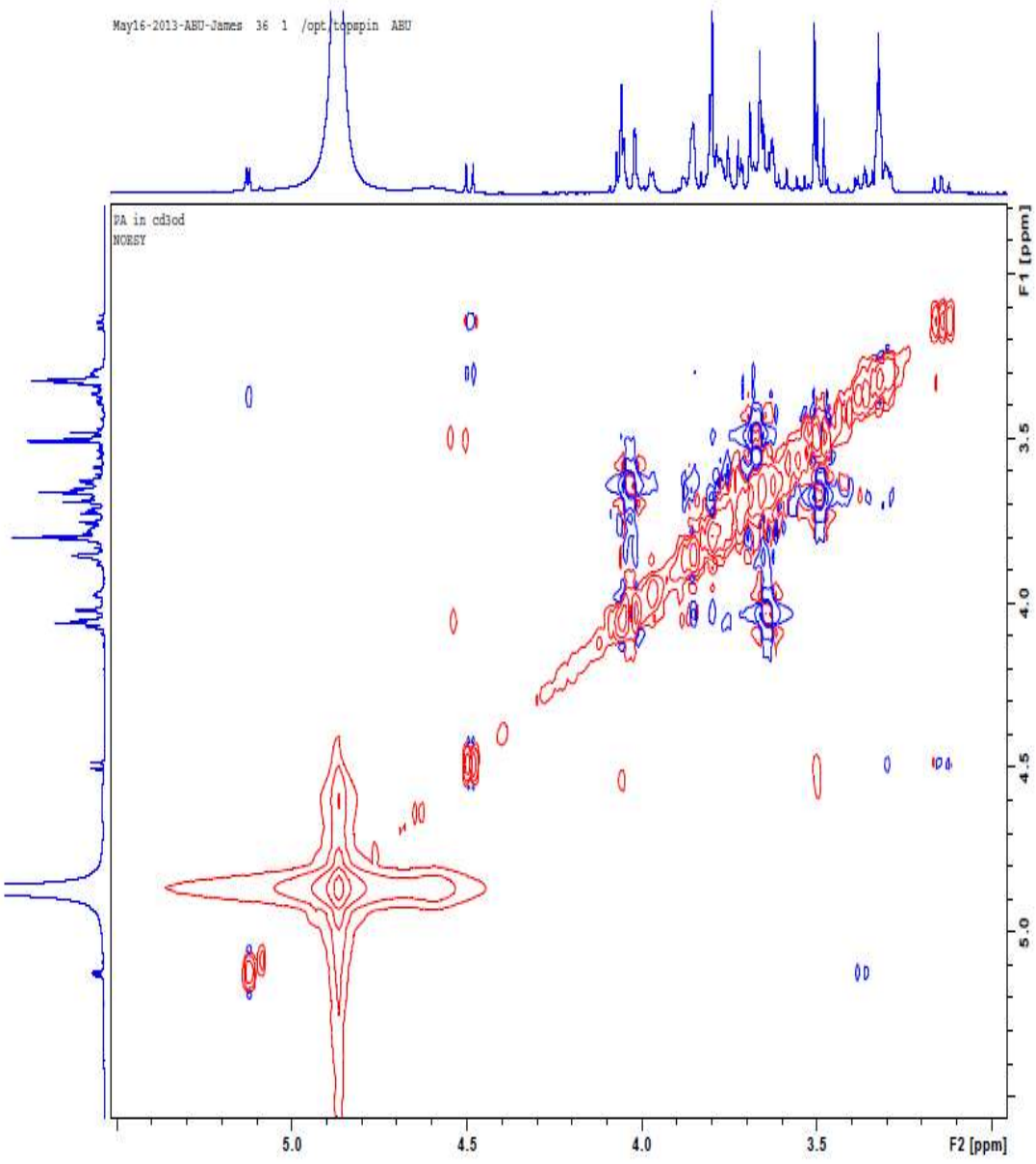


Figure 4.9 NOESY NMR Spectrum of PA

4.14 1D- and 2D-NMR Spectra of PA

Table 4.2 to 4.6 show the 1D- and 2D-NMR spectral results of PA.

Table 4.2 ^{1}H, ^{13}C, DEPT and HSQC NMR data of α-D-Glucopyranose G_1

S/No	δ_C	δ_H	DEPT
1	94.0	5.14	CH
2	73.0	3.37	CH
3	73.9	3.81	CH
4	69.4	3.78	CH
5	71.7	3.82	CH
6	62.6	3.69	CH_2

Table 4.3 ^{1}H, ^{13}C, DEPT and HSQC NMR data of β-D-Glucopyranose G_2

S/No	δ_C	δ_H	DEPT
1	98.2	4.50	CH
2	76.3	3.16	CH
3	77.5	3.99	CH
4	76.8	4.08	CH
5	71.9	3.84	CH
6	62.8	3.80	CH_2

Table 4.4 ^{1}H, ^{13}C, DEPT and HSQC NMR data of β-D-Fructofuranose F1

S/No	δ_C	δ_H	DEPT
1	64.6	3.60	CH_2
2	99.2	-	C
3	78.0	3.32	CH
4	71.2	3.82	CH
5	83.2	3.98	CH
6	62.9	3.73	CH_2

Table 4.5 ^{1}H, ^{13}C, DEPT and HSQC NMR data of α-D-Fructofuranose F2

S/No	δ_C	δ_H	DEPT
1	65.1	3.60	CH_2
2	103.2	-	C
3	78.0	3.32	CH
4	71.9	3.82	CH
5	83.3	3.98	CH
6	64.2	3.73	CH_2

Table 4.6 ^{1}H, ^{13}C, DEPT and HSQC NMR data of α-D-Fructofuranose F3

S/No	δ_C	δ_H	DEPT
1	65.9	3.50, 3.68	CH_2
2	105.9	-	C
3	78.1	3.32	CH
4	74.9	3.70	CH
5	84.2	4.03	CH
6	64.5	3.53, 3.65	CH_2

The HMBC correlations that established the linkage of the sugars are: C-2 of F3, C-1 of F2; C-2 of F1, C-2 of G2; C-4 of G2, C-1 of F3 and C-2 of G1, C-6 of F3. Some COSY correlations are: H-1 and H-2 of G1, H-1 and H-2 of G-2 and, H-5 and H-6 of F3. One NOESY correlation is that of H-6 and H-1 of F3.

4.15 Spectral Analysis of PX

Figure 4.10 to 4.18 show the UV, IR, 1D- and 2D-NMR spectra of PA.

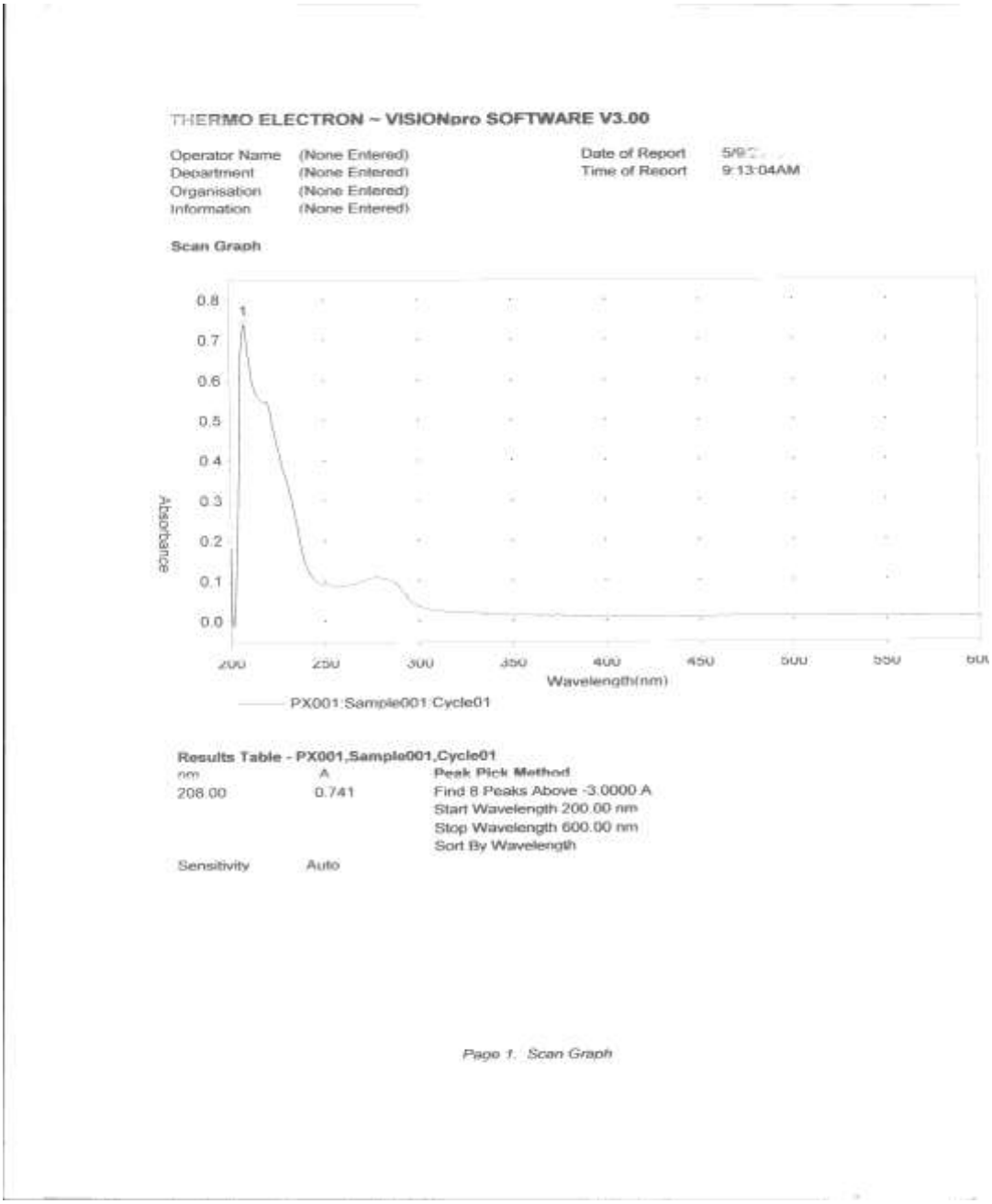
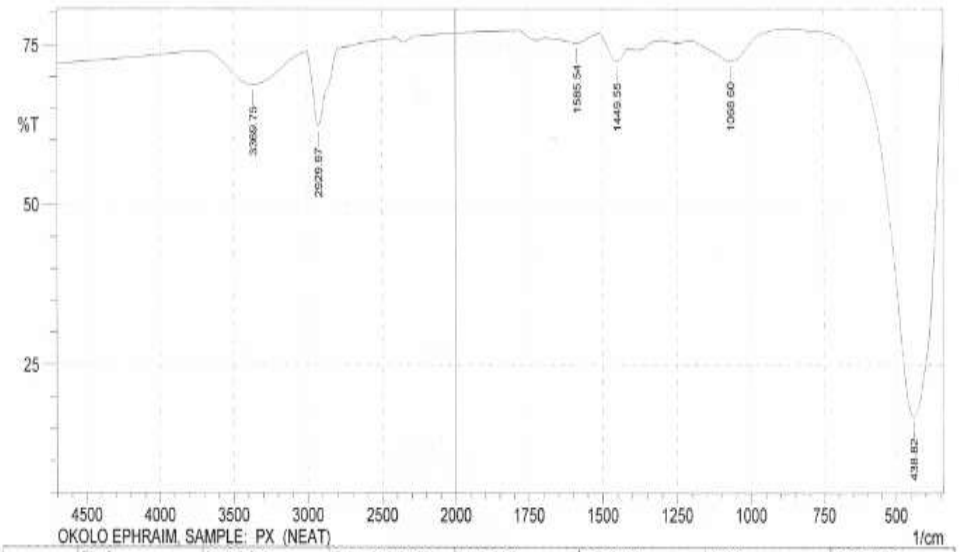


Figure 4.10 UV Spectrum of PX

SHIMADZU

FTIR ANALYSIS RESULT NARICT,ZARIA

FTIR- 8400S FOURIER TRANSFORM INFRARED SPECTROPHOTOMETER



OKOLO EPHRAIM, SAMPLE: PX (NEAT)

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	438.82	16.94	58.78	773.48	339.48	134.94	83.75
2	1068.6	72.58	3.93	1202.66	866.07	41.71	2.91
3	1449.55	72.52	2.9	1511.28	1411.94	12.9	0.89
4	1585.54	75.36	0.97	1648.23	1511.28	16.41	0.38
5	2929.97	62.23	12.13	3010.02	2408.21	82.75	8.04
6	3369.75	68.72	5.36	3694.77	3010.02	100.7	11.48

Comment;
OKOLO EPHRAIM, SAMPLE: PX (NEAT)

User; Administrator

No. of Scans;
Date/Time; 5/13/2013 11:54:47 AM
Resolution;

Figure 4.11 IR Spectrum of PX

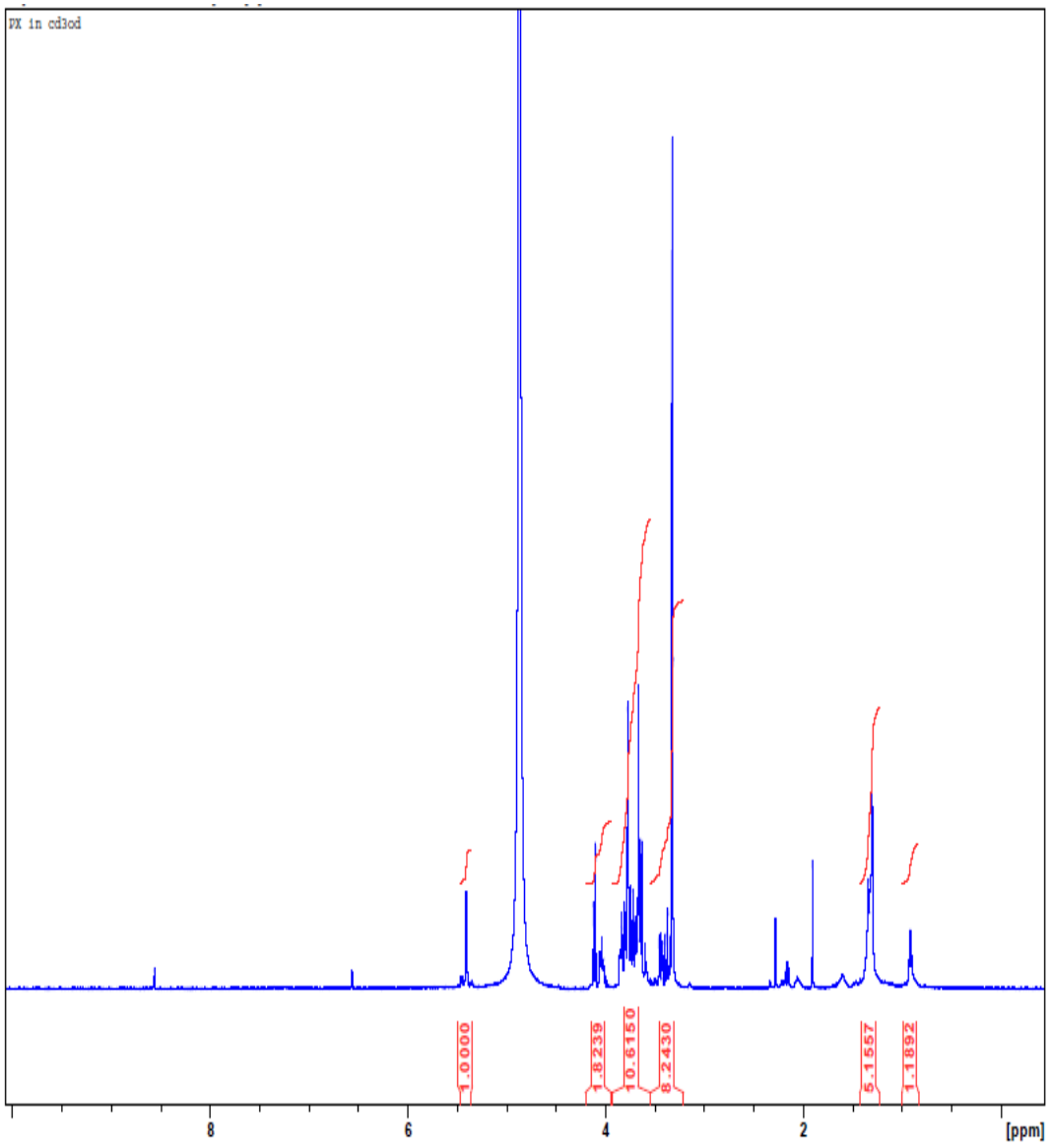


Figure 4.12 Proton NMR Spectrum of PX

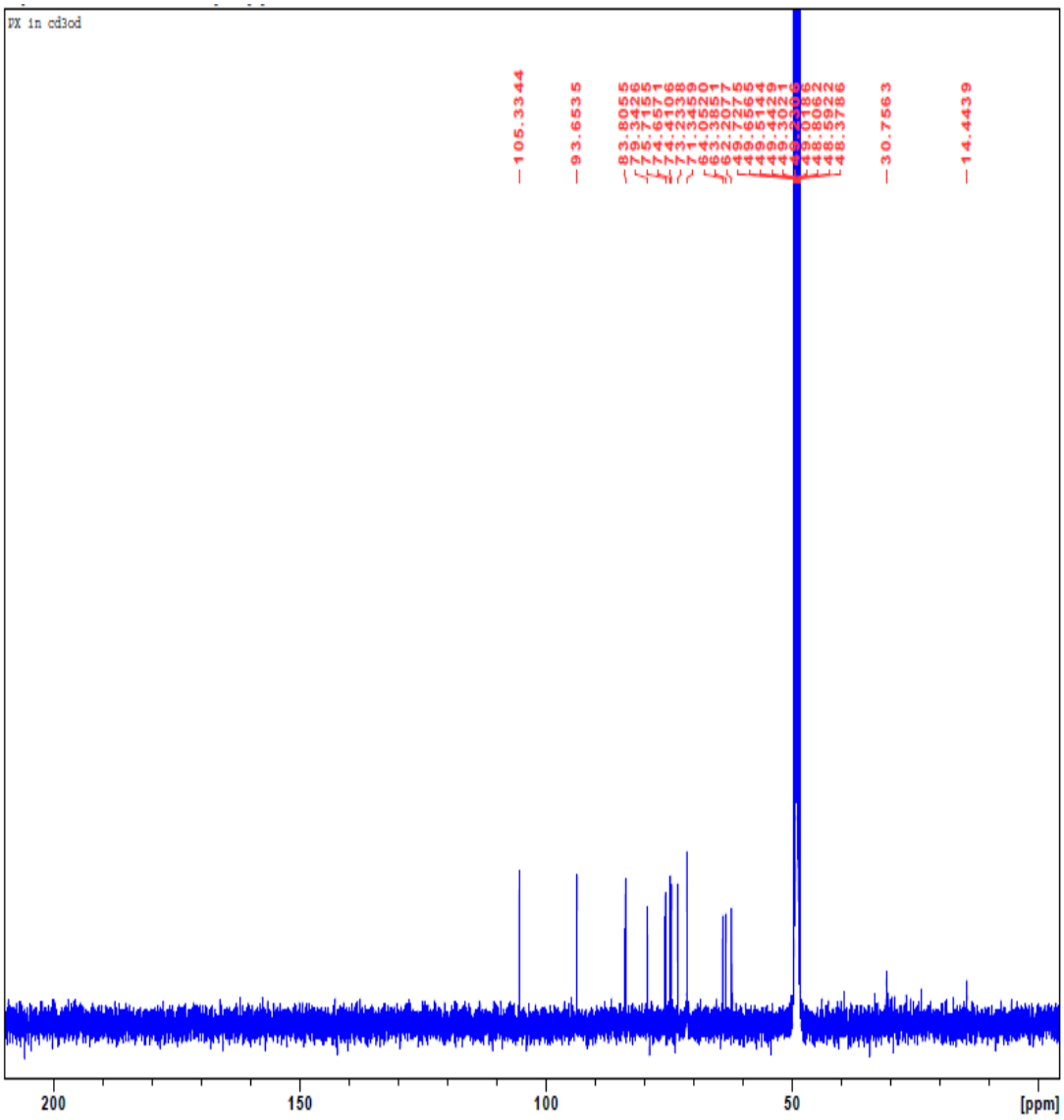


Figure 4.13 Carbon-13 NMR Spectrum of PX

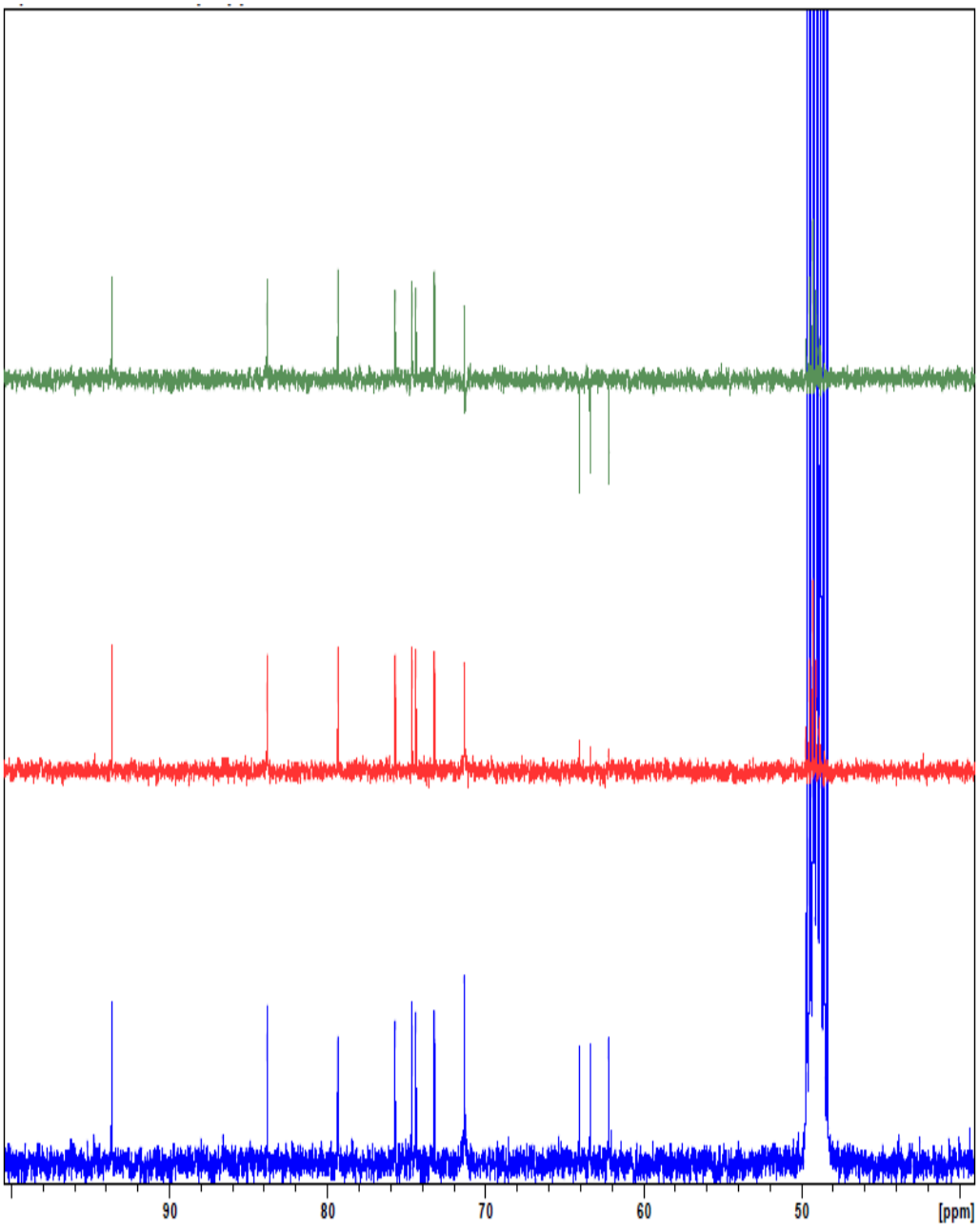


Figure 4.14 DEPT NMR Spectrum of PX

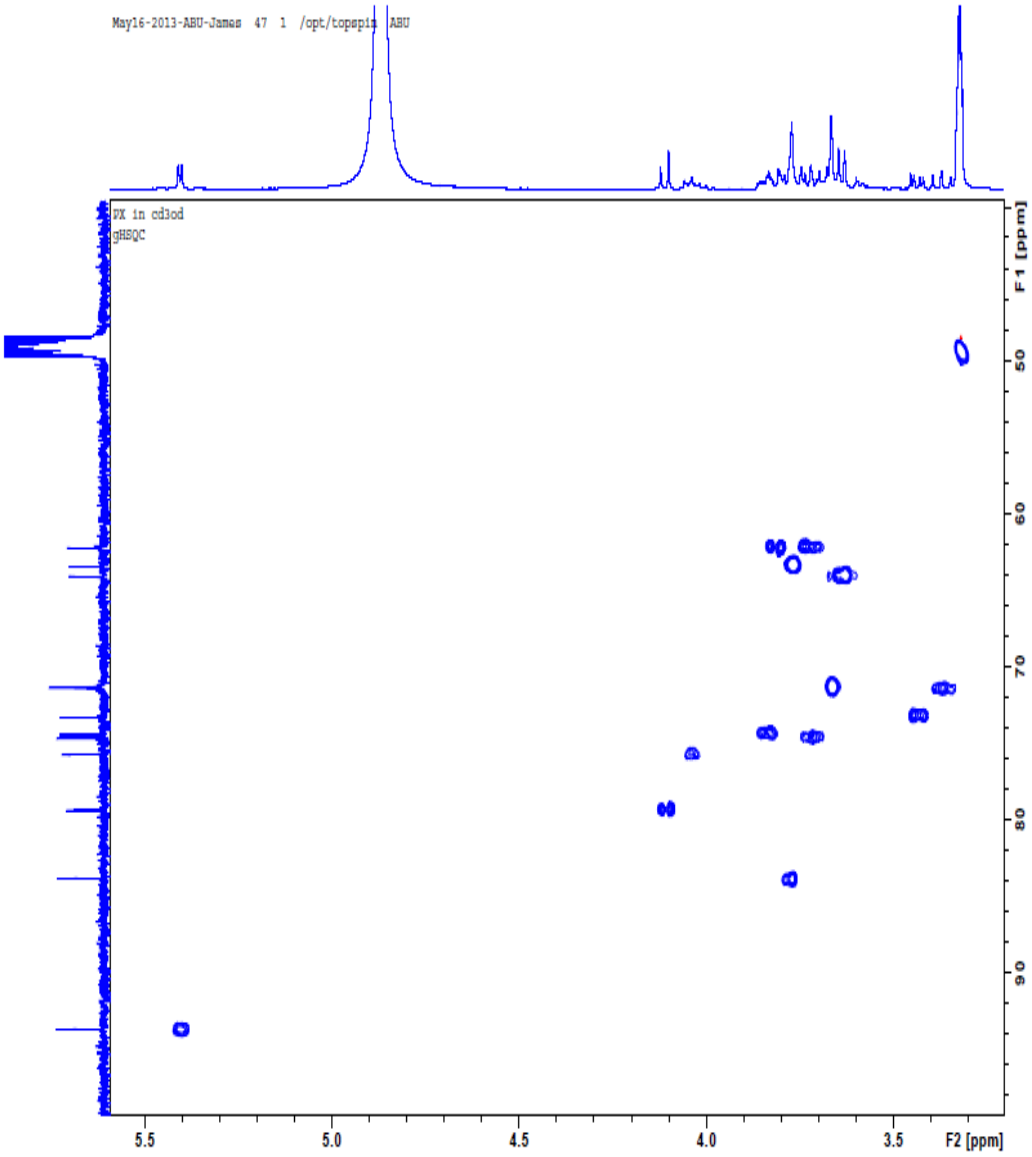


Figure 4.15 HSQC NMR Spectrum of PX

4.29

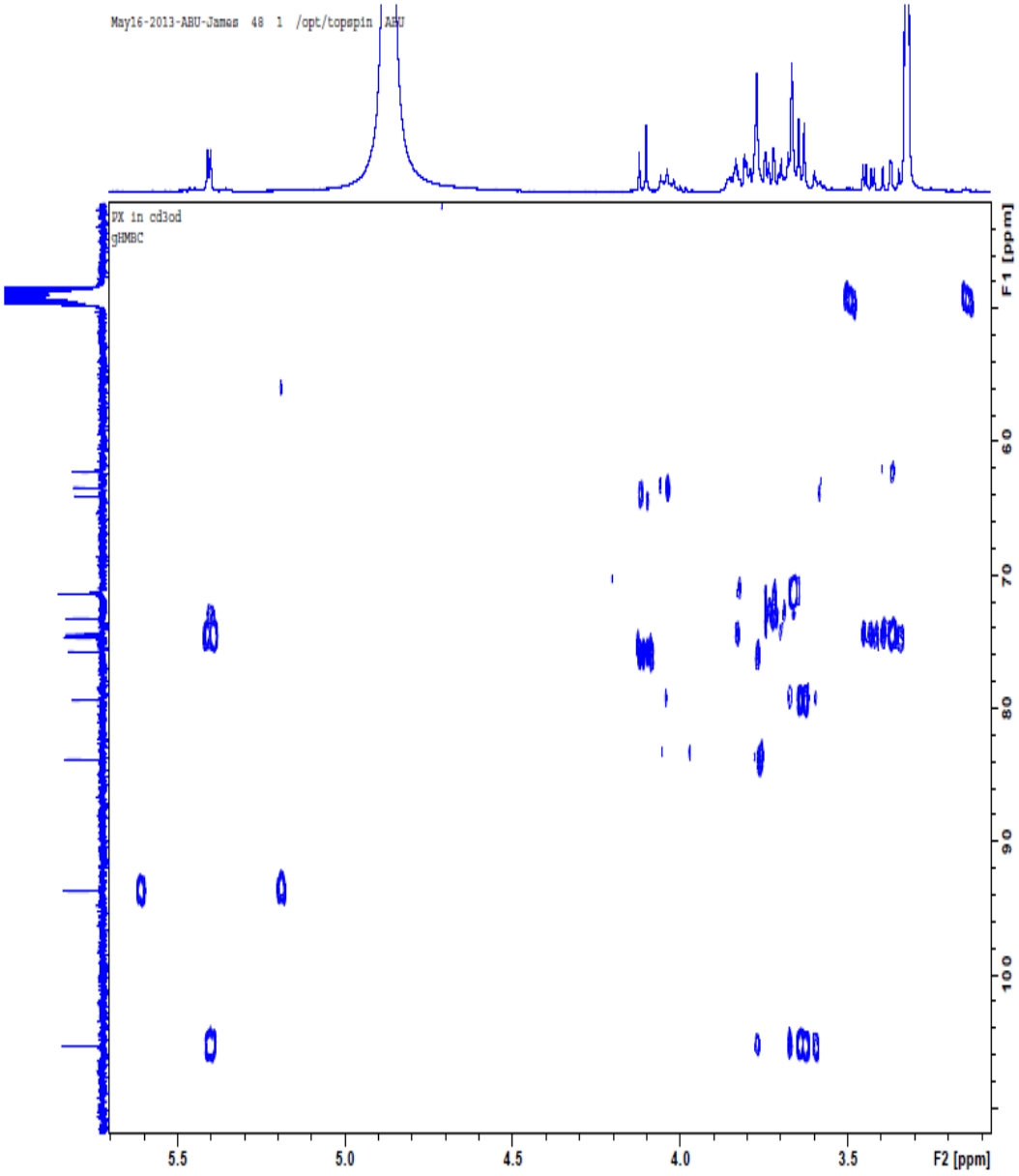


Figure 4.16 HMBC NMR Spectrum of PX

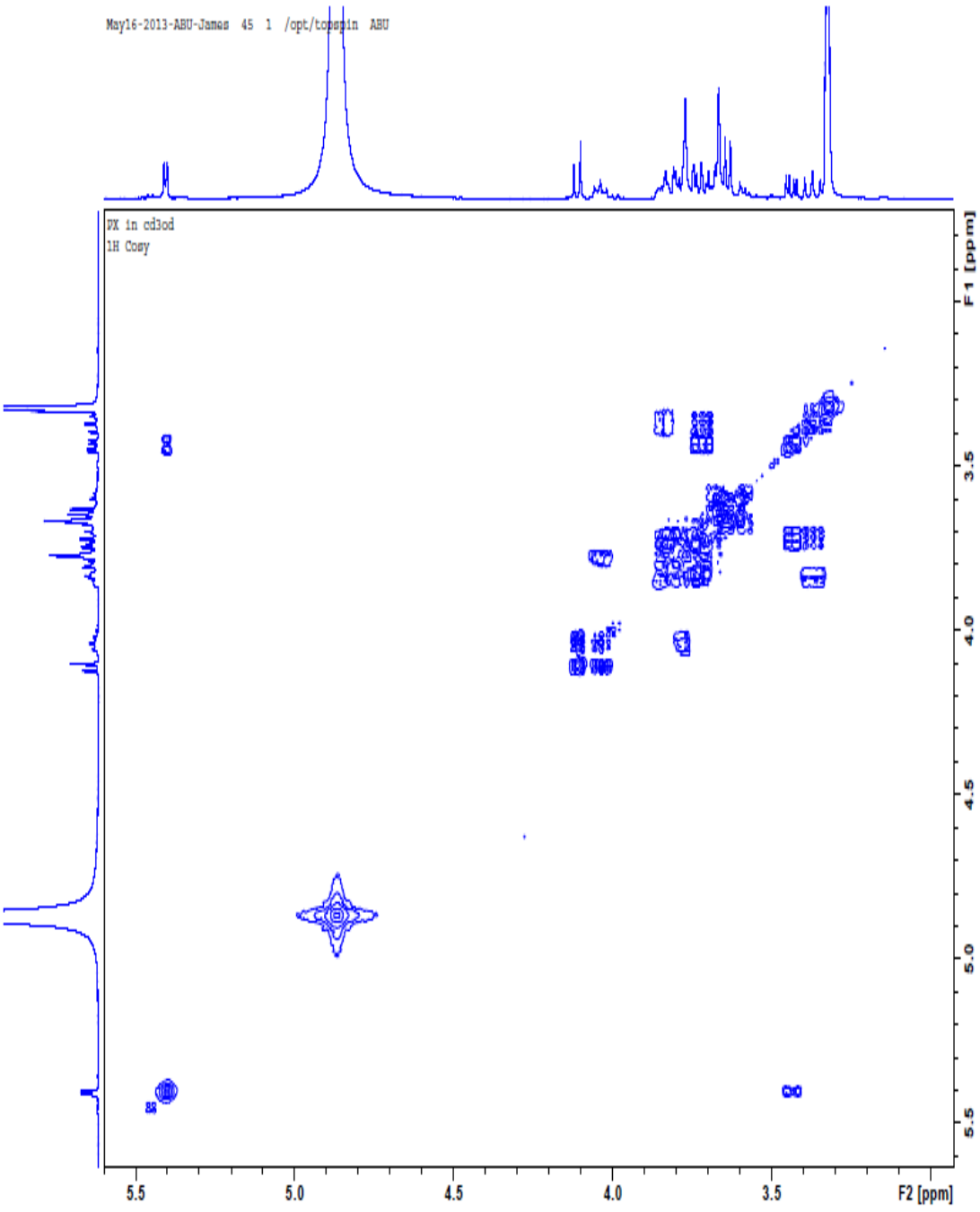


Figure 4.17 COSY NMR Spectrum of PX

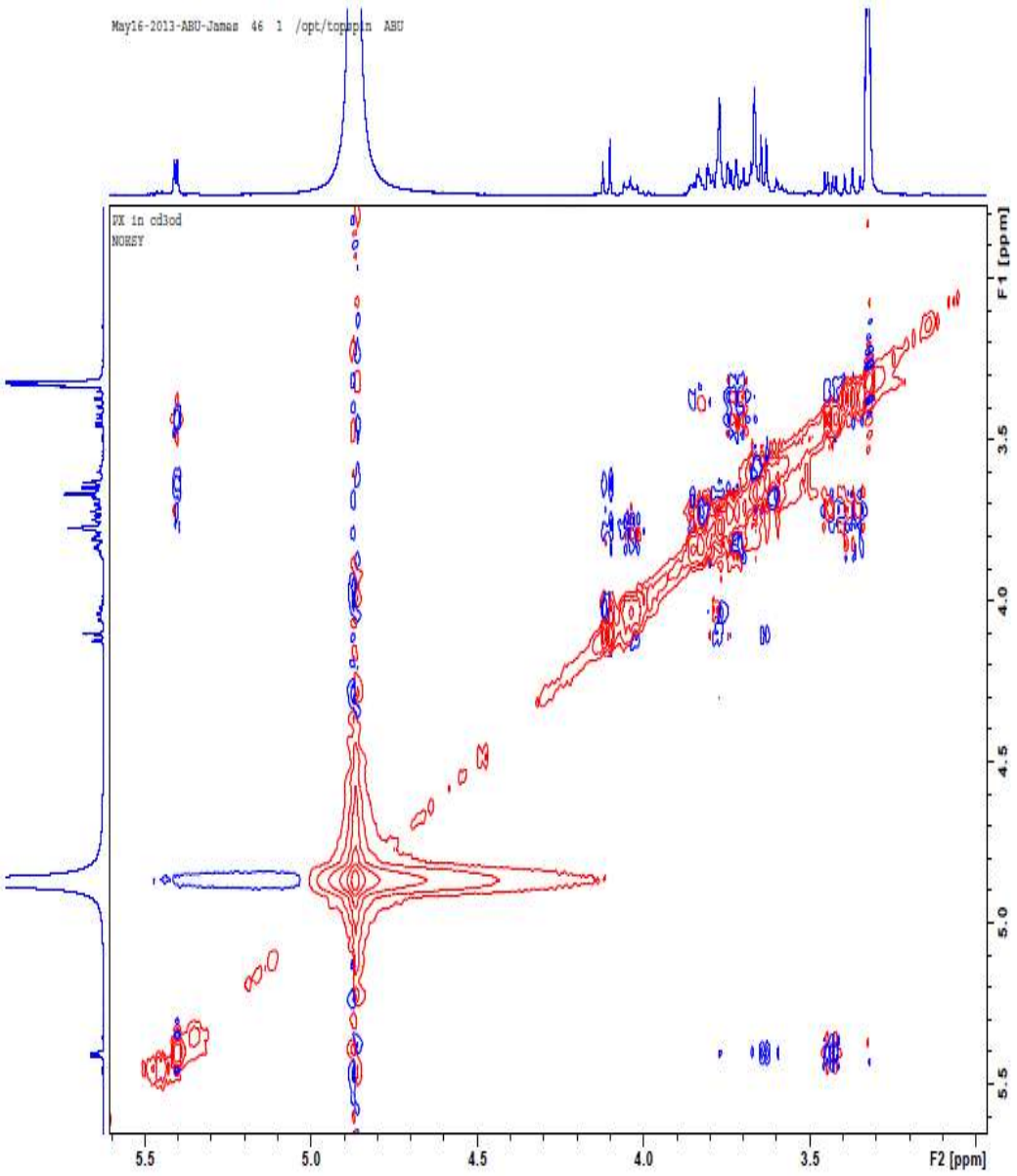


Figure 4.18 NOESY NMR Spectrum of PX

4.16 1D - and 2D – NMR Spectra of PX

Table 4.7 and 4.8 show the 1D- and 2D-NMR spectral results of PA.

Table 4.7 ^{1}H, ^{13}C, DEPT and HSQC NMR data of α-D-Glucopyranose G4

S/No	δ_C	δ_H	DEPT
1	93.7	5.40	CH
2	73.2	3.45	CH
3	74.7	3.72	CH
4	71.3	3.68	CH
5	74.4	3.83	CH
6	62.2	3.72, 3.83	CH_2

Table 4.8 ^{1}H, ^{13}C, DEPT and HSQC NMR data of α-D-Fructofuranose F4

S/No	δ_C	δ_H	DEPT
1	64.1	3.64	CH_2
2	105.3	-	C
3	79.3	4.04	CH
4	75.7	4.11	CH
5	83.8	3.78	CH
6	63.4	3.77	CH_2

The HMBC correlation that established the sugar linkage is C-2 of F4, C-1 of G4. Some COSY correlations are H-1, H-2 of G4 and H-3, H-4 of F4. A NOESY correlation is H-3, H5 of F4.

4.17 Spectral Analysis of CP1 as Shown in Figure 4.19 to 4.27

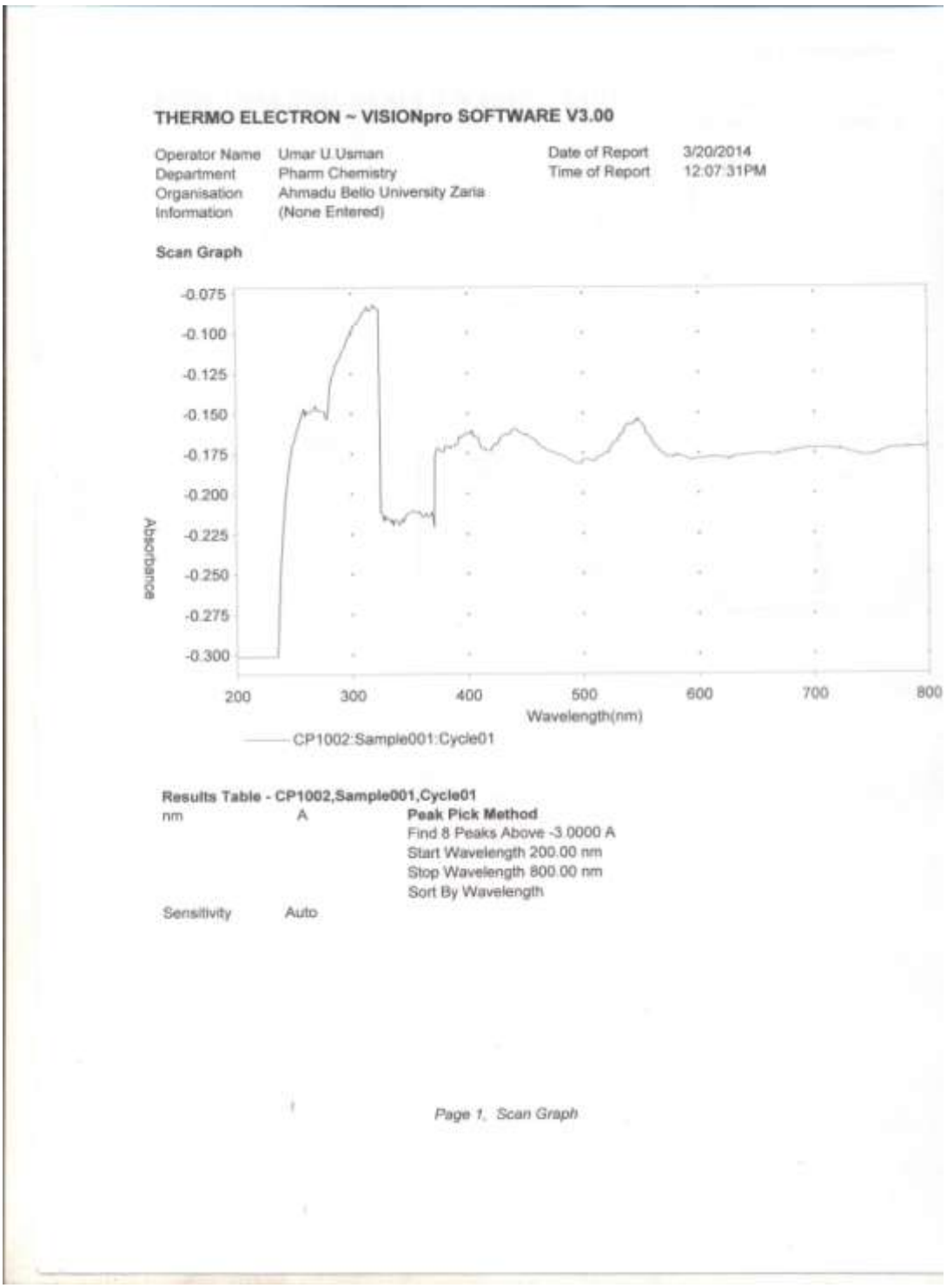
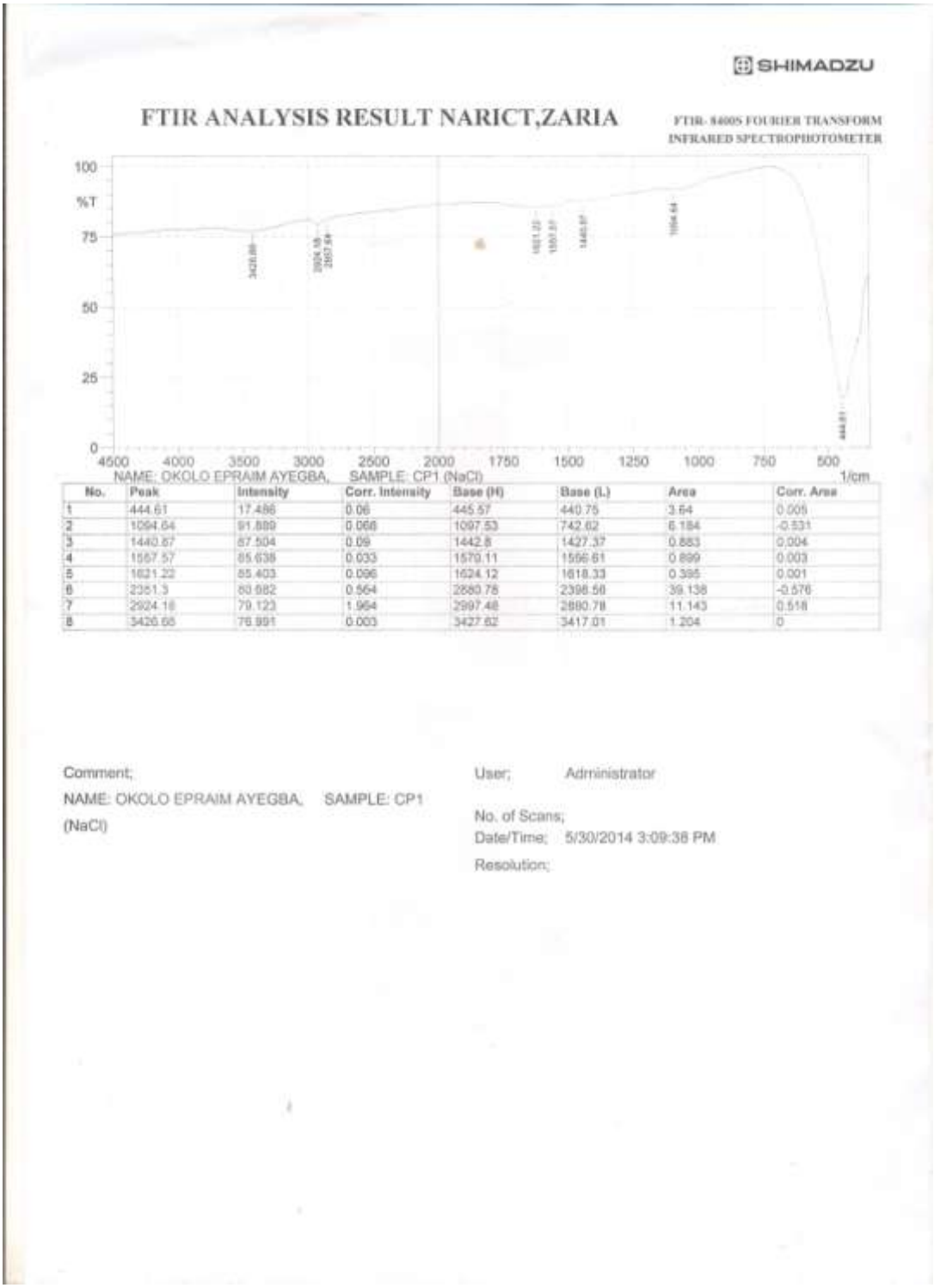


Figure 4.19 UV Spectrum of CP1



No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	444.61	17.486	0.06	445.57	440.75	3.64	0.005
2	1094.64	91.889	0.068	1097.53	742.62	6.184	-0.531
3	1440.87	87.504	0.09	1442.8	1427.37	0.883	0.004
4	1567.57	85.638	0.033	1579.11	1556.61	0.899	0.003
5	1621.22	85.403	0.096	1624.12	1618.33	0.385	0.001
6	2351.3	80.882	0.564	2380.78	2396.56	39.138	-0.576
7	2924.16	79.123	1.964	2997.48	2880.78	11.143	0.518
8	3426.65	76.891	0.003	3427.62	3417.01	1.204	0

Comment;
NAME: OKOLO EPRAIM AYEGBA, SAMPLE: CP1
(NaCl)

User; Administrator
No. of Scans;
Date/Time; 5/30/2014 3:09:38 PM
Resolution;

Figure 4.20 IR Spectrum of CP1

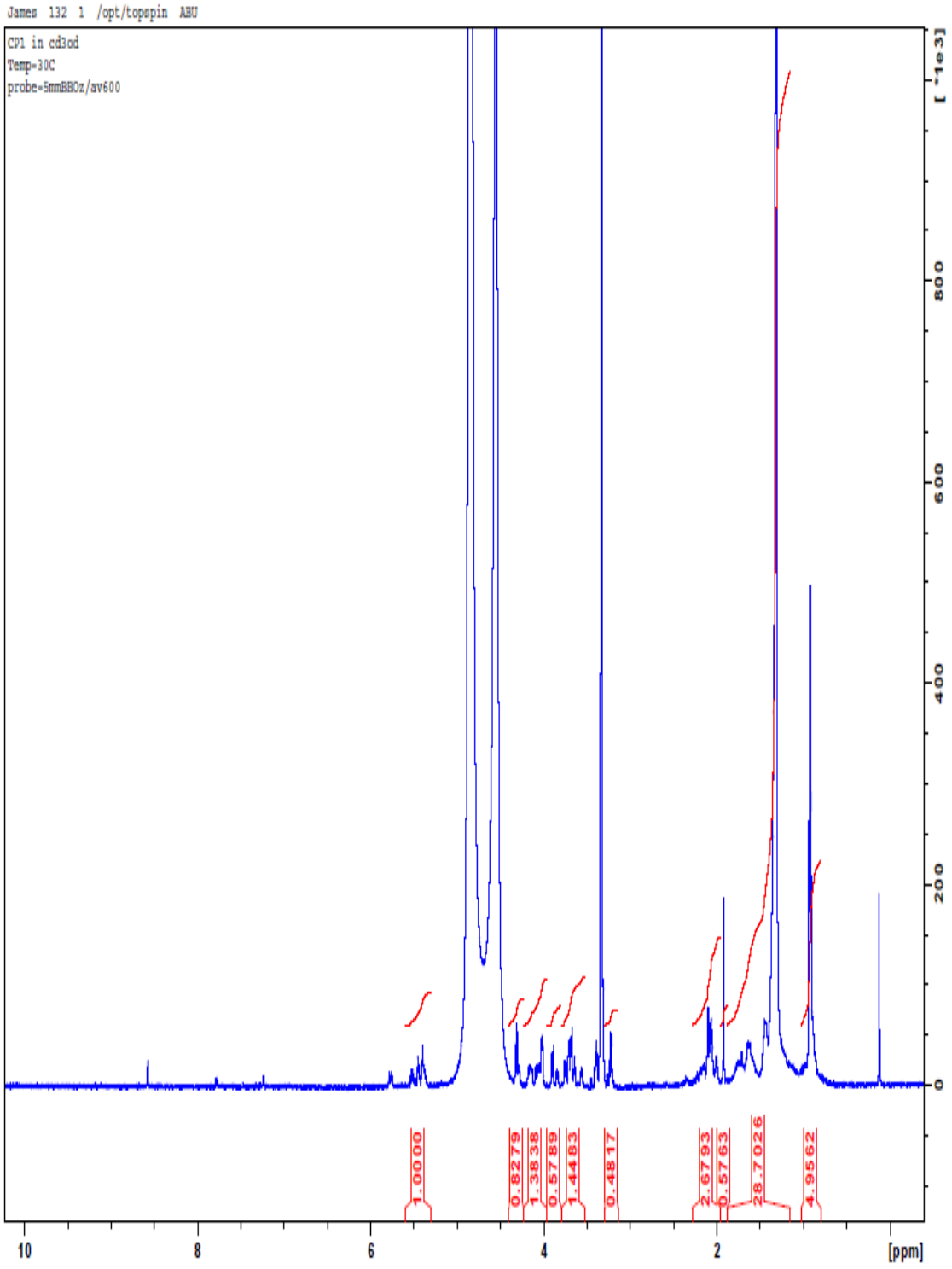


Figure 4.21 Proton NMR Spectrum of CP1

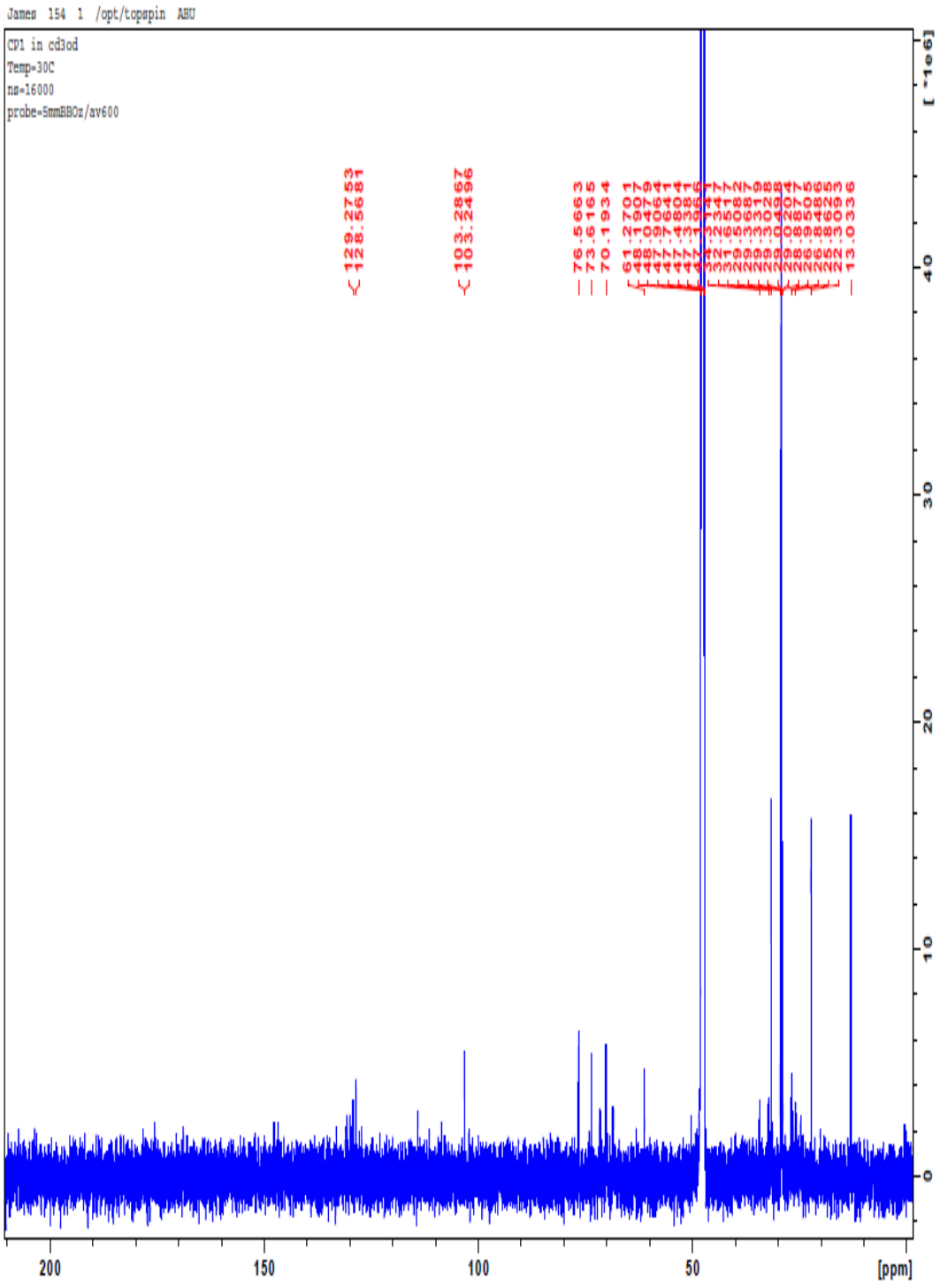


Figure 4.22 Carbon-13 NMR Spectrum of CP1

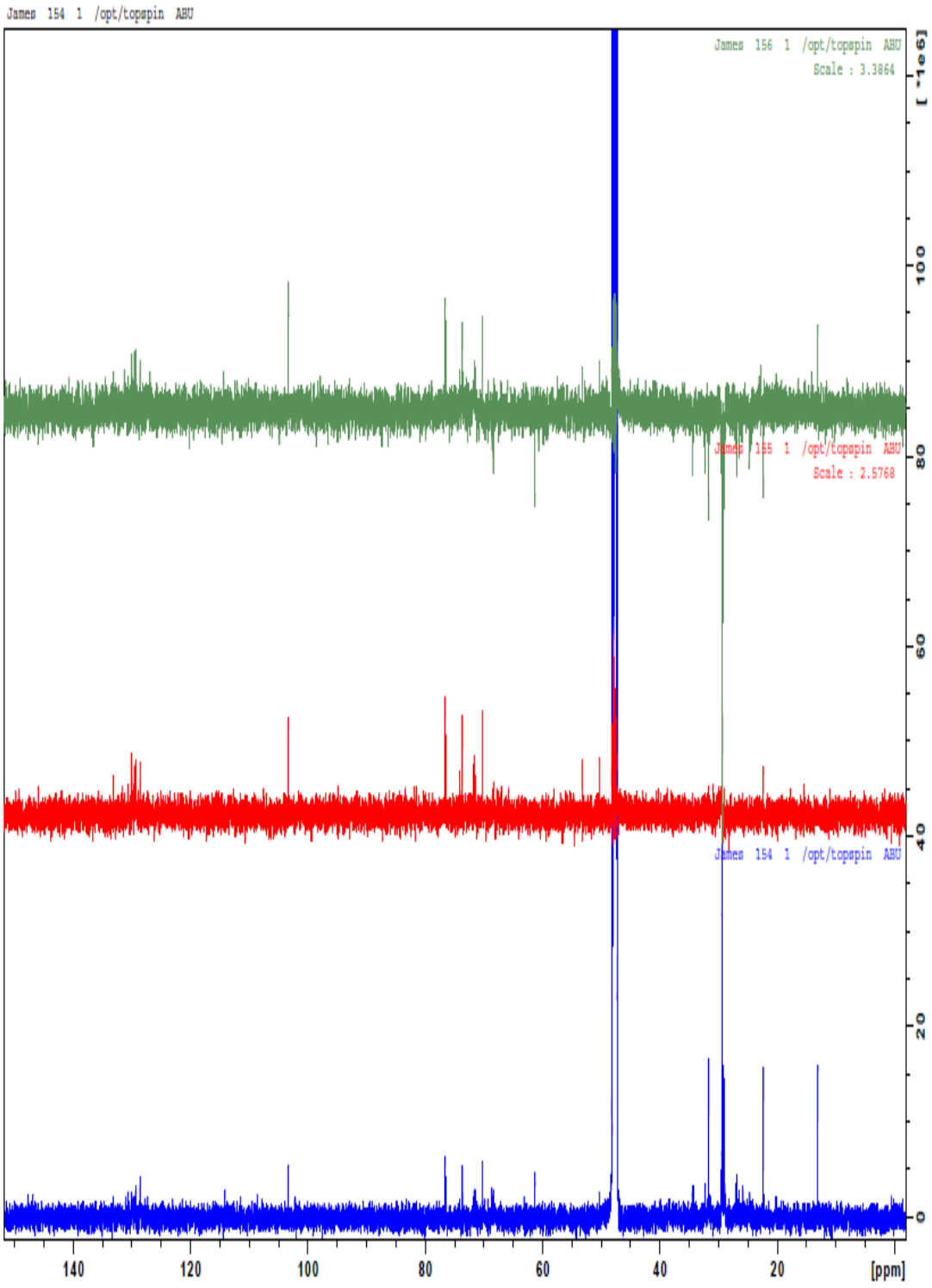


Figure 4.23 DEPT NMR Spectrum of CP1

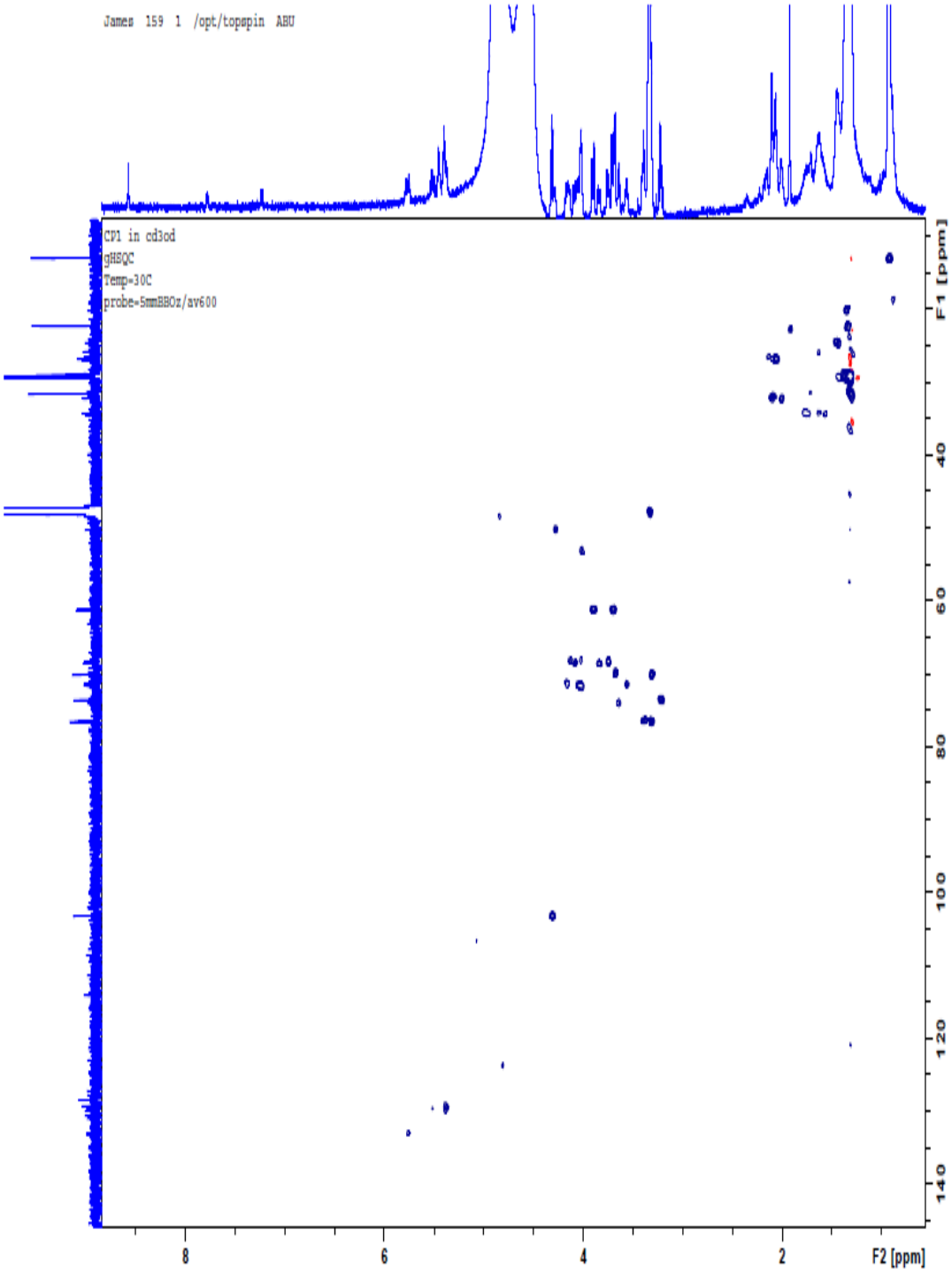


Figure 4.24 HSQC NMR Spectrum of CP1

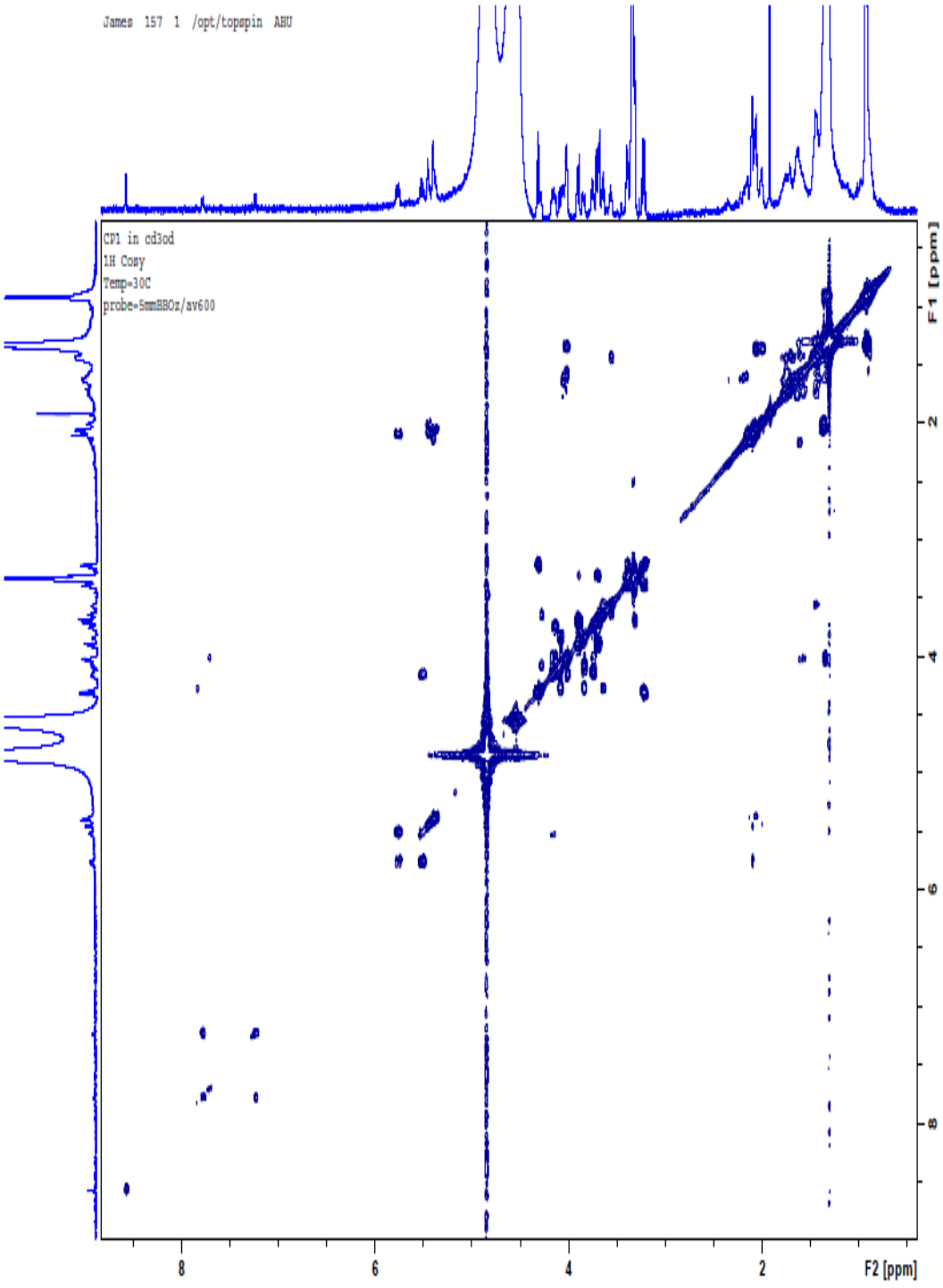


Figure 4.25 COSY NMR Spectrum of CP1

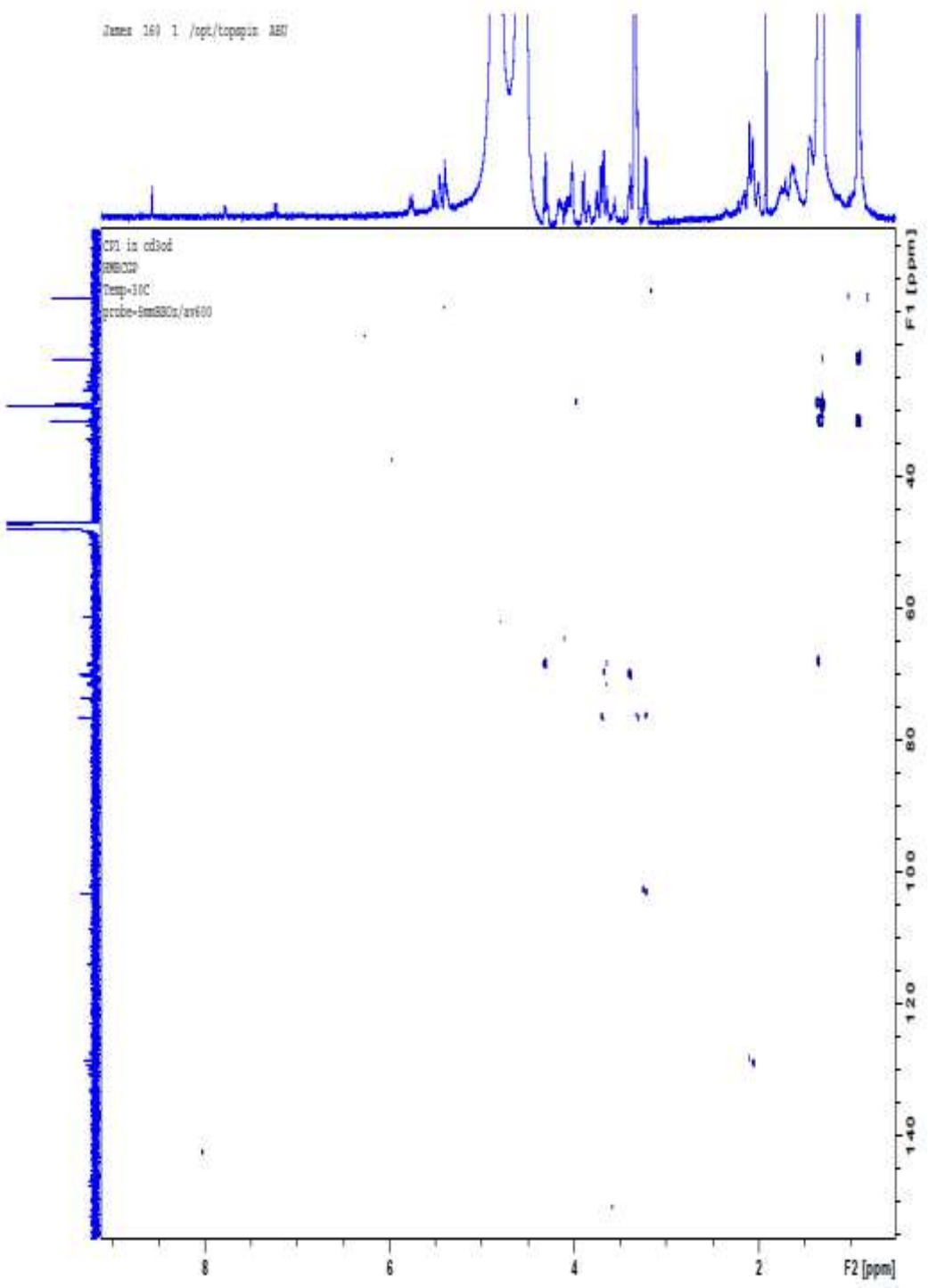


Figure 4.26 HMBC NMR Spectrum of CP1

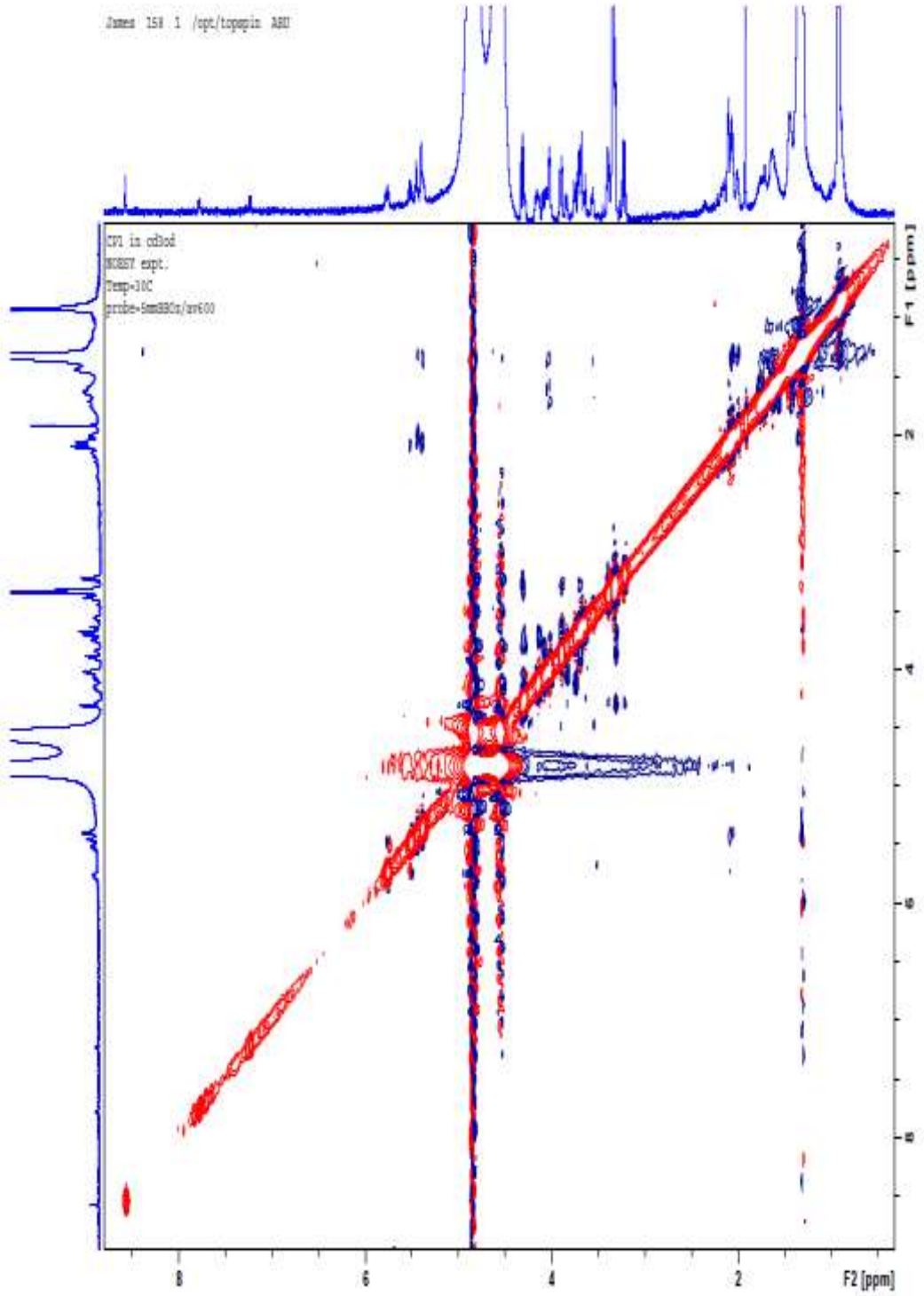


Figure 4.27 NOESY NMR Spectrum of CP1

4.18 Summary of ^{1}H, ^{13}C, DEPT and HSQC NMR Spectral Data of CP1

Table 4.9: ^{1}H, ^{13}C, DEPT and HSQC NMR data of CP1

	δC	δC (glycone)	δH	DEPT
a.	13.0		0.98	CH_3
b.	22.3		1.93	CH_2
c.	25.9		1.65	CH_2
d.	26.8		2.07	CH_2
e.	27.0		2.07	CH_2
f.	28.9		1.37	CH_2
g.	29.0		1.37	CH_2
h.	29.0		1.37	CH_2
i.	29.0		1.37	CH_2
j.	29.0		1.37	CH_2
k.	29.0		1.37	CH_2
l.	29.0		1.37	CH_2
m.	31.7		2.11, 2.02	CH_2
n.	32.2		1.85	CH_2
o.	34.3		1.31	CH_2
p.	61.3	61.3	3.89, 3.70	CH_2
q.	70.2	70.2	3.30	CH
r.	73.6	73.6	3.65	CH
s.	76.6	76.6	3.37	CH
t.	103.2	103.2	4.30	CH
v.	129.3		5.54	CH
w.	132.3		5.74	CH

x.	53.2		4.02	CH
y.	50.0		4.30	CH
z.	68.5		4.11, 4.00	CH_2
α.	71.5	71.5	4.00	CH

4.19 Summary of the COSY NMR Spectrum of CP1

The alphabets below represent the protons as shown in table 4.9 above. For example, Ha-H(f-l) means proton a is correlated to protons f-l which are in about the same chemical environment.

1. H(de) – Hw-Hv

   $(CH_2)_2 – CH = CH$

2. Ha- H(f – l)

   $CH_3(CH_2)_7$

3. Hb-H(f-l)

   $CH_2(CH_2)_7$

4. Hc-Hb

   CH_2-CH_2

5. Hx-Hc

   H_2N-CH-CH_2

6. Ho-Hz

   CH_2-CH_2 – O

4.20 Summary of the HMBC NMR Spectrum of CP1

The alphabets shown below still represent the respective protons as shown in the summary table of the ^{1}H, ^{13}C, DEPT and HSQC NMR spectra. C subscript any alphabet represents the carbon atom attached to the respective proton. For example, Hb represents proton b but C_b represents the carbon to which b is attached while H(f-l) - C_b – Ha means protons (f-l) correlate with C_b and proton a also correlates with $C_{b.}$

1. H(f-l) – C_b - Ha

   $(CH_2)_7$-CH_2-CH_3

2. H(f-l) - C_{f-l}
3. H(f-l) - C_n

   $(CH_2)_7$ – CH_2

4. Ht – C_z

   CH_2– O-Galactose

5. Hm - C_v

   CH_2-CH =

6. Ho – C_z

   CH_2-CH_2-O

7. Hy - C_z

   H_2NCH-CH_2-CH_2-O

8. Hx - C_{de}

   H_2N-CH- $(CH_2)_2$

4.21 Result of Acute Toxicity Studies

Table 4.10: Result of acute toxicity studies

First Phase			**Second Phase**		
Dose in mg/kg	Number of mice used	Number of deaths/ Number of mice used	Dose in mg/kg	Number of mice used	Number of deaths/ Number of mice used
10	3	0/3	200	1	1/1
100	3	0/3	400	1	0/1
1000	3	2/3	800	1	1/1
			1600	1	1/1

4.22 *In vitro* and *In vivo* Antisnake Venom Activity of the Methanol Extract of the Rootbark of *Phyllanthus muellerianus*

Table 4.11: Result of *in vitro* and *in vivo* antisnake venom studies

***In vitro* studies**				***In vivo* studies**		
Group	Treatment	Number of deaths/ Number of mice used	Percentage Survival within 24hrs	Treatment	Number of deaths/ Number of mice used	Percentage survival within 24hrs
1	LD	6/6	0%	LD	6/6	0%
2	A_3 + LD	2/6	67%	A_1 + LD	6/6	0%
3	A_4 +LD	1/6	83%	A_2 + LD	3/6	50%
4	A_5 + LD	4/6	33%	A_3 + LD	6/6	0%

LD = 10mg/kg of the venom; A_1-A_5 are 5, 10, 20, 40 and 80mg/kg of the extract respectively.

4.23 Free Radical Scavenging Activity of PA Compared to Vitamin C

Table 4.12: Free radical scavenging activity of PA compared to vitamin C

Concentration in µg/ml	% Free radical scavenging activity of Pure Compound PA	% Free radical scavenging activity of Vitamin C
1000	93.1 ± 0.0	95.5 ± 0.0
500	89.7 ± 0.0	89.4 ± 0.2
250	89.8 ± 0.2	92.0 ± 0.2
125	87.9 ± 0.5	92.8 ± 0.0
50	85.7 ± 0.9	96.5 ± 0.2
25	90.0 ± 0.9	94.8 ± 0.0
10	83.3 ± 0.5	89.6 ± 0.2
5	87.0 ± 0.2	62.2 ± 0.2

CHAPTER FIVE

5.0 DISCUSSION

5.1 General Discussion

The result of the preliminary phytochemical screening of the methanolic extract revealed the presence of terpenoids, tannins, alkaloids, flavonoids, saponins, and carbohydrate. They are known to have varying types and degrees of activity.

Compounds that have been isolated from the genus *Phyllanthus* fall into the class of alkaloids, flavonoids, terpenoids, steroids, lipids and sugars. Some of such compounds are scopoletin, campesterol, bergenin, xanthoxyline, laevulose and saccharose among many others (Joao *et al*, 1998).

Preparative thin layer chromatography carried out on fractions collected from gel filtration chromatography succeeding silica gel column chromatography of the n-butanol fraction of the methanolic extract yielded the two pure compounds, PA and PX. The TLC of the two compounds was respectively run in two different solvent systems to

ascertain their purity. The melting point of PA is 158-160^{0}C but PX charred in the process.

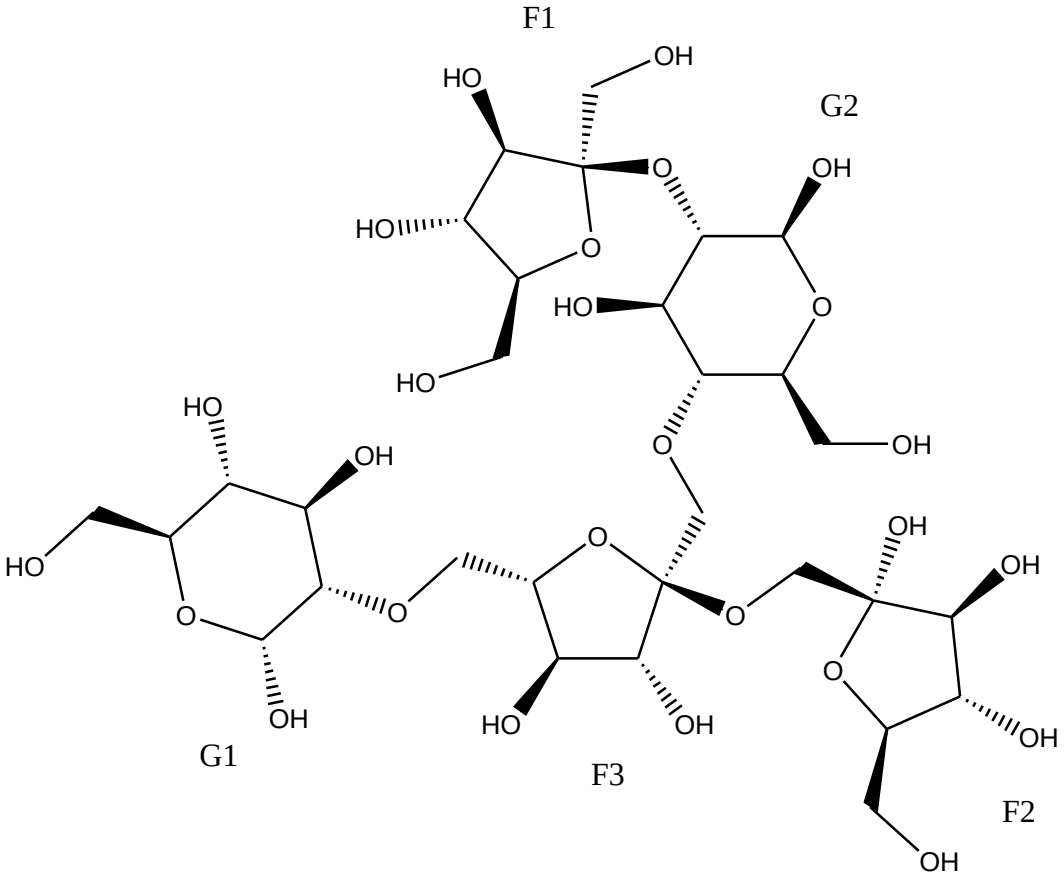
Also, preparative thin layer chromatography carried out on fractions collected from gel filtration chromatography of the n-butanol fraction of the dry methanolic extract yielded the pure CP1. The TLC of CP1 was also run in different solvent systems to ascertain its purity.

5.2 Structure Elucidation of PA

The UV spectrum of PA indicates 214nm as the wavelength of maximum absorption. The IR values at 3391cm^{-1}, 2928cm^{-1} and 1066cm^{-1} represent O-H, C-H and C-O stretching vibrations respectively.

The proton NMR of PA is characteristic of sugars. The peaks at 5.14ppm and 4.50ppm are characteristic of glucose while the oxymethine and oxymethylene peaks are found between 3.00ppm and 4.00ppm. Thirty peaks appear in the ^{13}C NMR spectrum within the range of sp^3 hybridized carbon atoms attached to highly electronegative elements (δ_C 62.6-105.9ppm) such as oxygen. Five of these carbon peaks are anomeric corresponding to an α-D-glucopyranose G1 (δ_C 94.0ppm), a β-D-glucopyranose G2 (δ_C 98.2ppm), a β-D-fructofuranose F1 (δ_C 99.2ppm) and two α -D-fructofuranose units, F2 (δ_C 103.2ppm) and F3 (δ_C 105.9ppm) respectively. The DEPT spectrum indicates the fact that among the 30 carbon atoms, 3 are quartenary (δ_C 99.2, 103.2 and 105.9ppm), 19 are methine (δ_C 69.4-98.2ppm) while 8 are methylene (δ_C 62.6-65.9ppm) carbon atoms (William, 2003; Carolyn *et al*, 1983).

The HSQC correlations indicate the hydrogen atoms that are bonded to their respective carbon atoms and their chemical shifts. The HMBC spectrum unravels the way the sugar molecules are linked: C-2 of F3 is correlated to C-1 of F2, C-4 of G2 is correlated to C-1 of F3, C-2 of F1 is correlated to C-2 of G2, and C-2 of G-1 is correlated to C-6 of



F3. Based on all these data, the proposed structure of PA is:

α-D-Glcp-(2→6)-[α-D-Fruf(1→2)]α-D-Fruf-(1→4)β-D-Glcp-(2→2)β-D-Fruf

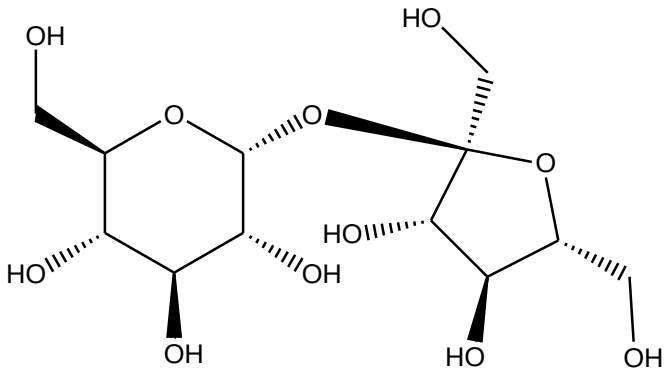
The COSY correlations H-1, H-2 of G1; H-1, H-2 of G-2 and H-5, H-6 of F3 and the NOESY correlation H-6, H-1 of F3 support the proposed structure.

5.3 Structure Elucidation of PX

The UV spectrum of PX indicates 208nm as the wavelength of maximum absorption. The IR values at 3370cm^{-1}, 2929cm^{-1} and 1068cm^{-1} represent O-H, C-H and C-O stretching vibrations respectively.

The proton NMR of PX is also characteristic of sugars. The peak at 5.40ppm is characteristic of glucose while the oxymethine and oxymethylene peaks are found between 3.25ppm and 4.00ppm. Twelve peaks appear in the ^{13}C NMR spectrum within the range of sp^3 hybridized carbon atoms attached to highly electronegative elements (62.2-105.3ppm) such as oxygen. Two of these peaks are anomeric corresponding to an α-D-glucopyranose unit G4 (93.7ppm) and an α-D-fructofuranose unit F4 (105.3ppm). The DEPT spectrum indicates the fact that among the 12 carbon atoms, 1 is quartenary (105.3ppm), 8 are methine (71.3-93.7ppm) while 3 are methylene carbon atoms (William, 2003; Seung-Kee and Alexander, 2008).

The HSQC correlations indicate the hydrogen atoms that are bonded to the various carbon atoms and their chemical shifts as represented in the table of results for G4 and F4. The HMBC spectrum unravels the way the sugar molecules are linked: C-2 of F4 is correlated to C-1 of G4. This linkage is known in nature for the disaccharide sucrose. Based on all these data, the structure of PX is:



Sucrose α-D-Glcp-(1→2)α-D-Fruf

The COSY correlations H-1, H-2 of G4; H-3, H-4 of F4; and the NOESY correlation H-3, H5 of F4 support this structure.

The comparison of the ^{13}C NMR chemical shifts of PX with that of a synthesized L-sucrose from the literature is shown below (Seung-Kee and Alexander, 2008).

Table 5.1: Comparison of the ^{13}C NMR of PX and a synthesized L-sucrose

S/No	δ_C for G4 (PX)	δ_C for G4 (Seung- Kee and Alexander, 2008)	δ_C for F4 (PX)	δ_C for F4 (Seung- Kee and Alexander, 2008)
1	93.7	93.7	64.1	63.9
2	73.2	73.2	105.3	105.4
3	74.7	74.7	79.3	79.2
4	71.3	71.3	75.7	75.7
5	74.4	74.4	83.8	83.7
6	62.2	62.2	63.4	63.5

5.4 Structure Elucidation of CP1

The UV spectrum of CP1 indicates 315nm as the wavelength of maximum absorption. The IR values at 3427cm^{-1}, 2924cm^{-1} and 1621cm^{-1} and 1095cm^{-1} represent O-H, C-H, C=C and C-O stretching vibrations respectively. The proton NMR of CP1 is characteristic of glycosides with the aglycone peaks showing between 0.98 – 2.11ppm.

The peaks at 5.54 and 5.74ppm represent those of the olefinic protons. The glycone peaks appear between 3.30 – 4.30ppm. Twenty six peaks have been accounted for from the ^{13}C and the DEPT NMR spectra out of which one is methyl, sixteen are methylene and nine are methine carbon atoms. Six of those carbon atoms have been recognized to be those of galactose with its anomeric carbon at 103.2ppm.

The HSQC spectrum was used to indicate protons that are bonded to their respective carbon atoms. COSY and HMBC spectra were used to bring out the various fragments and the proposed structure of the isolated compound CP1. The fragments and the proposed structure are shown below. The alphabets a, b to z represent the protons as illustrated in Table 4.8 and section 4.31.

1. a b f-l c x n

   CH_3CH_2 $(CH_2)_7$ – CH_2 – $CH(NH_2)$ - CH_2

   HMBC (J^2) COSY COSY HMBC (J^4)

2. de w v m

   $(CH_2)_2$ – CH = CH – CH_2

   COSY COSY HMBC (J^2)

3. y o z

   CH (NH_2) – CH_2 – CH_2 – O- Galactose

   HMBC (J^3) COSY HMBC (J^3)

4. a b f-l c x n de w v

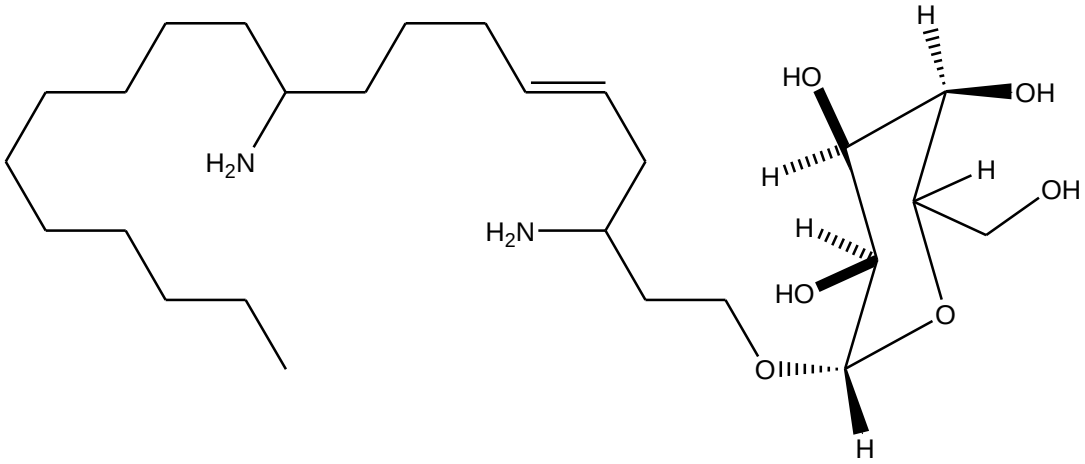
$CH_3CH2(CH2)7CH2CH(NH2)CH2$ —— $(CH_2)_2CH=CH$

HMBC J^3 |

$GalactoseOCH_2CH_2(NH_2)CHCH_2$

z o y m

Or



(2*S*,3*S*,4*S*,5*R*)-2-((*E*)-3,10-diaminoicos-5-enyloxy)-tetrahydro-6-(hydroxymethyl)-2*H*-pyran-3,4,5-triol

It is a galactoside of a sphingoid base-like compound. The sphingoid base sphingosine is the first member of the sphingolipids found in a wide variety of organisms. Families of plants known to contain sphingolipids include Zamiaceae, Auracariaceae, Ginkgoceae, Lauraceae, Winteraceae and Fagaceae among others (Sarah *et al*, 2008; Islam *et al*, 2011).

5.5 Antivenin and *in vitro* Antioxidant Studies

The LD_{50} of the methanol extract was determined from the acute toxicity studies to be 400mg/kg. It exhibited a significant *in vitro* activity against the venom of *Naja nigricollis* in mice. There was 67% protection against lethality at an administered dose of 20mg/kg, 83% at 40mg/kg and 33% at 80mg/kg of the extract. The *in vitro* activity suggests that the crude drug can be used at the site of bite. Since the activity is not dose dependent, the result also suggests that at 80mg/kg of the extract toxicity competes with activity while 20mg/kg of the extract is not an optimum dose in mice.

A significant *in vivo* activity in mice was observed at an administered dose of 10mg/kg of the extract (50% protection against lethality). At the other doses (5 and 20mg/kg), there was 0% protection against lethality. At 5mg/kg, the crude drug may not be potent enough while at 20mg/kg, toxicity competes with *in vivo* activity.

The results above confirm the traditional claim that *Phyllanthus muellerianus* root bark possesses antisnake venom properties. There are ethnomedicinal claims in the literature as to the antivenin properties of other *Phyllanthus* species of plants. However no scientific investigation was found in the literature to confirm such claims.

The antioxidant activity of the isolated pentasaccharide PA in the presence of DPPH is comparable to that of vitamin C. Using the independent sample student t-test, there is no significant difference between the free radical scavenging activity of PA and vitamin C. The antioxidant activity of PA could be as a result of its polyhydroxy nature that is capable of reducing free radicals. Oligosaccharides from fungi, land plants and algae are known to possess antioxidant, antitumor and immune-stimulation activities (Josiane, 2009)

The aqueous and methanol extracts of the leaf and fruit of *Phyllanthus niruri*, and the whole plant of *Phyllanthus amarus* have been found to have considerable DPPH

scavenging activity among other species (Harish and Shivanandappa, 2004; Chandan *et al*, 2012). The *Phyllanthus* genus could therefore be said to be rich in phytochemicals that could be used in managing or preventing various degenerative diseases such as cancer, brain dysfunction and other diseaes which could be caused by the oxidation of cell content.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The result of the preliminary phytochemical screening of the methanolic extract revealed the presence of terpenoids, tannins, alkaloids, flavonoids, saponins, and carbohydrate. Subjecting the partitioned n-butanol fraction to silica gel chromatography followed by gel filtration and finally preparative thin layer chromatography yielded two oligosaccharides, a pentasaccharide PA and sucrose PX. Subjecting also the n-butanol fraction, obtained by directly adding n-butanol to the methanol extract, to gel filtration and preparative thin layer chromatography gave an amino alcohol glycoside CP1.

The LD_{50} of the methanol extract of *Phyllanthus muellerianus* was found to be 400mg/kg intraperitoneally while that of the aqueous extract of *Phyllanthus fraternus* was found to be 692.8mg/kg intraperitoneally and that of the methanol extract of *Phyllanthus niruri* was found to be 471.2mg/kg orally (Matur *et al*, 2009; Okoli *et al*,

2010). The methanol extract of *Phyllanthus muellerianus* showed significant protection against lethality in mice *in vitro* through the administered doses of the extract and the venom. *In vivo*, protection against lethality was seen at 10mg/kg of the extract only. The free radical scavenging activity of the pentasaccharide PA is comparable to that of vitamin C.

6.2 Conclusion

The research has achieved the stated objectives. To the best of our search, this is the first time the isolation of the pentasaccharide PA and the amino alcohol glycoside, CP1 are being reported. This will add to the global natural products data base. PX which is sucrose is known to exist in nature.

Based on the findings, it can be concluded that the traditional use of the root bark of *Phyllanthus muellerianus* has scientific basis.

6.3 Recommendations

In order to fully validate the ethnomedicinal claim of the root bark of *Phyllanthus muellerianus*, it is recommended that:

1. More compounds should be isolated and tested for antisnake venom and antioxidant activity.
2. Detailed antisnake venom and antioxidant studies should be carried out on the extract and such isolated compounds, using other models.

REFERENCES

Abubakar M.S., Sule M.I., Pateh U.U., Abdurahman E.M., Haruna A.K., Jahun B.M.(2000). In vitro snake venom detoxifying action of the leaf extract of *Guiera senegalensis*, *Journal of Ethnopharmacology*, 69: 253-257.

Adesida G.A., Girgis P., Taylor D.A.H. (1972). Friedelin derivatives from *Phyllanthus muellerianus*, *Phytochemistry*, 11: 851-852.

Adzu B., Abubakar M.S., Izebe K.S., Akumka D.D., Gamaniel K.S.(2005). Effect of *Annona senegalensis* root bark extracts on *Naja nigricolis* venom in rats, *Journal of Ethnopharmacology*, 96 : 507-513.

Aliyu A.B., Musa A.M., Abdullahi M.S., Oyewale A.O., Gwarzo U.S. (2008). Activity of plant extracts used in Northern Nigeria Traditional Medicine against Methicillin-Resistant Staphylococcus aureus, *Nigerian Journal of Pharmaceutical Sciences*, Volume 7, Number 1 : 2-4.

Anas A.K., Rajendaran K., Durga J., Hari P.S., Ashutosh M., Dinesh C., Indranil K.Y., Jain D.A (2010). Antisnake venom activity of different extracts of *Pouzolzia indica* against Rusell viper venom, *International Journal of ChemTech Research*, Volume 2, No 1, pp 744-751.

Barbi, N.S. (1992). ContribuicËaÄo aÁ pesquisa de plantas antiofõÂdicas:estudo de polissacarõÂdeos presentes na casca de Casearia sylvestrisSw. M.S. thesis, Federal University of Rio de Janeiro.

Brussotti G., Cesari I., Frassa G., Grisoli P., Dacarro C., Caccialanza G. (2011). Antimicrobial properties of stem bark extracts from *Phyllanthus muellerianus* (Kuntze) Excell, *Journal of Ethnopharmacology* 135: 797-800.

Burkill H.M. (1994). The Useful Plants of West Tropical Africa, Volume 2, Second Edition, The Trustees of the Royal Botanical Gardens, Kew, pp 121-123.

Carolyn I.N., Helga J.C., Albert R.G., Paul D.E., Suzanne R.T., John W.B. (1983). ^{13}C NMR Investigation of Nonenzymatic Glucosylation of Protein, *The Journal of Biological Chemistry*, Volume 258, Number 23, 14279-14283.

Chandan S., Umesha S., Balamurugan V. (2012). Antileptospiral, antioxidant and DNA damaging properties of *Eclipta alba* and *Phyllanthus amarus*, *Open Access Scientific Reports*, Volume 1, Issue 4, 1-8.

De Smet P.A.G.M. (1997). Adverse effect of herbal remedies, *Adverse Drug Reactions Bulletin*, 183, 695-698.

Edeoga H.O., Okwu D.E., Mbaebie B.O. (2005). Phytochemical constituents of some Nigerian medicinal plants, *African Journal of Biotechnology* 4 : 685-688.

Harish R., Shivanandappa T. (2004). Antioxidant and hepatoprotective potential of *Phyllanthus niruri*, *Food Chemistry*, 95, 180-185.

Hutchinson J., Dalziel J.M., Keay R.W.J. (1958). Flora of West Tropical Africa, Second Edition, Crown Agents for Oversea Governments and Administrators, Millbank, London, pp384.

Inder K.M., Devang K. (2000). Antisnake venom properties of medicinal plants, *Der pharmacia lettre* 2(5) : 399-411.

Islam M.N., Marie-Pierre J., Sylvie C., Carl K-Y. N. (2011). Sphingosine in plants – more riddle from the sphinx? *New Phytologist*, 193: 51-57.

James R.H. (2003). The Classes of Natural Product and Their Isolation, Royal Society of Chemistry, www.rsc.org/pdf/tct/natprods1.pdf.

Joao B.C., Adair R.S.S., Valdir C.F., Rosendo A.Y. (1998). A Review of the plants of the Genus *Phyllanthus*: Their Chemistry, Pharmacology and Therapeutic Potential, *Medicinal Research Reviews* 18(4) : 225-258.

Josiane Courtios (2009). Oligosaccharides from land plants and algae: Production and application in therapeutics and biotechnology, *Current opinion in microbiology*, 12 : 261-273.

Kasturiratne A., Wickremasinghe A.R., de silver N., Gunawardena N.K., Pathmeswaran A., Premaratna R., Savioli L., Lalloo D.G., de Silva H.J. (2008). The Global Burden of Snakebite: A Literature Analysis and Modeling Based on Regional Estimates of Envenoming and Deaths. *PLoS Med* 5(11) : e218, 1591-1602.

Kew J., Morris C., Aihie A., Fysh R., Jones S., Brooks D. (1993). Arsenic and mercury intoxication due to Indian ethnic remedies, *British Medical Journal*, 306, 506-7.

Lorke D. (1983). A new approach to practical acute toxicity testing, *Archives of Toxicology*, 54: 275-287.

Mark Percival (1998). Antioxidants, *Clinical Nutrition Insights*, pp 1-4.

Markfarlane R.G. (1967). Russel's Viper Venoms, 1963-1964. *British Journal of Haematology*, 13, 437-451.

Matur B.M., Matthew T., Ifeanyi C.I.C. (2009). Analysis of the phytochemical and in vivo antimalarial properties of *Phyllanthus fraternus* Webster, *New York Science Journal*, 2(5): 12-19.

Mayer A.M.S., Rodriguez A.D., Berlinck R.G.S., Fusetani N. (2011). Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis and antiviral activities; affecting the immune and nervous system, and other miscellaneous mechanisms of action, *Comparative Biochemistry and Physiology*, Part C 153, 191-222.

Miller L.G. (1998). Herbal Medicinals: selected clinical considerations focusing on known potential drug-herb interactions. *Archives of Internal Medicine* 158, 2200-2211.

Murray M.T., Pizzorno J.E. Jr. (2000). Botanical medicine - a modern perspective. In: Pizzorno J.E. Jr., Murray M.T., Textbook of Natural Medicine, Volume 1 (eds.), Churchill Livingstone, pp 267-279.

Nakagawa M., Nakanishi K., Darko L.L., Vick J.A. (1982). Structures of cabenegrins A-I and A-II, potent anti snake venoms. *Tetrahedron letters* 23, 3855-3858.

Okoli C.O., Ibiam A.F., Ezike A.C., Akah P.A., Okoye T.C. (2010). Evaluation of antidiabetic potentials of *Phyllanthus niruri* in alloxan diabetic rats, *African Journal of Biotechnology*, Volume 9(2): 248-259.

Philipson, J.D. (2001). Phytochemistry and medicinal plants. *Phytochemistry* 2001, 56, 237-243.

Pugh R.N.H., Theakston R.D.G. (1980). Incidence and mortality of snake bite in savanna Nigeria, *Lancet* 2 : 1181-1183.

Reid H.A., Theakston R.D.G. (1983). The management of snake bite. *Bulletin W.H.O* 63 : 885-895.

Ron K., Abraham N. (2002). Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions and Methods for Their Quantification, *Toxicologic Pathology*, Volume 30, Number 6, pp 620-650.

Rusell F.E. (1980). Snake venom poisoning, Philadelphia J.B. Lippincott Company, pp 235-285.

Sanjoy K.P., Aparna G., Dasgupta S.C., Anthony G. (2002). Snake venom as therapeutic agents: From toxin to drug development, *Indian Journal of Experimental Biology*, Volume 40, pp 1353-1358.

Sanjoy K.P., Yogeshwer S. (2003). Herbal Medicine: Current Status and the Future, *Asian Pacific Journal of Cancer Prevention*, 4, 281-288.

Sarah T.P., Anatoliy B., Kerri H., Madhura A., Christopher A.H., Sullards M.C., Dennis C.L., Alfred H.M. (2008). Thematic Review Series: Sphingolipids. Biodiversity of sphingoid bases ("sphingosines") and related amino alcohols, *Journal of Lipid Research*, 49(8): 1621-1639.

Sasidharan S., Chen Y., Saravanan D., Sundram K.M., Latha L.Y. (2011). Extraction, Isolation and Characterization of Bioactive Compounds from Plants Extracts, *African Journal of Traditional Complementary and Alternative Medicines* 8(1) : 1-10.

Seung-Kee S., Alexander W. (2008). Probing Osmotic Effects on Invertase with L-sucrose: Supporting Information, *Org Biomol Chem*, 6(18) : 3362-3365.

Sheeja T.T. (2012). Taxonomy of the Genus *Phyllanthus*. In: Ramadasan K., Harikumar K.B. (eds.) Traditional Herbal Medicines for Modern Times (*Phyllanthus* species: scientific evaluation and medicinal applications), Taylor and Francis Group, Florida USA, pp 3.

Shirwakar A., Shirwakar A.R., Rajendran K., Punitha I.R.S. (2006). In Vitro Antioxidant Studies on the Benzyl Tetra Isoquinoline Alkaloid Berberine, *Biological and Pharmaceutical Bulletin*, 29, 1906-1910.

Sies H., Stahl W., Sundquist A.R. (1992). Antioxidant Function of Vitamins, *Annals of the New York Academy of Sciences*, 669, 7-20.

Silva G.L., Lee I., Douglas K.A. (1998). Special problems with extraction of plants. In: Cannell J.P.R (eds). Natural Products Isolation, Human Publishers, New Jersey USA, pp 251-293.

Singh B.R, Tu A.T. (1996). Overview of Snake Venom Chemistry. In: A.T. Tu (eds.) Natural Toxins II, Advances in Experimental Medicine and Biology, Plcnum Press, New York, Volume 391, pp 37-62.

Smith H.M., Smith R.B., Sawin H. L. (1977). A Summary of snake classification (Reptilia, Serpentes). *Journal of Herpetology*, 11 : 115-122.

Sofowora, A. (1982). Medicinal Plants and Traditional Medicine in Africa, 1st Edition, John Wiley and Sons, Chichester, pp 142-143.

Tailor R.H., Acland D.P., Attenborough S., Cammue B.P.A., Evans I.J., Osborn R.W., Ray J.A., Rees S.B., Broekaert W.F. (1997). A Novel Family of Small Cysteine-rich Antimicrobial Peptides from the Seed of I*mpatiens balsamina* is derived from a Single Precursor Protein, *The Journal of Biological Chemistry*, 272, 24480-24487.

Tan N.H., Gnanajothy P., (1990). A comparative study of the biological properties of venoms from snakes of the genus *Vipera* (true adders), *Comparative Biochemistry and Physiology*, 96: 683-688.

Theakston R.D.G., Reid H. A. (1983). The development of simple standard assay procedure for characterization of snake venoms, *Bulletin of W.H.O*. 61, 949-956.

Topic B., Tani E., Tsiakitzis K., Kourounaskis P.N., Dere E., Hasenohrl R.U., Hacker R., Mattern C.M., Huston J.P. (2002). Enhanced maze performance and reduced oxidative stress by combined extract of *Zingiber officinale* and *Ginkgo biloba* in the aged rat, *Neurobiology of Aging* 23(1) : 135-143.

Trease K., Evans W.C. (1996). Textbook of Pharmacognosy, 14th Edition, Balliere, Tindall, London, pp 251-293.

Vanhaecke M., den Ende W.V., Lescrinier E., Dyubankova N. (2008). Isolation and Characterization of a Pentasaccharide from *Stelleria media*. *The Journal of Natural Products*, 71, 1833-1836.

Ved H.S., Koenig M.L., Dave J.R., Doctor B.P. (1997). Huperazine A, a potential therapeutic agent for dementia, reduces neuronal cell death caused by glutamate, *Neuroreport* 8(4): 963-968.

Vickers A., Zollman C. (1999). ABC of complementary medicine: herbal medicine, *British Medical Journal*, 319, 1050-1053.

Vivek K.G., Surendra K.S. (2006). Plants as natural antioxidants. *Natural Product Radiance*, Volume 5(4) : 326-334.

Walter B.M., do Nacimento M.C., Bettina M., Pereira R., Nuno A.P. (2000). Plant natural products active against snake bite - the molecular approach, *Phytochemistry* 55 : 627-642.

WHO (2013). Traditional Medicine, www.who.int/topics/traditional_medicine/en/.

WHO News (2002). Traditional medicine strategy launched, 80, 610.

William A.B. (2003). NMR Spectroscopy in the Study of Carbohydrates: Characterizing the Structural Complexity, *Concepts in Magnetic Resonance*, Part A, Volume 19A (1): 1-19.

Winslow L.C., Kroll D.J. (1998). Herbs as medicine, *Archives of Internal Medicine*, 158, 2192-2199.

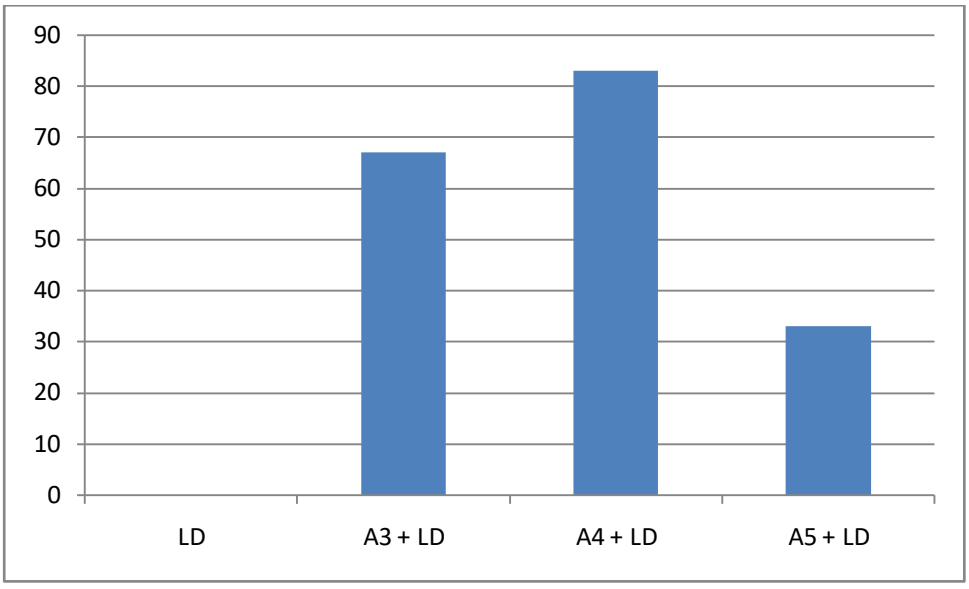
Wright A.H., Wright A.A. (1957a). Handbook of Snakes, Volume 1, Comstock Publishing Associates, Ithaca, NY, pp 564.

Wright A.H., Wright A.A. (1957b). Handbook of Snakes, Volume 2, Comstock Publishing Associates, Ithaca, NY, pp 1105.

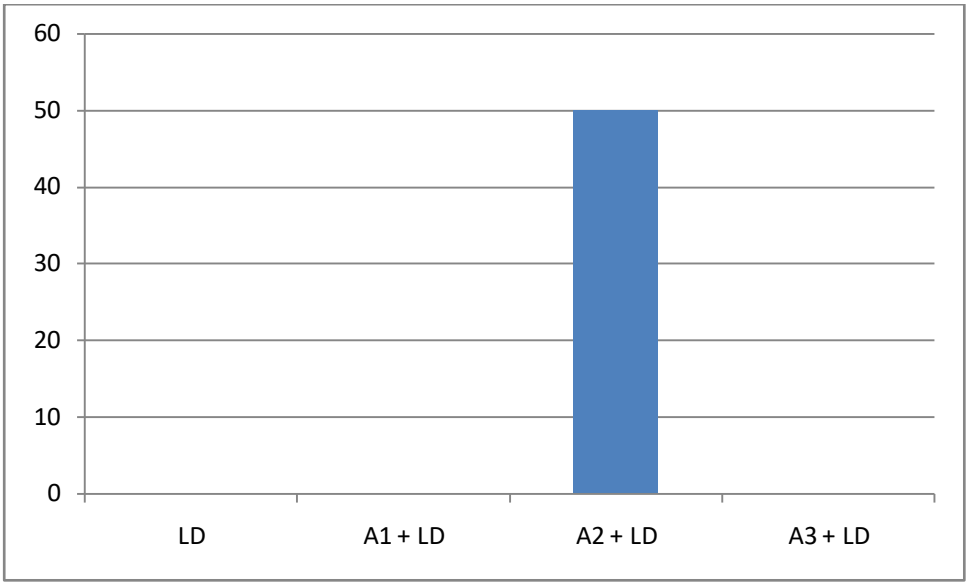
Zhang L., Yu H., Zhao X., Lin X., Tan C., Cao G., Wang Z. (2010). Neuroprotective effects of salidroside against beta-amyloid-induced oxidative stress in SH-SY5Y human neuroblastoma cells. *Neurochemistry International* 57(5) : 547-555.

Zhou L., Yang C., Li J., Wang S., Wu J. (2003). Heptasacchride and octasacharide isolated from *Paris polyphylla* var. yunnanensis and their plant growth-regulatory activity, *Plant Science* 165, 571-575.

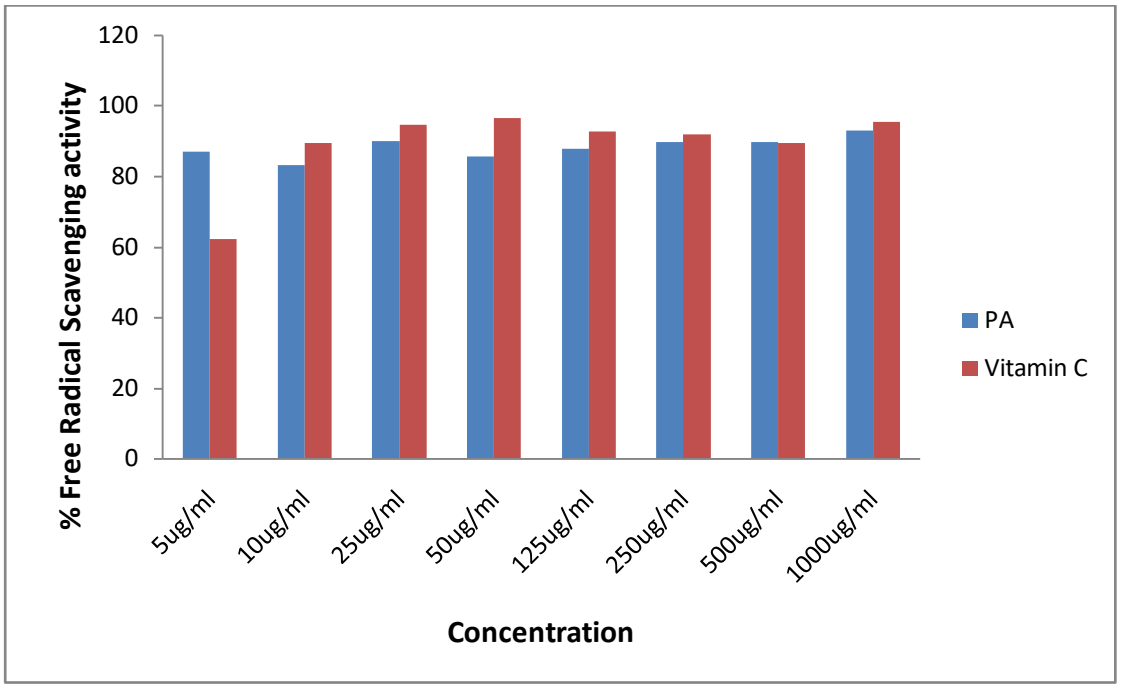
APPENDICES



Appendix I: A graph of administered dose versus percent survival (*in vitro* snake venom detoxifying test)



Appendix II: A graph of administered dose versus percent survival (*in vivo* snake venom detoxifying test)



Appendix III: A graph comparing the free radical scavenging activities of PA and vitamin C at various concentrations