PHYTOCHEMICAL AND SOME BIOLOGICAL STUDIES OF THE METHANOL EXTRACT OF THE AERIAL PARTS OF *AMPELOCISSUS GRANTII*(BAKER) PLANCH. (VITACEAE)

BY

YAHAYA MAIKAFI MUSA

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

JANUARY, 2016

PHYTOCHEMICAL AND SOME BIOLOGICAL STUDIES OF THE METHANOL EXTRACT OF THE AERIAL PARTS OF *AMPELOCISSUS GRANTII*(BAKER) PLANCH. (VITACEAE)

BY

Yahaya Maikafi MUSA B. Sc. Chem. (ABU) M. Sc. Pharm. Chem. (ABU)
Ph.D/Pharm Sci/16648/2007-2008

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY DEGREE (Ph.D) IN PHARMACEUTICAL AND MEDICINAL CHEMISTRY

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,
FACULTY OF PHARMACEUTICAL SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

JANUARY, 2016

DECLARATION

I do solemnly declare that, the work in this thesis entitled **PHYTOCHEMICALAND SOME BIOLOGICAL STUDIES OF THE METHANOL EXTRACT OF THE AERIAL PARTS OF *AMPELOCISSUS GRANTII*(BAKER) PLANCH. (VITACEAE)** has been performedby me in the Department of Pharmaceutical and Medicinal Chemistry under the supervision of Prof. M. I. Sule, Prof. U. U. Pateh and Prof. M. Ilyas.

The information derived from the literature has been duly acknowledged in the text and in list of references provided. No part of this dissertation was previously presented for another degree or diploma in this or any institution.

YahayaMaikafiMusa

………………………….   …………………………….   ……………………

Name of student   Signature   Date

CERTIFICATION

This thesis entitled **PHYTOCHEMICALAND SOME BIOLOGICAL STUDIES OF THE METHANOL EXTRACT OF THE AERIAL PARTS OF** ***AMPELOCISSUS GRANTII*****(BAKER) PLANCH. (VITACEAE) by YahayaMaikafiMUSA** meets the regulations governing the award of Doctor of Philosophy in Pharmaceutical and Medicinal Chemistry of Ahmadu Bello University, Zaria, and is approved for its' contribution to scientific knowledge and literary presentation.

Prof. M. I. Sule

…………………………. ……………………………. ……………………

Chairman, Supervisory Committee Signature Date

Prof.U. U. Pateh

…………………………. ……………………………. ……………………

Member, Supervisory CommitteeSignature Date

Prof. M. Ilyas

…………………………. ……………………………. ……………………

Member, Supervisory CommitteeSignature Date

Dr. A. M. Musa

…………………………. ……………………………. ……………………

Head of DepartmentSignature Date

Prof. K. Bala

…………………………. ……………………………. ……………………

Dean, Signature Date

School of Post Graduate Studies

DEDICATION

This research work is dedicated to SayyidaFatimat-uz-Zahra (sa)

AKNOWLEDGEMENT

In the name of Allah the Almighty who taught man about matter that he does not knows, prayers and peace be upon our Prophet Mohammed and his pure progeny (as). I am most grateful to Allah the Almighty for helping and guiding me throughout my life.

Firstly, I would like to extend my thanks to Professor M. I. Sule for his continuous encouragement, excellent supervision, valuable advice and constructive criticism that help me get through many intricate phases during the preparation of this thesis. I am also grateful to my second and third supervisors Professor U. U. Pateh and Professor M. Ilyaswhospared no efforts to guide me during the preparation of this thesis. I thank you for giving me so much of your precious time and teaching me the different ways to approach a research problem and the need to be persistent to accomplish any goal. Without your guidance and constructive comments, this dissertation simply could not be completed. Your patience with me and the difficult part of this study is sincerely treasured.

Special thanks go to the Head of Department, Department of Pharmaceutical and Medicinal Chemistry,Dr. A. M. Musa who is the most responsible for helping me to continue my Ph.D. study.

I am gratefully acknowledged the contributions of Dr.Musa Ismail Wara of the Department of Pharmaceutical and Medicinal Chemistry, UsmanDanfodio University, Sokoto, without your involvement, the structural elucidation work would have been very challenging, if not impossible.

I respectfully acknowledge the assistance, technical support and friendship of M. I. Salisu. You are great. My thanks are also extended to all Pharmaceutical and Medicinal

Chemistry department members for their encouragement, support and help throughout this study.

Special thanks must go to Dr. AbdullahiHamzaYaro of Faculty of Medicine, Bayero University, Kano for his kind helps and great efforts in assisting me in performing pharmacological studies. In addition, I would like to express my appreciation to Mika'il S. Abdullahi of the Institute for Leather Research and Science Technology, Zaria for his assistance in performing antimicrobial study and Dr. HamiduUsman of the Department of Chemistry, University of Maiduguri, Nigeria for assisting with the collection of the plant material.

My thanks are also extended to Dr. Musa Mohammed, Head of Basic Research Department, National Institute for Chemical Technology (NARICT) Zaria for the hospitality, advice and prayers during the course of preparation of this thesis.

I would like to thank all my colleagues(the present and those already graduated) particularly Muhammad AuwalTijjani and OgwuChinwe for thepeaceful and friendly academic environment. My thanks also go to my colleagues and friends in College of Agriculture, Lafia worthy of mention is Dr. M. M. Ari and Dr. M. M. Musa for their valuable advices and encouragements.

Above all, I would like to thank my family who tolerated my absence during the preparation of this thesis and for making my life so wonderful. May Allah bless them All.

I thank my late parents, HajiyaAisha Muhammad MukhtariSidi and Alhaji Musa Maikafi, for giving me life in the first place, for educating me and for unconditional support and encouragement to pursue my interest. I thank my brother Musa Danmusa for his financial support and advice that were very useful and all the rest of my brothers and sisters too.

Finally, I would like to thank College of Agriculture, Lafia for the study fellowship award which has enabled me pursue my PhD study.

ABSTRACT

The plant *Ampelocissus grantii* is used in traditional medicinal practice in the treatment of cough, wounds, venereal infections and other ailments.Phytochemical examination of the methanol extract of the aerial parts of *Ampelocissus grantii* (Baker) Planch (Family, Vitaceae) revealed the presence of carbohydrates, steroids, tannins, flavonoids, saponins and terpenoids. Three flavonoids, identified as 3□, 4□, 5, 7-tetrahydroxyflavone (Luteolin), isorhamnetin 3 - O – β – D – glucopyranosyl – (1□ □ □- 6□ □)– β – D – glucopyranoside,quercetin 3-O - α – L – glucopyranosyl – (1□ □ □- 6□ □)– β – D – glucopyranoside and one phenyl butanone, identified as 4 – O – β – D – rhamnopyranosyl – 4 – ethylphenylbut – 2 – one were isolated from the aerial parts of the plant. The structural identification was performed using detailed analysis of ^{1}H and ^{13}C NMR spectra including2D NMR spectroscopic techniques (COSY, NOESY, HSQC, and HMBC) and by comparison with reference data. The intraperitoneal LD_{50} of the crude methanol extract was determined to be 1250 mg/kg body weight *i.p.* in mice. Antinociceptive (acetic acid induced-writhing and hot plate induced-pain) and anti-inflammatory (carrageenan-induced oedema) effects of the crude methanol extract were studied. The results showed in the mouse writhing assay, the extract significantly ($P \leq 0.001$) and dose-dependently inhibited the abdominal constrictions. The highest inhibition was observed at dose of 300 mg/kg of the extract (95.19 %) while that of the ketoprofen 20 mg/kg was noted as 71.23 %, while in the pain latency test the extract exhibited no significant increase at a dose of 50mg/kg body weight, but at higher doses of 150 and 300 mg/kg body weight it increased significantly ($P< 0.05 – 0.01$) the pain threshold in dose related manner. Pentazocine (20 mg/kg; *i.p*) significantly ($p < 0.001$) prolonged the latency time much more than the extract. The extract significantly ($P < 0.01 - 0.001$) inhibited the paw oedema at all doses tested. The percentage anti-inflammatory effect (at

the peak of carrageenan-induced oedema) of the extract at 150 mg/kg was 80.95% comparable to that of ketoprofen (76.19%), the standard anti-inflammatory.The crude methanol extract was also screened for antimicrobial activity using the paper discs method against clinical isolates. The results showed that the methanol extract of the plant at concentration of 50 mg/ml, inhibited the growth of*Staphylococcus aureus, Streptococcus pyrogenes,Bacillus subtilis, Escherichia coli, Klebsiellapneumoniae, Pseudomonas aeruginosa,* and *Candida albicans*except *Corynebacterumulcerus,Salmonella typhi,* and *Neisseria gonorrhea* with zones of inhibition between15.0–27.0 mm.The result of this study has expanded our knowledge on the Phytochemistry of *Ampelocissus grantii,* confirming the rationale of the ethnomedicinal use of the plant.

TABLE OF CONTENT

Content	**Page**
Cover page	I
Title page	ii
Declaration	iii
Certification	iv
Dedication	v
Acknowledgement	vi
Abstract	viii
Table of Contents	ix
List ofFigures	xiv
List of Tables	xvi
List of Appendices	xviii
Abbreviations	xix
CHAPTER 1	
1.0 INTRODUCTION	1
1.1General Introduction	1
1.2The Value of Plants Used in Ethnomedicine for Drug Discoveries	4
1.3 Statement of Research Problems	5
1.4 Justification of Study	7
1.5 Aim of Study	8
1.6 Statement of Research Hypothesis	8
1.7 General Objective of the Study	8
CHAPTER 2	
2.0 LITERATURE REVIEW	
2.1 Vitaceae	10

2.2 Morphology of *Ampelocissus species* 10

2.3 Morphology of *Ampelocissus grantii* 11

2.4 EthnomedicinalUses of *Ampelocissus grantii* 12

2.5 Biological Actions of *Ampelocissus grantii* 13

2.6 Phytochemistry of the Genus *Ampelocissus* 14

2.7 Chemistry of Flavonoid Compounds 18

2.7.1 Introduction to flavonoids 18

2.7.2 Flavonoid structural classification 19

2.7.3 Chemical constituents of flavonoids 21

2.7.4 Detection and isolation of flavonoids 23

2.7.5 Structure elucidation of flavonoids 26

2.7.6 Biological activity of flavonoids 37

CHAPTER 3 40

3.0 MATERIALS AND METHODS 40

3.1 Materials/Reagents/Equipment 40

3.1.1 Apparatus and equipment 40

3.1.2 Chemicals/Reagents 40

3.1.3 Instruments 40

3.1.4 Experimental animals 41

3.1.5 Drugs used 41

3.1.6 Microorganisms tested 41

3.2 Analytical Procedures 42

3.2.1 Thin layer chromatography (TLC) 42

3.2.2 Column chromatography (CC) 42

3.2.3 Gel filtration (GF) 43

3.2.4 Preparative thin layer chromatography (PTLC) 44

3.2.5 Melting point (mp) determination 44

3.2.6 Spectral analysis 44
3.2.7 Spray/visualization reagents 44
3.2.8 Route of administration of drugs 44
3.3 Extraction Procedures 44
3.3.1 Collection, identification and preparation of plant material 44
3.3.2 Extraction 44
3.4 Preliminary Phytochemical Screening 47
3.4.1 Test for carbohydrates 47
3.4.2 Test for flavonoids 48
3.4.3 Test for alkaloids 49
3.4.4 Test for tannins 49
3.4.5 Test for anthraquinones 49
3.4.6 Test for saponins 50
3.4.7 Test for steroids 50
3.4.8 Test for cardenolides 50
3.5 Preliminary Thin Layer Chromatography of Crude Methanol Extract 51
3.6 Isolation of Compounds 1, ML 1 and ML 2 52
3.6.1 Thin layer chromatography of acetone soluble fraction of methanol extract of *A. grantii* 52
3.6.2 Column chromatography of acetone soluble fraction of methanol extractof*A. grantii* 52
3.6.3 Characterization of compounds 1, ML 1 and ML 2 54
3.7 Isolation of Compound ML 7 55
3.7.1 Thin layer chromatography of n-butanol soluble fraction of methanol extract of *A. grantii* 55
55
3.7.2 Column chromatographyof n-butanol soluble fraction of methanol extract of *A. grantii* 55
55

3.7.3 Characterization of ML 7 56
3.8 Pharmacological Studies 57
3.8.1 Acute toxicity studies 57
3.8.2 Antinociceptive and anti-inflammatory studies 57
3.9 Antimicrobial Study 59
3.9.1 Preliminary antimicrobial activities studies 59
3.9.2 Minimum inhibitory concentration (MIC) 60
3.9.3 Minimum bactericidal concentration (MBC) 61
3.10 Statistical Analysis 61
CHAPTER 4
4.0 RESULTS 62
4.1 Description and Yield of Extracts 62
4.2. Results Preliminary Phytochemical Screening of Crude Methanol Extract 62
4.3 Results of Thin Layer Chromatography of Crude Methanol Extract 65
4.4 Isolation of Compounds 1, ML 1 and ML 2 69
4.4.1 Results of thin layer chromatography of acetone soluble fraction 69
4.4.2 Results of column chromatography separation of acetone fraction 69
4.5 Characterization of Compound 1 73
4.5.1 Solubility test of compounds 1 73
4.5.2 Thin layer chromatography (TLC) examination of compound 1
4.5.3 Chemical studies of compound 1 73
4.5.4 Spectra of compound 1 73
4.6 Spectral Analysis of Compound 1 73
4.6.1 Proton nuclear magnetic resonance (^{1}H–NMR) of compound 1 73
4.6.2 ^{1}H – ^{1}H COSY spectral analysis of compound 1 73
4.7 Characterization of ML 1 73
4.7.1 Solubility test of ML 1 77

4.7.2 Thin layer chromatography (TLC) examination of ML 1 77

4.7.3 Chemical studies of ML 1 77

4.7.4 Spectra of ML 1 77

4.8 Spectral Analysis of ML 1 77

4.8.1 Proton nuclear magnetic resonance (^{1}H–NMR) of ML 1 77

4.8.2 Carbon – 13 nuclear magnetic resonance (^{13}C–NMR) of ML 1 78

4.8.3 DEPT ^{13}C-NMR spectrum of ML 1 78

4.8.4 ^{1}H – ^{1}H COSY spectral analysis of ML 1 84

4.8.5 HSQC NMR spectrum of ML 1 84

4.8.6 HMBC NMR spectrum of ML 1 84

4.8.7 ^{1}H – ^{1}H NEOSY spectral analysis of ML 1 84

4.9 Characterization of ML 2 93

4.9.1 Solubility test of ML 2 93

4.9.2 Thin layer chromatography (TLC) examination of ML 2 93

4.9.3 Chemical studies of ML 2 93

4.9.4 Spectra of ML 2 93

4.10 Spectral Analysis of ML 2 93

4.10.1 Proton nuclear magnetic resonance (^{1}H–NMR) data of ML 2 93

4.10.2 Carbon – 13 nuclear magnetic resonance (^{13}C–NMR) data of ML 2 94

4.10.3 DEPT ^{13}C-NMR spectral data of ML 2 94

4.10.4 ^{1}H – ^{1}H COSY spectral analysis of ML 2 103

4.10.5 HMQC NMR spectral data of ML 2 103

4.10.6 HMBC spectral data of ML 2 103

4.10.7 ^{1}H – ^{1}H NEOSY spectral analysis of ML 2 103

4.11 Isolation of Compound ML 7 115

4.11.1 Thin layer chromatography of n-butanol soluble fraction of methanol extract 115

4.11.2 Column chromatography of n-butanol soluble fraction of methanol extract 115

4.12 Characterization of ML 7 117

4.12.1 Thin layer chromatography (TLC) examination of ML7 117

4.12.2 Solubility test ML7 117

4.12.3 Chemical studies of ML7 117

4.12.4 Spectral data of ML7 117

4.13 Spectral Analysis of ML7 117

4.13.1 Proton nuclear magnetic resonance (^{1}H – NMR) of ML 7 117

4.13.2 Carbon – 13 nuclear magnetic resonance (^{13}C – NMR) of ML 7 117

4.13.3 DEPT ^{13}C-NMR of ML 7 117

4.13.4 HSQC spectrum of ML 7 124

4.13.5 ^{1}H – ^{1}H COSY spectral analysis of ML 7 124

4.13.6 ^{1}H – ^{1}H NEOSY spectral analysis of ML 7 124

4.14 Pharmacological Studies 126

4.14.1 Acute toxicity study 126

4.14.2 Effect of crude methanol extract of the aerial parts of Ampelocissus grantii on acetic acid-induced writhing in mice. 126

4.14.3 Effect of crude methanol extract of the aerial parts of Ampelocissus grantii on pain latency in mice exposed to hot plate 128

4.14.4 Effect of methanol extract of *Ampelocissus grantii* on carrageenan induced paw oedema in rats 130

4.15 Results of antimicrobial studies 132

CHAPTER 5

5.0 DISCUSSION 136

5.1 Phytochemical Studies 136

5.2 Pharmacological Studies 144

5.3 Antimicrobial Studies 149

CHAPTER 6

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS 152

6.1 Summary 152

6.2 Conclusion 152

6.3 Recommendations 153

REFERENCES 154

APPENDICES 166

LIST OF FIGURES

3.1:	Extraction and fractionation of plant material	46
4.1	Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of compound 1	71
4.2	^{1}H-^{1}H COSY of compound 1	72
4.3	Proton nuclear magnetic resonance (^{1}H–NMR) spectrum of ML 1	81
4.4	^{1}H–NMR of ML 1 (Expanded aromatic region)	82
4.5	^{1}H–NMR of ML 1 (Expanded sugar region)	83
4.6	Carbon – 13 nuclear magnetic resonance (^{13}C–NMR) of ML 1	84
4.7	DEPT ^{13}C-NMR spectrum of ML 1	85
4.8	^{1}H – ^{1}H COSY NMR spectrum of ML 1	86
4.9	HSQC NMR spectrum of ML 1	87
4.10	HMBC NMR spectrum of ML 1	88
4.11	^{1}H – ^{1}H NEOSY spectrum NMR of ML 1	89
4.12	Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of ML 2	90
4.13	^{1}H-NMR spectrum of ML 2 (Expanded aromatic region)	91
4.14	^{1}H-NMR) spectrum of ML 2 (Expanded sugar region)	92
4.15	Carbon-13 Nuclear Magnetic Resonance (NMR) spectrum of ML 2	93
4.16	DEPT Carbon-13 Nuclear Magnetic Resonance (NMR) spectrum of ML 2	94
4.17	^{1}H-^{1}H COSY NMR spectrum of ML 2	95
4.18	HSQC NMR spectrum of ML 2	96
4.19	HMBC NMR spectrum of ML 2	97
4.20	^{1}H – ^{1}HNOESY NMI spectrum of ML 2	101
4.21	Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of ML 7	102

4.22 Carbon-13 Nuclear Magnetic Resonance (NMR) spectrum of ML 7 103

4.23 DEPT Carbon-13 Nuclear Magnetic Resonance (NMR) Spectrum of ML 7 104

4.24 ^{1}H-^{1}H COSY NMR spectrum of ML 7 105

4.25 HSQC NMR spectrum of ML 7 106

4.26 ^{1}H – ^{1}HNOESY Spectrum of ML 7 107

108

109

LIST OF TABLES

4.1 Yield of the partition portions of the crude methanol extract of the aerial parts of *Ampelocissus grantii* 63

4.2 Phytochemical analysis of the crude methanol extract of the aerial parts of *Ampelocissus grantii* 64

4.3 TLC of the crude methanol extracts of the aerial part of *Ampelocissus grantii* using n-hexane: ethyl acetate (5:1) 66

4.4 TLC of the crude methanol extracts of the aerial part of *Ampelocissus grantii* using chloroform: ethyl acetate (5: 4) 67

4.5 TLC of the crude methanol extracts of the aerial part of *Ampelocissus grantii* using ethyl acetate : methanol : water (40: 5: 4) 68

4.6 Fractions obtained from column chromatography of the acetone fraction of the aerial parts of *Ampelocissus grantii* 71

4.7 Fractions obtained from column chromatography of the A4 fraction of the acetone portion of the aerial parts of *Ampelocissus grantii* 72

4.8 ^{1}H-NMR and ^{1}H-^{1}H correlation NMR (COSY) assignments of compound 1 76

4.9 ^{1}H-NMR and ^{13}C NMR assignments of compound ML 1 in CD_3OD 89

4.10 HSQC correlation in NMR spectra of ML 1 90

4.11 HMBC correlation in NMR spectra of ML 1 91

4.12 Proton-proton correlations in NMR spectrum of ML 1 using COSY and NOESY techniques and their assignments. 92

4.13 ^{1}H-NMR and ^{13}C-NMR assignments of compound ML 2 in CD_3OD 111

4.14 HSQC correlation in NMR spectra of ML 2 112

4.15 HMBC correlation in NMR spectra of ML 2 113

4.16 Proton-proton correlations in NMR spectrum of ML 2 using COSY and NOESY techniques and their assignments. 114

4.17 Fractions obtained from column chromatography of the n-butanol fraction of the aerial part of *Ampelocissus grantii* 116

4.18 ^{1}H-NMR and ^{13}C-NMR assignments of ML 7 in CD_3OD 125

4.19 Effect of crude methanol extract of the aerial parts of *Ampelocissus grantii* and ketoprofen on acetic acid-induced writhing in mice 127

4.20 Effect of crude methanol extract of the aerial parts of *Ampelocissus grantii* and pentazocine on pain latency in mice exposed to hot plate 129

4.21 Effect of crude methanol extract of the aerial parts of *Ampelocissus grantii* and ketoprofenon carrageenan-induced paw oedema in rats 131

4.22 Results of susceptibility test of *Ampelocissus grantii* methanol extract and standard antibiotics against various organisms. 133

4.23 Minimum inhibitory concentration (MIC) results of the *Ampelocissus grantii* methanol extract 134

4.24 Minimum bactericidal concentration (MBC) results of the *Ampelocissus grantii* methanol extract 135

LIST OF APPENDICES

1.0 Determination of LD_{50} of crude methanol extract of the aerial parts of *Ampelocissus grantii* 166

2.0 Effect of methanol extract of *Ampelocissus grantii* on acetic acid induced abdominal constriction in mice 167

3.0 Effect of methanol extract of *Ampelocissus grantii* on pain reaction time in mice 170

4.0 Effect of methanol extract of *Ampelocissus grantii* on Carrageenan induced paw oedema in rats. 172

5.0 Publication Title: Y.M. Musa, M.I. Sule, U.U. Pateh, M. Ilyas, A.M. Musa, H. Usman, M.S. Abdullahi (2010). PRELIMINARY PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF METHANOL EXTRACT OF AERIAL PARTS OF *AMPELOCISSUS GRANTII* (BAKER) PLANCH. *International Journal Chemical Sciences* 3(2): 167-174.

ABBREVIATIONSAND SYMBOLS

AGME	*Ampelocissus grantii* methanol extract
APG	Angiosperm Phylogeny Group
δ	Chemical shift (ppm) (NMR)
BuOH	Butanol
CC	Column Chromatography
$CHCl_3$	Chloroform
COSY	Double Quantum Correlation Spectroscopy (NMR)
d	Doublet (NMR)
DCM	Dichloromethane
dd	Double doublet (NMR)
DEPT	Distorsionless Enhancement by Polarization Transfer
DMSO	Dimethylsulfoxide
EtOAc	Ethyl acetate
EtOH	Ethanol
Fig.	Figure
g	Gram (s)
Glc	Glucose
s	Hour (s)
GF	Gel Filtration
^{1}H-NMR	Proton Nuclear Magnetic Resonance
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Gradient Heteronuclear Single Quantum Coherence
Hz	Hertz
IR	Infrared Spectroscopy
J	Coupling constant (NMR)
μl	Microlitre

LC	Liquid Chromatography
LD_{50}	Median Lethal Dose
LPLC	Low Pressure Liquid Chromatography
m	Multiplet (NMR)
MeOD	Deuteroited Methanol
MeOH	Methanol
mg	Milligram
MHz	Mega hertz
ml	Millilitre
Mp	Melting point
MPLC	Medium Pressure Liquid Chromatography
MS	Mass Spectrometry
MW	Molecular Weight
m/z	Mass per electronic charge
NMR	Nuclear Magnetic Resonance
nd	Not determined
NSAIDS	Non-steroidal anti-inflammatory drugs
PC	Paper Chromatography
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
ppm	Parts per million
PTLC	Preparative Thin Layer Chromatography
q	Quadruplet
R_F	Retardation factor
s	Second (s)
SEM	Standard Error of Mean
s	Singlet

spp	Species
t	Triplet
TLC	Thin Layer Chromatography
TMS	TetramethylSilane
UV	Ultra Violet Spectroscopy

CHAPTER 1

1.0 INTRODUCTION

1.1 General Introduction

The exploitation of plants by man for the treatment ofdiseases has been in practice for a very long time. The knowledge of the usage of herbs has been handed down from generation to generation for thousands of years (Newman and Cragg, 2012). Herbal medicine is an evolutionary process where communities and individuals continue to discover new techniques that can transform practice in the field of medicinal sciences (Ansari and Inamdar, 2010). Herbal drugs constitute a major part in all the traditional system of medicines (Higa *et al*., 1994).Their useis a triumph of popular therapeutic diversity. Plants above all other agents have been used formedicine from time immemorial because they have fitted the immediate personal need, are easilyaccessible and inexpensive (Mukherjee, 2008). For many years people have developed a store of empirical information concerning the therapeutic values of local plants before orthodox medical practice appeared (Karou *et al.,* 2007). Through periods of trials, error and success, these herbalist and their apprentices have accumulated a large body of knowledge about medicinal plants. Thefirst generation of plant drugs were usually simple botanicals employed in more or less their crude form (Iwu *et al.,*1999). Several effective medicines used in their natural state were selected as therapeutic agents based on empirical study of their applications by traditional societies from different part of the world(Ekeanyanwu, 2011).

Medicinal plants are those which in one or more of its organ contain substances that can be used for therapeutic purposes or which are precursor for the synthesis of useful drugs (Sofowora, 1993). A special feature ofhigher plants is their capacity to produce a large numberof organic chemicals of high structural diversity, the

socalledsecondary metabolites (Castello *et al*., 2002). Many of these compounds are differentially distributed among limited taxonomic groups within the plant kingdom and conversely, each plant species has a distinct profile of secondary metabolites (Farnsworth and Morris, 1976). Plant secondary metabolites have been variously referred to as natural products because of their historical significance as sources of dyes, polymers, fibers, glues, oils, waxes, flavoring agents, perfumes, drugs and their tremendous importance, both for the plant (for plant-environment interactions) as for humans, for their biological activities that can have high therapeutic value (Croteau, *et al*., 2000). The usual term used in referring to these chemical substances present in plants is constituent and the constituents which possess biological properties are called active constituents.

Phytochemistry is concerned with the chemical studies of these plant constituents and it involves the assay of plant extracts by chemical means or other means to determine the active constituent(s) of the plant. It gives account of the enormous variety of organic substances accumulated by plants, their biosynthesis, turnover and metabolism and their natural distribution and biological functions (Trease and Evans, 2002).

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plant are alkaloids, flavonoids, saponins, sterol, tannins, anthraquinones and phenoliccompounds (Edeoga *et al*., 2005).

Screening of organic substances accumulated by plants for their pharmacologicalactivities has indeed been the vast source of innumerable therapeutic agents representing molecular diversity engineered by nature.

Following the industrial revolution, a second generation of plant drugs emerged based on scientific processing of the plant extracts to isolate "their active constituents". Plant materials remain important components in combating serious diseases in the world; for therapeutic approach to several pathologies. In the recent past there has been a tremendous increase in the use of plant based health products in developing as well as developed countries resulting in an exponential growth of herbal products globally. Currently the global market for medicinal plants has been estimated to be around US $62 billion and the demand is growing rapidly. It is globally recognized that medicinal plants play a significant role in providing health benefits to human beings. An upward trend has been observed in the research on herbs. Herbal medicines have a strong traditional or conceptual base and the potential to be useful as drugs in terms of safety and effectiveness leads for treating different diseases. World Health Organization has made an attempt to identify all medicinal plants used globally and listed more than 20,000 species (Pandey*et al.,* 2008). Medicinal plants have played a key role in the world health care with about 80% of Africans depending on phytomedicine which has shown a wide range uses in the treatment of diseases, priority disease of Africa such as HIV/AIDS, malaria, sickle cell anaemia, diabetes and hypertension(Ekeanyanwu, 2011).Plants continue to serve as possible sources for new drugs and chemicals derived from various parts of plants (Calixto *et al.,* 2000).

Historically, plants have provided a good source of therapeutic agents. The isoquinoline alkaloid, emetine obtained from the underground part of *Cephalis ipecacuanha,* and related species, have been used for many years as an amoebicidal drug for the treatment of abscesses due to the spread of *Entamoeba histolytica*infections. Quinine, an alkaloid that occurs naturally in the bark of the *Cinchona* tree, is another important drug of plant origin with a long history of usage

against malaria. Scientists from divergent fields are investigating plants with an intention to discover valuable phytochemicals. Laboratories all over the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro.*

1.2 The Value of Plants Used in Ethnomedicine for Drug Discoveries

Medicinal plants provide a rich source of new materials for primary health care in Africa and other part of the developing world. The goals of using plants as source of therapeutic agents are: i) to isolate bioactive compounds for direct use as drugs; ii) to produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity/or lower toxicity; iii) to use agents as pharmacological tools; iv) to use the whole plant or part of it as a herbal remedy. Notable examples were quinine from *Cinchona pubescens,* reserpine from*Rauwolfia serpentina*and taxol from *Taxus spp.* (Farnsworth and Morris, 1976).

During the last few decades, there has been a resurgence of interest in plants as source of medicines and of novel molecules for use in the elucidation of physiological/biochemical phenomena. There is the worldwide green revolution, which is reflected in the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs. Furthermore, underlying this upsurge of interest in plants is the fact that many important drugs in use today were derived from plants or from starting molecules of plant origin. Digoxin/digitoxin, the vinca alkaloids, reserpine and tubocurarine are some important examples (Iwu *et al.*, 1999). Laboratories around the world are engaged in screening plants for biological activity with therapeutic potentials. The potential of higher plants as sources of new drugs is unexplored (Hostettman,1998). Among more than 258,000 species of higher plants

only about 5-10% has been investigated chemically for the presence of biological active compounds (Balandrin *et al.,* 1993).

In recent time there has been a marked shift towards herbal cures because of the pronounced cumulative and irreversible reactions of modern drugs. However, due to over population, urbanization and continuous exploitation of these herbal reserves, the natural resources along with their related traditional knowledge are depleting day by day (Pande *et al.,* 2007). In the present era of drug development and discovery of newer drug molecules, many plant products are evaluated on the basis of their traditional uses.

1.3 Statement of Research Problem

Pain is a kind of symptom and disease with emotional changes and tissue damage. Instant relief is essential since severe pain can cause metabolism disorder and other diseases (Staud, 2011). Analgesics are mainly divided into two classes: opiate receptor agonists and non-steroidal anti-inflammatory drugs (NSAIDs). The former may lead to drug dependence even though it is effective for various pains. NSAIDs are widely used in the treatment of pain, but they have side effects, especially on the gastrointestinal tract (Odabsoglu *et al.,* 2006).

The search for analgesics continues because there is still a lack of potent analgesics with minimal or no side effects (Besson, 1999). Currently, the most utilized drugs for the management of acute and chronic pain are opiates like morphine and its derivatives (Wang *et al.* 2006), which are frequently under-prescribed in apprehension of drug tolerance, dependence, and addiction (Hunt and Mantyh, 2001).

To develop new synthetic compounds in this category is an expensive venture and again may have problems of side effects. On the contrary, many medicines of plant

origin had been used and are in use successfully since long time without any serious effects (Kumar *et al.,* 2010). Thus, natural products from plants have the potential to be developed into new and effective analgesic drugs (Calixto *et al*. (2000).

Inflammation as one major cause of pain is related to cancer, diabetes and cardiovascular disease (Lucas *et al.,* 2006). It is the body's immediate response to damageof its tissues and cells by pathogens, noxious substances, or physical injury. These instigators induce activation of inflammatory mediators such as kinins, cyclooxygenase products and cytokines, which have become key targets for therapeutic intervention in a range of diseases including pain (Medzhitov, 2008).

NSAIDS are among the most commonly used drugs worldwide. They are prescribed for orthopaedic conditions such as osteoarthritis, soft-tissue injuries and fractures etc(Malizos, 2009). NSAIDS e.g Ibuprofen and naproxen etc. are used in the above said conditions. The other class of drugs is glucocorticoids e.g cortisone and prednisone etc. However, besides their high costs, severe adverse reactions and toxicity, including some risk of infections in subsets of patients being treated with biological response modifiers e.g Tumour necrosis factor, alpha blocking agents (Barnes, 2002). The side-effects with currently used drugs are G.I ulceration and bleeding, renal damage, hypertension, hyperglycemia. Besides the above side-effects, the greatest disadvantage in presently available potent synthetic drugs lies in their toxicity and reappearance of symptoms after discontinuation. Therefore, the screening and development of drugs for their anti-inflammatory activity is the need of hour and there are many efforts for finding anti-inflammatory drugs from indigenous medicinal plants (Srinivasan *et al*., 2011).

Resistance to antimicrobial agents is a major global public health problem. In the developing countries bacterial infections are still the main cause of deaths (Iwu *et al*.,

1999). Despite the progress made in the understanding of microorganisms and their control in industrialized nations, incidents due to drug resistant microorganisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns (Iwu *et al*., 1999). *Streptococcus pneumonia, Streptococcus pyogenes* and *Staphylococci*, the organisms that cause respiratory and cutaneous infections, as well as *Pseudomonads* and members of the *Enterobacteriaceae*, causing diarrhea, urinary tract infections, and sepsis, are resistant to virtually all of the older antibiotics (Harold, 1992). The search for new antibacterial compounds with improved activity to replace those that have become inactive is therefore necessary. Traditional healers use many plants to treat diseases that appear to be of bacterial aetiology and plants may afford valuable antimicrobial compounds (Cowan, 1999).Over 75% of the antibacterial drugs in clinical use are of natural origin and most of them are obtained from fungal sources (Newman and Crag, 2012). Screening of plant extracts for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective compounds. Plant derived antimicrobial compounds might inhibit bacteria through different mechanisms than conventionally used antibiotics, and could therefore be of clinical value in the treatment of infections caused by resistant microbes (Parekhand Chanda, 2007). Only a small fraction of the known plant species of the whole world have been evaluated for the presence of antimicrobial compounds, and thus it is necessary to increase the efforts in collecting and screening plants for the development of novel and environmentally safe antimicrobial agents (Stein *et al*., 2005).

1.4 Justification of Study

It has been shown that the approved and regulated chemical armamentarium available today is failing to provide effective health care for even one-third of the human

population (Cordell *et al.,* 2001). In addition, a number of significant global disease states, including cancer, tuberculosis, malaria and certain viral, fungal and bacterial infections, are showing significant pattern of resistance to the known therapeutic agents (Henry, 2000). Therefore, there is a need to develop alternative therapeutic agents from various sources such as medicinal plants.

Naturally occurring compounds from medicinal plants play an essential role in drug discovery. From 1981 to 2010, 64%of new approved therapeutic agents were inspired or directly derived from natural products (Newman and Cragg,2012; David, *et al.,* 2014). Theexploration of natural biodiversity has led to the identification of a remarkable variety of chemicalentities that possess highly selective and specific biological activities and unique modes of action (Cragg and Newman, 2013).
Of the several hundred thousand plant species around the globe, only a small proportion has been investigated both phytochemically and pharmacologically (Hostettmann, 1998). This has created the need to search for bioactive compounds from plants that have been reported for use in condition related to pains, inflammation as well as infections caused by microorganisms. Thus, when one consider that a single plant may contain up to thousands of constituents, the possibilities of making new discoveries become evident. The crucial factor for the ultimate success of an investigation into bioactive plant constituents is thus the selection of plant material. Aspart of these opportunities we selected a medicinal plant, *Ampelocissus grantii,* an African medicinal plant widely used in folk medicine to treat many diseases including bacterial, parasitic, viral, protozoa and fungal diseases.

1.5 Aim of Study

The aim of this study is about the potential of a select species of plants from the family Vitaceae, *Ampelocissus grantii* (Baker) Planch. This plant have already

demonstrated potential biological activities(Gessler *et al.,* 1994; Koné*, et al., 2004;* Bizimana *et al*., 2006; Aderbauer *et al*., 2008) and has been reported to be of profound utility for the health of human kind in African traditional medicine. It is used to treat varieties of ailments which include shigellosis, fever including malaria, urinary schistosomiasis, old wound (2 to 3 years), diarrhea, cancer, trypanosomiasis, rheumatism and muscular pain (Dalziel, 1937; Burkill, 2000; Adjanohoun *et al*., 1980; Inngjerdingen *et al*., 2004; Muhammad and Amusa, 2005; Bah *et al*., 2006; Bizimana *et al*., 2006; Etuk *et al*., 2009); a highly potential factor in drug discovery and development.

Keeping in mind the folkloric reputation, the plant was chosen to scientifically validate some of these claims through phytochemical and some biological screening of the aerial parts of this plant, with a view to separate, isolate and characterize some bioactive compounds, and validating the plant anti-rheumatic claim using laboratory animal models as well as its use ass antimicrobial agent using laboratory microorganisms which will serve as leads for the development of clinically useful products for better health care system of common people of Africa and third world countries.

1.6 Statement of Research Hypothesis

The aerial parts of *Ampelocissus grantii* contain bioactive compounds with analgesic, anti-inflammatory and antimicrobial activities.

1.7 General Objectives of Study

The primary objectives of this study were to identify, isolate and characterize bioactive compounds from the aerial parts of *A. grantii* using standard phytochemical methods and to investigate the analgesic, anti-inflammatoryand

antimicrobialproperties using biological models to validate some ofthe claimed ethnomedicinal uses.

Specific Objective 1:

1. To conduct preliminary phytochemical screening of the crude methanol extract
2. To establish the chromatographic profile of the crude methanol extract and partition portions of the methanol extract
3. To isolate and characterize some bioactive compounds from partition portions of the methanol extract.
4. To determine the analgesic and anti-inflammatory activities of the crude methanol extract
5. To determine antimicrobial activities of the crude methanol extract

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Vitaceae

Vitaceae is a family of dicotyledonous flowering plants including the grape and Virginia creeper. The family name is derived from the genus Vitis. The name sometimes appears as Vitidaceae, but Vitaceae is a conserved name and therefore has priority over both Vitidaceae and another name sometimes found in the older literature, Ampelidaceae (Jansen *et al*. 2006).

The relationships of Vitaceae are unclear and the family does not appear to have any close relatives. In the Cronquist system, the family was placed near the family Rhamnaceae in order Rhamnales. The family was placed in the Rosid clade, but not classified in an order, by the Angiosperm Phylogeny Group (APG). The Angiosperm Phylogeny Web places Vitaceae in its own order, Vitales. Recent phylogenetic analyses support Vitaceae as the sister-group to all other rosids (Jansen *et al*. 2006).

Vitaceae may be recognized by their caducously stipulate leaves with palmately compound, -lobed or -veined blades and often rather coarse teeth, cymose inflorescences, and smallish flowers with valvate petals. The stamens are equal in number to and opposite the petals, the usually 4-ovulate, bicarpellate gynoecium has more or less sessile stigmas, and the berry often has ruminate seeds. The stems are often strongly lenticellate, with the bark ultimately flaking, and the leaf blades are very brittle when dry, the fine details of the venation being obscure. Leaf-opposed tendrils or inflorescences are common in the family. Even individual seeds are distinctive, the endosperm being T-shaped in transverse section, the arms often being incurved (Jansen *et al*. 2006).

Plants of the family Vitaceae belong to the specific life form of climbing plants. Based on their characteristic adaptive strategy regarding water supply, use of light energy, and some other ecological factors, their structural and functional adaptations define them as a specific ecological type(Jansen *et al*. 2006).

2.2Morphology of *Ampelocissus species*

Ampelocissus is a genus of Vitaceae having 90 or more species found variously in tropical Africa, Asia, Central America, and Oceania. Species of Ampelocissus are herbaceous or woody,hermaphrodite (sp) or polygamo-dioecious flowering plants with tendrils for climbing. Fruits are grape-like berries having 1-4 seeds.

2.3*Ampelocissus grantii*

Taxonomy of *Ampelocissus grantii*

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Super division:	Spermatophyta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Rosidae
Order:	Rhamnales
Family:	Vitaceae
Genus:	*Ampelocissus*
Species:	*Ampelocissus grantii* (Baker) Planch.

Morphology of *Ampelocissus grantii*

Ampelocissus grantii is a strong climber with robust branches 3-5 mm thick and a long half-succulent root. There are numerous varieties according to ecological conditions.

Leaves:they are sub-orbicular in outline with 3-5 lobes rounded at apex or ovate orbicular and only slightly lobed. Cordate at base, 5-17 x 6-25 cm margins rather sharply dentate, each tooth forms the end of a leaf nerve. Petioles 3-14 cm long, hollow, striated, under surface of younger leaves sometimes covered by a loose woolly whitish indumentums, only the nerves and veins pubescent, or nearly gluberous, 5 base nerve, tertiary nerve net-like(Jansen *et al*. 2006).

Flowers:they are small, yellowish, in densely-flowered cymes of 4-10 cm total length, flowers massed together in hall 6 mm in diameter, 5 lobes flowers 1.5-2 m long, 1.5-1.7 mm broad, only distinctly lobed.

Fruit: Fruit is an ovoid berry 1 cm,

Seed: Seed ovate-elleptic 7-9 x 5-6 mm in a thin surgery pulp.

Distribution: Senegal to Sudan, Somalia, East Africa, Zimbabwe, and Victoria falls. Commonly found in savannah zone especially among rocks and red reforestation.

Vernacular: the plant is known as “rogon daji” or “lanbi” in Hausa and “eteku” in Yoruba

2.4Ethnomedicinal Uses of *Ampelocissus grantii*

The plant *Ampelocissus grantii* is used in traditional medicinal practice in the treatment of cough, wounds, venereal infections and other ailments.Ethnobotanical studies revealed that the plant is used alone or mixed with other medicinal plants to

treat shigellosis and fever including malaria (Adjanohoun *et al*., 1980) and urinary schistosomiasis (Bah *et al*., 2006). It is also used to cure old wound (2 to 3 years) (Inngjerdingen *et al*., 2004), cancer (Muhammad and Amusa, 2005), diarrhea(Etuk *et al*., 2009), trypanosomiasis, rheumatism and muscular pain (Bizimana *et al*., 2006). The root decoction of *Ampelocissus grantii*combined with *Khaya senegalensis* and soda is taken with food as a medication against pyaemicconditions accompanied by ulcers and abscesses. Boiled in millet gruel, the root is used against skin diseases. In Sudan, a leaf infusion is mixed with onions and drunk and used as a wash to relieve fever, pains in head and neck. In Tanzania the leaf sap is drunk as a remedy for malaria (Burkill, 2000; Dalziel, 1937). In Nigeria, the root is used by women to increase lactation; whole plant decoction applied to breast(Burkill, 2000).

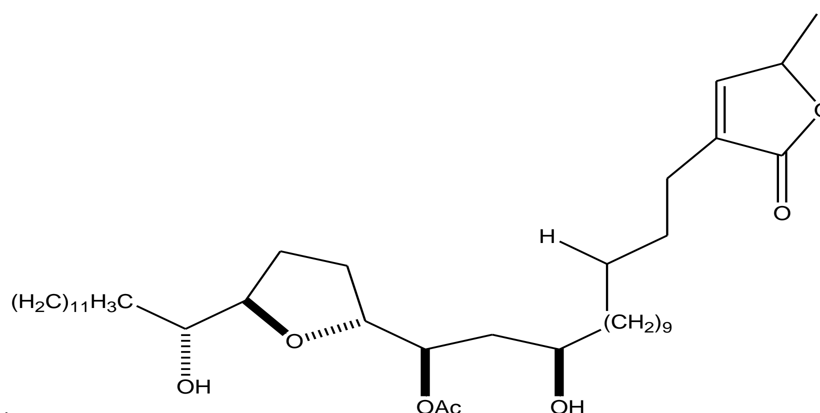
2.5Biological Actions of *Ampelocissus grantii*

Biological activitiesreported indicated that the plant showed marked antimalarial activity. The most active fraction against the multidrug-resistant *Plasmodium falciparum* strain KI *in vitro* was the ethyl acetate (EtOAc) fractionof the leaves with an IC_{50} value at 9.0 mg/ml (Gessler *et al.,* 1994).The trypanocidal activity has been demonstrated by in vitro and in vivo studies (Bizimana *et al*., 2006; Aderbauer *et al*., 2008), and anthelmintic activity (Koné*, et al., 2004).*

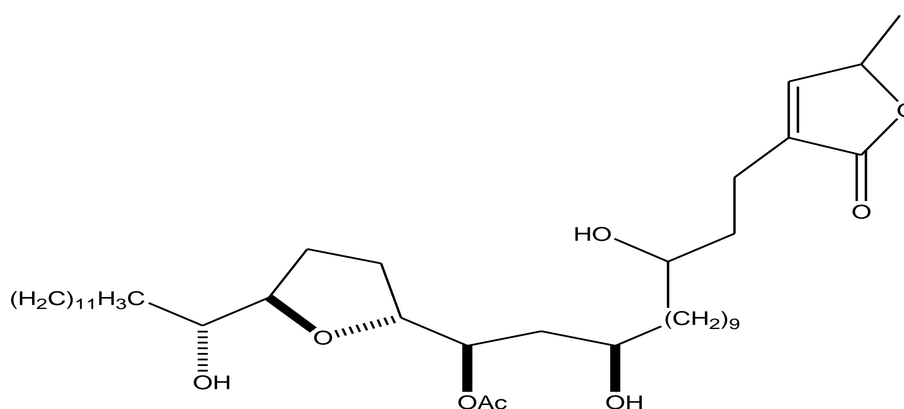
In 2008,acetogenin characterized as 22-epicalamistrin B (I), acetogenin, uvaribonin (II) and a chalcone, 3′-formyl-2′,4′,6′-trihydroxy-5′-methyldihydrochalcone (III)were isolated from methanol extract of the rhizomes of *Ampelocissus* sp. from Philippines. All thecompounds possessed significant cancer cell growth inhibitory activity against a panel of human cancer cell lines (Pettit *et al.,* 2008). The phenol content, antimicrobial and antioxidant activities of acetone and water extracts from the rhizomes of *Ampelocissus grantii* was also reported (Zongo *et al.,* 2010).

2.6Phytochemistry of the Genus *Ampelocissus*

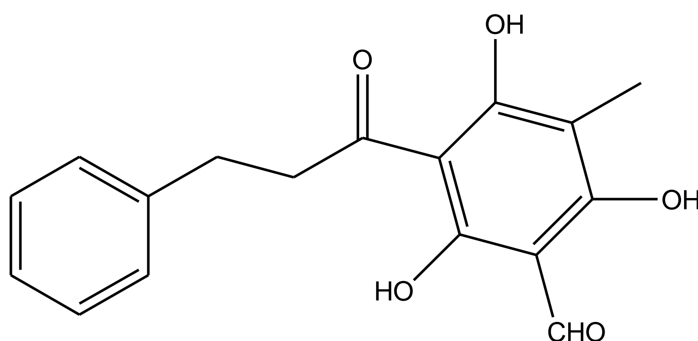
An investigation of the Philippine *Ampelocissus species* roots for cancer cell growth inhibitory components led to the isolation of a new acetogenin characterized as 22-epicalamistrin B (I) by employing primarily 2D NMR and high-resolution mass spectral analysis, a known acetogenin, uvaribonin (II) and a chalcone, 3′-formyl-2′,4′,6′-trihydroxy-5′-methyldihydrochalcone (III) were also isolated. The constituents were all found to show significant cancer cell growth inhibitory activity against a panel of human cancer cell lines (Pettit *et al.,* 2008).



I



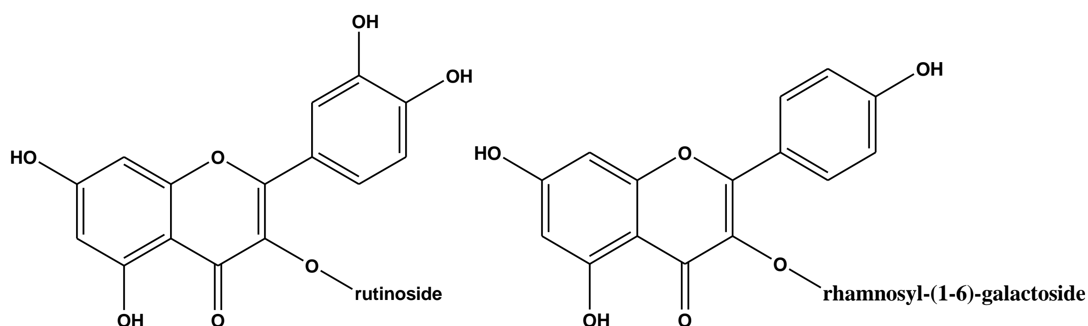
II



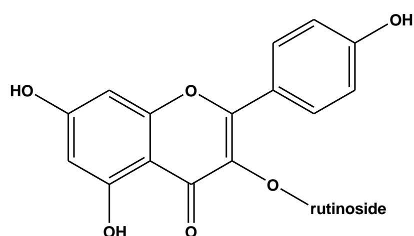
III

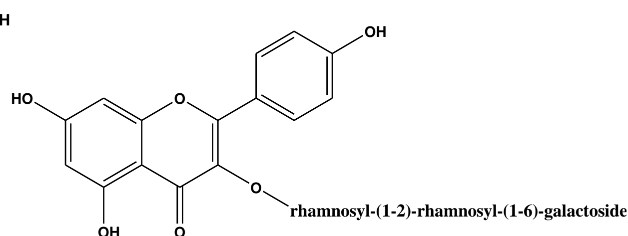
While this genera has not been investigated much before, investigation on various genus from the family have been described. From the genus Cisssus, it was reported that the leaves of *C. rhifolia* contain quinolizidine alkaloids, flavonoids and terpenoids (Siafah *et al*., 1983). The stem bark of *C. isimmer*showed the presence of Stilbenes, triterpenoids and steroids (Khan *et al*., 1986), while stilbenoids have been isolated from *C. quandrangularis* (Singh *et al*., 2007).

It was reported that the bioactive N-butanol fraction of the ethanol extract of the leaves of *Cissus ibuensis* when fractionated over silica Gel column gave Quercetin 3-O-rutinoside (IV) and mixture of Flavonoids which was purified using reverse phase HPLC to give Kaempferol 3-O-α-rhamnopyranosyl (1→6)-β-D-galactopyranoside(V), Kaempferol 3-O-rutinoside(VI)and Kaempferol3-O-α-rhamnopyranosyl(1→6)-α-rhamnopyranosyl(1→2) –β-D-galactopyranoside (VII) (Ahmadu *et al*.,(2010)).



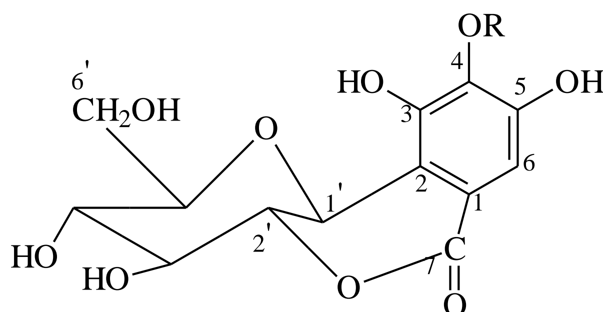
IV V





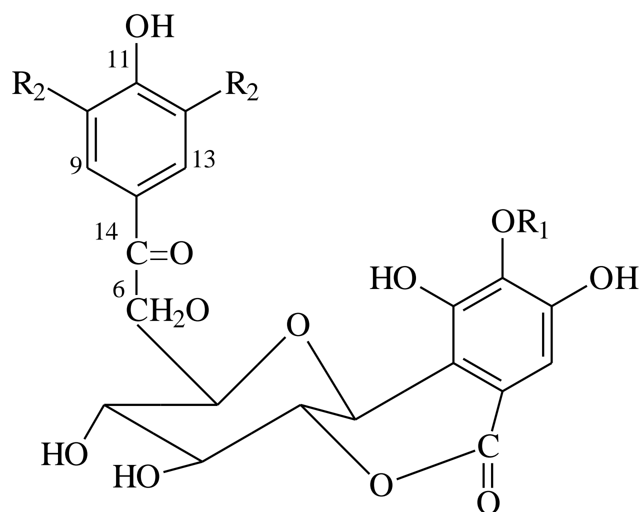
VIVII

Investigation of the roots and stems of *Cissus pteroclada* (Hayata) resulted in isolation of fourteen compounds including five isocoumarins, bergenin (VIII), norbergenin (IX), 6-*O*-galloylbergenin (X),6-*O*-(4-hydroxy benzoyl) bergenin (XI), and 6-*O*-galloylnorbergenin (XII) (Lin*et al.,* 2012).



VIII, R = CH_3

IX, R = H

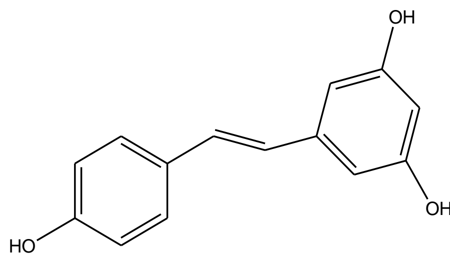


X, $R_1 = CH_3$, R_2, OH

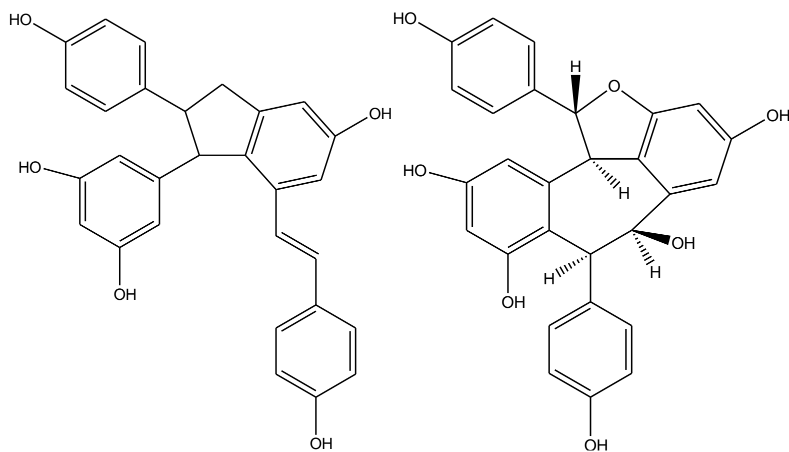
XI, $R_1 = CH_3$, R_2, H

XII, R_1 = H, R_2, OH

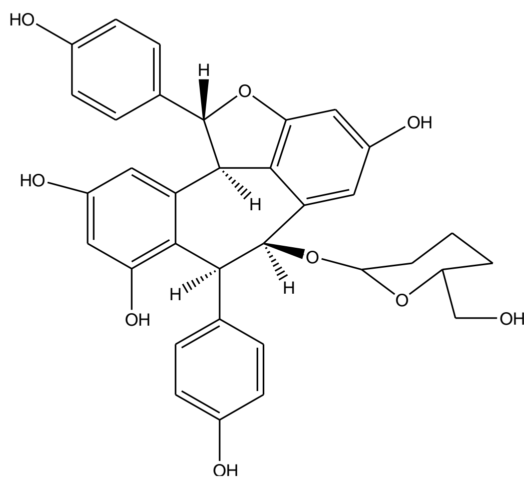
From the genus *Vitis,* the chemical study of the extracts of the branches of *Vitis vinifera* led to the isolation of resveratrol (XIII),and its three isomers : ε-viniferin (XIV), balanocarpol (XV) and a new compound β- glucopyranosyl 8- balanocarpol (XVI) (Felicio *et al*., 2001)



XIII



XIV XV



From the literature review discussed above, chemical investigation of the plant established the occurrence of flavonoids, saponins, phenolics and tannins. A chemical investigation of the acetone and n-butanol soluble portion of the methanol extract of the aerial parts has led to isolation of phenolics particularly flavonoids. It is therefore desirable to look in the chemistry of flavonoid compounds.

2.7 Chemistry of Flavonoid Compounds

2.7.1 Introduction to flavonoids

The recent past saw a slow but steady rise in the interest in flavonoids, and their benefits in the treatment of a vast number of diseases and conditions, including pregnancy toxaemia, rheumatic fever, diabetes and cancer. In the late 1980s and throughout the 1990s flavonoids were intensely studied concerning their actions as mutagenic agents and as antioxidants and pro-oxidants as their likely roles in biological systems (Aviram and Fuhrman, 1998; Lambert and Elias, 2010). In the early 1990s the antioxidant activity of flavonoids was extensively studied *in vitro*, and it was assumed that such activity would be at the basis of the health promoting benefits of these compounds. However, in the late 1990s and early 2000s the metabolism of flavonoids was deeply scrutinized, and the results indicated that their antioxidant activity *in vivo* could not account for the overall actions attributed to them (Fraga *et al*., 2010). The paradigm for flavonoid action changed towards the establishment of flavonoids as inflammation modulators, and more recently their role in neuroprotection, memory and cognition has been under scrutiny (Gomes *et al*., 2008; Spencer *et al*., 2009; Spencer, 2010). However, exact mechanisms for many of the actions attributed to flavonoids have not yet been established, but the relationship between their activity and the presence of specific functional groups in the molecules

is undeniable. Moreover, each role attributed to flavonoids has been linked to different structural features – for example, while antioxidant activity depends essentially on the number and location of OH groups in the molecules, their antagonist effect towards adenosine receptors depends more on the overall planarity than on the hydroxyl groups; in fact, the latter even appear to be counter-productive (González *et al*., 2007).

2.7.2 Flavonoid structural classification

Flavonoids are polyphenols of plant origin that are among the most important compounds in human diet due to their widespread distribution in foods and beverages. They can occur both infreestates (aglycones) and as glycosides, and differ in their substituents (type, number and position) and in their unsaturation pattern. The most common classes are the flavones, flavonols, flavanones, catechins, isoflavones and anthocyanidins, which account for around 80 % of flavonoids.

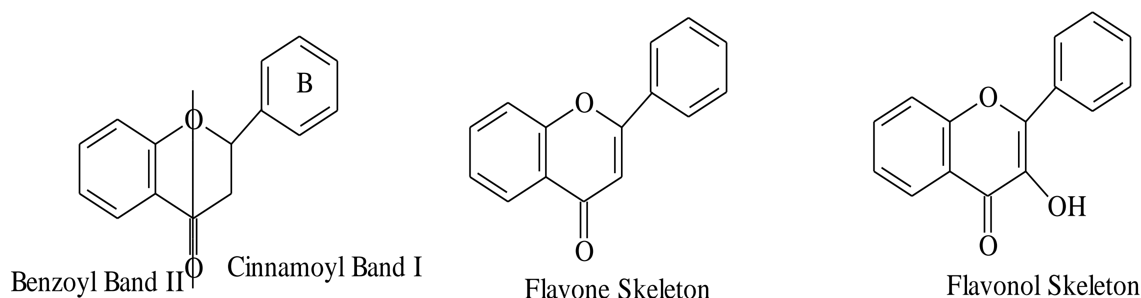


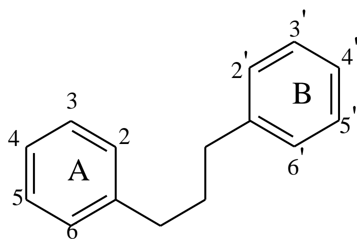
Fig. 2.1: Flavone and flavonol skeleton with benzyl and cinnamoyl moieties.

The flavonoids are polyphenolic compounds possessing 15 carbon atoms based on a skeletal structure of two benzene rings joined by a linear three carbon chain, which can be represented as C_6- C_3 - C_6phenyl-benzopyran backbone (XVII). The position of the phenyl ring relative to the benzopyran moiety allows a broad separation of these compounds into flavonoids (2-phenyl-benzopyrans), isoflavonoids (3-phenyl-

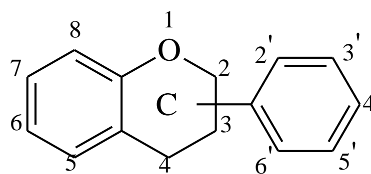
benzopyrans) and neoflavonoids (4-phenyl-benzopyrans) (Figure 2.1). Division into further groups is made on the basis of the central ring oxidation and on the presence of specific hydroxyl groups. Most common flavonoids are flavones (with a C2-C3 double bond and a C4-oxo function), flavonols (flavones with a 3-OH group) and flavanones (flavone analogues but with a C2-C3 single bond), and abundant isoflavonoids include isoflavones (the analogue of flavones). 4-arylcoumarin (a neoflavonoid with a C3-C4 double bond) and its reduced form, 3, 4-dihydro-4-arylcoumarin, are the major neoflavonoids. Other natural compounds, such as chalcones and aurones also possess the C6-C3-C6 backbone, and are henceforth included in the general group of flavonoids.

Flavonoids generally consist of two fused rings (a CHROMANE ring). The first ring A is aromatic, the second ring C is an oxygen-containing heterocyclic bearing a second aromatic ring B in position 2, 3, or 4 (XVIII), The fused A and C rings arc often considered the flavonoid nucleus (XIX). The numbering of the ring atoms follows the usual conventions. Thus the heteroatom in ring C is designated 1, and numbering continues clockwise around the heterocyclic through 10. Ring B is numbered 1', 2', 3' etc., beginning with the bridging carbon and proceeding clockwise through 6'.

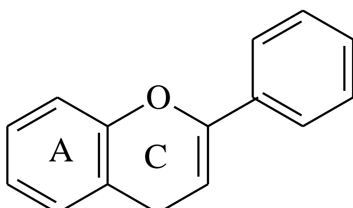
In few cases the six-member heterocyclic ring C occurs in an isomeric open form or is replaced by a five-member ring as in case- of aurones, e.g. 2-benzyl-coumarone (XX). The oxygen bridged involving the central carbon atom (C_2) of the 3C-chain occurs in a rather limited number of cases, where the resulting heterocyclic is of the furan type.



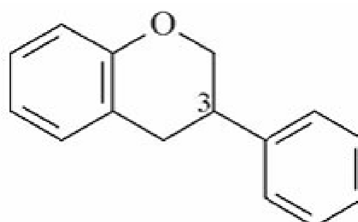
XVII



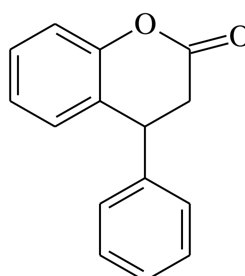
XVIII



XIX



XX



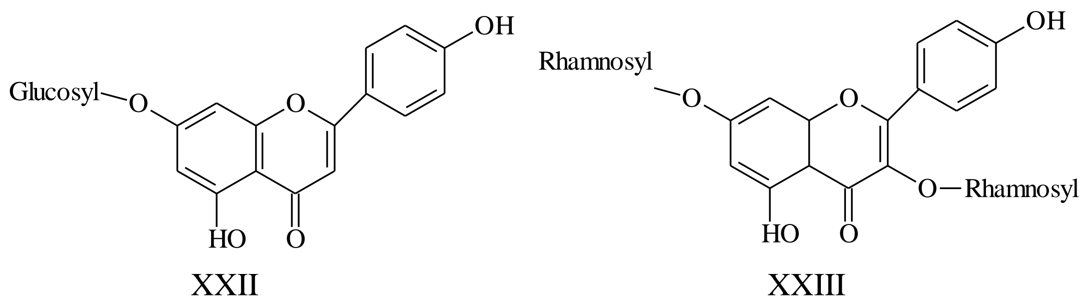
XXI

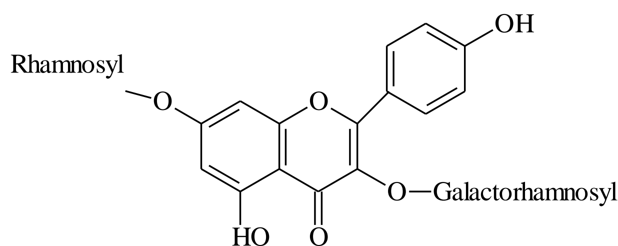
Flavones, flavonols, flavanones and anthocyanins bear ring B in position 2 of the heterocyclic ring. In isoflavonoids, ring B occupies position 3. Biflavonoid consist of two flavonoids molecules. A group of chromane derivatives with ring B in position 4 (4-phenyl-coumarins, XXI) are known as neoflavonoids. The chalcones are considered by many authorities to members of the flavonoid family, despite lacking the heterocyclic ring C (Swain, 1965).

2.7.3 Chemical constituents of flavonoids

Flavonoids occur as aglycone, glycosides and methylated derivatives (Middleton, 1984). Most often, they occur in plants as glycosides, which yield on hydrolysis (a) one or more sugar units and (b) sugar free compounds. The sugar-free compounds are

referred to as "aglycones". In the glycoside form, a least one of the OH groups of the aglycone is glycosylated by one or more saccharides. The glycosidic linkage is normally located in the 3 or 7 position of flavonoid aglycone. The ease with which flavonoid glycosides are hydrolyzed into aglycones and sugars varies from case to case; usually refluxing with mineral acids is necessary for a complete breakdown. The sugars components have been usually identified by the conventional method of paper chromatography in various solvent systems. The sugar units are linked to the aglycone by a semi-acetal linkage. The sugars which have been found in flavonoids glycosides include simple hexoses and pentoses (monsides), di- (biosides) and tri- (triosides) saccharides, always combined through the C_1 position usually by a β-linkage. D-glucose, occurring either alone or as part of a disaccharide, is the most common sugar in glycosides and while L-arabinose and D-xylose are rare. An example of a monoglycoside is cosmosin (Apigenin-7-glucoside) (XXII), kaempferrtin (Kaempferol-3, 7-dirhamnosidc) (XXIII) is an example of a diglycoside while robinin (Kaempferol-7-rhamnoside-3-galactosyl-rhamnoside) (XXIV) is an example of triglycoside. Attachment of these sugars renders glycosides their solubility in polar solvents (especially water).Flavonoids occurring with the majority of their hydroxyl groups being protected by methylation are lipid soluble (Harbone, 1973).





XXIV

2.7.4 Detection and isolation of flavonoids

Flavonoids are usually detected by means of the characteristic colours they produce with various reagents. An advantage of the flavonoids is that they are so readily detected on chromatograms (PC and TLC) without the use of chromogenic sprays. Many are coloured (e.g. anthocyanins, chalcones and aurones) and the others which are sufficiently coloured to be visible may be visualized by means of UV light (flavonols fluorescence, others absorbs and appear as dark spots) and in iodine vapour (flavones appear as yellowish-brown, anthocyanins as blue-gray, chalcones and aurones as orange-red). Other spray reagents include aluminium chloride (2% in methanol), (Spiegl, 1969), ferric chloride, ammonia vapour (Mabry *et al.*, 1970).

The colour reactions of various classes of flavonoids are carried out by adding the reagents (except conc. sulphuric acid in which the solid substance is dissolved) to an alcoholic solution of the sample.

Method of isolation depends on the type of flavonoid being isolated. Usually, a pre-extraction with petroleum ether or hexane is frequently carried out to rid the plant material of sterols, chlorophylls, carotenoids etc. (Bhutani *et al.*, 1969). Majority of flavonoids, especially glycosides and polar aglycone can be extracted with water or a 1:1 mixture of water and methanol. Those which are only slightly soluble in water are moderately polar to be well extracted with acetone, ethanol or methanol or a combination of these solvents (Robinson, 1967). The less polar aglycones such as

isoflavones, flavanones and dihydroflavonols or flavones which are highly methylated are usually extracted with solvents such as benzene, chloroform or ethyl acetate (Herz *etal*., 1972). Various precipitating reagents such as neutral or basic lead ethanoate has been used to help precipitate out flavonoids. The isolated flavonoid can be free from the lead ethanoate by adding sulphuric acid or hydrogen sulphide leaving the lead as insoluble sulphate or sulphide. Other precipitating reagents are picric acid, pyridine, and barium hydroxide (Robinson, 1967).

Various techniques have been used for the separation and isolation of flavonoids. For separation, the best analytical method at present is high-performance chromatography (HPLC), which separates compounds by their differential preference for the solvent or the column. HPLC requires a smaller sample and is more complete and precise than other methods. Analysis of flavonoids with reversed phase HPLC was reviewed (Daigle and Conkerton, 1988). Ultraviolet (UV) was the most popular detection method due to the presence of strong flavonoid chromophores and availability of detection systems (Daigle and Conkerton, 1988). A binary HPLC gradient system applied to *Ginkgo biloba*forseparation of naturally occurring flavonoids was developed (Pietta *et al*., 1991). Thirty three flavonol glycosides found in *Ginkgo biloba*andextracts were separated from fingerprinting as well as quantification of aglycones after acid hydrolysis (Hasler *et al*.. 1990). In 1993, Sticher suggested using this approach to standardize phytomedicines containing flavonol components (Sticher, 1993). Li and Fitzloff (2002) used HPLC with photodiode array (PDA) detection to compare flavonol aglycone content in various pharmaceutical products and published a method to simultaneously determine terpene lactones and flavonol aglycones using HPLC with evaporative light scattering detection (ELSD).

Other analytical techniques include older, less precise methods, such as gas chromatography, column chromatography, thin layer chromatography (TLC) and paper chromatography; in decreasing order of precision. The problem with gas chromatography is that gas is the solvent and therefore the flavonoid compounds to be separated must be either volatile at relatively low temperature or derivatives to be made volatile. In column chromatography, solvent and alumina or silica are placed in a column, the compounds to be studied are 'added at the top, and then solvent moves through the column. With column chromatography larger amounts of the compound can be analyzed, and it is easier to recover the compounds of interest, than with gas chromatography (Mabry *et al.*, 1970). In TLC the compound is on a plastic or glass plate that is coated with silica or alumina, and then the plate is placed in a container with solvent, which is allowed to run up the plate. The compounds to be characterized separate out by their affinity for the silica/alumina or the solvent. The compounds arc then visualized by ultraviolet light and recovered. TLC is still used today for rapid analysis of compounds and to determine the solvent systems that are needed for HPLC and on silica gel is very favourable for the analysis of flavonoids(Harbone, 1973).

Paper chromatography, the most widely used in flavonoid analysis is suitable for the separation of complex mixtures of all type of flavonoids and their glycosides. Paper chromatographic analysis arc mostly carried out on Whatman No. 1 or No. 3 and for optimum resolution, two-dimensional chromatography is recommended (Harbone. 1973).

2.7.5 Structure elucidation of flavonoids

Structural identification of flavonoid compounds is more accurate with mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR) or diode array spectrometry. MS breaks the compound and provides both its molecular weight and a characteristic fragmentation spectrum. NMR identifies the molecule by characteristic pattern of hydrogen relationships. Both the MS and NMR are very precise and sensitive. Diode array spectrometry measures the ultraviolet spectrum simultaneously over several wavelengths at 10^{-9} to 10^{-12} g compound. Other standard techniques such as infrared (IR) and ultraviolet (UV) spectroscopy have also been for structure analysis of flavonoids(Fossen and Andersen, 2005).

2.7.5.1Ultraviolet spectroscopy (UV)

Ultraviolet and visible spectroscopy was one of the earliest techniques routinely used for flavonoid analysis. The UV spectra of flavones and flavonols in methanol exhibit two major absorption peaks in the region 240-400 nm. The existence of these two characteristic peaks are commonly referred to as band I in the 300 to 550 nm range, arising from the B ring(cinnamoyl system), and band II in the 240 to 285 nm range, arising from the A ring(benzoyl system)(Fig. 1.3). Particularly, the position of band I in the methanol spectrum provides information about the type of flavonoid as well as its oxidation pattern (Mabry *et al.*, 1970).

Flavonols with a substituted 3-hydroxy group (methylated or glycosylated), band I appears in the range 328-357 nm which overlaps the region of band I in flavones, and the general shape of the spectral curves approach those of flavones.

Oxidation pattern of the B-ring in flavones could be identified from the nature of the peaks. In this, 3', 4'- or 3', 4', 5'- oxygenated flavones usually exhibit two absorption

peaks (or one maximum with a shoulder) between 250-275 nm, while the 4'-oxygenated equivalents have only one peak in this range(Mabry *et al*., 1970).

The utility of UV has been extended by measuring spectra of an alcoholic solution of the flavonoid in the presence of inorganic salts (shift reagents). Flavonoids, depending on the number and position of hydroxyl and other substituents give characteristic spectra., Shift reagents, such as sodium methoxide and aluminium chloride, lead to shifts in the maximum wavelength of these bands due to methoxide-induced deprotonation of OH groups or Al^{3+} complexation by OH groups, were routinely used to study flavonoid structure. Nowadays these techniques are not routinely used but still continue to be applied in some cases, in particular to HPLC eluates - a hyphenated LC-UV-MS has been developed using post-column UV shift reagents for the flavonoid analysis of crude extracts(Mabry *et al*., 1970).

The UV spectra of flavones and flavonols in the presence of sodium methoxide (NaOMe) provide information on the hydroxylation pattern of the flavonoid. Sodium methoxide is a strong base and ionizes to some extent all hydroxyl groups of the flavonoid nucleus. The addition of NaOMe to flavones in methanol usually produces bathochromic shifts in all absorption bands. However, a large bathochromic shift of band I of about 40-65 nm, with an increase in intensity, is diagnostic for the presence of a free 4'-hydroxy group(Mabry *et al*., 1970).

Sodium acetate is a weaker base than NaOMe, and as such ionizes only the more acidic hydroxyl groups in flavones and flavonols, i.e., the 3-, 7- and 4'-hydroxy groups (Mabry, 1970). The ionization of the 7-hydroxy group mainly affects band II. Whereas the ionization of the 3- and/or 4'-hydroxy groups mainly affects band I. Particularly, NaOAc is a useful diagnostic reagent for the specific detection of 7-hydroxy groups. The UV spectra of flavones containing free 7-hydroxy groups

exhibit a diagnostic 5-20 nm bathochromic shift of band II in the presence of NaOAc (Mabry, 1970). However, when 6 and 8 oxygen substituted, the bathochromic shift with NaOAc is often smaller presumably because of the reduced acidity of the 7-hydroxy group. Certain 3", 4'-dioxygenated derivatives without the 7-OH group showed bathochromic shifts of 20-25 nm(Mabry *et al*., 1970).

The presence or absence of an ortho-dihydroxy group at all locations of the flavonoid nucleus could be detected from the effect of NaOAc/H_3BO_3 in the UV spectrum. In the presence of NaOAc, boric acid forms chelate complexes with ortho-dihydroxy groups. Flavones containing a B-ring ortho-dihydroxy group show a consistent 12-30 nm bathochromic shift of band I in the presence of NaOAc/H_3BO_3, whereas a bathochromic shift of about 5-10 nm in band I in the presence of NaOAc/ H_3BO_3 is diagnostic for the presence of an ortho-dihydroxy group ('at C-6, 7 or C-7, 8) in the A-ring(Mabry *et al*., 1970).

Aluminum chloride forms acid stable .Complexes with flavones and flavonols which contain hydroxyl groups at C-3 or C-5, whereas the aluminum chloride complexes with ortho-dihydroxy groups are not stable. The presence of an ortho-dihydroxy group in the B-ring of flavones and flavonols can be detected by comparison of the spectrum of the flavonoid in the presence of $A1C1_3$ with that obtained in $A1C1_3$/HC1. 30-40 nm hypsochromic shift observed in band I of the $A1C1_3$ spectrum on the addition of acid results from the decomposition of the complex of $A1C1_3$ with the ortho-dihydroxy group. The presence of three adjacent hydroxyl groups in the B-ring gives only a 20 nm hypsochromic shift on the addition of acid to the AlCl3 solution (Mabry, 1970).

The addition of acid to a methanolic solution of flavones or flavonols which already contain $AlC1_3$ decomposes the complexes between $AlC1_3$ and ortho-dihydroxy

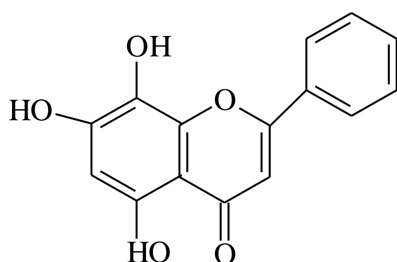
groups. Hence, any shift still remaining in band I or band II relative to the methanolic spectrum will be due to the presence of free 3- and/or 5-hydroxy groups in the flavonoid, Regeneration of the methano1 spectrum on the addition of acid indicates that both the 3-and 5-hydroxy groups are either absent or substituted (Markham, 1975).

The $AlCl_3$/HCl spectrum of a 5-hydroxy flavone typically consists of four major absorption peaks, band Ia, Ib, IIa and IIb, which are all bathochromically shifted relative to their band of origin (presumably la end Ib originate from I; and IIa and IIb from II)(Mabry *et al*., 1970).

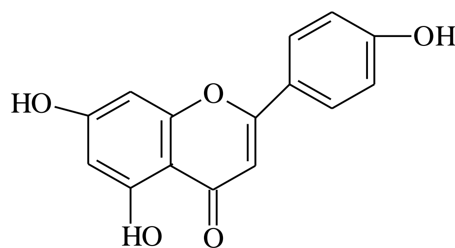
2.7.5.2Vibrational spectroscopy

Vibrational spectroscopy, in its infra-red and Raman variants, is a spectroscopic technique that analyses the vibrational modes of molecules and molecular groups, allowing bond characterization, and, by comparison with known tabulated data, identification of functional groups; in the case of flavonoids, vibrational spectroscopy has been systematically used to study hydroxyl and carbonyl groups, but more recent technical developments have allowed its application to a broader set of research goals. Infrared spectral determinations have been mainly used with flavones for fingerprint identification, rather than for structural determination, because interpretation is generally difficult and shift were unpredictable. However, it is still useful in structural elucidation of flavonoid compounds. For example, flavones lacking a 5-hydroxy1 group are readily distinguished from those with a 5-hydroxyl because the frequency of the carbonyl band absorption is at 1620-1639 cm^{-1} instead of at 1653 cm^{-1} or above (Harbone, 1976). Also, C-H vibrations of flavones

unsubstituted in the B-ring which occurs in the 680-870 cm^{-1} region differs from 4'-substituted flavones. For example, Norwoqonin (XXV) has two bands at 690 and 765 cm^{-1} while apigenin (XXVI) has a single band at 831 cm^{-1}(Wagner, 1965). Raman spectra are much less complex than the IR spectra of the same molecules, and for that reason Raman spectroscopy has been gradually taking over IR spectroscopy, although it is common to use both techniques as complement of each other. Vibrational spectroscopy is seldom used alone, and most studies are accompanied by other spectroscopic approaches and/or quantum chemical computations (Siebert and Hildebrandt, 2007).



XXV



XXVI

2.7.5.3Nuclear magnetic resonance spectroscopy (NMR)

Nuclear Magnetic Resonance spectroscopy (NMR) is one the most powerful research techniques used to investigate the structure and some properties of molecules. One of the main applications of NMR in flavonoid research is the structural elucidation of novel compounds, for which nothing is known; although NMR traditionally requires large amounts of sample, which is not easy to obtain when analysing novel compounds, the technical developments in the last decade, both in NMR instrumentation, pulse programs and in computing power, have allowed the complete assignment of all proton and carbon signals (Fossen and Andersen, 2005).

Protonnuclear magnetic resonance spectroscopy (^{1}H-NMR)

The ^{1}H-NMR spectrum of flavonoids appears predominantly in the range δH0-10 ppm downfield. The chemical shift establishes the nature of the hydrogen, and coupling constants determine the presence of the ortho, meta and vicinal protons. The ortho and metacouplings have ranges of 6.5-9.0 Hz and 1.5-2.5 Hz, respectively and are of value in establishing the aromatic substitution pattern.

A-Ring Protons: The protons at C-6 and C-8 of flavones and flavonols and isoflavones which contain the common 5,7-dihydroxy substitution pattern gives rise to two doublets (J= 2 - 2.5Hz) in the range 6.0-6.5 ppm. The; H-6 doublet occurs consistently at higher field than the signal for the H-8. However, there are small but predictable variations in the chemical shifts of the C-6 and C-8 proton signals depending on the 5- and 7-substitutions. In the spectra of luteolin derivatives, Iuteolin-7-rhamnoglucoside, and luteolin-7-glucoside where sugar is attached to the oxygen at the C-7 the signals for both H-8 and H-6 are shielded downfield (Mabry *et al*., 1970)

In flavanones (3,4-dihydroflavones), the H-6 and H-8 give a singlet near 5.95 ppm; with the addition of a 3-hydroxy group to give 3-hydroxyflavanones, the chemical shifts of these protons are slightly altered and the pattern change to a very strongly coupled pair of doublets. In the catechins, the absence of the 4-carbonyl group causes the signal of the H-6 proton to move upfield, while the H-8 proton is little affected, this change is clearly associated with the effect upon the 5-hydroxyl group, which is strongly hydrogen bonded to the 4-carbonyl in compound possessing this feature. That the proton ortho to the 5-hydroxyl is more affected than that para is in accord with previous experience (Mabry *et al*., 1970).

The spectral patterns produced by the A-ring protons can thus be seen to reveal the position of oxygen substituents of the ring, and often the character of ring C.

B-Ring protons: The protons of ring B usually appears in the range 6.7-7.9 ppm, which is downfield from the region where A- ring protons absorb. The signal pattern observed for the B-ring protons is characterized for the substitution pattern of that ring and, in addition suggests the oxidation level of ring C. If ring B is oxygenated at C-4', a typical four peak pattern of two doublets (each J=8.5 Hz) is observed. The doublet for the C-3' and C-5' protons (which are shielded by the C-4' oxygen substituent) always appear upfield from the C-2' and C-6' protons and generally falls in the range 6.65-7.1 ppm for all types of flavonoids. The position of the C-2' and C-6' doublet depends to some extent on the oxidation level of ring C, however, it consistently appears at lower field 7.1-8.1 than the C-3' and C-5' doublet(Mabry *et al*., 1970).

The NMR spectra of flavonoids with 3', 4'-oxygenation pattern in the B-ring are more complex than for compounds with the 4-oxygenation.

The C-5' proton of 3', 4'-oxygenated flavones and flavonols appears as a doublet centered between 6.7 and 7.1 ppm (J=8.5 Hz) and the C-2' and C-6" protons signals, which often 'overlap, usually occur between 7.2 and 7.9 ppm. The relative positions of the signals for the C-2' and C-6' may be used to distinguished the 3'-methoxy-4'-hydroxy from the 4'-methoxy-3'-hydroxy B-ring substitution pattern in flavonols. The C-2' proton signal is usually centered at slightly higher field than the C-6’ proton signal in flavonoids containing the 4-methoxy groups. In contrast, their positions are reversed when a 3'-methoxyl group is present in a 3', 4'-oxygenated flavonol.

Different spectral patterns are observed for 3', 4'-oxygenated isoflavones, flavanones and dihydroflavonols. These compounds give complex multiplet, usually two peaks, for the C-2', C-5' and C-6' protons in the region 6.7-7.1 ppm. The chemical shift for B-ring protons in these flavonoids depend upon whether or not the proton is ortho or

para to an oxygen function. In 3', 4-dioxygenated isoflavones, flavanones .and dihydroflavonols, the C-2', C-5' and C-6' protons are either ortho or para to an oxygen substituents and therefore have similar chemical shifts. The C-2' and -6' proton signals usually overlap in the region 6.5-7.7 ppm in flavonoids having 3',4',5'-oxygenation pattern(Mabry *et al.*, 1970).

C-Ring Protons: The chemical shifts of the C-ring protons among the different flavonoids classes depends upon the oxidation level of the C-ring. The C-3 proton in flavones gives sharp singlet near 6.3 ppm which usually overlaps the signals produced by the A-ring protons. On the other hand, the C-2 proton in isoflavones, which is beta position to the C-4 keto function, occurs in the range 7.6-7.8 ppm (in CCl_4), a range downfield from where most aromatic proton signals appear. The position of the signal obtained for the C-2 proton when the isoflavone (or its TMSether) is examined in either CCl_4or $CDC1_3$ shifts about 1 ppm downfield when the compound is analyzed in DMSO-d_6.(Mabry *et al.*, 1970).

The H-α and H-β protons of chalcones occur as doublets (J=17Hz) in the range 6.7-7.4 ppm (H-α) and 7.3-7.7 ppm (H-β), while the aurones benzylic proton appears as a singlet at 6.5-6.7 ppm. The signal for the C-2 proton of flavanones appears as quartet (two doublets, J=5Hz; .1=1 Hz or near 5.2 ppm as a result of the coupling of the C-2 proton with the C-3 proton. The C-3 protons couple with each other (J=17Hz) in addition to their spin-spin interaction with the C-2 proton, thus giving rise to two overlapping quartets near 2.8 ppm. Two of the signals of each quartet however, are weak and are often not observed.(Mabry *et al.*, 1970).

2.7.6Structural Elucidation of Compounds Using Nuclear Magnetic Resonance (NMR) Technique

Identification of compounds usually involve a combination of different techniques including nuclear magnetic resonance (NMR), mass spectrometry (MS), ultra violet (UV) and infrared (IR). Other ways of confirming the identification of compounds include calculation of retardation factor (R_f) value in different solvent systems and determination of melting point. In this study NMR technique was used as a tool for identifying the structure of compounds.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

One-Dimensional Spectroscopy (^{1}H and ^{13}C):

The two most basic NMR experiments are the ^{1}H and the ^{13}C NMR experiments, which are aimed at the determination of the resonance frequency of each ^{1}H or ^{13}C nucleus in the molecule.

^{1}H NMR experiments register the chemical shifts (δ) and spin-spin couplings, the latter described by the coupling constants (*J*). This provides valuable information about the relative number of hydrogen and also their type, by comparison of the recorded chemical shifts with compiled data. This is particularly useful in establishing the aglycone type and the acyl groups attached to it, as well as in identifying the number and the anomeric configuration of the glycoside moieties attached to the aglycone.

^{13}C NMR data is used to complement ^{1}H NMR data, and is particularly useful at establishing the type of group present in the samples' molecules by comparison with compiled data; however, it must be noted that ^{13}C NMR is much less sensitive due to the abundance of ^{13}C (1.1 %) when compared to ^{1}H (99.9 %) (Claridge, 1999).

Together, these two 1D experiments are used primarily to identify aglycone types and substituent groups, but a definite structural elucidation, which the accurate location of the various groups, requires various 2D experiments.

Two-Dimensional Spectroscopy

Two-dimensional (2D) NMR spectrum contains signals dispersed according to two characteristic frequencies rather than one so that the number of distinct signals that can be resolved are more than in normal 1D spectrum. In resolved 2D experiments, chemical shifts and hetero or homonuclear spin couplings are separated into two dimensions. The correlation experiments meanwhile differ from resolved experiments in that they contain a mixing period during which coherence is transferred or evolves in the spin system. The result is a 2-D spectrum exhibiting connectivities, i.e. cross peaks between signals from coupled spins. Thus, the 2-D spectrum generate contour maps that show the correlations between different nuclei in the molecules, and can be either homonuclear or heteronuclear, depending on whether the interacting nuclei are of the same or different elements (Friebolin, 1998; Claridge, 1999).

Homonuclear Correlations

^{1}H-^{1}H Correlation Spectroscopy (COSY)

^{1}H-^{1}H Correlation Spectroscopy was one of the first multidimensional systems. COSY cross peaks are between protons that are coupled to each other, usually two bonds apart ($^{2}J_{HH}$), but sometimes also three and four bonds apart ($^{3}J_{HH}$ and $^{4}J_{HH}$); the intensity of coupling affects the intensity of the peak. DQF-COSY (Double Quantum Filter COSY) is an improvement of the COSY experiment in which non-coupled proton signals, such as those from solvent, are eliminated as they may overlap signals from the analyte (Claridge, 1999; Neri and Tringali, 2001).

A further improvement is the TOCSY (Total Correlation Spectroscopy) experiment, which creates correlations between all protons in a given spin system, as long as there are couplings between every intervening protons; this is extremely useful to identify protons on sugar rings – every proton from one sugar ring will have a correlation with all other protons from the same ring but not with those of other rings. Magnetization is transferred over up to 5 or 6 bonds, and is interrupted by small or null ^{1}H-^{1}H couplings and hetero-atoms; also, the number of transfer steps can be adjusted by changing the spin-lock time (Fossen and Andersen, 2005).

Selective 1D TOCSY (also known as HOHAHA, homonuclear Hartman-Hahn) is particularly useful in compounds with more than one sugar moiety, in which overlap occurs; in this experiment, one peak is selected and that magnetization is transferred step wisely to the protons in the same spin system; instead of crosspeaks, transfer is shown by increased multiplet intensity (Fossen and Andersen, 2005).

Hoeteronuclear Correlations

Heteronuclear 2D NMR experiments correlate nuclei of different elements. The most powerful techniques of all are undoubtedly the 2D proton–carbon experiments HMQC/HSQC (Heteronuclear Multiple Quantum Coherence/Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) as they provide an opportunity to do detail proton and carbon NMR data directly.

HMQC and HSQC establish one bond correlations between the protons of a molecule and the carbons to which they are attached ($^{1}J_{CH}$). Both these are much more sensitive than the correspondent 1D ^{13}C experiments; while in 1D experiment the low abundance of the isotope leads to a low signal-to-noise ratio, in the heteronuclear 2D

experiment the initial magnetization occurs on the highly sensitive ^{1}H nuclei and is then transferred to the ^{13}C atoms that are connected to each proton.

^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation) is an experiment in which long-range interactions (typically $^{2}J_{CH}$ and $^{3}J_{CH}$) are analyzed. HMBC is usually more sensitive to 3-bond correlations than to 2-bond correlations, but this depends on the overall signal-to-noise ratios and on the adjustable parameters of each experiment (Claridge, 1999; Krishnamurthy, 2000; Fossen and Andersen, 2005).

HBMC application to flavonoids usually addresses assignment on nonprotonated C atoms, from both the aglycones and acyl groups. Unlike TOCSY, HMBC transfer is not stopped by heteroatoms, and so it can also be used to determine the linkage points of heteroatom containing groups such as sugar residues. HMBC is also useful to distinguish some classes of flavonoids, as flavones from aurones, which have similar 1H and 13C NMR spectra but very different HMBC spectra. Currently, only enhanced variants of the HSQC and HMBC experiments, namely gradient enhanced (*ge*) ones, are used, due to their higher sensitivity and capacity. These have been used to establish strong intramolecular H bonding between the 4-oxo and 5-hydroxy groups in flavonoids (Exarchou *et al.*, 2002; Kozerski *et al.*, 2003).

Further developments in NMR experiments, using new 2D and 3D techniques, have been developed in recent years, and are starting to be used for flavonoid analysis. In particular, 2D and 3D HSQC-TOCSY experiments are capable of assigning all ^{13}C signals of individual glycosides in polyglycosilated flavonoids (Fossen and Andersen, 2005).

Connectivity through space-the nuclear overhauser effect

While the above mentioned 2D techniques are useful to establish the connectivities between atoms through bonds, the Nuclear Overhauser Effect (NOE), which can be summarized as “A change in the intensity of an NMR signal from a nucleus, observed when a neighbouring nucleus is saturated”, is useful at establishing non-bonded connectivities, or connectivities through space. The cross peaks in a ^{1}H-^{1}H NOESY (NOE Spectroscopy) spectrum correspond to correlations between protons that are close to each other in space (up to 4 Å) but not necessarily connected through bonds; these correlations may arise from both intramolecular and intermolecular proton interactions, and has make a major contribution to the determination of stereochemistry (Neri and Tringali, 2001). A 2D NOE experiment, ROESY (Rotating Overhauser Effect Spectroscopy), has been used in flavonoid research mainly to establish the stereochemistry of various flavonoids (Claridge, 1999; Fossen & Andersen, 2005).

Friebolin, H. (1998). *Basic One- and Two-Dimensional NMR Spectroscopy*, Third Edition.

Neri, P. and Tringali, C. (2001). Application of modern NMR techniques in the structureelucidation of bioactive natural products, Tringali 69-128.

2.7.7Biological activity of flavonoids

Medicinal plants are well-known biosynthetic laboratories of bioactive substances, thus they can magically provide us with the key to our awful health problems in life. Flavonoids constitute a large group of plant secondary metabolites that enjoy a widespread accumulation throughout the plant kingdom and are commonly found in fruits, vegetables and certain beverages (López-Lázaro, 2009). Their wide occurrences in nature have aroused considerable interest in their use and considerable data has been accumulated concerning their pharmacological actions and other

properties (Cushnie and Lamb, 2005). They have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, anti-tumor and antioxidant activities. Recent studies also support a protective effect of flavonoids consumption in cardiovascular diseases and cancer (Tanwar and Modgil, 2012).

2.7.7.1Antioxidant activity

Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers (Pal *et al.,* 2009). This activity is attributed to their hydrogen-donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available ‘‘H” atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure (Tripoli *et al*., 2007).

Free radical scavenging capacity is primarily attributed to high reactivities of hydroxyl substituents that participate in the reaction (Heim *et al*., 2002). Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical as in fig (V), thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain (Ferreira *et al*., 2010).

Many disease processes involve the generation of reactive oxygen species (superoxide anionic radical O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl-radical (OH^-) etc). If not neutralized, these oxidative radicals cause cellular oxidative stress, increase lipid per oxidation and cause instability of cellular membranes. The antioxidant-defense mechanism of the body includes enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, but also nonenzymatic counterparts such as glutathione, ascorbic acid, and α-tocopherol. The increased production of reactive oxygen species during injury results in consumption and depletion of the

endogenous scavenging compounds (Havsteen, 2002). The best-described property of almost every group of flavonoids is their capacity to act as antioxidants (Pal *et al.,* 2009). The flavonones and catechins seem to be the most powerful flavonoids for protecting the body against reactive oxygen species.

2.7.7.2Anti-inflammatory effect

Flavonoids have been found to be prominent inhibitors of COX or LOX (Bitis *et al*., 2003; Pal *et al*., 2009). Flavonoids prevent synthesis of PGs that suppress T-cells. The immune cells communicate with chemical signals called cytokines which are controlled by flavonoids. Flavonoids inhibit the activity of PKC at ATP-binding site. Flavonoids also promote IFN synthesis (Havsteen, 2002).Various flavonoids (e.g., quercetin, apigenin, tea catechins) have also been shown to have anti-inflammatory activity by inhibiting cycloxygenase-2 (COX2) and inducible nitric oxide synthase, which is related to antioxidant activity. Flavonoids also inhibit cytosolic and tyrosine kinase (Middleton *et al*., 2000; Kang *et al*., 2009) and also inhibit neutrophil degranulation (Middleton *et al*., 2000). Citrus flavonoids, hesperidin is known to possess anti-inflammatory and analgesic effects. Apigenin, luteolin, quercetin are known to possess anti-inflammatory activity (Tapas *et al*., 2008).

2.7.7.3 Antiviral effects

The antiviral activity of flavonoids was shown in a study by Wang *et al*. (1998). The flavonoid quercetin gave some protection to animals experimentally inoculated with many viruses. It also inactivated *Herpes simplex*, polio, rabies and some flu viruses. Catechin is more potent than quercetin against herpes virus. Quercetin was reported to exhibit both anti-infective and antireplicative abilities. The interaction of flavonoids with the different stages in the replication cycle of virus was previously described (Kaul *et al*., 1985). For example, some flavonoids work on the intracellular

replication of virus, whereas others inhibit the infectious properties of the viruses. By far, most studies of the effects on viruses were performed *in vitro* and little is known about the antiviral effect of flavonoids *in vivo*. Flavonones having sugar moiety showed antimicrobial activity while none of the flavonols and flavonolignans showed inhibitory activity on microorganisms. Quercetin has been reported to completely inhibit growth of Staphylococcus aureus (Tapas *et al*., 2008).

2.7.7.4 Anticancer effects

Flavonoids for a long time have been part of the herbal treatment by lay practitioners, but they were recognized only recently as effector substances. Examples of herbal preparations owing their growing recognition as effective anticancer drugs to flavonoids are propolis and Essiac (Havsteen, 2002). Flavonoids are potent bioactive molecules that possess anticarcinogenic effects since they can interfere with the initiation, development and progression of cancer by the modulation of cellular proliferation, differentiation, apoptosis, angiogenesis and metastasis (Ramos, 2007). Flavonoids have emerged as potential chemopreventive candidates for cancer treatment due to their ability to induce apoptosis (Ramos, 2007).

2.7.7.5 Antithrombotic effects

Flavonoids are used as antithrombotic due to their ability to scavenge free radicals. They inhibit cyclooxygenase and lipooxygenase pathway. The main antiaggregatory effect is by the inhibition of thromboxane A2 formation. Flavonoids like quercetin, kaempferol and myricetin are known to possess antiaggregatory properties (Middleton *et al*., 2000; Tapas *et al*., 2008).

2.7.7.6 Antidiabetic effects

All flavonoids cannot cure diabetes mellitus because most types of this disease are basically genetic and no single drug can correct an inborn error. However, flavonoids

can ameliorate some of the consequences of diabetes mellitus (Havsteen, 2002) and have been identified to be good inhibitors of aldose reductase (Patra and Chua, 2010). It has been reported by several researchers that quercetin possess antidiabetic activity and it has been found that it brings about regeneration of pancreatic islets and increases insulin release in streptozotocin-induced diabetes. Also, it has been reported to stimulate Ca2+ uptake from isolated islet cells thus suggesting it to be effective even in non-insulin dependent diabetes (Tapas *et al*., 2008). Li *et al*. (2009) indicated that flavonoids in *Ipmoea batalas* leaf possesses antidiabetic activity against alloxan-induced diabetes at a dose of 100 mg/kg (Li*et al*., 2009). Sriram and Subramanian reported fisetin to be a therapeutic agent for treatment of diabetes mellitus at a dose of 10 mg/kg (Sriram and Subramanian, 2011).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Materials/Reagents/Equipment

3.1.1Apparatus and equipment

i. Soxhlet apparatus
ii. Column (Sintered glass) of various size
iii. TLC tank of various size
iv. Oven
v. Distillation apparatus
vi. Evaporating dish
vii. Rotary evaporator
viii. Water bath
ix. Stop watch

3.1.2 Chemicals/Reagents

Solvents were obtained from Sigma-Aldrich chemicals Germany and were redistilled twice before used. Silica gel (Merck: 60-120μ Mesh) and Sephadex LH-20 (Pharmacia Biotech) were used for column chromatography while polyester and aluminium foil backed pre-coated silica gel plate (Whatman) wereused for thin layer chromatography.

3.1.3 Instruments

1. Melting Point (mp)
Gallenkamp (capillary) melting point apparatus.
2. Nuclear Magnetic Resonance Spectroscopy (NMR)

Brucker CX (600 MHz) spectrophotometer

3. Hot plate
Gallenkamp (England) thermostat hot plate apparatus (cat no: HL-054)

3.1.4 Experimental animals

Adult Swiss albino mice of either sex weighing between 18-25 g and rats weighing between 150-200 g were procured from the Animal house facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria were used for these studies. The animals were housed in standard cages and were fed on standard laboratory animal diet and water, *ad libitum*. Food was withdrawn during the experimental hours. All experimental protocols were in accordance with the Ahmadu Bello University, Zaria research policy.

3.1.5 Drugs used for Pharmacological studies

The following chemicals and drugs were used: Carrageenan (Sigma-Aldrich), acetic acid (RambaxyLaboratories Ltd., Punjab), Ketoprofen (Lek Pharmaceuticals company, Slovenia), Pentazocine (Simpha). All drugs were freshly prepared to the

desired concentration with distilled water just before use. The extract was also freshly prepared using distilled water.

3.1.6 Microorganisms tested and media used antimicrobial study

Clinical isolates of *Staphylococcus aureus, Streptococcus pyrogenes, Corynebacterium ulcerus, Bacillus subtilis, Escherichia coli, Salmonella typhi, Klebsiella pneumonia, Pseudomonas aeruginosa, Neissera gonorrhea* and *Candida albicans* obtained from the Department of Medical Microbiology, Ahmadu Bello University, Teaching Hospital, Zaria, Nigeria were used in this study. Standard susceptibility antibiotic discs used were Chloramphenicol (5 µg/disc), Erytromycin (5 µg/disc) and Sparfloxocin (2 µg/disc) produced by Oxoid Ltd., Hamshire, England. The powdered nutrient agar and nutrient broth medium were obtained from a chemical store in Zaria Nigeria.

All isolates were purified and kept in slants of blood agar medium.

3.2 Analytical Procedures

3.2.1 Thin layer chromatography (TLC)

Techniques: One way ascending

*Stationary phase:*Polyester and aluminium foil backed pre-coated silica gel plate (250 µm layer) wereused as stationary phase for thin layer chromatography.

Spotting and development: Spots were applied to the base line manually using a fine glass capillary tube and plates were dried using an air blower, developed at room temperature using a Shandon chromatographic tank.

*Solvent systems:*Various solvent systems were used as mobile phase. For details see text.

*Detection:*Developed plates were usually air-dried before visualization. Spots on TLC plates were visualized on spraying with 10% Sulphuric acid followed by heating at 110°C for 5-10 min.

3.2.2 Column chromatography (CC)

The following conditions were employed in running the column chromatography.

*Techniques:*Gradient elution

*Column:*Sinteredglass column (75 cm x 2 cm); (20 cm x 1.2).

*Stationary phase:*Silica gel 60 – 120 mesh size

*Column packing:*Columnswere packed by the wet slurry method and eluates were collected manually.

*Sample loading:*Samples were applied using dry load method, sample was dissolve in small amount of suitable organic solvent, and it was then mixed with a small quantity of silica gel, triturated and the loaded on top of the column.

*Solvent for elution:*Various solvents systems were used for elution. Elution was carried out using one or mixture of the following solvents:n-Hexane, Chloroform, Dichloromethane, Ethyl acetate and Methanol. For details see text.

3.2.3 Gel filtration (GF)

Gel filtration was carried out using Sephadex LH-20 (Sigma)

*Packing method:*The Sephadex LH-20 was suspended in methanol and allowed to swell for 24 hours prior to use. It was then poured to the column and allowed to settle tightly.

*Sample application:*Samples were dissolved in small volume of the methanol and applied on top of the column.

*Solvent:*Methanol

3.2.4 Preparative thin layer chromatography (PTLC)

PTLC was carried out using Merck silica gel precoated glass plate 20x20 cm with layer thickness of 25 µm. A thin line about 1.5 cm from the bottom of the plate was drawn with a pencil. The sample to be separated was dissolved in minimum amount of solvent to give an approximate concentration of 20 mg/ml. It was then applied uniformly along the thin line using capillary tube. The plate was allowed to dry after which it was developed using appropriate solvent system. The developed plate was air dried in a fume cupboard and the position of the band of interest was marked with pencil and scraped off the backing of the plate on a foil. The scrapped sorbent was sized reduced using pestle and mortar, transferred to a sintered glass funnel and washed repeatedly with appropriate solvent and the solution obtained was evaporated to give the compound (Gibbons and Gray, 1998).

3.2.5 Spectral analysis

NMR spectra (both 1D and 2D) were obtained on a Brucker CX (600 MHz) spectrophotometer. Spectra's were referenced to the CD_3OD solvent signal at δ3.80 (^1H) and 49.00 (^{13}C) with Tetramethylsilane(TMS) as internal standard. Chemical shift values (δ) were reported in parts per million (ppm) relative to TMS and coupling constants (J values) are given in Hertz (Hz). NMR spectrophotometer at the University of Kwa-Zulu Natal Durban, South Africa was used for the NMR analysis.

3.2.6 Spray/Visualization reagents

Developed TLC plates were usually air-dried and were visualized on spraying with 10% Sulphuric acid followed by heating at 110^0C for 5-10 min.

3.2.7 Route of administration of drugs

The *A. grantii* methanol extract and the standard drugs were administered to the animals intraperitoneally (*i.p.*) in all the experiments.

3.3 Extraction Procedures

3.3.1 Collection, identification and preparation of plant material

The whole plant was collected from Gwoza Wakane village, Gwoza, Borno State, Nigeria in October, 2008. The plant was confirmed and authenticated by Mallam Muhammad Musa at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria by comparing with an existing voucher specimen (No. 045). A voucher specimen was deposited there for further reference. The aerial parts of the plant were air dried under shade, powdered to fine powder using a porcelain mortar and pestle and subsequently referred to as powdered plant material.

3.3.2 Extraction

Powdered aerial parts (1.2 Kg)of *Ampelocissus grantii* was extracted exhaustively with methanol (5.0 litres) using Soxhlet apparatus. The resultant extract was concentrated *in vacuo* to afford a dark green mass which weighed 165.00 g (13.75% w/w) and was referred to as *Ampelocissus grantii* methanol extract (AGME). The methanol extract (140 g) was dry partitioned successively with n-hexane, dichloromethane and acetone to obtain n-hexane, dichloromethane and acetone soluble fractions respectively. The marc was dissolved in water and the insoluble filtered and the filtrate was partition with n-butanol to obtain the n-butanol soluble fraction.

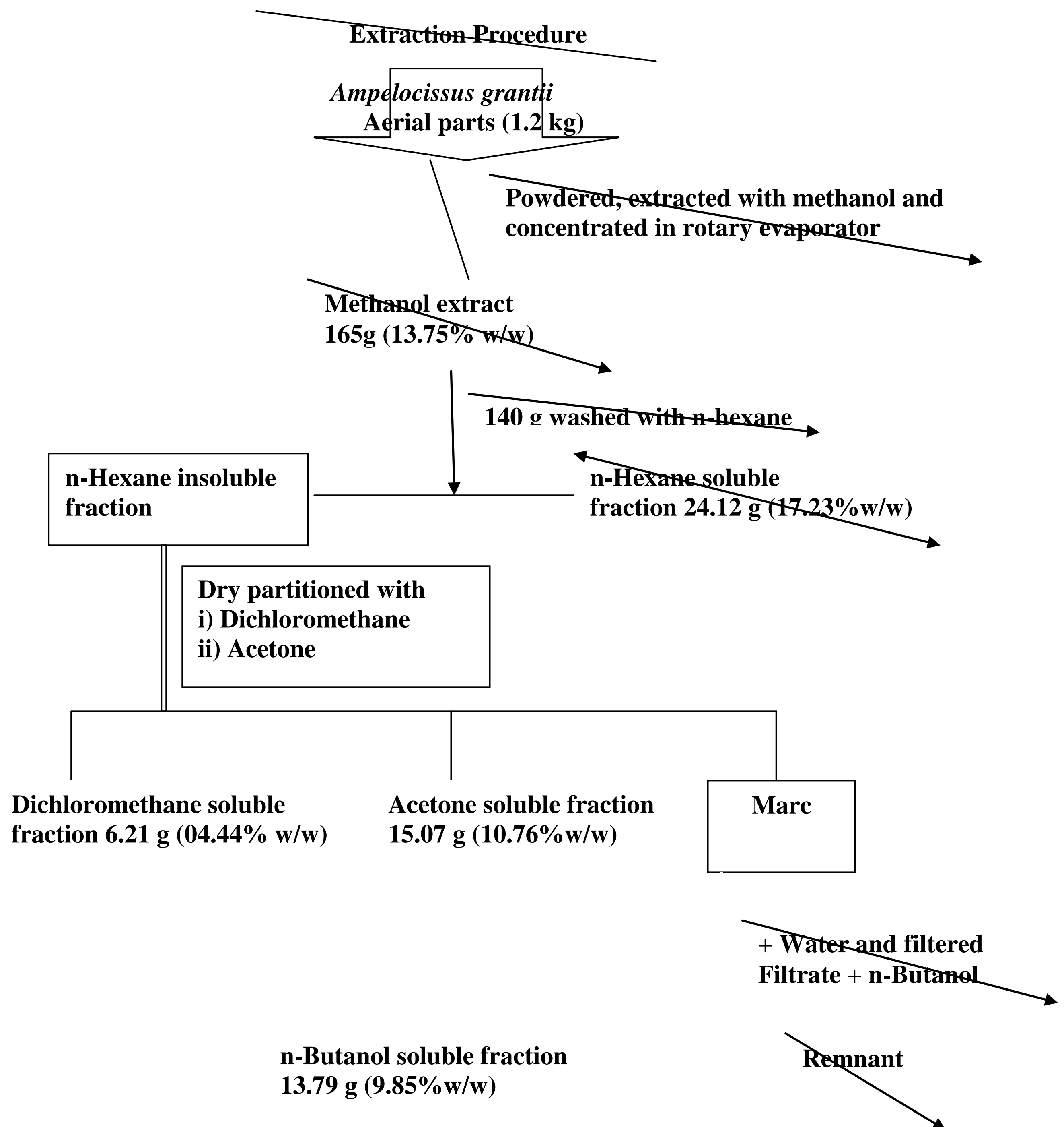


Figure 3.1: Extraction and fractionation of plant material

3.4 Preliminary Phytochemical Screening

The qualitative phytochemical screening of the crude methanol extract was carried out in order to ascertain the presence of its constituents using standard conventional methods as summarized below:

3.4.1 Test for carbohydrates

(i) Molisch's test (General test for carbohydrates)

A few drops of Molisch reagent was added to extract dissolved in water, followed by addition of 1 ml of concentrated sulphuric acid down the side of the test tube. The mixture was allowed to stand for 2 minutes, and then diluted to 5 ml with water. The formation of red or dull violet at the interphase of the layer is taken as positive test for the presence of carbohydrate (Trease and Evans, 2002).

(ii) Barfoed'stest(Test for monosaccharides)

About 0.2 g of the extract was dissolved in water and then filtered. The filtrate was mixed with 1 ml of Barfoed's reagent in a test tube and heated on a water bath for 2 minutes; a red precipitate of cupric oxide is taken as positive test (Brain and Turner, 1975).

(iii) Fehling's

(a) Test for reducing sugars

About 0.2 g of the extract was dissolved in water and then filtered. The filtrate was heated with 5 ml equal volume of Fehling's solution A and B. formation of a red precipitate of cuprous oxide indicates the presence of reducing sugars (Trease and Evans, 2002).

(b) Combined reducing sugar

About 0.2 g the extract was hydrolyzed by boiling with 5 ml of dilute hydrochloric acid, HCl, and resulting solution neutralized with 10% sodium hydroxide, NaOH, solution. Two drops of Fehling's solution A and B was added and heated on a water bath for 2 minutes. Formation of reddish-brown precipitate indicates the presence of combined reducing sugar (Trease and Evans, 2002).

3.4.2 Test for flavonoids

(i) Shinoda test

About 0.5 g the extract was dissolved in ethanol warmed and filtered. To the alcoholic solution of the extract three pieces of magnesium chips were added followed by a few drops of concentrated hydrochloric acid. Appearance of an orange, pink or red to purple colour indicates the presence of flavonoids (Markham, 1975).

(ii) Ferric chloride test

Little portion of the extract was boiled with water and filtered. To 2 ml of the filtrate, two drops of freshly prepared ferric chloride, $FeCl_3$, solution was added; green, blue or violet colouration indicates the presence of phenolic hydroxyl group (Trease and Evans, 2002).

(iii) Sodium hydroxide test

2 ml of the extract was dissolve in 10% aqueous sodium hydroxide solution and filtered to give yellow colour, a change in colour from yellow to colourless on addition of dilute hydrochloric acid indicate the presence of flavonoids (Trease and Evans, 2002).

3.4.3 Test for alkaloids

0.5 g of the extract was stirred with 5 ml of 1% aqueous hydrochloric acid on a water bath and filtered. 3 ml of the filtrate was divided into three portion of 1ml each. To the first 1 ml few drops of freshly prepared Dragendoff's reagent was added and observed for formation of orange to brownish precipitate as an evidence for the presence of alkaloids. To the second, 1 drop of Mayer's reagent was added and observed for formation of white to yellowish or green colour precipitate indicating the presence of alkaloids. To the third, 1 ml drop of Wagner's reagent was added to give a brown or reddish-brown precipitate indicating the presence of alkaloids (Silva *et al.,* 1998; Trease and Evans, 2002).

3.4.4 Test for tannins

A small quantity of the extract was boiled with 10ml of water, cooled and filtered. Two drops of ferric chloride was added to the filtrate, formation of a blue-black, or green precipitate was taken as evidence for the presence of tannins (Trease and Evans, 2002).

3.4.5 Test for anthraquinones

(i) Free anthraquinones

The extract was shaken with 10 ml of benzene, the content was filtered, and 5 ml of 10% ammonia solution (NH_4OH) was added to the filtrate, the mixture was shaken. Presence of a pink, red or violet colour in the ammoniacal layer (lower phase) indicates the presence of free anthraquinones (Trease and Evans, 2002).

(ii) Combined anthraquinones:

The extract was boiled with 10 ml of aqueous sulphuric acid and filtered hot. The filtrate was shaken with 5 ml benzene, the benzene layer was separated and half its volume, 10% ammonia solution was added. A pink, red or violet colouration in the ammonia phase (lower phase) indicates the presence of combined anthraquinones derivatives (Trease and Evans, 2002).

3.4.6 Test for saponins

About 0.5 g of the extract was shaken with water in a test tube. Frothing which persisted for 15 minutes indicates the presence of Saponins (Silva *et al.,* 1998).

3.4.7 Test for steroids

(i) Liebermann-Burchard's test

To a small quantity of the extract was added to 1 ml chloroform and filtered. The filtrate was mixed with 2ml acetic acid and cooled in ice. Concentrated sulphuric acid was added down the side of the test tube. A colour change from violet to blue or bluish green indicates the presence of steroidal nucleus (Silva *et al.,* 1998).

(ii) Salkwoski's test

A little quantity of the extract was dissolved in 1 ml chloroform and to it 1 ml of concentrated sulphuric acid was added down the test tube to form two phases. Formation of red or yellow colouration was taken as an indication for the presence of steroidal ring (Silva *et al.,* 1998).

3.4.8 Test for cardenolides

(i) Legal test

Solution I: 0.5% of recently prepared sodium nitroprusside in water and solution II: 2N sodium hydroxide. About 2 mg of the extract was dissolved in three drops of

pyridine. 1 drop of solution I was added followed by 4 drops of solution II, one at a time. A deep red colour was taken as the presence of cardenolide aglycone (Silva *et al.,* 1998).

(ii) Keller-Killani's test

0.5 g the extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of ferric chloride. This was then under laid with 1ml of concentrated sulphuric acid. A reddish-brown ring obtained at the interphase indicates the presence of a digitaloxose sugar component characteristic of cardenolides. A violet ring may appear below the ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer (Richard, 1998).

3.5 Preliminary Thin Layer Chromatography (TLC) of the Crude Methanol Extract of *Ampelocissusgrantii*

The crude methanol extract was subjected to thin layer chromatography examination using polyester backed precoated thin layer chromatography plates using the solvent system: ethyl acetate: methanol water 40:5:4 [EMW]; chloroform: ethyl acetate 5:4 [CE] and n-hexane: ethyl acetate 5:1 [HE] .

After the development of each plate with each of the above solvent system, the plates were allowed to dry, and then visualized onspraying with 10% Sulphuric acid followed by heating at 110^{0}C for 5-10 min.In each of the plates, the number of spots and their $R_f$values were recorded.

3.6 Isolation of Compounds 1, ML 1 and ML 2

3.6.1 Thin layer chromatography (TLC) of acetone soluble fraction of the methanol extract of the aerial parts of*Ampelocissus grantii*

The acetone soluble fraction of the methanol extract was subjected to thin layer chromatography examination using the solvent system: ethyl acetate: methanol water (100:16.5:13.5) After the development of the plate with the above solvent system, the plate was allowed to dry, and then visualized by spraying with 10% sulphuric acid and heated in oven for 5-10 minutes. The number of spots and their R_f value were recorded.

3.6.2 Column chromatographyof acetone soluble fraction of the methanol extract of the aerial parts of*Ampelocissus grantii*

The acetone soluble fraction (10 g)was subjected to column chromatography (column size, 100 cm x 4 cm) over silica gel (60 -120 mesh; 250 g) and eluted with n-hexane, dichloromethane, ethyl acetate and methanol in order of increasing polarity. A total of 55 fractions of 100 ml volumeswere collected and their composition monitored by thin layer chromatography (TLC) using the solvent systems;

i.) n-hexane-ethyl acetate (5:1)

ii.) n-hexane-ethyl acetate (7:3)

iii.) chloroform-ethyl acetate (5:4)

Similar fractions were pooled together and reduced to six major fractions designated as A1- A6. The progress of the elution is presented in Table 4.6.

Fraction A3 (28 – 38) was subjected to gel filtration using Sephadex LH-20 and eluted with methanol to obtained 40 fractions. The fractions were reduced to 13 (subtractions $A3_{1-13}$). Sub fraction $A3_6$gave a single spot when subjected to TLC using two solvent systems of ethyl acetate: methanol: water (100: 16.5: 13.5) and chloroform-methanol-water (3: 3: 1) to afford ML 1. It was recrystallized in methanol to give yellow amorphous powder 5 mg.

Fraction A4 (39 – 40) was further subjected to column chromatography (column size 75 cm x 2 cm) over silica gel (60 -120 mesh; 75 g) using gradient elution starting from chloroform, chloroform-methanol mixture and methanol as outlined in Table 4.7.

240 fractions of20 ml volumewere collected and monitored by TLC using the following solvent systems:

i. ethyl acetate: methanol: water (4: 1:1)
ii. ethyl acetate: methanol: water (100: 16.5: 13.5)
iii. chloroform: methanol: water (3: 3:1)

Similar fractions were pooled and reduced to eight sub-fractions which were coded ($A4_{1-8}$). The sub fraction $A4_1$eluted from chloroform-methanol (90; 10) consisting of one major and two minor spots was submitted to gel filtration to afford 5 fractions ($A4_{1a-e}$). Fraction $A4_{1c}$ (6-18) gave a single spot when subjected to TLC using chloroform: methanol: water (3: 3:1) and ethyl acetate: methanol: water 100: 16.5: 13.5). Evaporation of the solvent afforded a yellow amorphous powder which weight 4 mg and was coded as compound 1.

Sub fraction $A4_4$eluted from chloroform-methanol (70: 30) was resubmitted to repeated gel filtration over Sephadex LH – 20 and eluted with methanol to afford 10 fractions ($A4_{4a-j}$). Fraction $A4_{4e}$(25-28) with one major spot was purify by preparative thin layer chromatography using ethyl acetate: methanol: water 100: 16.5: 13.5) to afford ML 2.Thiswas furtherrecrystallized in methanol to give 4 mg of the compound.

3.6.3 Characterization of compounds 1, ML 1 and ML2

3.6.3.1 Thin layer chromatography (TLC) examination of compounds1, ML 1 and ML 2

The isolated compounds were subjected to thin layer chromatographic examination in two different solvent systems to prove their homogeneity. The solvent systems used as mobile phase are as follows:

i. chloroform: methanol: water (3: 3: 1)

ii. ethyl acetate: methanol water (100:16.5:13.5)

Developed chromatograms were dried and then visualized by spraying with 10% sulphuric acid and heated in oven for 5-10 minutes. The R_f values for the spots were calculated and results recorded.

3.6.3.2 Solubility test of compounds1, ML 1 and ML 2

The solubility of each compound isolated was determined in chloroform, ethyl acetate, acetone and methanol at room temperature.

3.6.3.3Chemical studies of compounds1, ML 1 and ML 2

Chemical studies of compound 1, ML 1and ML2 using the standard procedure to test for flavonoids nucleus was carried out.

i.) **Ferric chloride test:** To small methanol solution of each compound, a few drops of $FeCl_3$ solution was added and colour changed noted (Trease and Evan, 2002).

ii.) **Shinoda's test:** To small ethanol solution of each compound, three pieces of magnesium turnings were added followed by a few drops of conc. HCl and colour change was noted(Markham, 1975).

3.6.3.4 Spectral analysis of compounds 1, ML 1 and ML2

The ^{1}H-NMR spectrum of each compound was recorded in CD_3OD on a Bruker CX instrument operated at 600 MHz.

3.7Isolation of Compounds ML7

3.7.1 Thin layer chromatographic (TLC) examination of n-butanol soluble fraction of the methanol extract of the aerial partsof *Ampelocissus grantii*

The n-butanol soluble portion of the methanol extract was subjected to thin layer chromatographic examination using the solvent system: ethyl acetate: methanol water (65:20:15) After the development of the plate with the above solvent system, the plate was allowed to dry, and then visualized by spraying with 10% sulphuric acid and heated in oven for 5-10 minutes. The number of spots and their R_f value were recorded.

3.7.2 Column chromatography of n-butanol soluble fraction of methanol extract of the aerial parts of *Ampelocissus grantii*

The n-butanol fraction of the methanol extract of *A. grantii* (5 g)was mounted over glass column (75 cm x 2.0 cm) packed with silica gel(150 g, 60-120 mesh). The column was eluted gradiently with ethylacetate, ethylacetate-methanolmixtures and methanol. The progress of column was monitored on thin layer chromatographyusing ethylacetate: methanol: water (15: 3: 2) as solvent system. The result of the column is summarized in table (4.17).

Based on TLC profile the fractions were reduced toeight fractions (A – H). Fraction E obtained from ethyl acetate: methanol (70:30) when submitted to repeated gel filtration on Sephadex LH-20. This was finally purified by preparative thin layer chromatography (PTLC) using ethyl acetate-methanol-water (15: 3: 2) as solvent system to afford a single spot coded as ML 7.

3.7.3 Characterization of ML 7

3.7.3.1 Thin layer chromatography (TLC) examination of ML7

ML7 was subjected to thin layer chromatography examination using the solvent system: ethyl acetate: methanol: water (15: 3: 2) to confirm its homogeneity.

3.7.3.2 Solubility test of ML7

The solubility of ML 7 was determined in chloroform, ethyl acetate, acetone and methanol at room temperature.

3.7.3.3Chemical studies of ML7

Chemical studies of ML7 using the standard procedure to test for flavonoids nucleus was carried out.

i.) **Ferric chloride test:** To small methanol solution of each compound, a few drops of $FeCl_3$ solution was added and colour changed noted (Trease and Evan, 2002).

ii.) **Shinoda's test:** To small ethanol solution of each compound, three pieces of magnesium turnings were added followed by a few drops of conc. HCl and colour change was noted(Markham, 1975).

3.7.3.4 Spectral analysis of ML7

The ^1H-NMR spectrum of ML7 was recorded in CD_3OD on a Bruker CX instrument operated at 600 MHz.

3.8Pharmacological Studies

3.8.1 Acute toxicity study (LD_{50})

The study was divided into two phases. In the first phase, nine mice of either sex were divided into three groups of three mice each. Three doses of the *A. grantii* methanol extract (10, 100 and 1000 mg/kg) were administered intraperitoneally (*i.p.*) to group I, II and III respectively. The mice were observed for signs and symptoms of toxicity

and mortality for 24 hours after treatment. In the second phase, 3 mice were divided into 3 groups of one mouse each and treated based on the result of the first phase. Three doses of the *A. grantii* methanol extract (1600, 2900, and 5000 mg/kg) were administered through the same route to groups I, II and III respectively. The mice were also observed for 24 hours. The median Lethal dose (LD_{50}) was calculated as the square root of the product of the lowest lethal dose and the highest non-lethal dose i.e. the geometric mean of consecutive doses for which 0 and 100% survival rates were recorded (Lorke, 1983).

3.8.2 Antinociceptive and anti-inflammatory studies

3.8.2.1 Acetic Acid – induced Abdominal Constriction in Mice

Mice were divided into 5 groups of 6 mice each. The first group served as control, were given 10 ml/kg of normal saline, the *A. grantii* methanol extract at 75, 150 and 300 mg/kg body weight were given to the second, third and fourth groups respectively. The fifth group received the reference drug, Ketoprofen at 20 mg/kgbody weight intraperitoneally. 30 minutes later, all the groups were treated with acetic acid (0.7 %, at a dose of l0 ml/kg body weight intraperitoneally). Mice were placed in individual cage and the number of abdominal constrictions was counted for each mouse 5 minutes after acetic acid injection for a period of 10 minutes (Koster *et al*., 1959). Percentage inhibition of writhing was obtained using the formula.

$$Inhibition\ (\%) = \frac{\text{Mean Number of writhes (control) - Mean Number of writhes (test)}}{\text{Mean Number of writhes (control)}}\ x\ 100$$

3.8.2.2 Hot plate test in mice

The method of Tunner (1965) as modified by Lanhers *et al.* (1992) was used for the study. Mice were placed on a hot plate maintained at temperature of 50 ± 0.5^{0}C and

the pain response latency was determined using a stopwatch. The interval between the time (in seconds) the mouse was placed on the hot plate and the time it started shaking/licking its paw or jumped off the plate was considered as an index of pain response latency. Mice that showed initial nociceptive response within 20 seconds were selected and used for the study. Mice were divided into five groups each containing six mice. Normal saline served as control and was given to the first group at a dose of 10 ml/kg, the extract at 75, 150 and 300 mg/kg body weight were given to the second, third and fourth groups respectively. The fifth group received the reference drug, Pentazocine at 30 mg/kg body weight intraperitoneally. 30 minutes post-treatment, each mouse was placed gently on the hot plate and the reaction time recorded. Measurements were taken at 0, 30, 60, 90, 120 and 150 minutes after the treatment of animals(Tunner 1965; Lanhers *et al.,* 1992). The final test mean value (Ta) for each treatment group was calculated which represented the after treatment reaction time and was subsequently used to determine the percentage thermal pain stimulus or protection.

3.8.2.3 Carrageenan-induced paw oedema in rats

Rats were divided into 5 groups each containing six rats. The first group received 1 ml/kg normal saline which serves as control. The *A. grantii* methanol extract at 75, 150 and 300 mg/kg body weight was given to the second, third and fourth groups respectively. The fifth group received the reference drug, ketoprofen at 20 mg/kg body weight intraperitoneally. Thirty minutes later, 0.1 ml of freshly prepared carrageenan suspension (1% w/v in 0.9% normal saline) was injected into the sub plantar region of the left hind paw of each rat. The paw diameter was measured with the aid of a vernier calliper at 0, 1, 2, 3 and 4 hour after the injection of carrageenan.

The difference between the readings at time 0 hour and the different time intervals was taken as the thickness of oedema (Winter *et al*., 1962).

3.9Antimicrobial Study

3.9.1 Preliminary antimicrobial activities studies

The sensitivities of all the organisms to the extract and the antibiotics were tested using the paper discs method. Using stock concentration of 50 mg/ml, (prepared by dissolving 0.5 g of the *A. grantii* methanol extract into 10 ml of sterilized distilled water).Blood agar base was the medium used as growth medium prepared according to manufacturer's instructions and was sterilized at 121^0C for 15 minutes and cooled. 20 ml of the sterile medium was poured into sterile Petri dishes covered and allowed to solidify. The plates were then seeded with the test microbes by spread plate techniques and left to stabilize for half an hour. Filter paper discs were cut and sterilized at 160^0C for 30mins, then soaked into the solution of the extract. The discs were dried at 45^0C, and planted on the surface of the medium previously seeded with the test microbes. Chloramphenicol (5 µg/disc), Erytromycin (5 µg/disc) and Sparfloxocin (2 µg/disc) standard discs were also place on the agar plate. The plates were incubated at 37^0C for 24 hours after which they were inspected for zones of inhibition of growth which were measured and recorded (in millimeters) (Bauer-Kirby, 1966).

3.9.2 Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration test was carried out on the microorganisms that were sensitive to the *A. grantii* methanol extract using the broth dilution technique. Nutrient broth agar was prepared according to manufacturer's instruction. 10ml each of the medium was dispensed into 5 screw cap test tubes and the medium was

sterilized at 121^0C for 15 minutes. MC – Farland's turbidity standard scale No. 0.5 was prepared (by adding 9.95mls of 1% H_2SO_4 and 0.5ml of 1% $BaCl_2$)to give a turbid solution. 10 ml normal saline was used to make a turbid suspension of the microbes, dilution of the microbes was carried out continuously using the normal saline until the turbidity marched that of the MC – Farland's scale by visual comparison, the microbes' concentrations at that point was $3x10^8$ cfu/ml.Two fold serial dilution of the *A. grantii* methanol extract in the nutrient broth was carried out and the following concentrations were obtained, 50, 25, 12.5, 6.25 and 3.125mg/ml. Having obtained the different concentrations and dilutions, 3 drops of the microbes in the normal saline were inoculated into the dilution of the extract in the broth; the culture was incubated at 37^0C for 24 hours. The lowest concentration of the extract which inhibited the growth of the microbes' was recorded as the minimum inhibitory concentration (MIC).

3.9.3 Minimum bactericidal concentration (MBC)

MBC were determined by using the broth dilution technique. The contents of the MIC tubes in the serial dilution were sub-cultured on the plates of the blood agar medium by dipping a sterile wire into each test tube and streaking the blood agar plates, the plates were then incubated at 37^0C for 24 hours after which they were observed for growth. The MBC was the plates with lowest concentrations of the extracts without growth.

3.10Statistical Analysis

All data were expressed as mean $\pm$ SEM. The mean values of control groups were compared with the mean values of treated groups using the Student's *t*-test. Results were considered significant at $P < 0.05$.

CHAPTER 4

4.0 RESULTS

4.1 Description and Yield of Extracts

Extraction of the aerial parts of *Ampelocissus grantii* with methanol gave a greenish gummy material weighing 165 g, (13.75% w/w) while the n-hexane, dichloromethane, acetone and n-butanol partition portions of the methanol extract gave yields of 24.12,6.21,15.07 and 13.79 representing 17.23, 04.44, 10.76, and 09.85%respectively are shown in the table 4.1.

4.2. Results of Phytochemical Constituents of Crude Methanol Extract of the Aerial Parts of A. *grantii*

The results of the preliminary phytochemical studies of the aerial parts of *Ampelocissus grantii* is presented in Table 4.2. The extract contains saponins, glycosides, steroids, terpenoids and flavonoids. Alkaloids were not detected in the extract (Table 4.2).

Table 4.1 Yield of the Partition Portions of the Crude Methanol Extract of the Aerial Parts of *Ampelocissus grantii*

Extract	Yield (g)	% Yield(% w/w)
n-Hexane	24.12,	17.23
Dichloromethane	6.21,	04.44
Acetone	15.07	10.76
n-Butanol	13.79	09.85

Table 4.2 Phytochemical Analysis of the Crude Methanol Extract of the Aerial Parts of *Ampelocissus grantii*

Group Constituents	Test	Observation	Inference
Carbohydrates			
General test	Molisch's Test	Dull violet colouration at interphase	+
Monosaccharide	Barfoed's Test	Red precipitate formed	+
Reducing sugar	Fehling (reducing sugar) Test	Red precipitate formed	+
	Fehling (combine reducing sugar) Test	Reddish brown precipitate formed	+
Alkaloids	Dragendorff's Test	-	-
	Wagner's Test	-	-
	Mayer' Test	-	-
Cardiac Glycosides	Legal's Test	Deep red colour formed	+
	Keller-Killiani's Test	Reddish-brown colour formed	+
Flavonoids	Shinoda's Test	Pink colouration	+
	Lead ethanoate' Test	A buff colour precipitate	+
	Iron (III) chloride($FeCl_3$)	Blue green colouration	+
	NaOH Test	Change in colour from yellow to colourless on addition of dil. HCl	+
Saponins	Frothing's test	FrothingPersist on warming	+
Steroidal Nucleus	Salkwoski's Test	Reddish-brown colour at interphase	+
			+
Tannins	Iron (III) chloride	Blue-black precipitate	+
	Lead ethanoate	White precipitate	+
Terpenoids	Lieberman Burchard's Test	Brown ring with brown interphase	+

Key**:** + = present, - =absent

4.3Results of Thin Layer Chromatography of Crude Methanol Extract

Thin layer chromatography of the crude methanol extract of *A. grantii* usingthe solvent system: n-hexane: ethyl acetate 5: 1 [HE]; chloroform: ethyl acetate 5:4 [CE] and ethyl acetate: methanol: water 40: 5: 4 [EMW]; revealed several spots(Table 4.3-4.5).

Table 4.3 TLC Profile of the Crude Methanol Extracts of the Aerial Parts of *Ampelocissus grantii* using n-Hexane: Ethyl acetate (5: 1)

Spots	R_f
1	0.90
2	0.82
3	0.75
4	0.65
5	0.48
6	0.28
7	0.22
8	0.15
9	0.12
10	0.00

Table 4.4 TLC Profileof the Crude Methanol Extracts of the Aerial Part of *Ampelocissus grantii* Using Chloroform: Ethyl acetate (5: 4)

Spots	R_f
1	0.92
2	0.83
3	0.75
4	0.63
5	0.48
6	0.37
7	0.07
8	0.00

Table 4.5 TLC Profileof the Crude Methanol Extracts of the Aerial Parts of *Ampelocissus grantii* Using Ethyl acetate: Methanol: Water (40: 5: 4)

Spots	R_f
1	0.97
2	0.95
3	0.92
4	0.83
5	0.77
6	0.69
7	0.65
8	0.49
9	0.37
10	0.23
11	0.17
12	0.00

4.4Isolation of Compounds 1, ML 1 and ML 2

4.4.1 Results of thin layer chromatographic profile of acetone soluble fraction of the methanol extract of *A. grantii*

Thin layer chromatography of the acetone soluble fraction of the methanol extract of *A. grantii* usingthe solvent system: ethyl acetate: methanol: water 100: 16.5: 13.5 revealed several spots.

4.4.2 Results of column chromatographic separation of acetone soluble fraction of the methanol extract of *A. grantii*

Fractionation of the acetone soluble fraction of the methanol extract of the aerial parts of *A. grantii* using silica gel column chromatography yielded six major fractions (A1 – 6) when eluted with n-hexane, dichloromethane, ethyl acetate and methanol (100%) summarized and presented in Table 4.6.

Repeated gel filtration over Sephadex LH-20 of fraction A3 and eluted with methanol gave 40 fractions which were reduced to 13 ($A3_{1-13}$). Sub fraction $A3_6$ gave a single spot when subjected to TLC using two solvent systems; ethyl acetate: methanol: water (100: 16.5: 13.5) and chloroform-methanol-water (3: 3: 1) to afford ML 1. It was recrystallized in methanol to give yellow amorphous powder 5 mg.

Further fractionation of A4 oversilica gel column chromatography and eluted with chloroform, chloroform -methanol mixture and methanol gradiently afforded eight subfractions ($A4_{1-8}$). The result is summarized in Table 4.7.

Sub fraction $A4_1$eluted from chloroform -methanol (90\; 10) consisting of one major and two minor spots was further purified by repeated gel filtration to afford compound 1 (4 mg).

Repeated gel filtration over Sephadex LH – 20 and eluted with methanolof $A4_4$and final purificationby preparative thin layer chromatography using ethyl chloroform - methanol-water (100: 16.5: 13.5) as solvent system was performedto afford ML 2 (4 mg).

Table 4.6 Fractions Obtained from Column Chromatography of the Acetone Fraction of the Methanol Extract of the Aerial Part of *Ampelocissus grantii*

No.	Fractions	Spots	Weight (mg)	Code
1	1-4	2	12	A1
2	5-27	5	15	A2
3	**28-38**	**3**	**140**	**A3**
4	**39-40**	**2**	**214**	**A4**
5	41-50	4	87	A5
6	51-55	2	20	A6

Table 4.7 Fractions Obtained from Column Chromatography of the A4 Fraction of the Acetone Portion of the Aerial Part of *Ampelocissus grantii*

No.	Eluting solvent	Fractions	Spots
1	$CHCl_3$ (100)	1-28	Nil
2	$CHCl_3$: MeOH (9:1)	**29-60**	**2**
3	$CHCl_3$: MeOH (8:2)	61-115	4
4	$CHCl_3$:MeOH (7:3)	**116-147**	**2**
5	$CHCl_3$: MeOH (6:4)	148-162	3
6	$CHCl_3$: MeOH (1:1)	163-175	4
6	$CHCl_3$: MeOH (4:6)	176-184	3
8	$CHCl_3$: MeOH (3:7)	185-205	3
9	$CHCl_3$: MeOH (2:8)	206-210	2
10	$CHCl_3$: MeOH (1:9)	211-220	4
11	MeOH (100%)	221-240	2

4.5.Characterization of Compound 1

4.5.1 Solubility test of compound 1

Compound 1was found to be soluble in methanol, ethyl acetate and acetone

4.5.2 Thin layer chromatography (TLC) examination of compound 1

Thin layer chromatography examination of compound 1using the solvent systems: chloroform: methanol: water 3:3:1 and ethyl acetate: methanol water 100:16.5:13.5 gave single spot with R_f value of 0.63 and 0.67 respectively.

4.5.3 Chemical test for compound 1

Chemical studies of compound 1using the standard procedure revealed the compound to be flavonoid (positive $FeCl_3$ test and Shinoda's test).

4.5.4 Spectral data of compound 1

The ^{1}H-NMR spectrum of the compound was recorded in CD_3OD on 600 MHz spectrophotometer.

4.6 Spectral Analysis of Compound 1

4.6.1Proton nuclear magnetic resonance (^{1}H–NMR) of compound 1

The^{1}H – NMR spectra data of compound 1 displayed six signals at δ 6.48, 6.50, 6.80, 7.60, 7.80 and 7.90 ppm(Fig. 4.1).

4.6.2 ^{1}H – ^{1}H COSY spectral analysis of compound 1

The ^{1}H- ^{1}H COSY (Fig. 4.2) showed correlations between the signals at δ7. 90 and 6.40, 6.45 and 6.85, 7.90 and 7.65, 7.80 and 7.65.

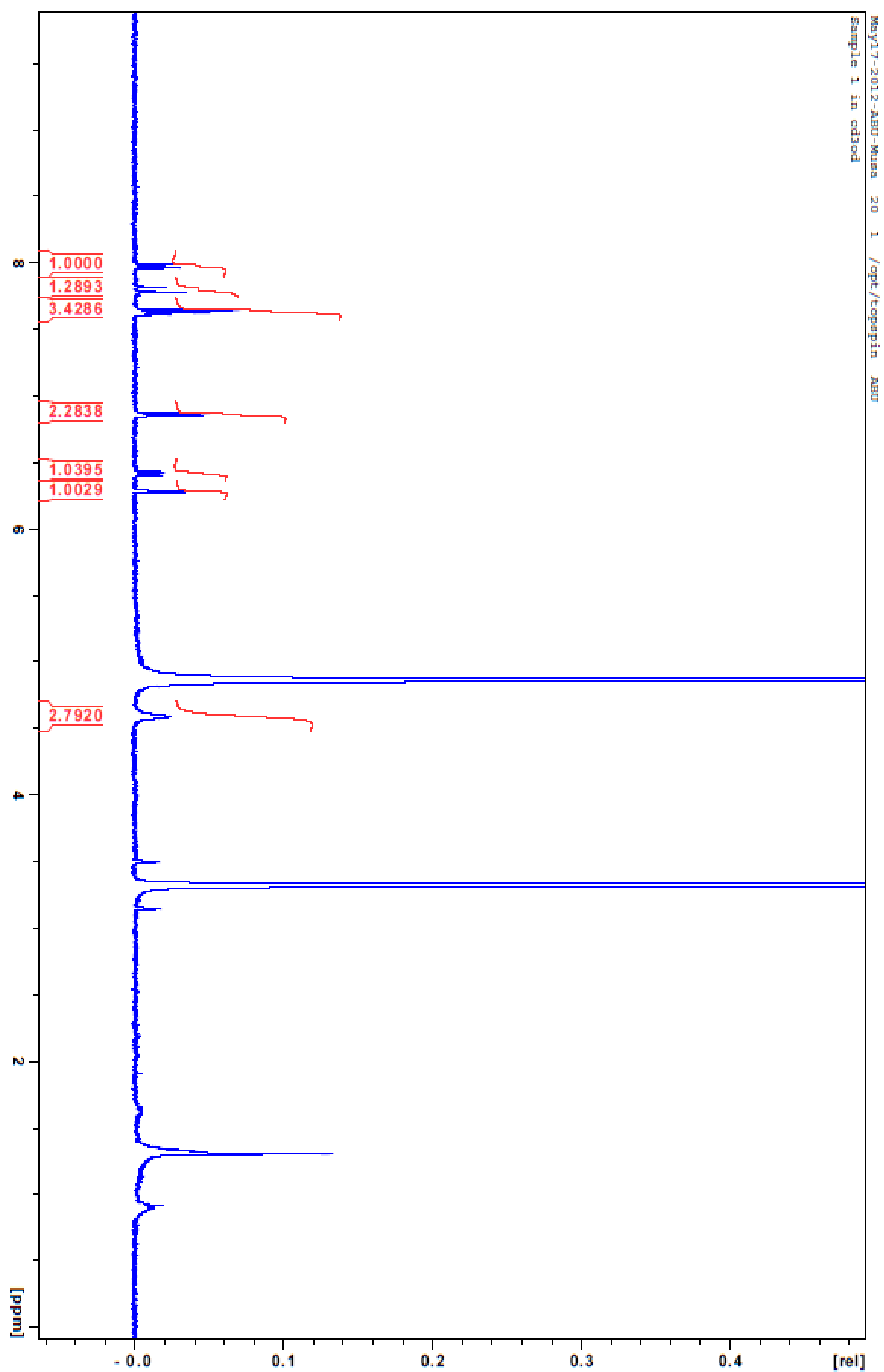


Figure4.1: ^{1}H-NMR spectrum of compound 1

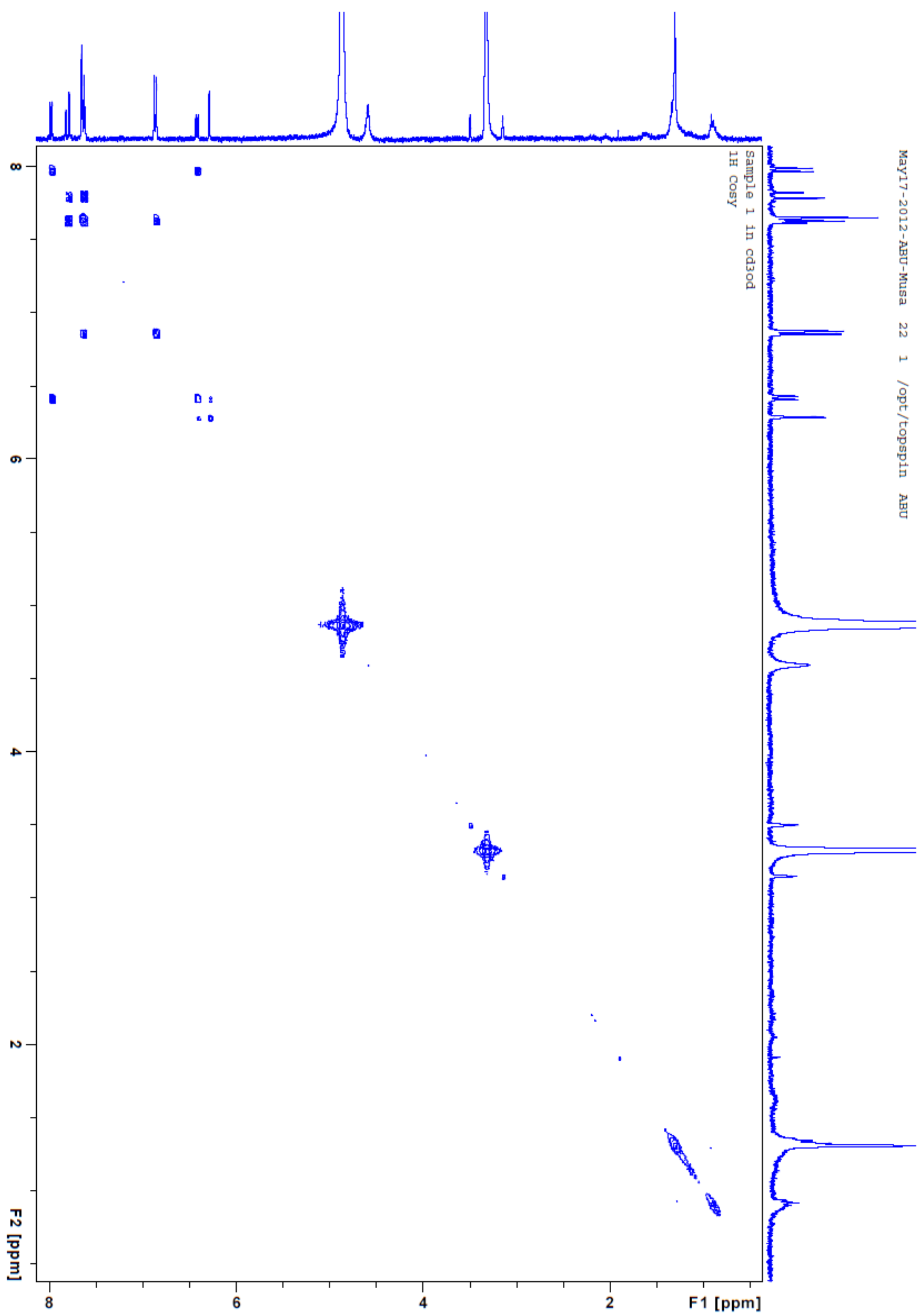


Figure4.2: ^{1}H-^{1}H COSY of compound 1

Table 4.8 ^{1}H-NMR and ^{1}H-^{1}H Correlation NMR (COSY) Assignments of Compound 1

Position	^{1}H NMR	^{1}H-^{1}Hcorrelation NMR (COSY)
	Chemical shift (δ ppm)	Correlation (chemical shift)
3	6.40 (s)	2’ (7.90)
6	6.45 (d)	8 (6.85)
8	6.85 (d)	6 (6.45)
2′	7.90 (d)	5’ (7.6)
5′	7.65 (d)	6’ (7.80)
6′	7.80 (dd)	5’ (7.65)

4.7 Characterization of ML 1

4.7.1 Solubility test of ML 1

ML 1was found to be soluble in methanol only at room temperature.

4.7.2 Thin layer chromatography (TLC) examination of ML 1

Thin layer chromatography examination of ML1using the solvent systems: chloroform: methanol: water 3:3:1 and ethyl acetate: methanol water 100:16.5:13.5 gave single spot with R_f value of 0.43

4.7.3 Chemical test for ML 1

Chemical studies of ML 1using the standard procedure revealed the compound to be flavonoid (positive $FeCl_3$ test and Shinoda's test).

4.7.4 Spectral data of ML 1

The ^{1}H-NMR spectrum of the compound was recorded in CD_3OD on 600 MHz spectrophotometer.

4.8 Spectral Analysis of ML 1

4.8.1 Proton nuclear magnetic resonance (^{1}H–NMR) of ML 1

The ^{1}H-NMR spectrum ML 1(Figure 4.3 – 4.5; Table 4.9) showed the presence of three ABX protons signals at δ 7.97, 1H, d, J = 2.22 H-2′; δ 6.92, 1H, d, J = 8.4 Hz, H-5′; δ 7.67, 1H, dd, J= 2.10; 6.0 Hz, H-6′, two protons signals at δ 6.45, 1H, d, J = 2.20, H-8 and δ 6.22, 1H, d, J=2.04 Hz, H- 6), and two anomeric protons representing two sugar moieties at δ 5.14 (1H, d, J = 6.8 Hz) and 4.12 (1H, d, J = 6.8 Hz).Signals between 3.00 – 3.84 (10H, multiplets) assigned to sugar protons.The ^{1}H-NMR spectrum also showed signal at δ 3.93 (3H, *s*), assigned as methoxy group.

4.8.2 Carbon – 13 nuclear magnetic resonance (^{13}C–NMR) of ML1

^{13}C-NMR spectrum (Figure 4.6; Table 4.9) showed signals for one OCH_3, two CH_2 (all aliphatic), fourteen CH (five aromatic, nine aliphatic) and ten quaternary carbons (one carbonyl, five O-bearing and four aliphatic)

4.8.3 DEPT ^{13}C-NMR spectrum of ML 1

The DEPT spectrum of ML 1 (Figure 4.7) clearly displayed 28 carbon signals (2 x CH_2, 15 x CH and 9 C) of which 12 could be assigned to two sugar moieties, one to methoxy group and the remaining 15 carbon signals were assigned to a flavonoid aglycone.

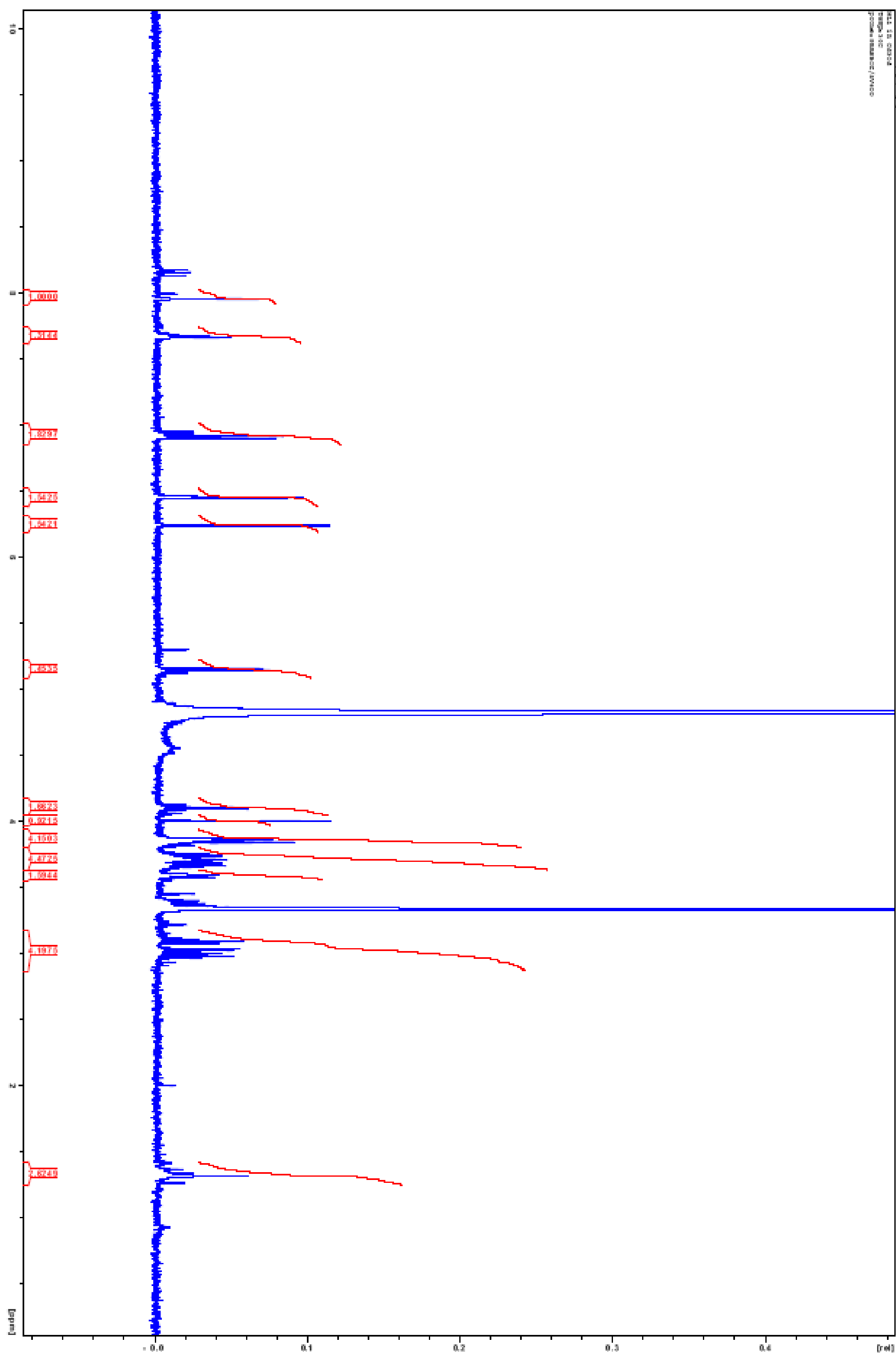


Figure 4.3: ^{1}H-NMR spectrum of ML 1

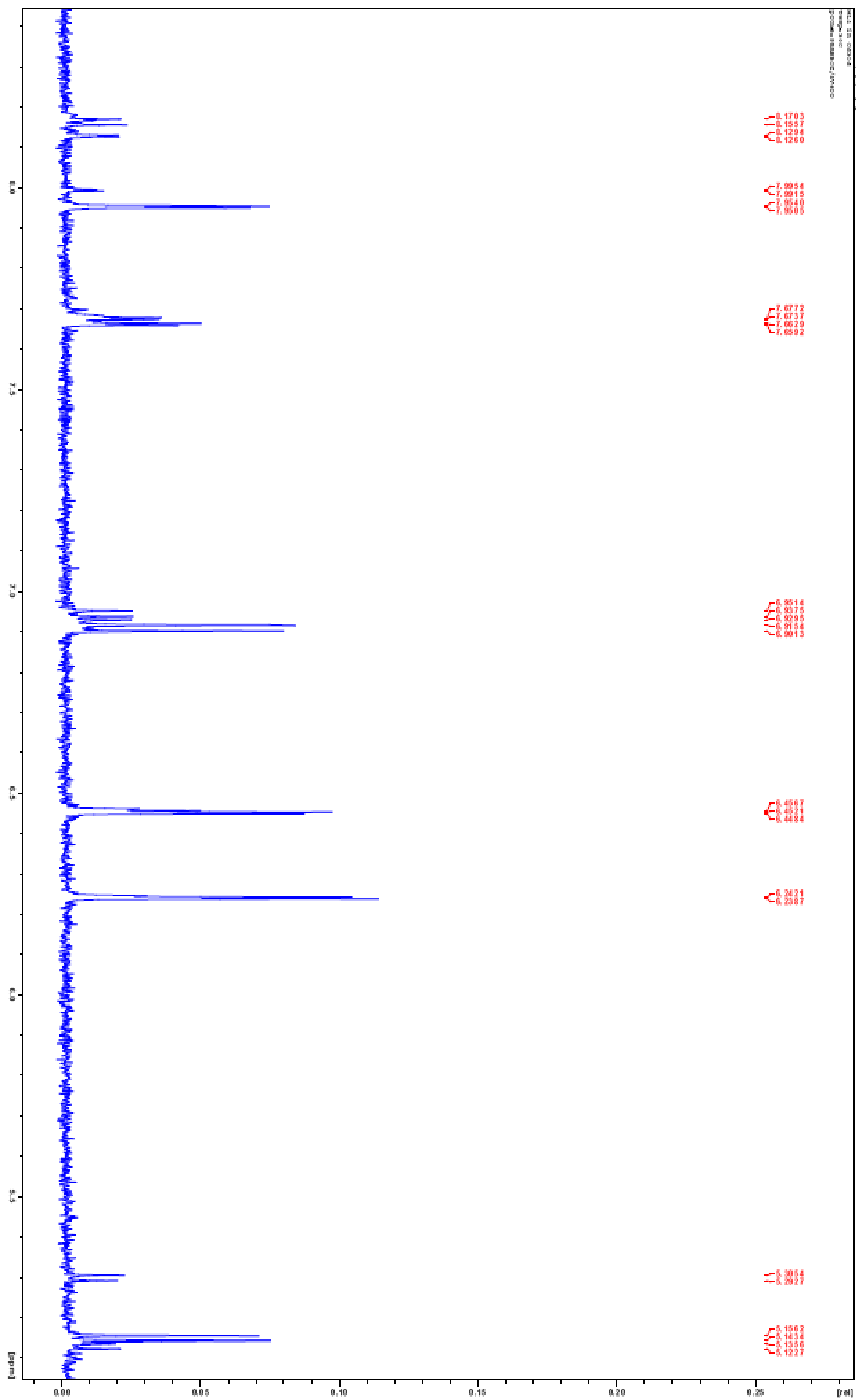


Figure 4.4: ^{1}H-NMR spectrum of ML 1 (Expanded aromatic region)

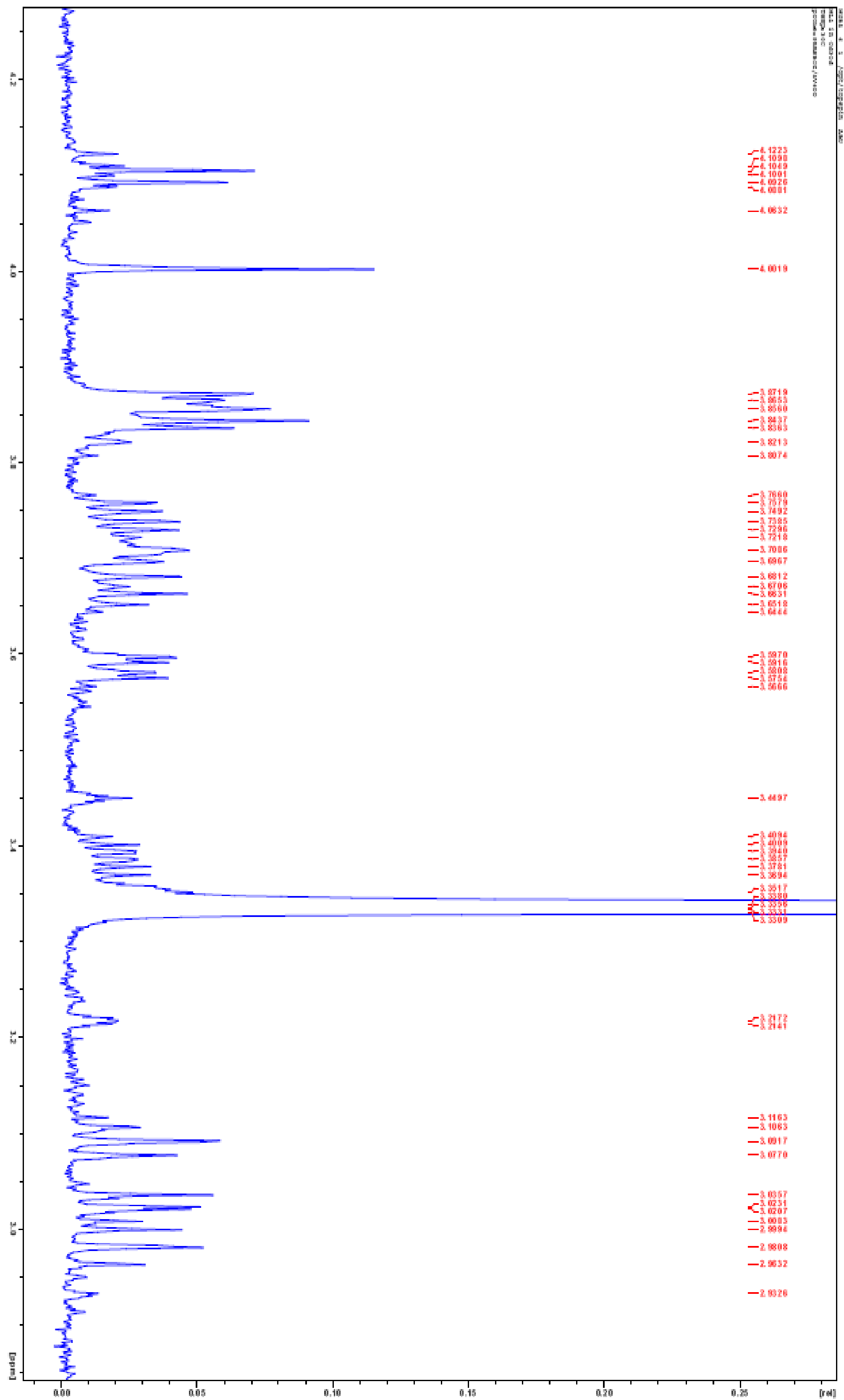


Figure 4.5: ^{1}H-NMR spectrum of ML 1 (Expanded sugar region)

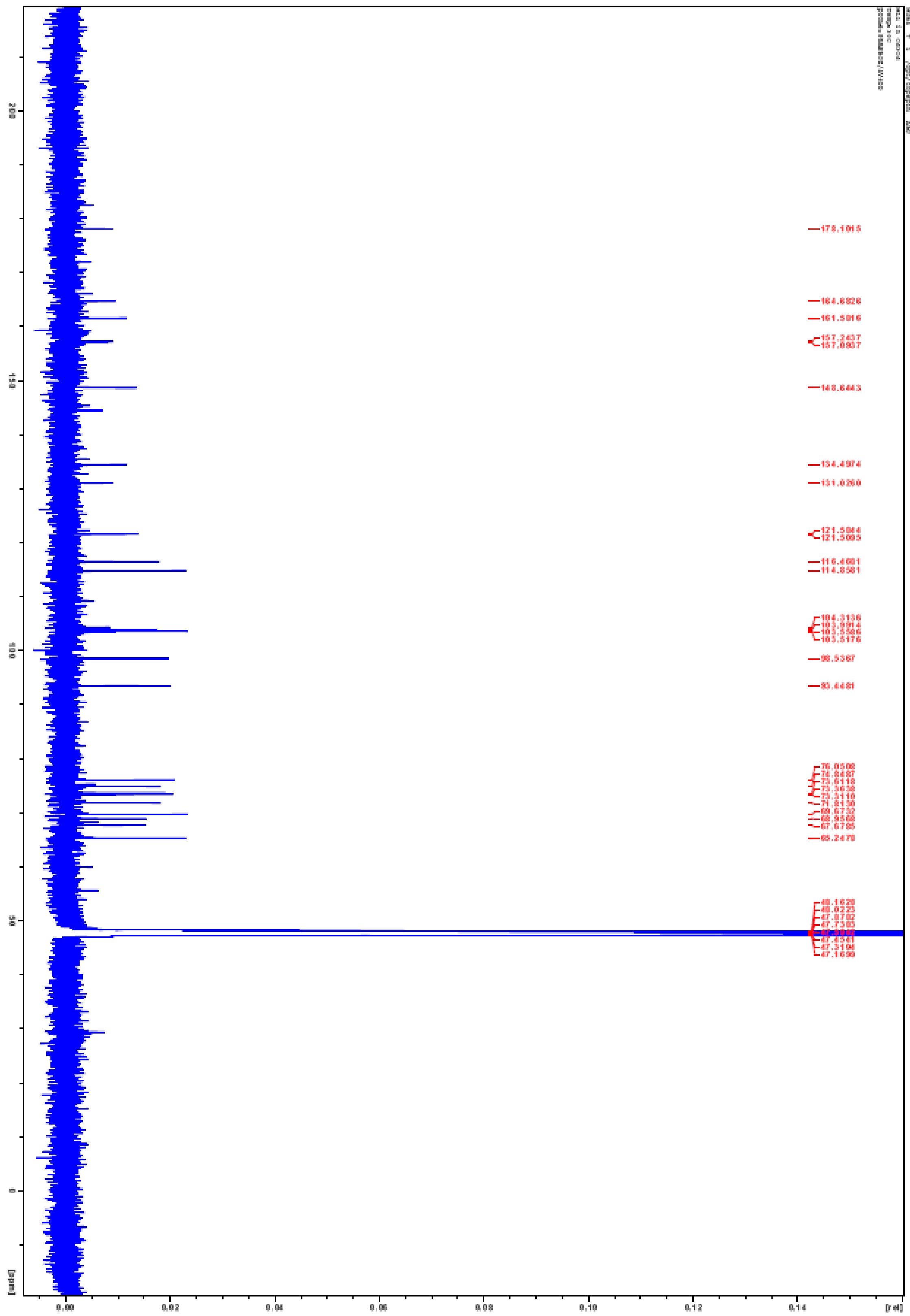


Figure 4.6: Carbon-13 Nuclear Magnetic Resonance (NMR) spectrum of ML 1

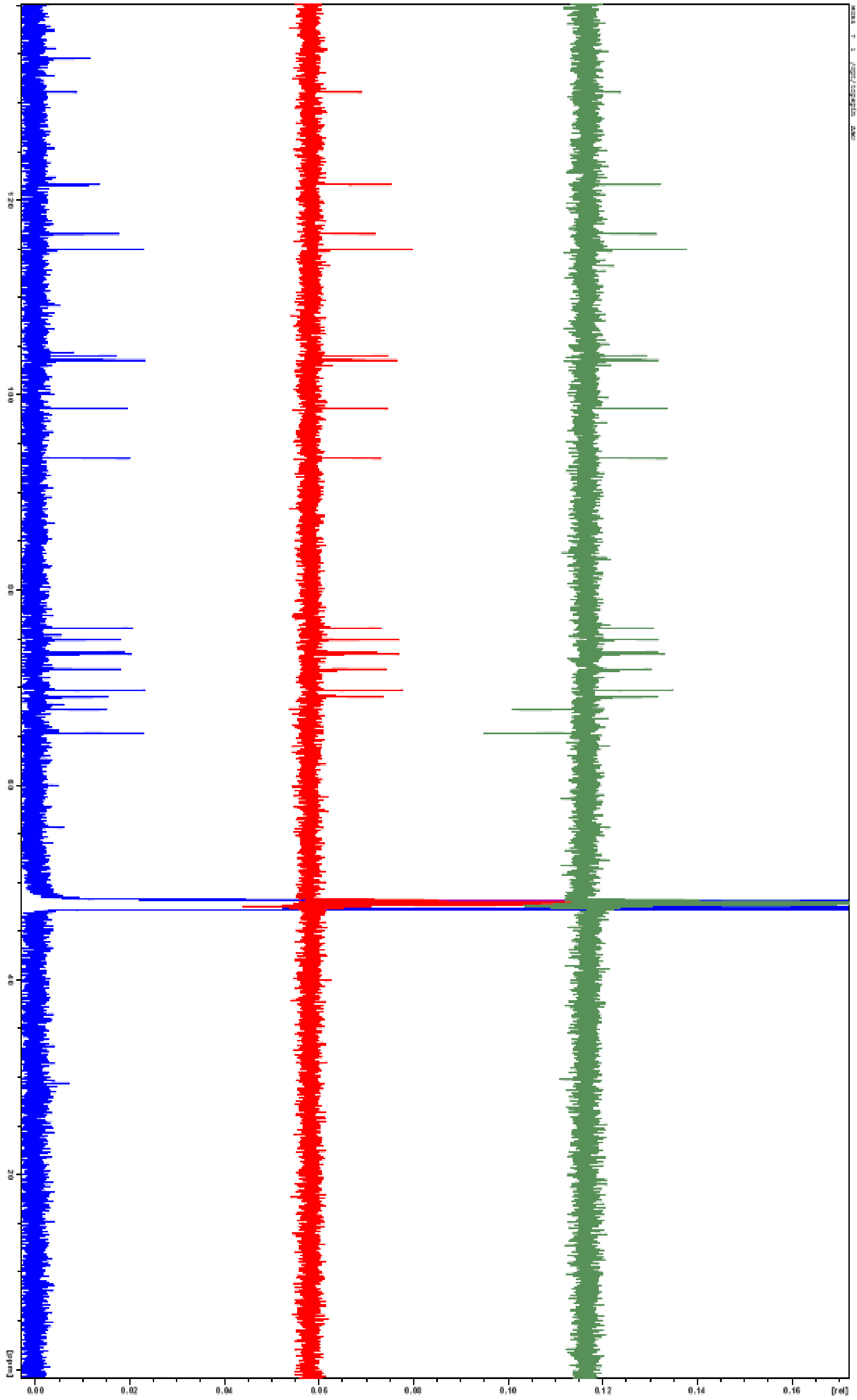


Figure 4.7: DEPT Carbon-13 Nuclear Magnetic Resonance (NMR) Spectrum of ML 1

4.8.4 ^{1}H – ^{1}H COSY spectral analysis of ML 1

The COSY spectrum of ML1 (Figure 4.8; Table 4.12) showing the proton-proton (^{1}H-^{1}H) correlations situated on the same environment and exhibiting the same nature of substitution on the aglycone and the sugar moieties.

4.8.5 HSQC NMR spectrum of ML 1

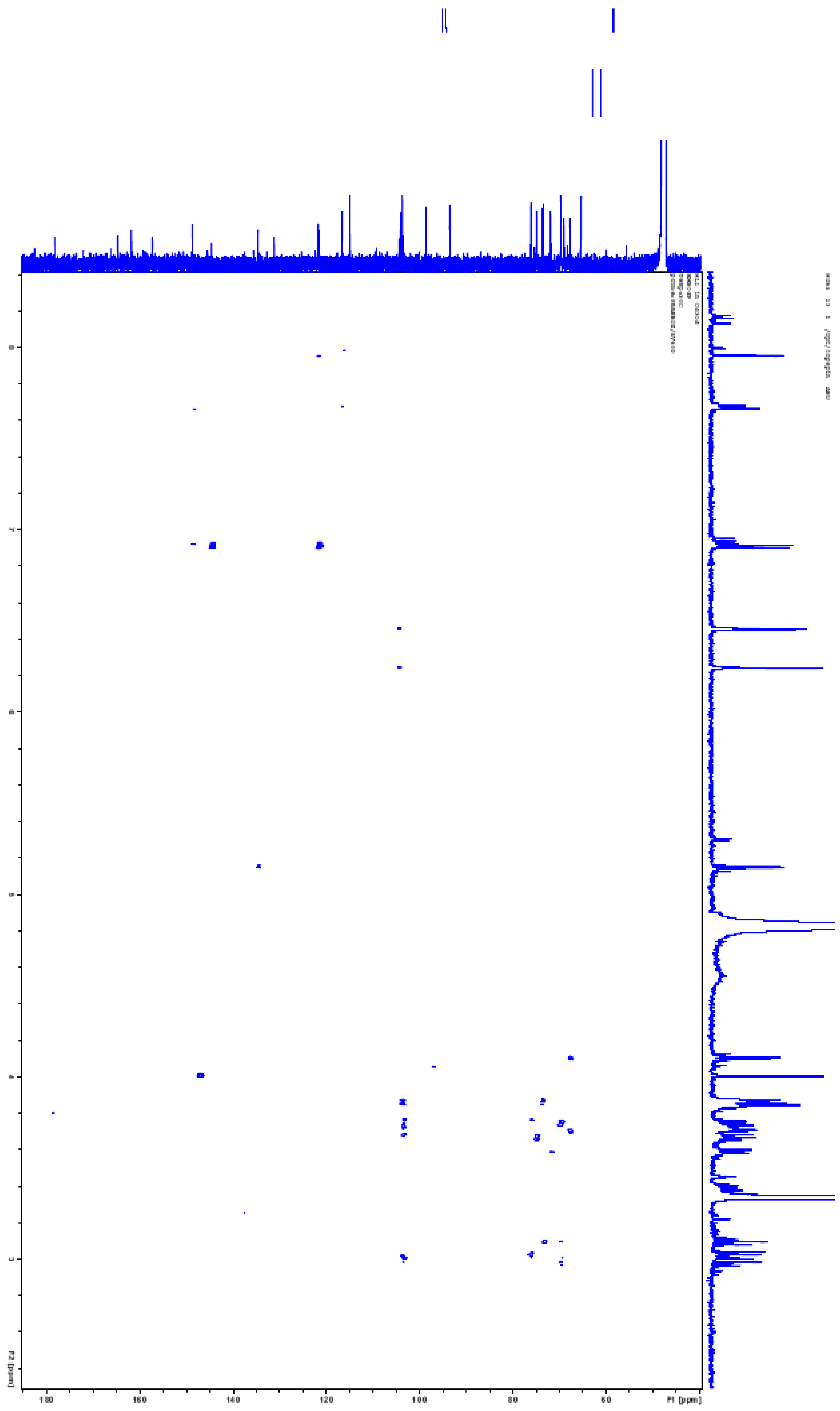
Figure 4.9(Table 4.10) shows the HSQC spectrum of ML 1 showing the assignment of protons to their respective carbons.

4.8.6 HMBC NMR spectrum of ML 1

The HMBC analysis confirmed the linkage of the sugar to the aglycone, linkages between sugars and other linkages in the compound. The HMBC NMR spectrum of ML 1 (Figure 4.10; Table 4.11) indicated three major cross peaks. The cross peak between C-3 at δ 134.49 ppm of the aglycone and H-1″at δ 5.14 ppm of sugar moiety, cross peak between the two sugars C-6″at 67.34 ppm and H-1‴ at 4.12 ppm and the cross peaks between C-3′ at δ 148.64 ppm of the aglyconeand the methoxy group. The spectrum also showed cross peaks between C-3′ at δ 148.64, C-4′ at δ 144.40 and C-2′ at δ 121.50 at H-5′ at 6.92 ppm, C-10 at δ 104.31 and H-6 and H-8 at 6.24 and 6.45 ppm respectively, C-3′ at 148.64 at H-7.67 ppm, and C-121.58 at 121.58 at H-7.97. C-6″ at 67.68 ppm at H-3.75.

4.8.7 ^{1}H – ^{1}H NEOSY spectral analysis of ML 1

Figure 4.11; Table 4.12Showed in Neosy spectral analysis of ML1



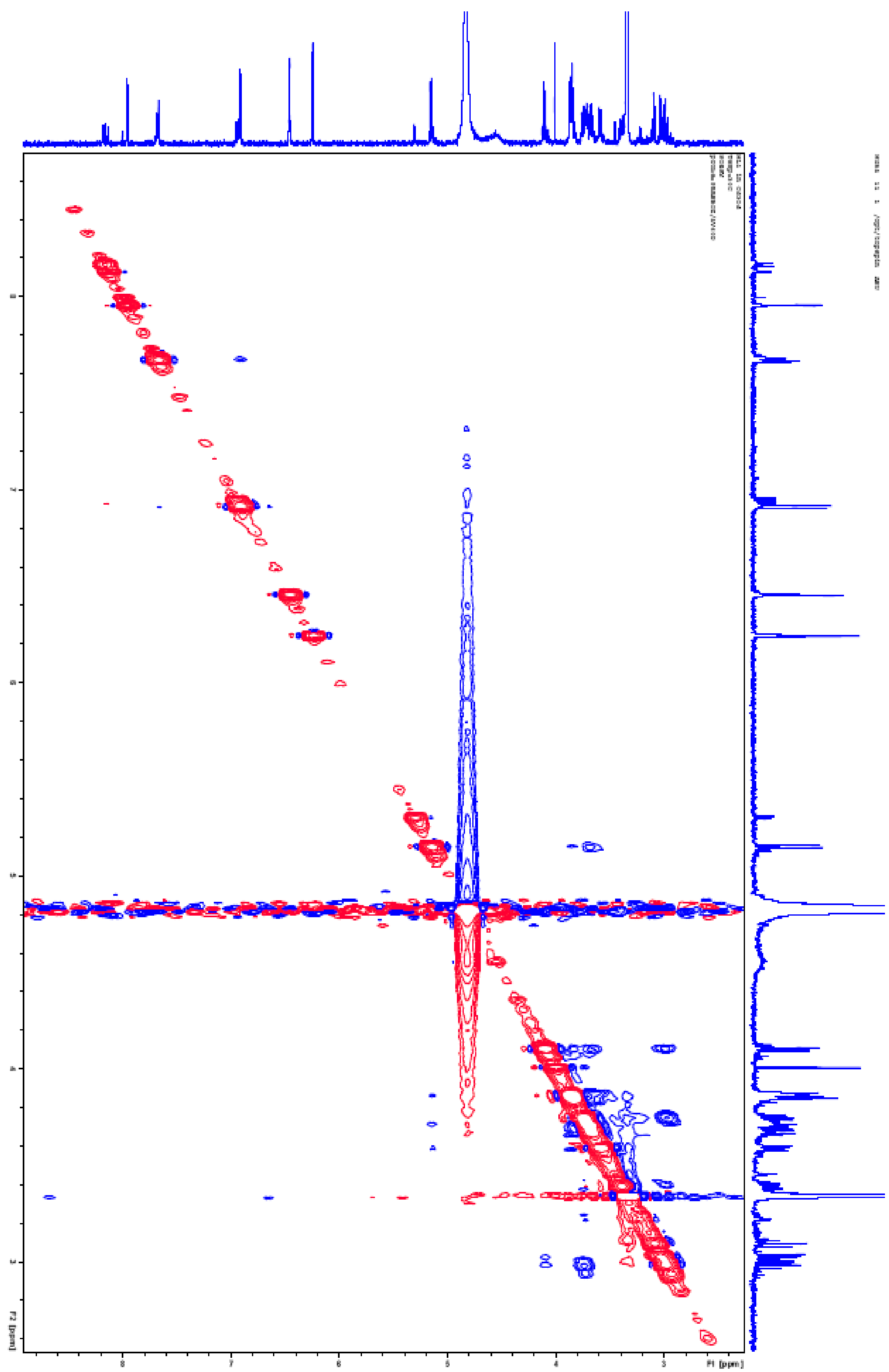


Table 4.9 ^{1}H-NMR and ^{13}C-NMR Assignments of Compound ML1 in CD_3OD

Position	Chemical shift (δ ppm)		DEPT
	^{1}H NMR	^{13}C NMR	
2		157.24	C
3		134.49	C
4		178.10	C
5		161.58	C
6	6.24 (d, J = 2.04)	98.53	CH
7		164.68	C
8	6.45 (d, J = 2.22)	93.44	CH
9		157.09	C
10		104.31	C
1′		121.58	C
2′	7.97 (d, J = 2.22)	116.46	CH
3′		144.40	C
4′		148.64	C
5′	6.92 (d, J = 8.42)	114.85	CH
6′	7.67 (dd, 2.10, 6.0)	121.50	CH
3′-OCH_3	4.00 (s)	55.50	C
Glucose			
1″	5.14 (d, J = 7.71)	103.55	CH
2″	3.00-3.84		CH
3″	3.00-3.84		CH
4″	3.00-3.84		CH
5″	3.00-3.84		CH
6″	3.84a; 3.65b	67.68	CH_2
Glucose			
1‴	4.15 (J = 6.68)	103.50	CH
2‴	3.00-3.84	76.05	CH
3‴	3.00-3.84		CH
4‴	3.00-3.84		CH
5‴	3.00-3.84		CH
6‴	3.75a; 3.00b	65.25	CH_2

Table 4.10 HSQC Correlation in NMR Spectra of ML1

Position	Chemical shift of correlated carbon
6	98.53
8	93.44
2′	116.46
5′	114.85
6′	121.50
3′-OCH_3	55.50
1″	103.99
2″	
3″	
4″	
5″	
6″	67.68
1‴	103.55
2‴	76.05
3‴	
4‴	
5‴	
6‴	65.25

Table4.11 HMBC Correlation in NMR Spectra of ML1

Proton position (chemical shift)	Carbon position (chemical shift)
6 (6.24)	10 (104.31)
8 (6.45)	10 (104.31)
2′ (7.97)	1’ (121.58); 6’ (121.50)
5′ (6.92)	3’ (144.40); 4’(148.64); 6’ (121.50)
6′ (7.67)	4’ (148.64)
1″ (5.14)	3 (134.49)
2″	-
3″	-
4″	-
5″	-
6″a(3.84)	-
6″b (3.65)	-
1‴ (4.12)	6’’ (67.68)
2‴	-
3‴	-
4‴	-
5‴	-
6‴ (3.75)	-
6‴ (3.00)	-
3’-OCH_3	3’ (148.64)

Table 4.12Proton-proton Correlations in NMR Spectrum of ML 1Using COSY and NOESY Techniques and Their Assignments.

Position (chemical shift)	Correlation (chemical shift)	
	COSY	NOESY
5′ (6.92)	6’ (7.67)	
6′ (7.67)	6’ (7.67)	
1″ (5.14)	6’’ (3.84)	
2″	-	
3″	-	
4″	-	
5″	-	
6″a(3.84)	-	
6″b (3.65)	-	
1‴ (4.12)	2’’’ (3.15)	
2‴	-	
3‴	-	
4‴	-	
5‴	-	
6‴ (3.75)	-	
6‴ (3.00)	-	
3′-OCH_3	-	

4.9Characterization of ML2

4.9.1 Solubility test of ML 2

ML 2 was found to be soluble in methanol only at room temperature.

4.9.2 Thin layer chromatography (TLC) examination of ML 2

Thin layer chromatography examination of ML 2 using the solvent system: ethyl acetate: methanol water 15:3:2 gave single spot with R_f value of 0.45.

4.9.3 Chemical test for ML 2

Chemical studies of ML 2 using the standard procedure revealed the compound to be flavonoid (positive $FeCl_3$ test and Shinoda's test).

4.9.4 Spectral data of ML 2

The ^{1}H-NMR spectrum of the compound was recorded in CD_3OD on 600 MHz spectrophotometer.

4.10 Spectral Analysis of ML 2

4.10.1 Proton nuclear magnetic resonance (^{1}H–NMR) data of ML 2

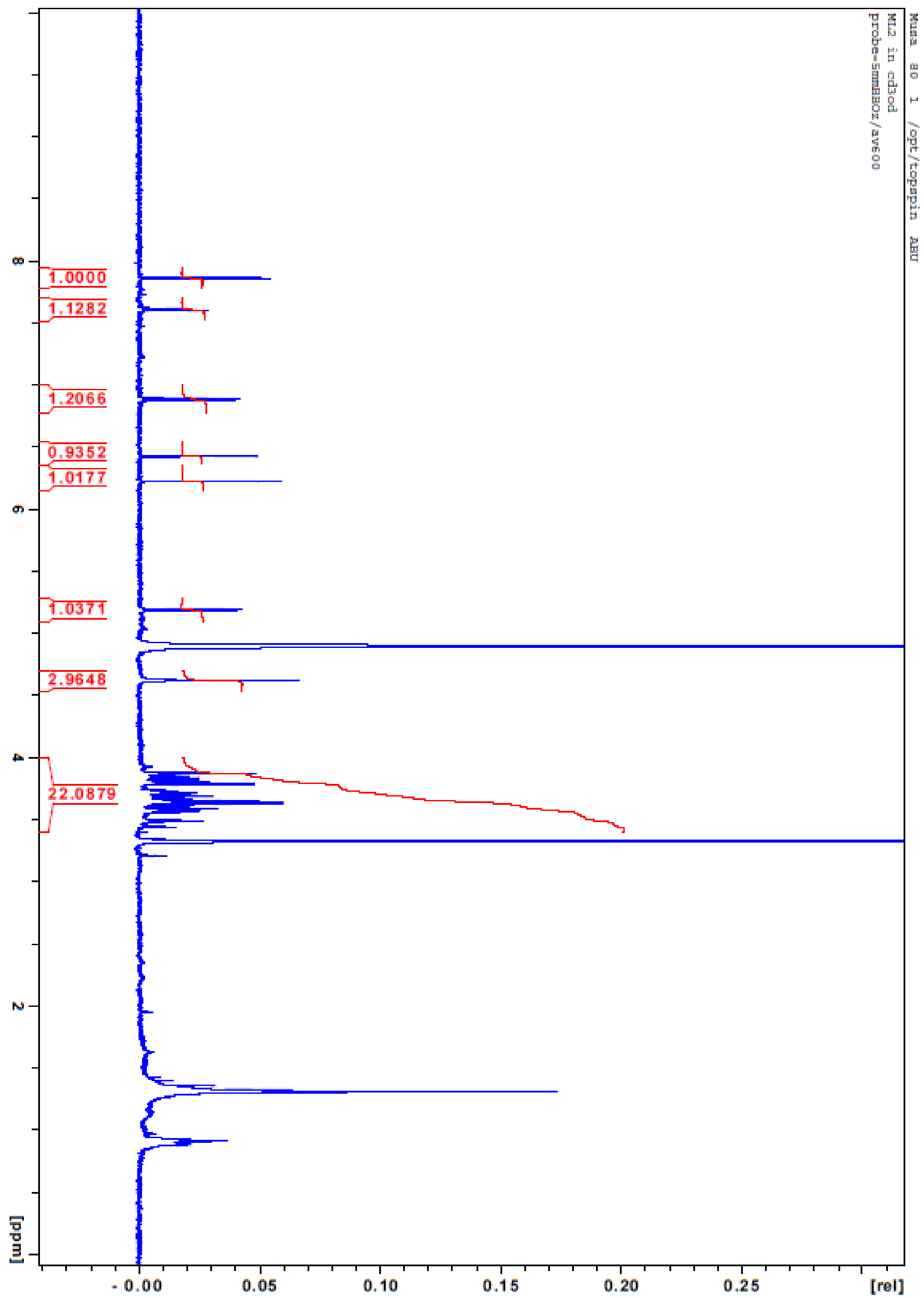
The ^{1}H-NMR of ML 2 (Figure 4.12 -4.14; Table 4.13)showed two signals at δ 6.20 (1H, d, J = 2.16) and δ 6.40(1H, d, J = 1.98) protons assigned to H – 6 and H – 8. Signals at δ 6.89 (d, J = 8.82 Hz), δ 7.60 (dd, J = 2.04, 8.4Hz) and δ 7.88 (1H, d, J = 2.22) are assigned to H – 5′, H – 6′ and H – 2′ protons respectively of ring B. The low field protons signals at δ 5.20 (1H, d, J = 8.1) and a singlet at δ 4.61are due to anomeric protons of 2 sugars units. Signals between 3.40 – 3.88 (10H, multiplets) assigned to sugar protons.

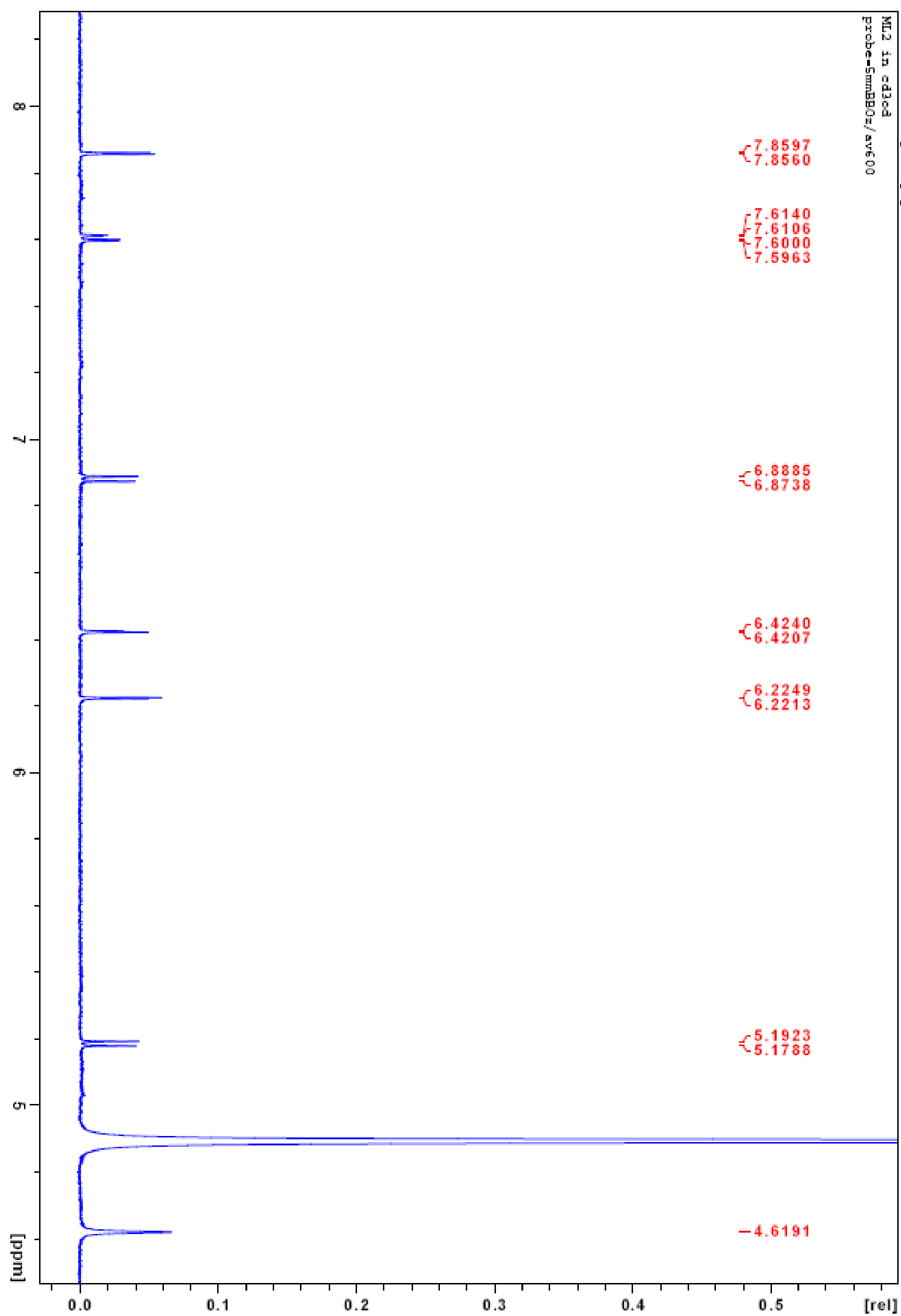
4.10.2 Carbon – 13 nuclear magnetic resonance (^{13}C–NMR) data of ML 2

The ^{13}C-NMR spectrum of ML 2 presented in Figure 4.15 indicated the presence of ten carbon signals between δ 60 and δ 76, namely δ 75.8, 73.7, 72.3, 71.8, 71.6, 69.5, 68.6, 63.4, 62.7, 60.5 as well as δ 103.9 (anomeric carbon signal of glucose). (Table 4.13).

4.10.3 DEPT ^{13}C-NMR spectral data of ML 2

Figure 4.16 shows the DEPT spectrum of **ML 2.** The spectrumdisplayed 27 carbon signals which includefive CH-groups of the aglycone, Seven CH-groups and three CH_2-groups signals among the sugar carbons and the remaining ten quartnery carbon signals are for the aglycone.


Musa 80 1 /opt/topspin ABU
ML2 in cd3od
probe-smmBBOz/av600
1.0000
1.1282
1.2066
0.9352
1.0177
1.0371
2.9648
22.0879
8
6
4
2
[ppm]
- 0.00
0.05
0.10
0.15
0.20
0.25
[rel]


Musa 90 1 /opt/topspin ABU
ML2 in cd3od
probe=5mmBBOz/av600
7.8597
7.8560
7.6140
7.6106
7.6000
7.5963
6.8885
6.8738
6.4240
6.4207
6.2249
6.2213
5.1923
5.1788
4.6191
[ppm]
[rel]

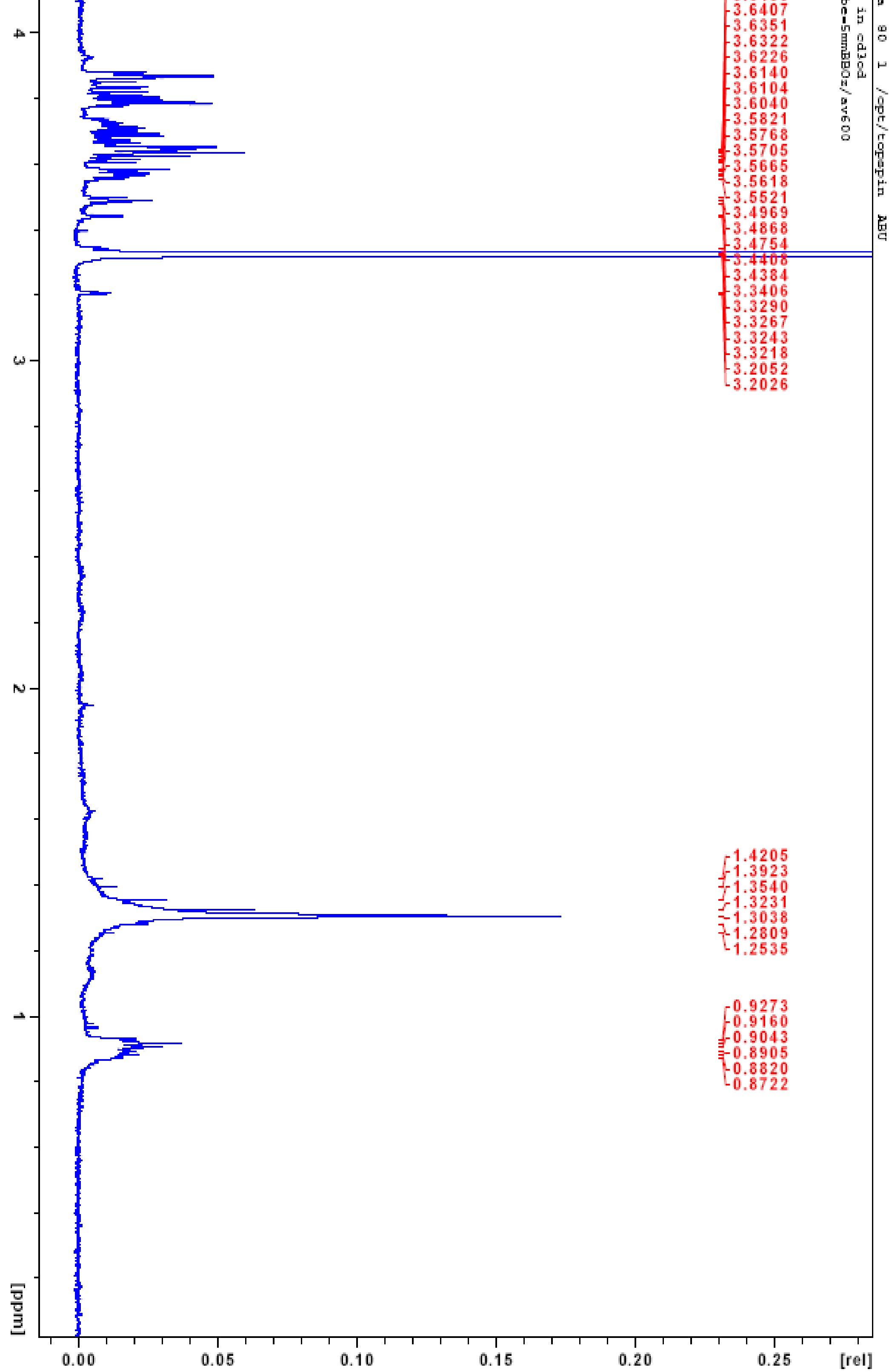

3.6407
3.6351
3.6322
3.6226
3.6140
3.6104
3.6040
3.5821
3.5768
3.5705
3.5665
3.5618
3.5521
3.4969
3.4868
3.4754
3.4408
3.4384
3.3406
3.3290
3.3267
3.3243
3.3218
3.2052
3.2026
1.4205
1.3923
1.3540
1.3231
1.3038
1.2809
1.2535
0.9273
0.9160
0.9043
0.8905
0.8820
0.8722
4
3
2
1
[ppm]
0.00
0.05
0.10
0.15
0.20
0.25
[rel]

Figure 4.14 ^{1}H-NMR spectrum of ML 2 (Expanded sugar region)

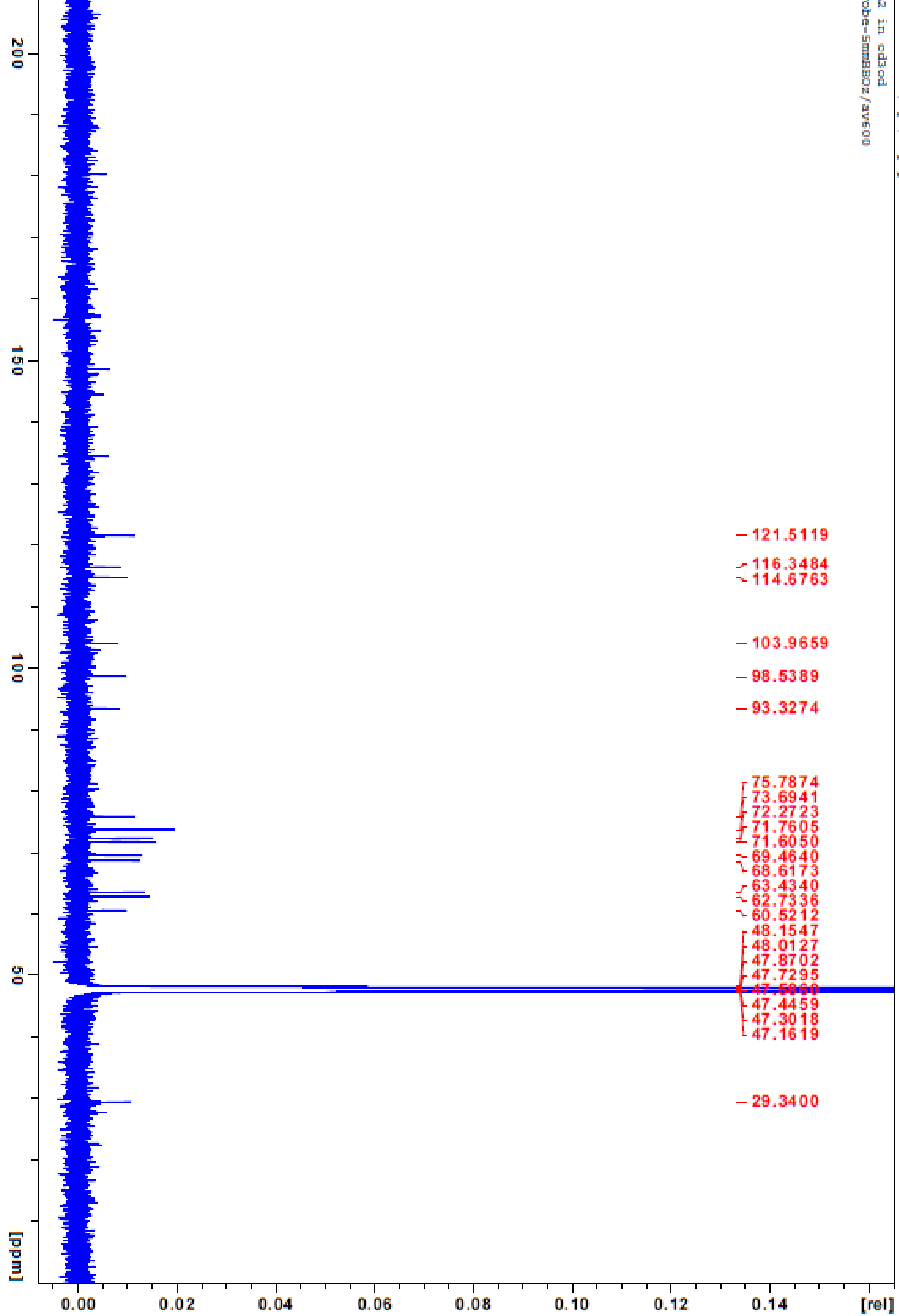

121.5119
116.3484
114.6763
103.9659
98.5389
93.3274
75.7874
73.6941
72.2723
71.7605
71.6050
69.4640
68.6173
63.4340
62.7336
60.5212
48.1547
48.0127
47.8702
47.7295
47.4459
47.3018
47.1619
29.3400
[ppm]
[rel]

Figure4.15: ^{1}HN-MR spectrum of ML 2 (Expanded sugar region)

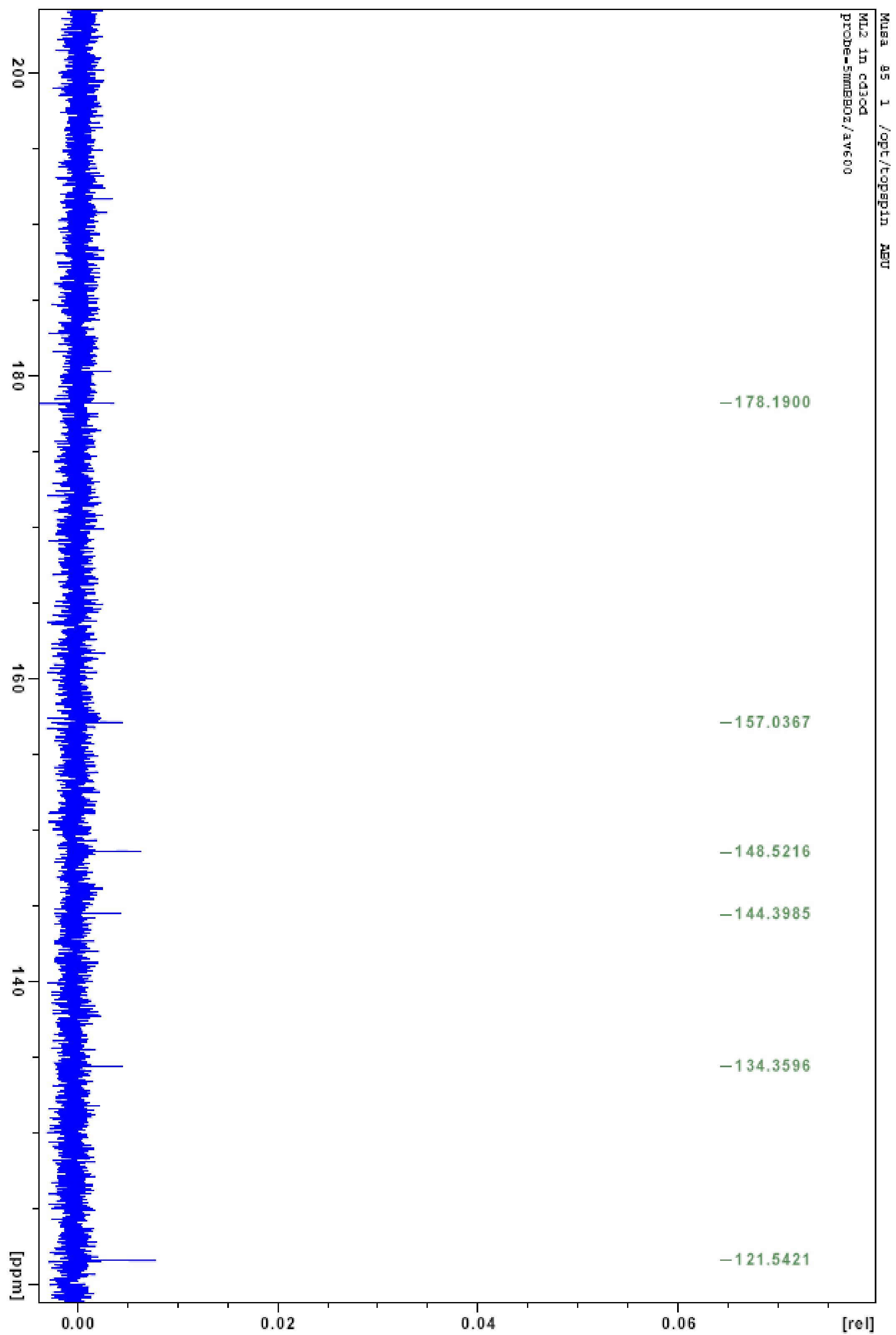

Musa 85 1 /opt/topspin AEU
ML2 in cd3od
probe-5mmBBOz/av600
178.1900
157.0367
148.5216
144.3985
134.3596
121.5421
200
180
160
140
[ppm]
0.00
0.02
0.04
0.06
[rel]

Figure 4.15aCarbon-13Nuclear Magnetic Resonance (NMR) spectrum of ML 2

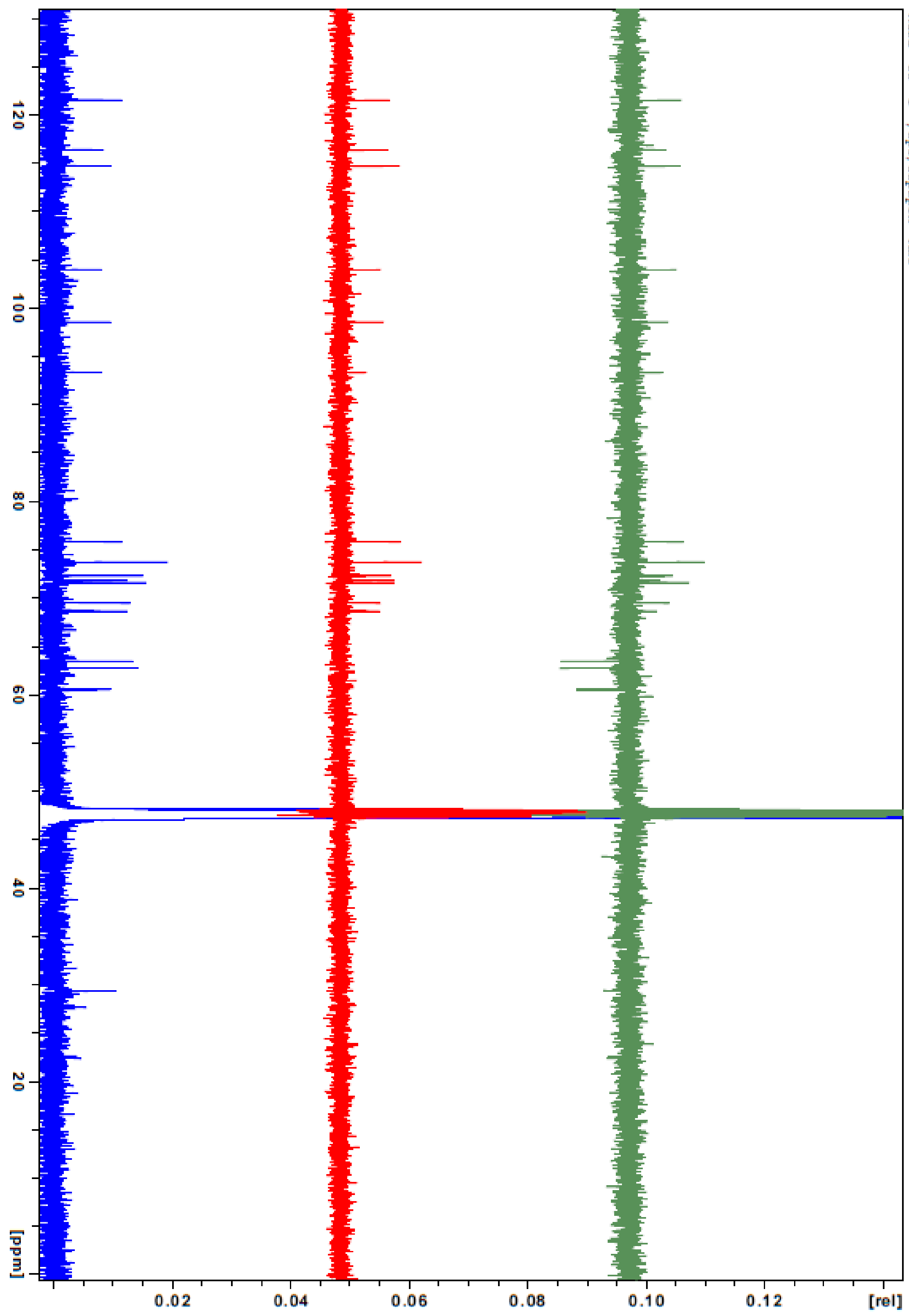

120
100
80
60
40
20
[ppm]
0.02
0.04
0.06
0.08
0.10
0.12
[rel]

Figure 4.15bCarbon-13Nuclear Magnetic Resonance (NMR) spectrum of ML 2

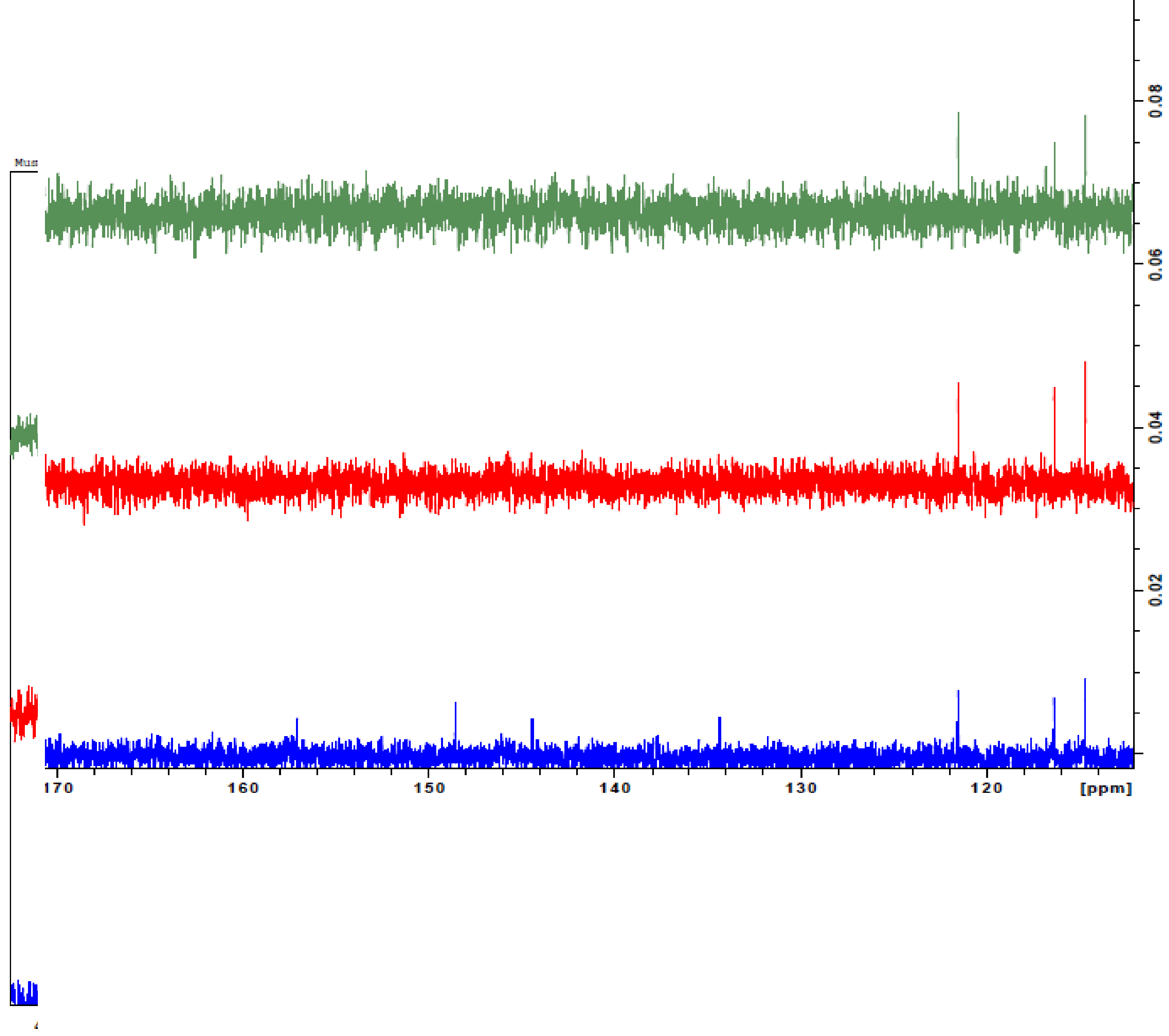

170
160
150
140
130
120
[ppm]
0.02
0.04
0.06
0.08

Figure 4.16bDEPT Carbon-13Nuclear Magnetic Resonance (NMR) Spectrum of ML 2

4.10.4 ^{1}H-^{1}H COSY Spectrum of ML 2

Figure 4.17 shows the spectrum of ML 2 and exhibited the ^{1}H- ^{1}H COSY showed correlation between the signals at δ7. 60 and 6.85 this means that the protons at 7.60 and 6.85 are located on adjacent carbons. There is also correlation between signals at δ 5.20 (H – 1″) and 3.88 (H – 2″) of sugar moiety suggesting that the signal at 5.20 belong to sugar molecule (Table 4.16).

4.10.5 HSQC NMR spectral data of ML 2

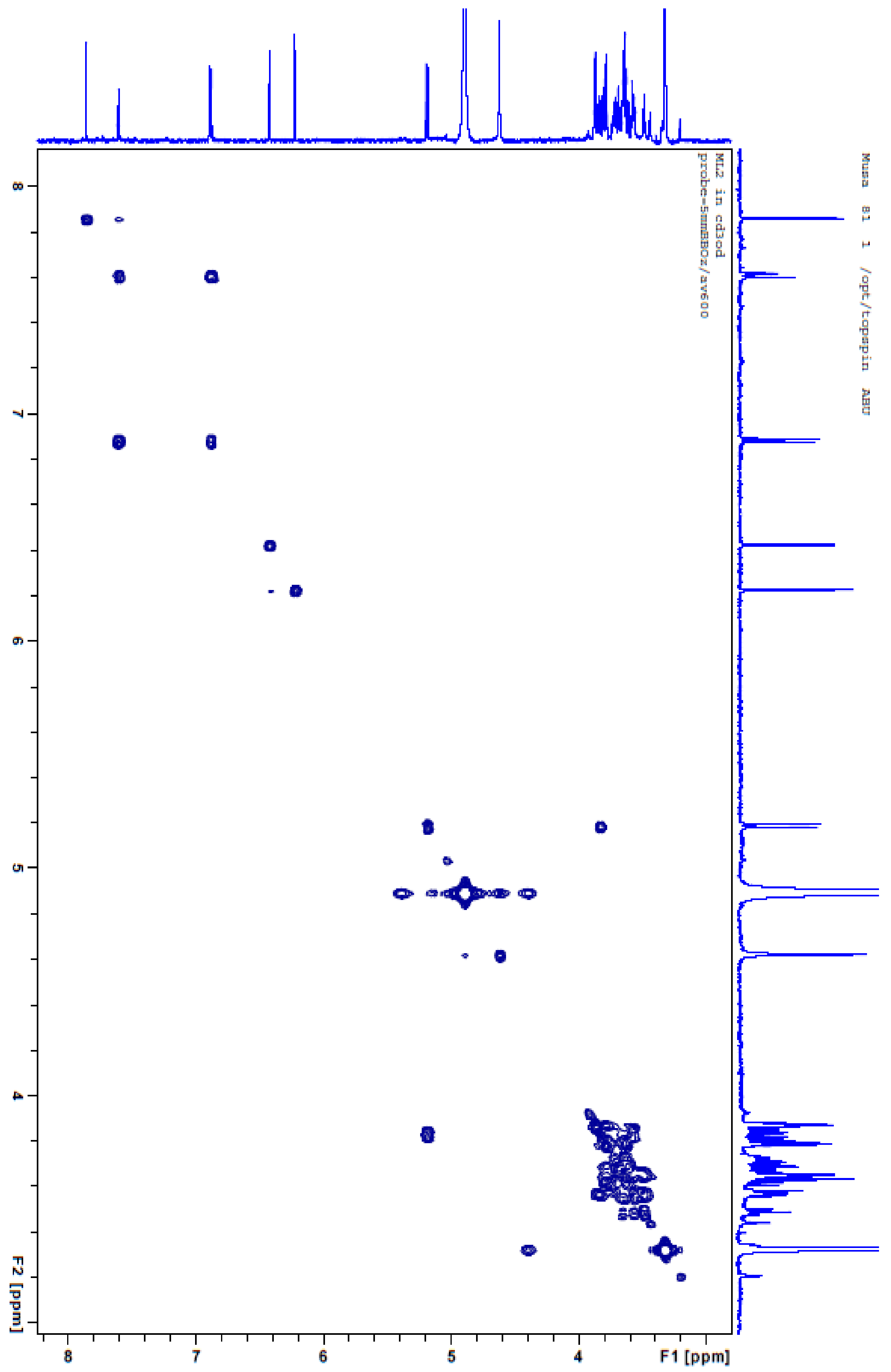
Figure 4.18 shows the HSQC spectrum of ML 1 showing the assignment of protons to their respective carbons(Table 4.14).

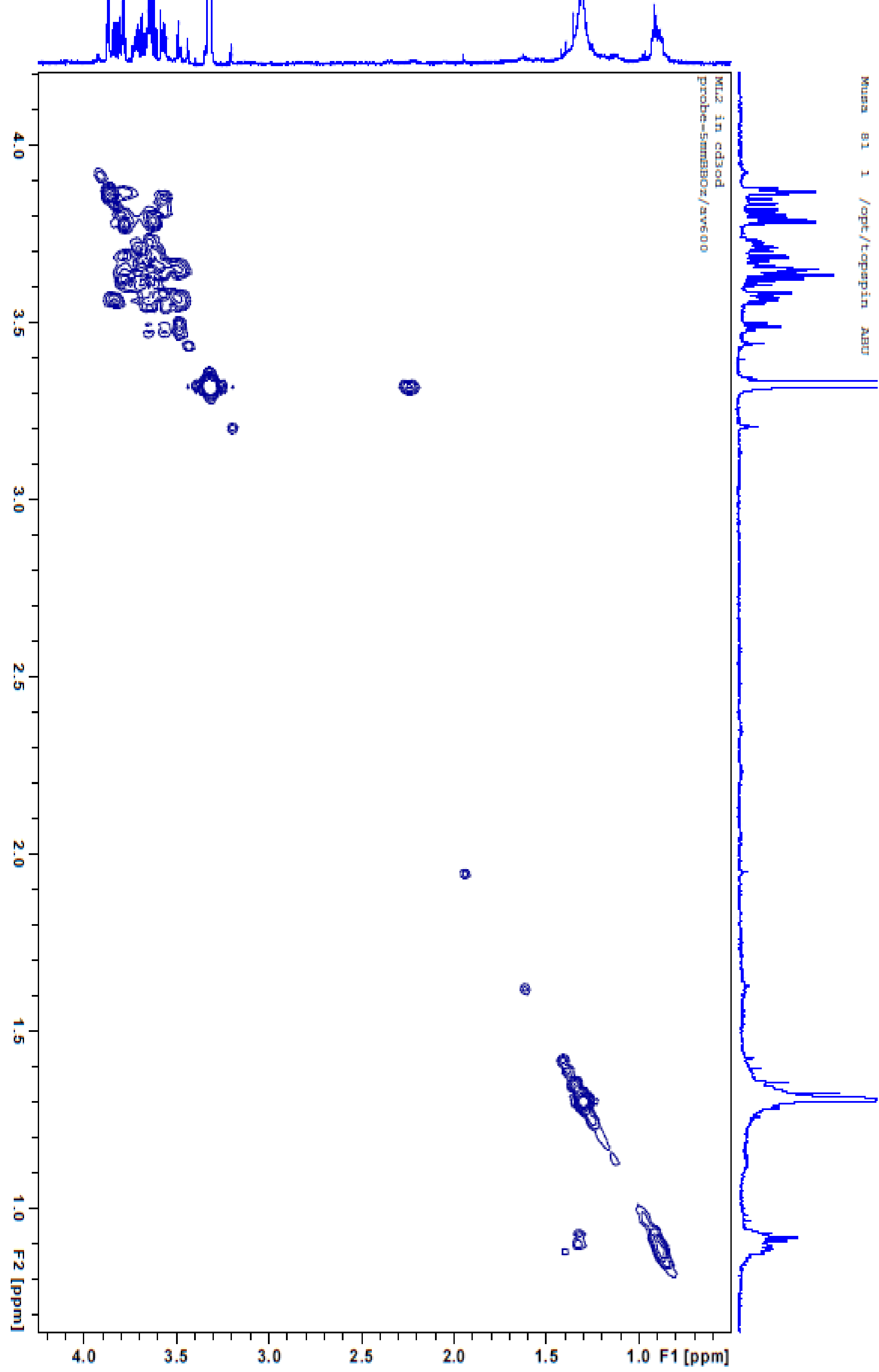
4.10.6 HMBC spectral data of ML 2

The HMBC NMR spectrum of ML 2 (Figure 4.19; Table 4.15) indicated two major cross peaks. The cross peak between C-3 at δ 134.4 ppm of the aglycone and H-1″at δ 5.20 ppm of sugar moiety and cross peak between the two sugars C-6″at 63.4 ppm and H-1‴ at 4.61 ppm.

4.10.7 ^{1}H – ^{1}H NEOSY spectrum of ML 2

The NOESY spectrum of ML 2(Figure 4.20; Table 4.16)showed correlation between the signals at δ5.20 and δ 3.48 of sugar molecule confirming the signal at 5.20 belong to sugar molecule.





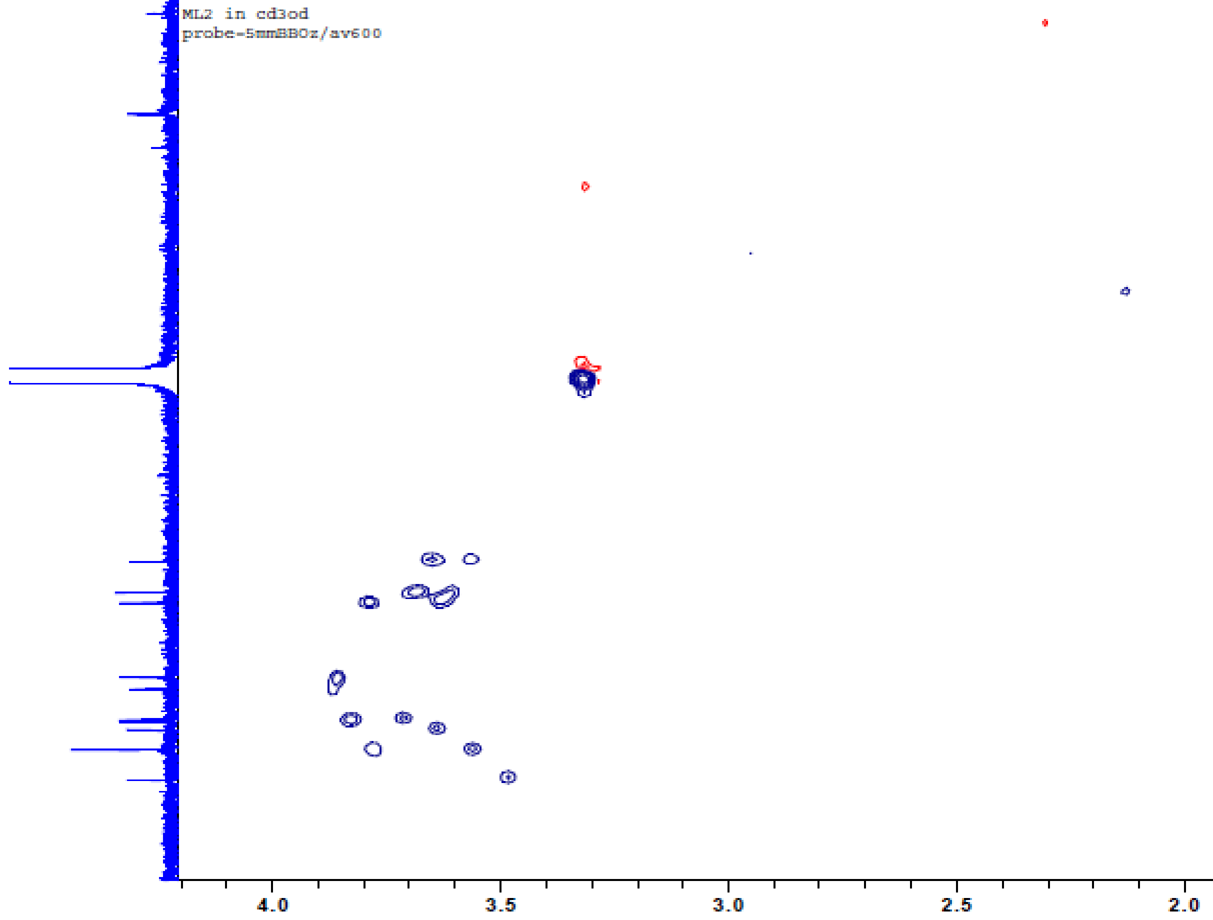

Musa 83 1 /opt/topspin ABU
ML2 in cd3od
probe=5mmBBOz/av600
4.0
3.5
3.0
2.5
2.0

Figure 4.18a: HSQC of ML 2

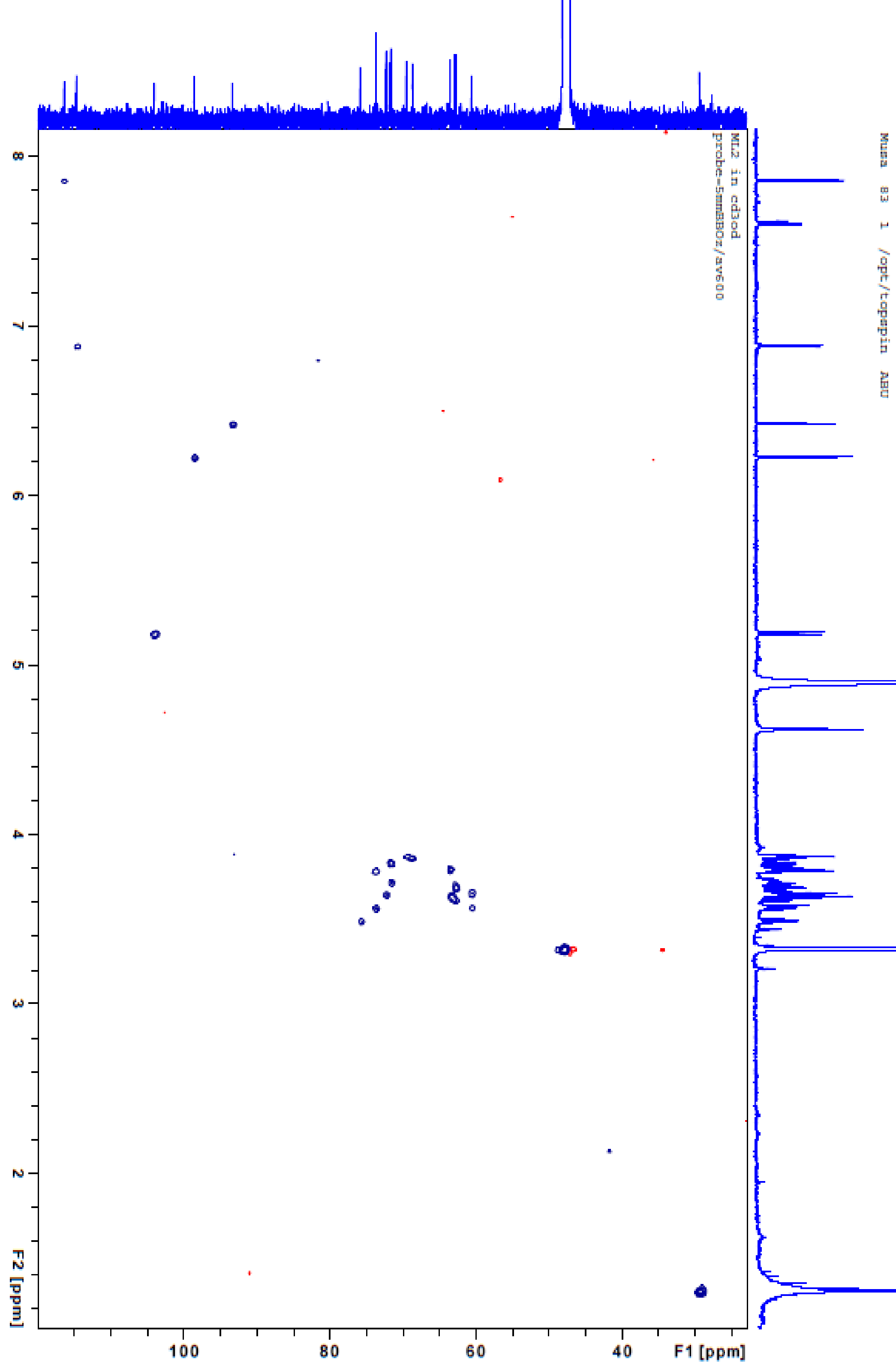


Figure 4.18b: HSQC of ML 2

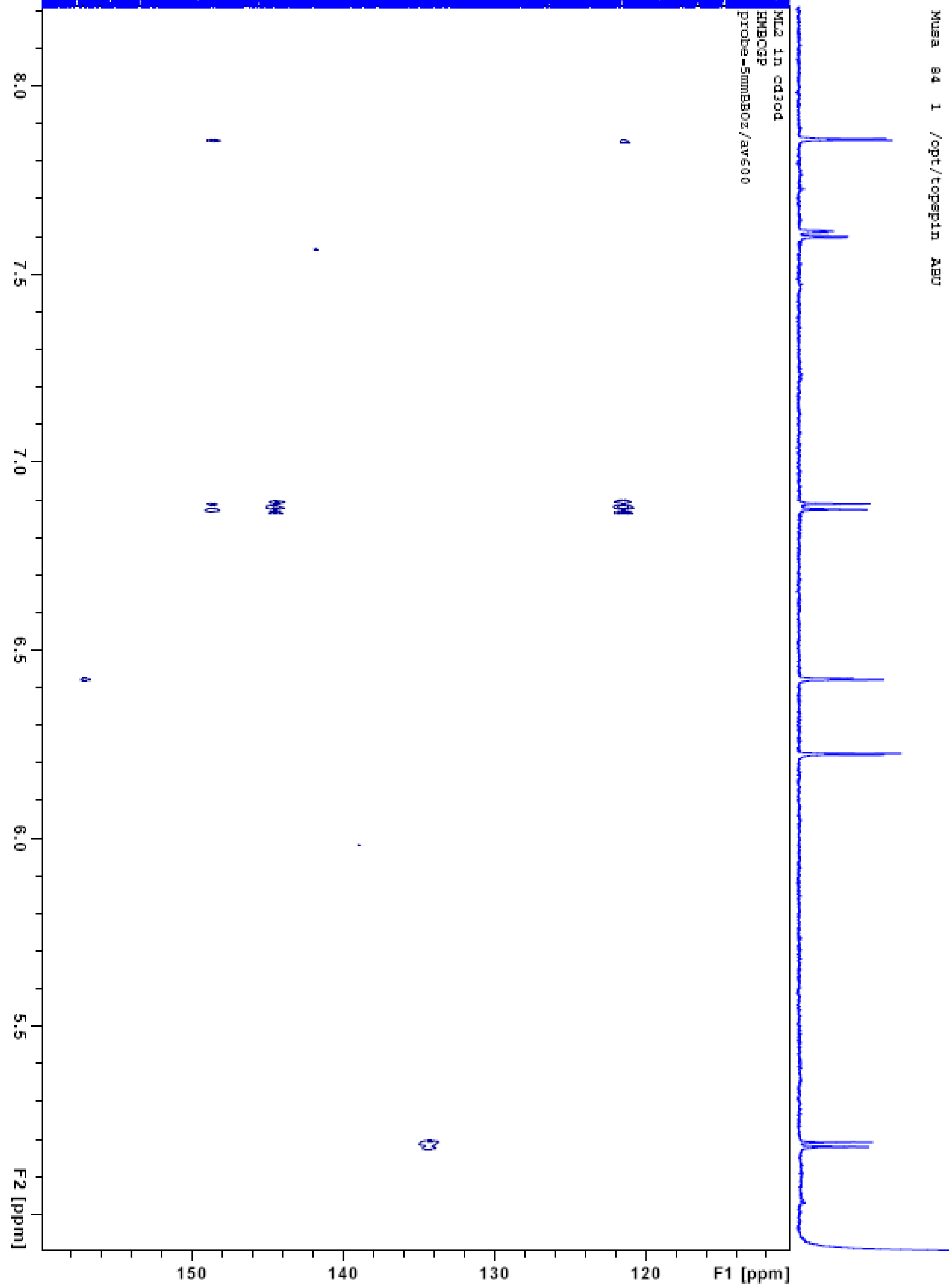

Musa 84 1 /opt/topspin ABU
ML2 in cd3od
HMBCGP
probe=5mmBBOz/av600
8.0
7.5
7.0
6.5
6.0
5.5
F2 [ppm]
150
140
130
120
F1 [ppm]

Figure 4.19a: HMBC NMR spectrum of ML 2

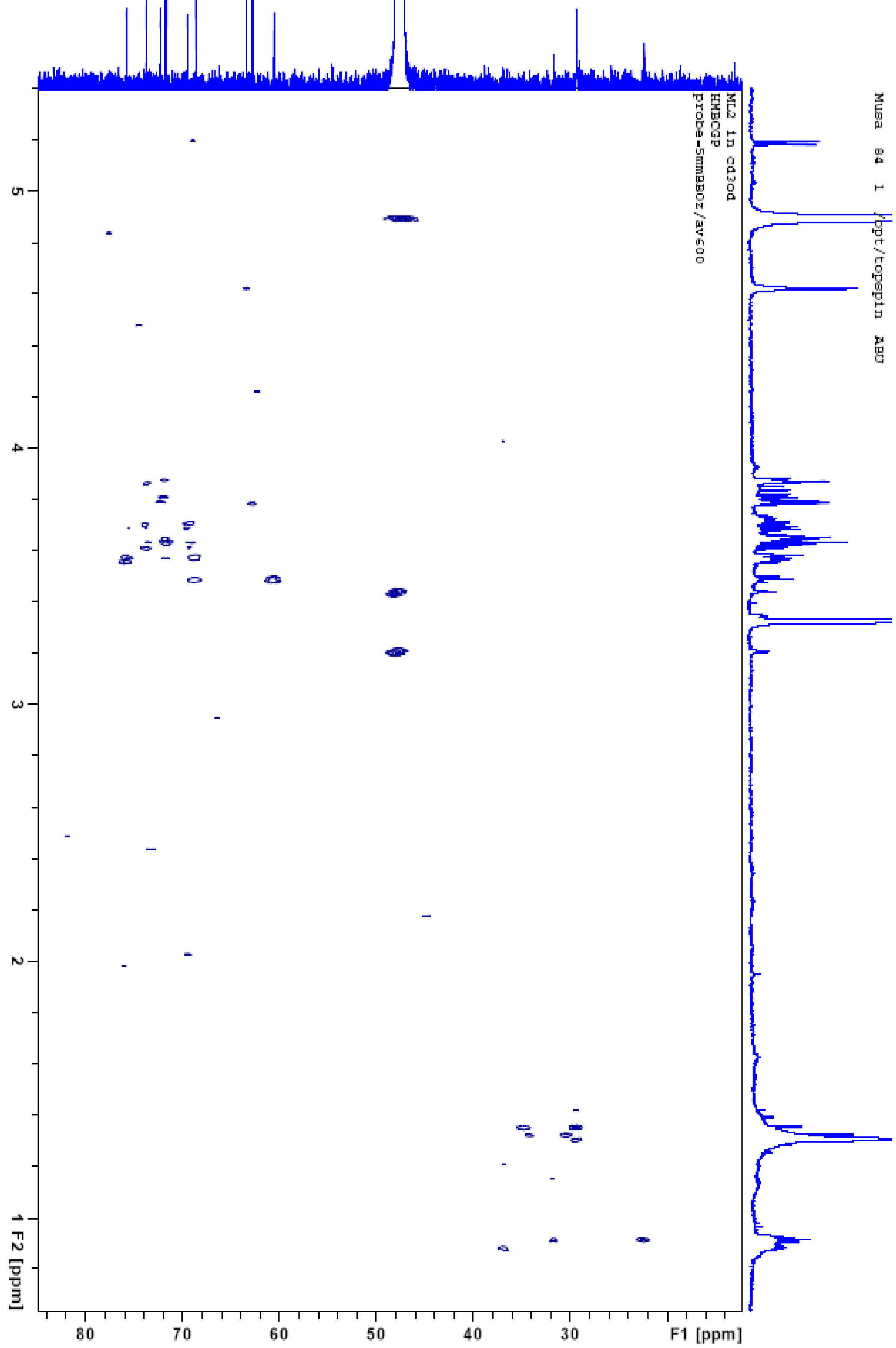

Musa 84 1 /opt/topspin ABU
ML2 in cd3od
HMBCGP
probe=5mmBBOz/av600
F1 [ppm]
F2 [ppm]

Fig 4.19b: HMBC NMR spectrum of ML 2

Fig 4.20: NOESY Spectrum of ML 2

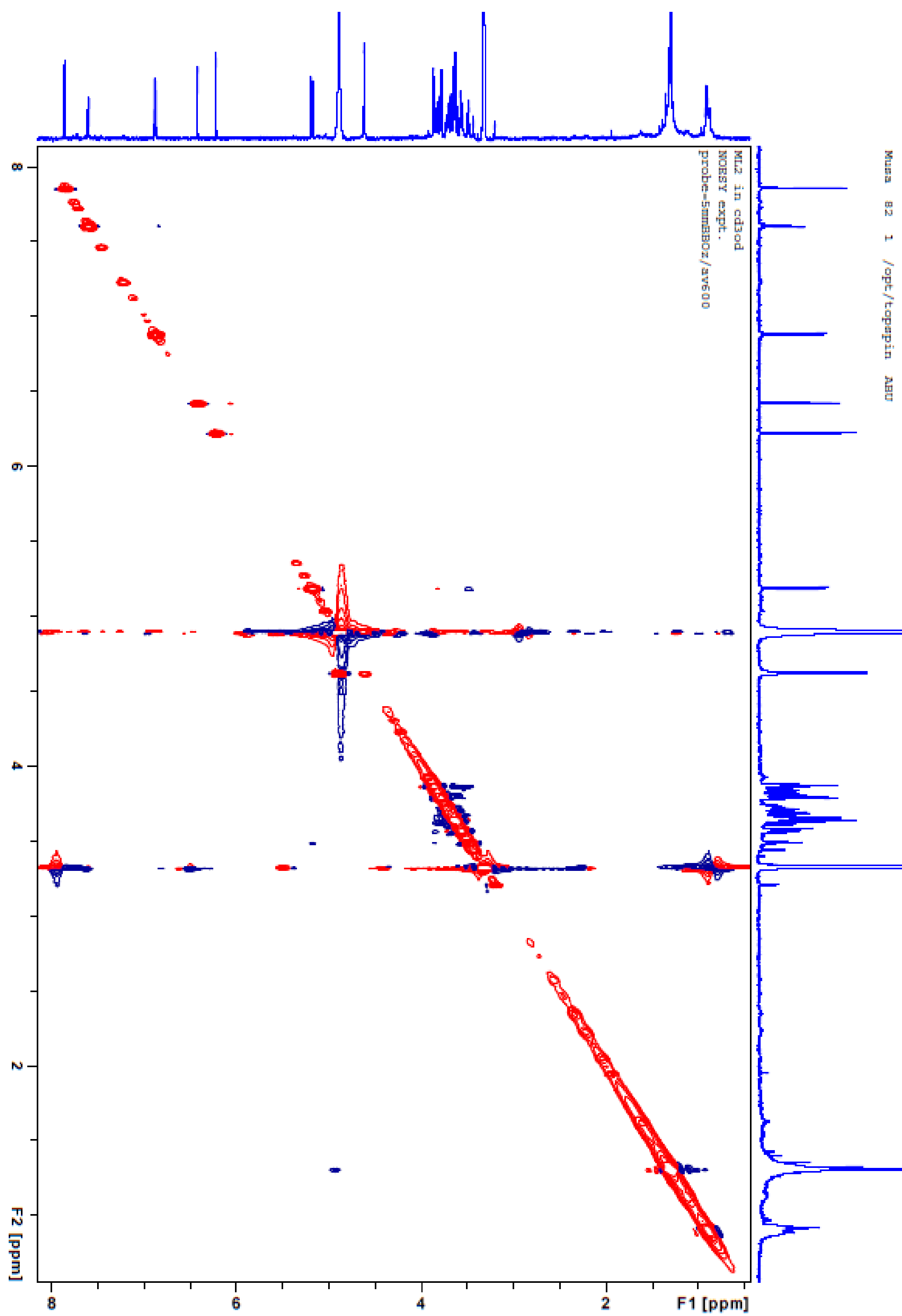


Table 4.13 ^{1}H-NMR and ^{13}C-NMR Assignments of Compound ML2 in CD_3OD

Position	Chemical shift (δ ppm)		DEPT
	^{1}H NMR	^{13}C NMR	
2		156.4	C
3		134.4	C
4		178.2	C
5		161.2	C
6	6.20 (d, J = 2.16)	98.5	CH
7		167.4	C
8	6.40 (d, J = 1.98)	93.3	CH
9		157.0	C
19		103.9	C
1′		121.5	C
2′	7.86 (d, J = 2.22)	116.3	CH
3′		144.4	C
4′		148.6	C
5′	6.89 (d, J = 8.82)	114.7	CH
6′	7.60 (dd, 2.04, 8.4)	121.5	CH
Glucose			
1″	5.20 (d, J = 8.1)	103.9	CH
2″	3.88	68.6	CH
3″	3.78	73.7	CH
4″	3.64	71.8	CH
5″	3.48	75.4	CH
6″	3.79a 3.65b	63.4	CH_2
Glucose			
1‴	4.61 (s)	----	CH
2‴	3.48	75. 4	CH
3‴	3.78	71.6	CH
4‴	3.72	69.5	CH
5‴	3.64	72.3	CH
6‴	3.56a3.65b	60.5	CH_2

Table 4.14 HSQC Correlation in NMR Spectra of ML2

Position	Chemical shift of correlated carbon
6	98.6
8	93.3
2′	116.3
5′	114.7
6′	121.5
1″	103.9
2″	68.6
3″	73.7
4″	71.8
5″	75.4
6″	63.4
1‴	----
2‴	75. 4
3‴	71.6
4‴	69.5
5‴	72.3
6‴	60.5

Table4.15 HMBC Correlation in NMR Spectra of ML2

Proton position (chemical shift)	Carbon position (chemical shift)
6	C-10 (103.9)
8	C-9 (157.0)
2′	C-6′(121.5), C-4′ (148.5)
5′	C-6′(121.5), C-3 (144.4), C-4′ (148.5)
6′	-
1″ (5.20)	C-3(134.4); C-4″ (75.8); C-2″ (68.6)
2″	-
3″	-
4″	-
5″	-
6″	-
1‴ (4.61)	C-6″ (63.4)
2‴	-
3‴	-
4‴	-
5‴	-
6‴	-

Table 4.16Proton-protonCorrelations in NMR Spectrum of ML2 Using COSY and NOESY Techniques and Their Assignments.

Position (chemical shift)	Correlation (chemical shift)	
	COSY	NOESY
5′	H-6’ (6.89)	
6′	H-5’ (7.60)	
1‴	H-2’’ (3.88)	H-2’’’ (3.48)

4.11 Isolation of ML 7

4.11.1 Thin layer chromatographic profile of n-butanol soluble fraction of the methanolextract

Thin layer chromatography of the n-butanol soluble fraction of the methanol extract of *A. grantii* usingthe solvent system: ethyl acetate: methanol: water (65: 20: 15) revealed several spots.

4.11.2 Column chromatographic separation of n-butanolsoluble fraction of the methanol extract

Fractionation of the n-butanol fraction of the methanol extract of the aerial parts of *A. grantii* using silica gel column chromatography yielded eight major fractions (A – H). Fraction E obtained from ethyl acetate: methanol (70:30) when submitted to repeated gel filtration and final purification by preparative thin layer chromatography using ethyl acetate: methanol: water (15:3:2) yielded ML 7 (4 mg).

Table 4.17 Fractions Obtained from Column Chromatography of the n-Butanol Soluble Fraction of the Aerial Part of *Ampelocissus grantii*

Eluent No.	Eluting solvent	Fraction No.	No. of spots	Weight (mg)
1	EtOAc (100%)	1 – 24	-	-
2	EtOAc:MeOH (9:1)	25 – 44	3	15
3	EtOAc:MeOH (8:2)	45 – 60	4	20
4	**EtOAc:MeOH (7:3)**	**61 – 82**	**2**	**134**
5	EtOAc:MeOH (6:4)	83 – 102	3	25
6	EtOAc:MeOH (1:1)	103 – 122	2	55
6	EtOAc:MeOH (4:6)	123 – 141	3	67
8	EtOAc:MeOH (3:7)	142 – 161	3	48
9	EtOAc:MeOH (2:8)	162 – 181	4	15
10	EtOAc:MeOH (1:9)	182 – 201	3	24
11	MeOH (100%)	202 – 210	2	-

4.12 Characterization of ML7

4.12.1Thin layer chromatography (TLC) examination of ML 7

Thin layer chromatography examination of ML 7using the solvent system: ethyl acetate: methanol water 100:16.5:13.5 gave single spot with R_f value of 0.43.

4.12.2 Solubility test of ML 7

The isolated compound, ML 7 was found to be soluble in methanol only at room temperature.

4.12.3 Chemical test for ML 7

Phytochemical studies of ML7 using the standard procedure (Trease and Evans, 2002) revealed the compounds to be phenolic compound (positive $FeCl_3$ test).

4.12.4 Spectral data of ML 7

4.13 Spectral Analysis of ML 7

4.13.1 Proton nuclear magnetic resonance (^{1}H–NMR) of ML 7

The ^{1}H-NMR spectrum of ML 7(Figure 4.21; Table 4.18)displayed signals at 7.40 (d), 7.30 (d), 7.20 (t) and 7.00 (t). The spectrum also displayed signals at 4.60 (m) 2.50, 2.40 and 2.07.

4.13.2 Carbon – 13 Nuclear Magnetic Resonance (^{13} C–NMR) of ML 7

The ^{13}C-NMR of ML 7 (Figure 4.22; Table 4.18) shows the presence of two methylene signals at δ72.33 and 60.36.

4.13.3 DEPT ^{13}C-NMR of ML 7

Figure 4.23 shows the DEPT of ML 7

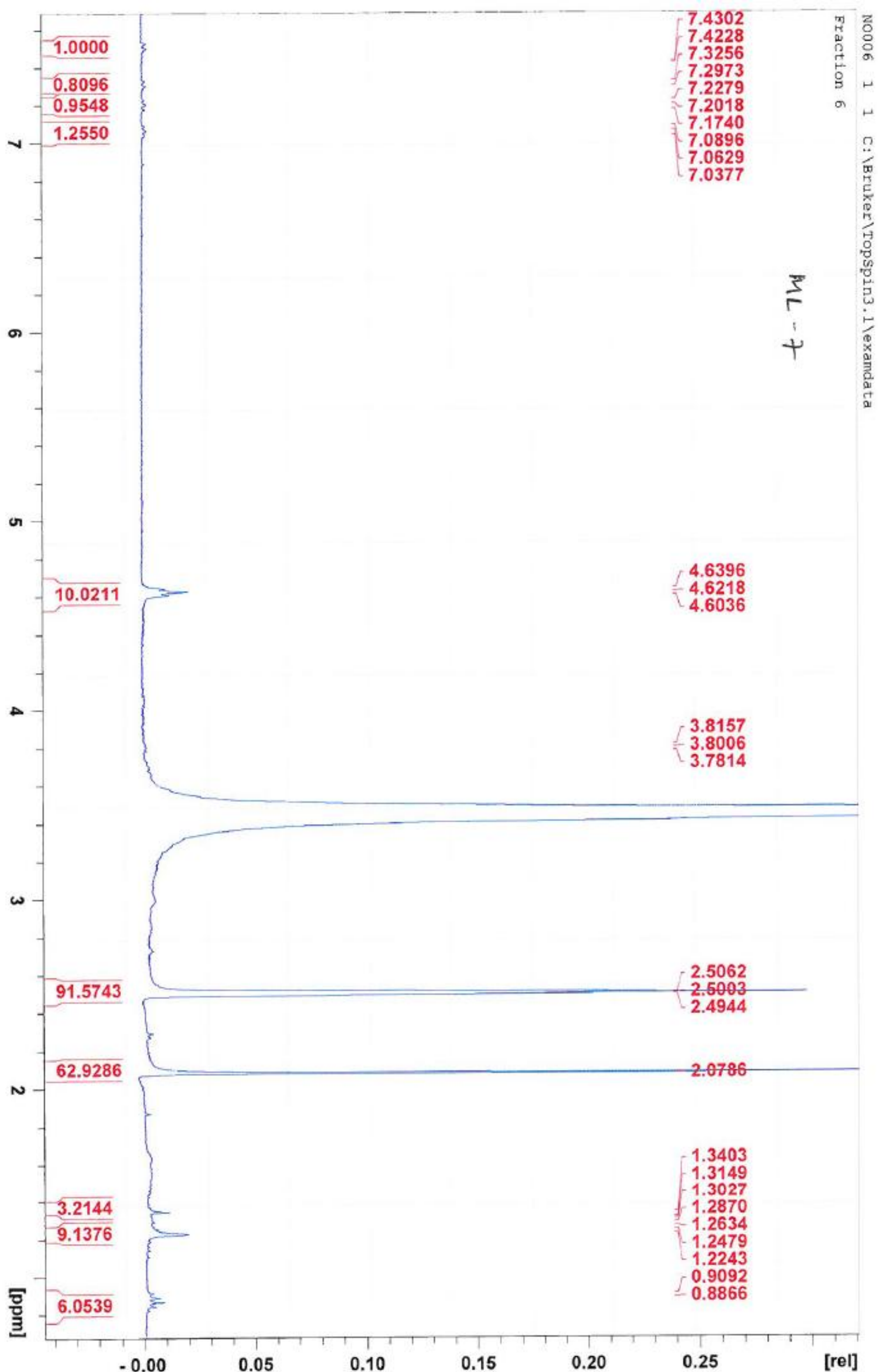


Figure4.21 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of ML 7

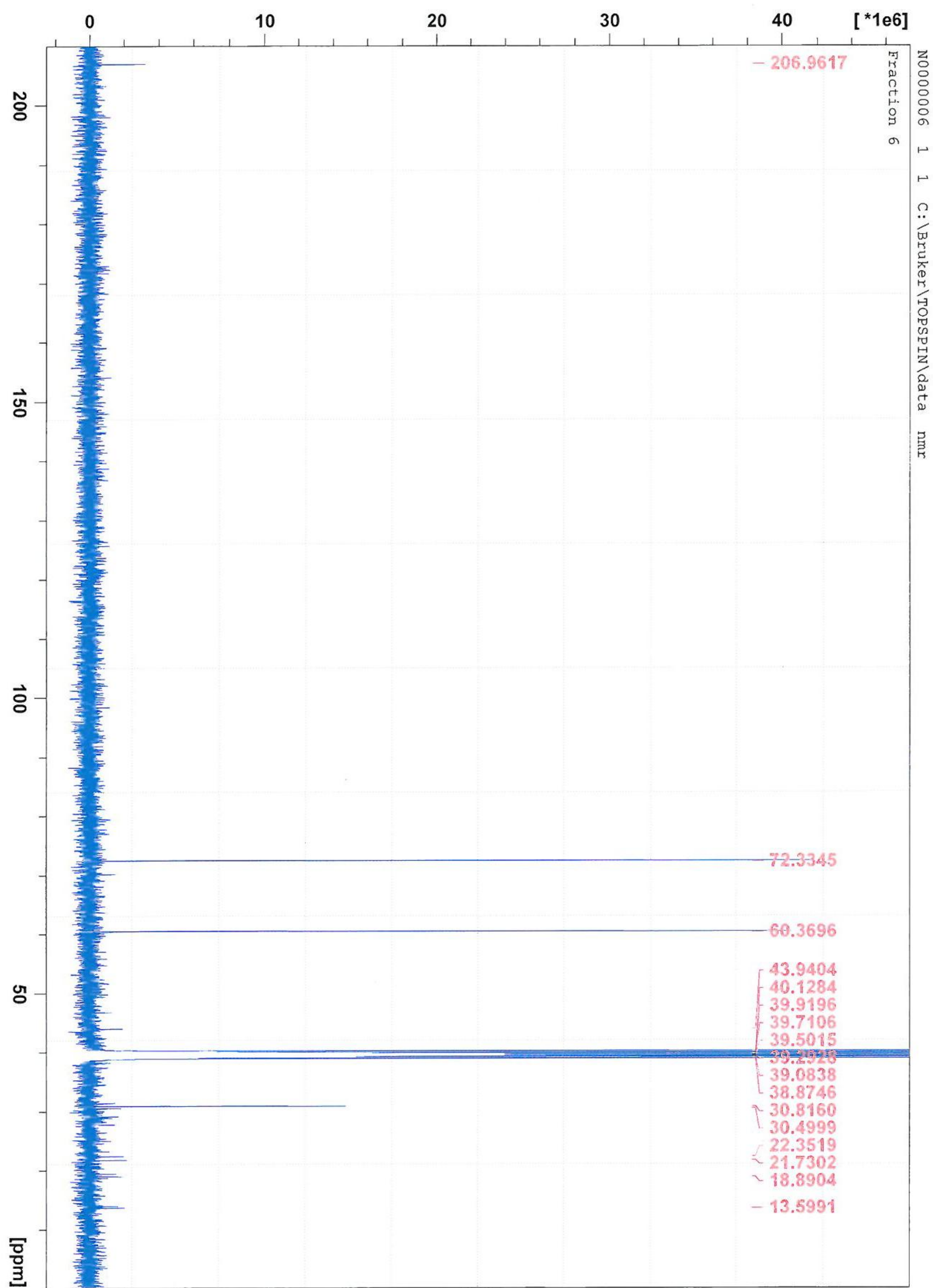


Figure 4.22 Carbon-13 Nuclear Magnetic Resonance (NMR) spectrum of ML 7

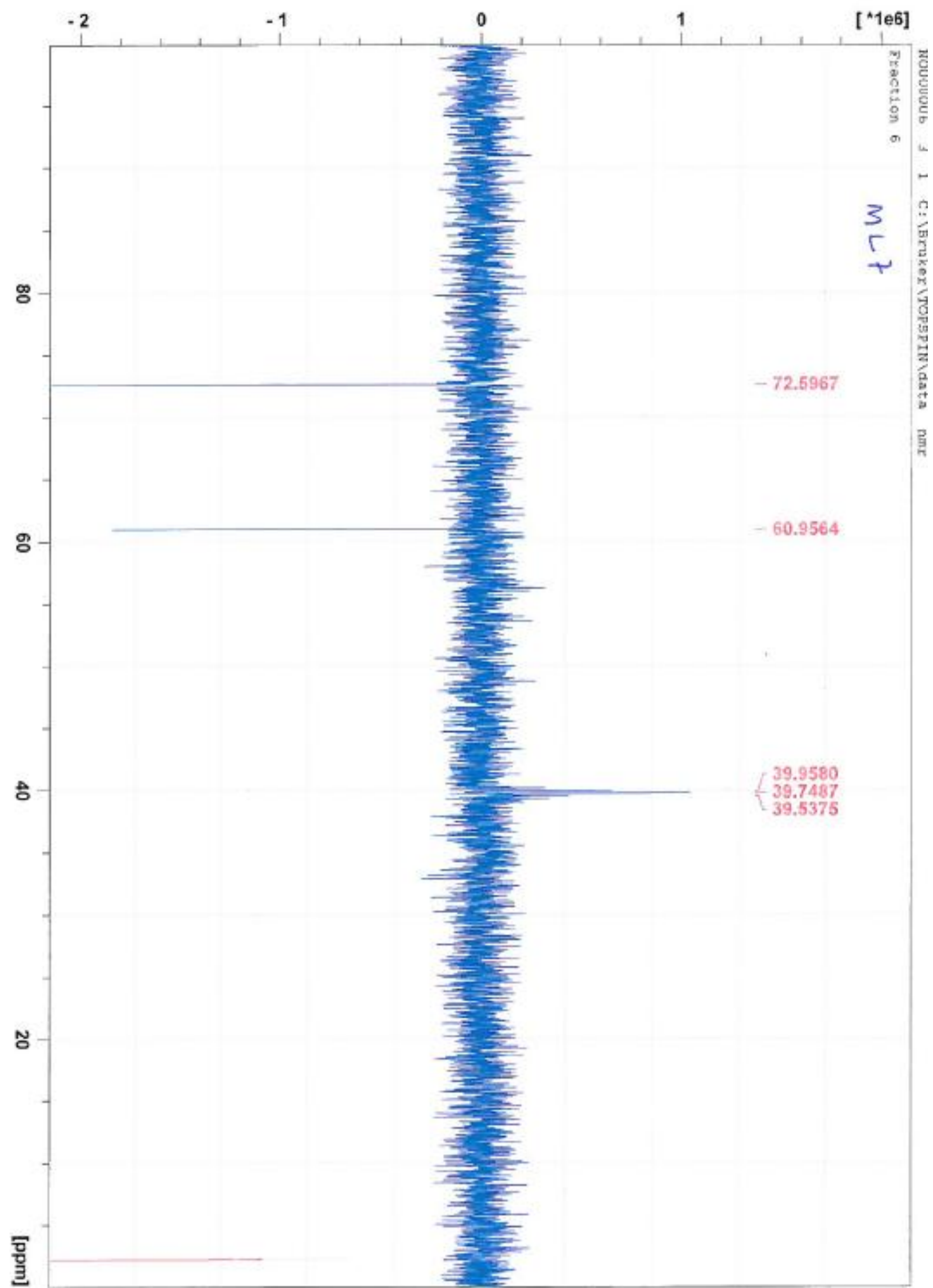


Figure4.23 DEPT Carbon-13 Nuclear Magnetic Resonance (NMR) Spectrum of

ML 7

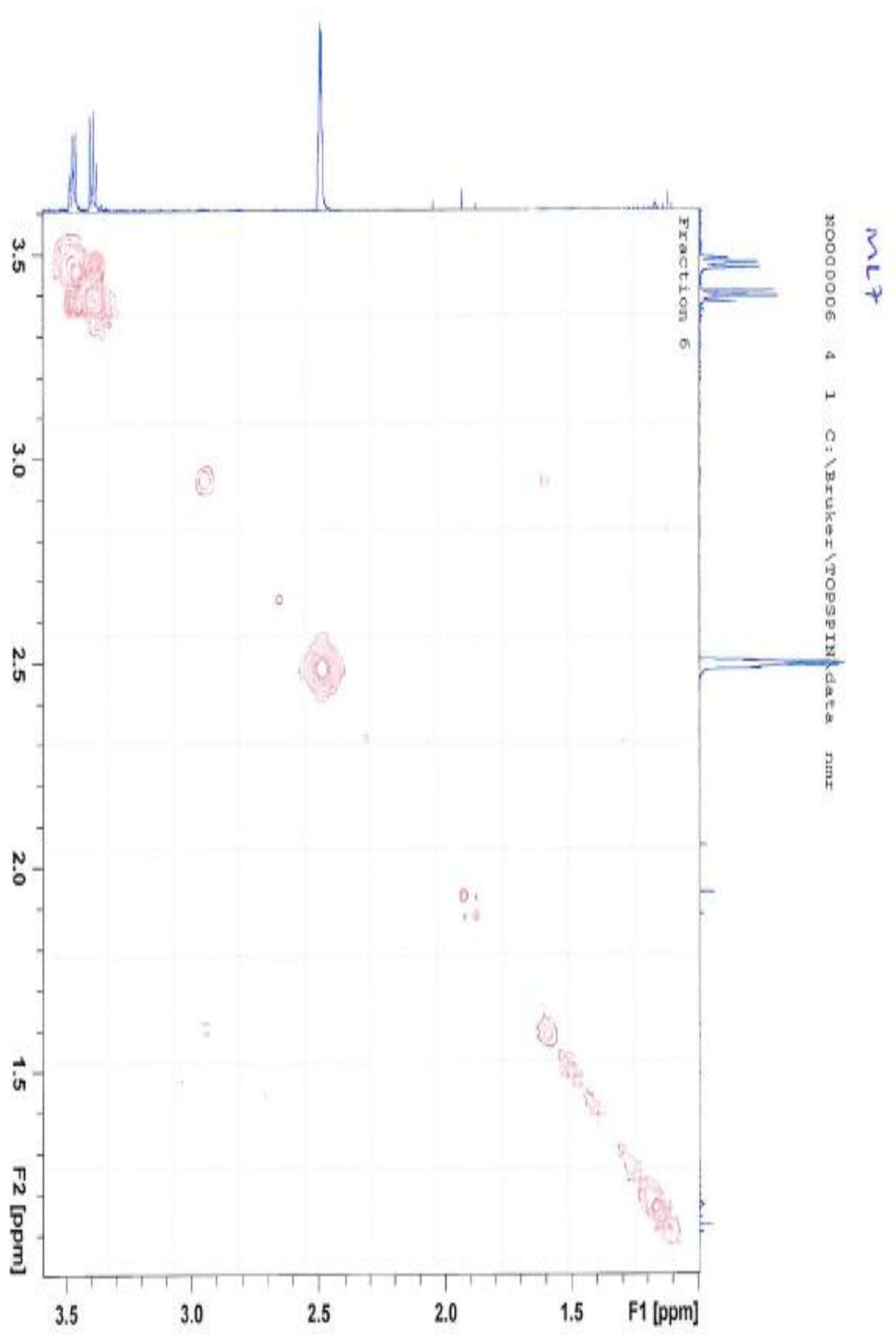


Figure4.24 ^{1}H-^{1}H COSY of ML 7

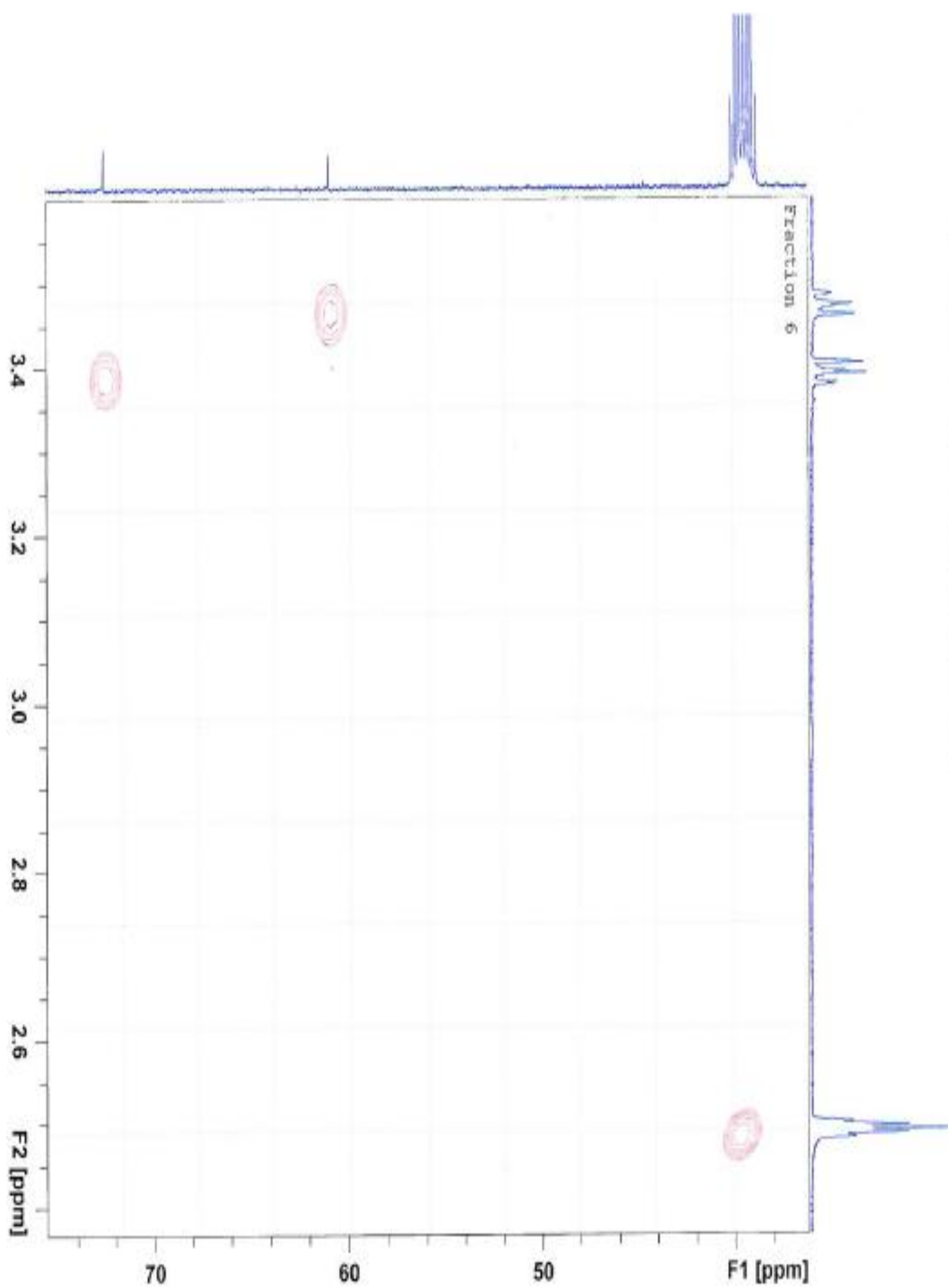


Figure4.25 HSQC of ML 7

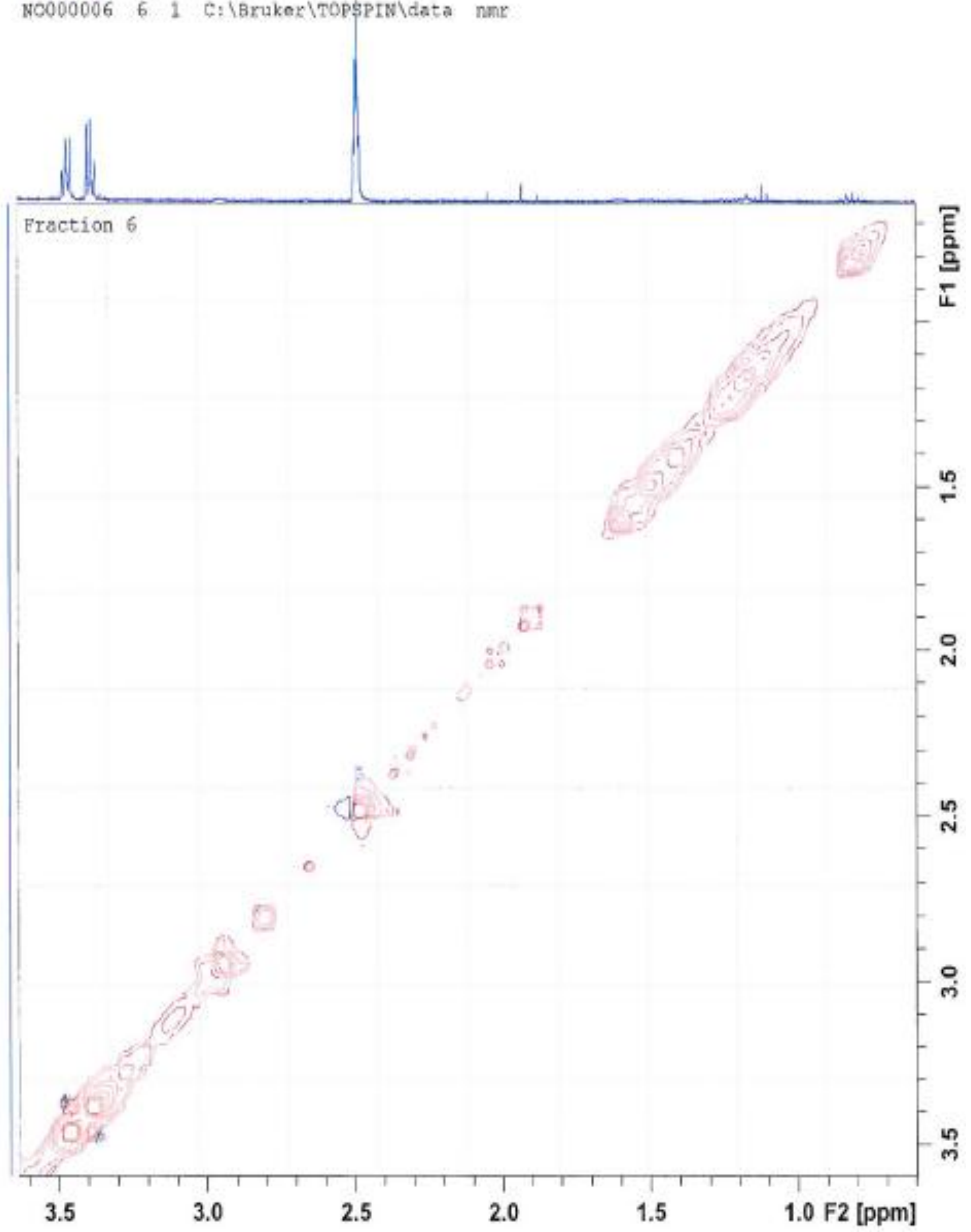


Figure4.26 NOESY Spectrum of ML 7

4.13.4 C – H Correlation (HSQC) of ML 7

Figure 4.25 shows the HSQC spectrum of ML 7 showing the assignment of protons to their respective carbons.

4.13.5 ^{1}H – ^{1}H COSY spectral analysis of ML 7

Figure 4.24showed the ^{1}H- ^{1}H COSY correlation of ML 7.

4.13.6 ^{1}H – ^{1}H NEOSY spectral analysis of ML 7

The NOESY spectrum of ML 7(Figure 4.26)showed correlation between the signals of protons in the same environment.

Table 4.18 ^{1}H-NMR and ^{13}C-NMR Assignments of ML 7 in CD_3OD

Position	Chemical shift (δ ppm)	
	^{1}H-NMR	^{13}C-NMR
1	7.40 d	-------
2	7.30 d	-------
3	7.20 t	-------
4	7.0 0 t	-------
5	2.50 t	-------
6	2.40 s	-------
7	2.07	30.50
8	3.40	43.12
9	3.45	206.96
10	4.60	30.8
1’	--------	72.33
2’	-------	60.36
Rhamnose		
1″	-------	-------
2″	-------	-------
3″	-------	-------
4″	-------	-------
5″	-------	-------
6″	0.91	18.89

4.14Pharmacological Studies

4.14.1 Acute toxicity study

The intraperitoneal LD_{50} of the extract was found to be 1265 mg/kg body weight via intraperitoneal route in mice.

4.14.2Effect of crude methanol extract of the aerial parts of*Ampelocissus grantii* on acetic acid-induced writhing in mice.

In the acetic acid induced writhing assay, the extract significantly ($P < 0.001$) and dose-dependently inhibited the writhes induced by acetic acid (Table 4.19). The highest percentage inhibition was observed at dose of 300 mg/kg of the extract (95.19 %) while that of the ketoprofen 20 mg/kg was noted as 71.23 %.

Table 4.19Effect of Crude Methanol Extract of the Aerial Parts of *Ampelocissus grantii* and ketoprofen on Acetic Acid-induced Writhing in Mice

Treatment	Mean number of writhes ± SEM	% inhibition
Normal saline (10ml/kg)	24.33±1.76	-
Extract (75 mg/kg *i.p.*)	5.83±1.195***	76.03
Extract (150 mg/kg *i.p.*)	2.00±0.856***	91.78
Extract (300 mg/kg *i.p.*)	1.17±0.601***	95.19
Ketoprofen (20 mg/kg *i.p.*)	7.00±2.671***	71.23

Data presented as Mean ± SEM; n=6;

*** P≤0.001 (compared with control values, Student's-test)

4.14.3 Effect of crude methanol extract of the aerial parts of *Ampelocissus grantii* on pain latency in mice exposed to hot plate

The mean latency of nociceptive responses to thermal stimuli is summarized in Table 4.20. *Ampelocissus grantii* extract exhibited no significant increase in pain latency at a dose of 50mg/kg body weight, but at higher doses of 150 and 300 mg/kg body weight it increased significantly ($P < 0.05 – 0.01$) the pain threshold in dose related manner. Pentazocine (20 mg/kg; *i.p*) significantly ($p < 0.001$) prolonged the latency time much more than the extract.

Table 4.20 Effect of Crude Methanol Extract of the Aerial Parts of *Ampelocissus grantii* and Pentazocine on Pain Latency in Mice Exposed to Hot Plate

Treatment	Mean pain latency (Seconds) ± SEM					
	0 (min)	30 (min)	60 (min)	90 (min)	120 (min)	150 (min)
Normal saline (10 ml/kg*i.p.*)	1.25±0.047	1.47 ±0.086	2.09 ±0.242	1.81 ±0.376	1.46 ±0.112	1.15 ±0.051
Extract (75 mg/kg *i.p.*)	1.21±0.088	2.10 ±0.113 (30.00)	2.85 ±0.200 (26.67)	2.66 ±0.421 (31.95)	1.35 ±0.105 (00.00)	1.52 ±0.143 (23.68)
Extract (150 mg/kg *i.p.*)	1.28±0.070	2.72±0.190* (45.96)	2.97 ±0.372 (29.63)	2.04 ±0.286 (11.27)	1.47 ±0.181 (00.68)	1.55 ±0.156 (25.16)
Extract (300 mg/kg *i.p.*)	1.23±0.095	2.83 ±0.465** (48.06)	2.99 ±0.482 (30.10)	3.05 ±0.593 (40.66)	1.87 ±0.322 (21.93)	1.72 ±0.109* (32.56)
Pentazocine (20 mg/kg *i.p.*)	1.33±0.114	4.07 ±0.257*** (63.88)	4.58 ± 0.490*** (54.37)	4.71±0.299*** (61.57)	2.84 ±0.199*** (48.59)	1.85 ±0.083** (37.30)

Data presented as Mean ± SEM; n=6;

Figure in parentheses represent percentage antinociceptive activity;

* P≤0.05; ** P≤0.01; *** P ≤0.001 compared with control values.

4.14.4 Effect of crude methanol extract of the aerial parts of *Ampelocissus grantii* on carrageenan-induced paw oedema in rats

In the normal saline treated rats, Subplantar injection of 1% carrageenan produced a local oedema reaching its maximum at 3 hours (Table 4.21). The extract significantly ($P < 0.01 - 0.001$) inhibited the paw oedema at all doses tested. The anti-inflammatory effect of the extract at 150 mg/kg (80.95%) was greater than that of the reference drug, ketoprofen (76.19%) at the 3rd hour (the peak of inflammation).

Table 4.21Effect of Crude Methanol Extract of the Aerial Parts of *Ampelocissus grantii* and Ketoprofen on Carrageenan-induced Paw Oedema in Rats

Treatment	Mean Paw Oedema Diameter (mm) ±SEM			
	1h	2h	3h	4h
Normal saline (10ml/kg)	0.13±0.0259	0.19±0.022	0.21±0.029	0.13±0.020
Extract (75 mg/kg *i.p.*)	0.03 ±0.004*** (76.92)	0.07 ±0.026** (63.16)	0.07 ±0.011*** (66.67)	0.04 ±0.007*** (69.23)
Extract (150 mg/kg *i.p.*)	0.02 ±0.007*** (84.62)	0.04 ±0.013*** (78.95)	0.04 ±0.005*** (80.95)	0.03 ±0.008*** (76.92)
Extract (300 mg/kg *i.p.*)	0.04 ±0.007*** (69.23)	0.05 ±0.016*** (73.68)	0.06 ±0.009*** (71.43)	0.05 ±0.006*** (61.54)
Ketoprofen (20 mg/kg *i.p.*)	0.07 ±0.005* (46.15)	0.06 ±0.013*** (68.42)	0.05 ±0.015*** (76.19)	0.05 ±0.013** (61.54)

Data presented as Mean ± SEM; n=6;

Figure in parentheses represent percentage anti-inflammatory activity;

* P≤0.05, ** P≤0.01, ***P≤0.001,

4.15Results of Antimicrobial Studies

The *in vitro* antimicrobial screenings presented in Table 4.22 showed that methanol extract of the aerial parts of *A. grantii* possesses inhibitory activity against some of the organisms tested *(Staphylococcus aureus,Streptococcus pyogenes, Bacillus subtilis, Escherichia coli, Klebsiellapneumonia,Psudomonas aeruginosa* and *Candida albicans).*The most susceptible organisms were *Streptococcus pyogenes* and*Klebsiellapneumonia* and the least susceptible was *Candida albicans,* shown by high and low zones of inhibition respectively. The extract did not inhibit the growth of *Corynebacterum ulcerus Salmonella typhi,* and *Neosseria gonorrhea.* The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results are shown in Tables 4.23 and 4.24 respectively. The results revealed that the MIC and MBC values of the methanol extract are 6.25mg/ml and 12.5mg/ml for the bacterial strains respectively and 12.5 mg/ml and 25 mg/ml for the fungal strain respectively.

Table 4.22 Results of Susceptibility Test of *Ampelocissus grantii* Methanol Extract and Standard Antibiotics againstVarious Organisms

Test Organism	Zone of inhibition of extract and antibiotics against the microbes (mm)			
	Extract	Chloramphenicol	Erytomycin	Sparfloxin
Staphylococcus aureus	23.0	0.0	22.0	0.0
Streptococcus pyrogenes	27.0	0.0	24.0	0.0
Corynebacterum ulcerus	0.0	0.0	19.0	30.0
Bacillus subtilis	26.0	20.0	30.0	34.0
Escherichia coli	21.0	24.0	20.0	20.0
Salmonella typhi	0.0	30.0	20.0	20.0
Klebsiella pneumonia	27.0	0.0	30.0	22.0
Pseudomonas aeruginosa	20.0	17.0	19.0	21.0
Neosseria gonorrhea	0.0	0.0	14.0	0.0
Candida albicans	17.0	NT	NT	NT

Table 4.23 MinimumInhibitory Concentration (MIC) Results of the *Ampelocissus grantii* Methanol Extract

Test Organism	Concentration of crude methanol extracts in mg/ml				
	50	25	12.5	6.25	3.125
Staphylococcus aureus	-	-	-	#	+
Streptococcus pyrogenes	-	-	-	#	+
Corynebacterium ulcerus	NT	NT	NT	N	NT
Bacillus subtilis	-	-	-	#	+
Escherichia coli	-	-	-	#	+
Salmonella typhi	NT	NT	NT	N	NT
Klebsiella pneumonia	-	-	-	#	+
Pseudomonas aeruginosa	-	-	-	#	+
Neisseria gonorrhea	NT	NT	NT	NT	NT
Candida albicans	-	-	#	+	++

= Clear solution (MIC), - = No growth, + = Cloudy solution (slight growth),
++ = Turbid solution (strong growth), NT = Not tested.

Table 4.24 Minimum Bactericidal Concentration (MBC) Results of the *Ampelocissus grantii*Methanol Extract

Test Organism	Concentration of crude methanol extracts in mg/ml

	50	25	12.5	6.25	3.125
Staphylococcus aureus	-	-	#	+	++
Streptococcus pyrogenes	-	-	#	+	++
Corynebacterium ulcerus	NT	NT	NT	NT	NT
Bacillus subtilis	-	-	#	+	++
Escherichia coli	-	-	#	+	++
Salmonella typhi	NT	NT	NT	NT	NT
Klebsiella pneumonia	-	-	#	+	++
Pseudomonas aeruginosa	-	-	#	+	++
Neisseria gonorrhoea	NT	NT	NT	NT	NT
Candida albicans	-	#	+	++	+++

# = MBC, - = no growth, + = scanty growth, ++ = moderate growth, +++ = heavy growth,
NT = Not tested.

CHAPTER 5

5.0DISCUSSION

5.1 Phytochemical Studies

Preliminary phytochemical studies of the methanol extract of the aerial parts of *Ampelocissus grantii* revealed the presence of saponins, glycosides, steroids, terpenoids and flavonoidswhich are associated with pharmacological activities of plants.

A. grantii is a plant well known in traditional medicine for its properties. It has been reported that the family Vitaceae include 14 genera and 800 species described but only a few number of them contain no significant alkaloids. Like in this study, previous studies did not detect alkaloids with *Ampelocissus* extracts (Raffauf, 1996; Zongo *et al.,* 2010).

The ^{1}H-NMR spectrum of flavonoids appears predominantly in the range 0-10 ppmdownfield. The chemical shift establishes the nature of the hydrogen, and coupling constants determine the presence of the ortho, meta and vicinal protons. The ortho andmeta couplings have ranges of 6.5-9.0 Hz and 1.5-2.5 Hz, respectively and are of value in establishing the aromatic substitution pattern (Mabry *et al.,* 1970).

In flavonoids glycosides, anomeric configurations, linkage sites and sequence of sugars in theflavonol glycosides can be determined using various NMR experiments, ^{1}H-NMR chemical shifts and vicinal coupling constants, ^{13}C-NMR chemical shifts and ^{13}C-^{1}H coupling constants, interresidue NOE and long range homo- and heteronuclear correlations. Usually the anomeric resonances of α-glycosides resonate at a downfield position by 0.3-0.5 ppm compared with that of the corresponding β-glycosides. Thus, resonances at the lowest field (4.5-5.5 ppm), which are doublets with $^{3}J_{1,2}$in the range 1-4 Hz, are of α-anomericprotons, whereas β-anomeric protons appear as doublets between 4.0 and 4.8 ppm with $^{3}J_{1,2}$ in the range6-8 Hz in monosaccharides stereochemistry.

Compound 1, was recrystallized from methanol to give pure yellow amorphous powder (4 mg) and1 was found to be soluble in ethyl acetate, acetone and methanol. Thin layer chromatography examination of compound 1 using the solvent systems chloroform: methanol: water (3:3:1) and ethyl acetate: methanol water (100:16.5:13.5) gave single spot with R_f value of 0.63 and 0.54 respectively. Chemical studies of compound 1, using the standard procedure revealed the compounds to be flavonoid (positive Shinoda's test).

As shown in Figure 3.1, The ^{1}H-NMR spectrum (600 MHz, CD_3OD) of compound 1 show two doublets resonating at around δ 6.45 and 6.85 ppm indicating the 5-OH and 7-OH substituted pattern for ring A. Ring B showed a pattern of three one-proton signals at δ 7.90 (d), 7.65 (*dd*) and 7.80 ppm (*d*) assigned to 2′, 5′, and 6′ protons respectively (Mabry*et al.,* 1970).The ^{1}H-NMR spectrum of compound 1 also exhibited a singlet at δ 6.40 ppm assigned to H-3. These suggest a luteolin nucleus. The protons on the flavonoids nuclei of glycosides and their aglycones produce similar ^{1}H-NMR spectra patterns, the spectra of luteolin and its C-3 glycosides all show identical absorption patterns for A-ring. Absences of signals in the sugar region δ 3.00 – 5.00ppm suggest absence of sugar moiety. Based on the above findings, the ^{1}H-NMR spectrum of compound 1 (Mabry, 1970), the structure of compound 1 was concluded as 3′, 4′, 5, 7 – tetrahydroxyl flavone (Luteolin).

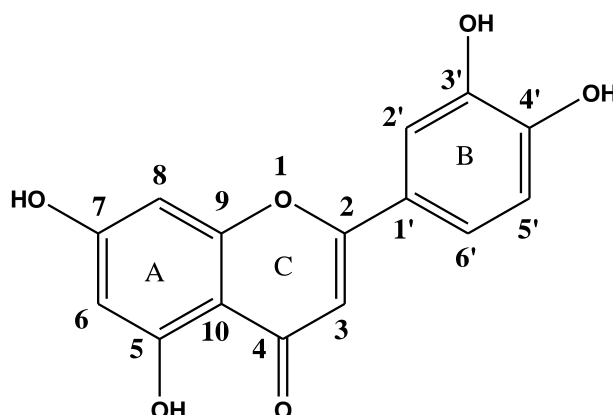


Figure 5.1: Structure of Compound 1

ML 1 was isolated by repeated column chromatography on silica gel and Sephadex LH – 20 from acetone soluble portion of the methanol extract of the aerial parts of*A. grantii*. ML 1 was obtained as yellow powder and was found to be soluble in methanol at room temperature. Thin layer chromatography examination of ML1using the solvent systems chloroform: methanol: water (3:3:1) and ethyl acetate: methanol water 100:16.5:13.5 gave single spot with R_F value of 0.49 and 0.42. The compound gave positive Shinoda's test.

The ^{1}H-NMR spectrum ML 1in CD_3OD(Figure 4.3 – 4.5; Table 4.9) showed the presence of three at δ 7.97 (1H, d, J = 2.22 H-2′), δ 6.92 (1H, d, J = 8.4 Hz, H-5′), and δ 7.67 (1H, dd, J = 2.10; 6.0 Hz, H-6′), two protons signals at δ 6.45 (1H, d, J = 2.22, H-8) and δ 6.22 (1H, d, J = 2.04 H- 6), and two anomeric protons representing two sugar moieties at δ 5.14 (1H, d, J = 7.71 Hz) and 4.12 (1H, d, J = 6.68 Hz). Signals between 3.00 – 3.84 (10H, multiplets) assigned to sugar protons. The ^{1}H-NMR spectrum also showed signal at δ 4.00 (3H, *s*), assigned as methoxy group. The ^{13}C-NMR and DEPT spectra of ML 1 clearly displayed 28 carbon signals (2 x CH_2, 17 x CH and 9 C) of which 12 could be assigned to two sugar moieties, one to methoxy group and the remaining 15 carbon signals were assigned to the aglycone.

The ^{1}H- and ^{13}C-NMR spectra of ML 1 were compared with the data of known compounds and showed a typical flavonol pattern with an isorhamnetin aglycone (Markham and Geiger, 1994; Rosch *et al*., 2004; Zou *et al*., 2007). In addition to the isorhamnetin moiety, the NMR spectra showed signals corresponding to two sugar moieties. The ^{1}H-NMR spectrum of ML 1 displayed two doublets at δ 5.14 (1H, d, J = 7.71 Hz) and δ 4.12 (1H, d, J = 6.68 Hz) for the anomeric protons. Based on the coupling constant J = 7.71 Hz and 6.68 Hz greater than 4.0 Hz, the two sugars

configurations could be identified as β-D-glucose, which correlated respectively with signals at 103.55, 103.50 in the HSQC spectrum. In the HMBC, the anomeric proton signal of the internal glucose at δ 5.14 (H-1") correlated with the carbon signal at δ 134.49 (C-3), the proton signal of the external glucose at δ 4.12 (H-1"') correlated with the anomeric carbon signal of the internal glucose at δ 67.68 (C-6″). These indicated that the internal glucose is attached to C-3 of the aglycone, while the external glucose is attached to C-6″ of the internal sugar. Based on the above evidence and detailed analysis of the NMR spectra, the structure of ML 1 was determined as isorhamnetin3-O-β-D-glucopyranose-(-1″→6″-)-β-D-glucopyranoside, which had not been reported previously from the plant.

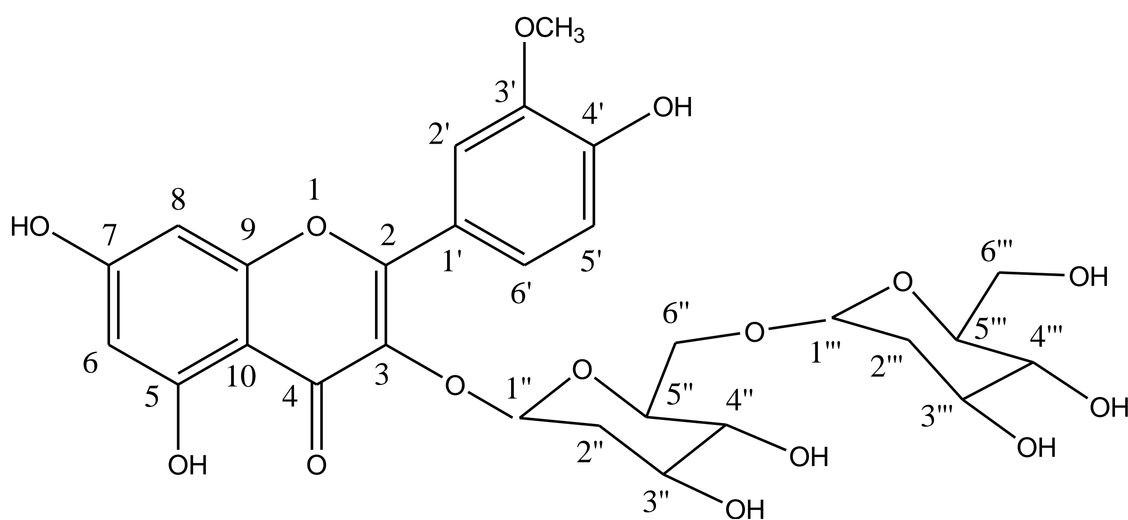


Figure 5.2: Structure of ML 1

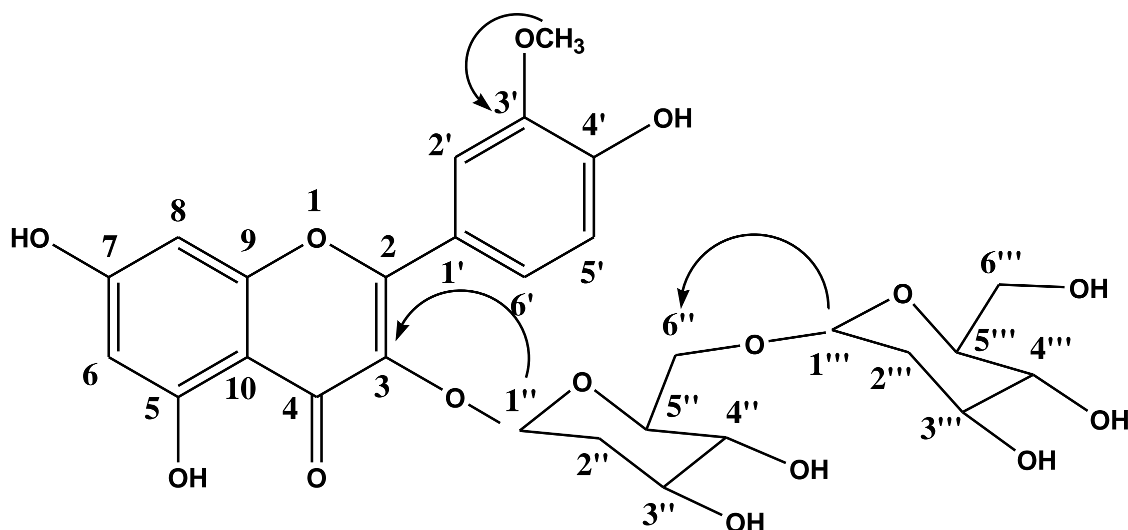


Figure 5.3: The key HMBC correlation of ML 1

ML2,was recrystallized from methanol to give pure yellow amorphous powder. ML 2was found to be soluble in methanol andThin layer chromatography examination of ML 2using the solvent system chloroform: methanol: water (3:3:1) and ethyl acetate: methanol water 100:16.5:13.5 gave single spot with R_F value of 0.63 and 0.59. Chemical studies of the compound using the standard procedure revealed the compound to be flavonoid (positive Shinoda's test).

The ^{1}H NMR spectrum (600 MHz, CD_3OD) of ML 2 (Figure 3.4) indicated a 3, 5, 7, 3′, 4′ -pentaoxygenated flavone. In the spectrum, two doublets resonated at around δ 6.40 and 6.20 ppm (J = 1.6 Hz). These signals, through HSQC analysis, correlate with the carbon of C-8 (δ 93.3) and C-6 *(*δ 98.6) and were assigned to H-8 and H-6 for ring A. Ring B showed a pattern of three one-proton signals at δ 7.89 (d; J = 2.2), 7.60 (*dd*; J = 2.0, 8.4 Hz) and 6.85 ppm (*d*; J = 8.8 Hz). These signals correlate with the carbons of C-2′ (δ 116.3), C-6′ (121.5) and C-5' (114.7), respectively. Protons C'_2-H and C'_5-H appeared as doublets with J = 2.2 and 8.8 Hz, respectively, confirming *meta* and*ortho* coupling with C'_6-H. The proton at C'_6-H appeared as a dd (J = 2.0 and

8.4 Hz), as expected. The coupling of such pattern is the characteristic of 3′, 4′ disubstituted flavonoids. The low field protons signals at δ5.20 (1H, d, J = 8.1) and a singlet at δ 4.61(1H) are due to anomeric protons of 2 sugars units. Signals between 3.40 – 3.88 (10H, multiplets) assigned to sugar protons. The signal at δ5.20 were assigned to the anomeric protons (H-1″) of glucose moiety, with a coupling constant (J = 8.2 Hz) indicating a β-configuration. The singlets at δ 4.61 were assigned to second anomeric protons of the glucose (H-1‴) indicating α-configuration for the sugar (Mabry *et al.,* 1970).

The ^{13}C-NMR spectrum of ML2also indicated thatthe compound is a diglycoside of quercetin with the sugars link to each other on the basis of one substituted glucose C – 6 (63.4) and one unsubstituted C-6 of glucose resonates at (60-62 ppm) (Mabry, 1970), and confirming one terminal glucose. These indicate that ML2 is a monosubstituted quercetin derivative. The ^{13}C-NMR of the aglycone part corresponds well to that of quercetin, the only differences being an upfield shift of the C-3 (approx. 3 ppm) and a downfield shift of the *ortho* related C-2 and C-4. These shifts are analogous to those reported when the C-3 hydroxyl group is substituted in flavonoids. Also, the ^{13}C-NMR spectrum showed the presence of signals at δC 103.9 for C-1″ of one of the glucose moiety. These observations support the ^{1}H-NMR data. Furthermore, the signal at δC 63.4 wasassigned to C-6″. This is in agreement with the observation that β-glucosidation of the glucose C-6 in 3 – O – glucosides provides a downfield shift of approximately 1.3ppm. Glucosylation of the glucose C-6 (e.g. diglucoside) infers a 1.3ppm downfield shift of the glucose C-6 (Mabry*et al.,* 1970). Thus, the signal at 63.4 was assigned to C-6″.

The HMBC spectrum was further utilized to identify the linkage between the aglycone and sugar units. A long range correlation between the *δ*5.20 (H-1″, glucose)

and the δ134.4 (C-3 of the aglycone) confirmed that the glucosyl was linked at C-3 of the flavonol skeleton. In the HMBC spectrum, a cross-peak between the δ63.4 (C-6″, glucose) and the δ 4.61 (H-1‴ glucose) further confirmed this result. Correlation of the^{1}H and ^{13}C NMR data by HSQC, HMBC and ^{1}H-^{1}H COSY experiments indicated the presence oftwo glucose moieties. Due to the good correlation of the ^{13}C-NMR signals of the aglyconewith quercetin-3-O-glycosides (Agrawal 1992;Merfort *et al.*, 1997), and those of the sugar moietieswith 6-O-glucosyl-glucose. Thus, ML 2was therefore identified as Quercetin 3-O-[-α-L- glucopyranosyl-(1'''→6″)-β- D-glucopyranoside.

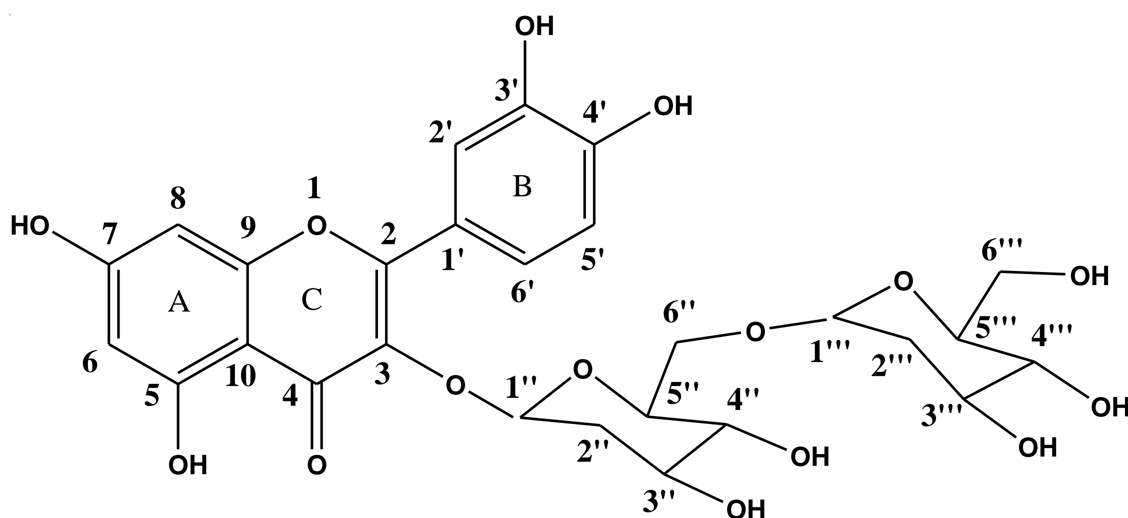


Figure 5.4: Structure of ML 2

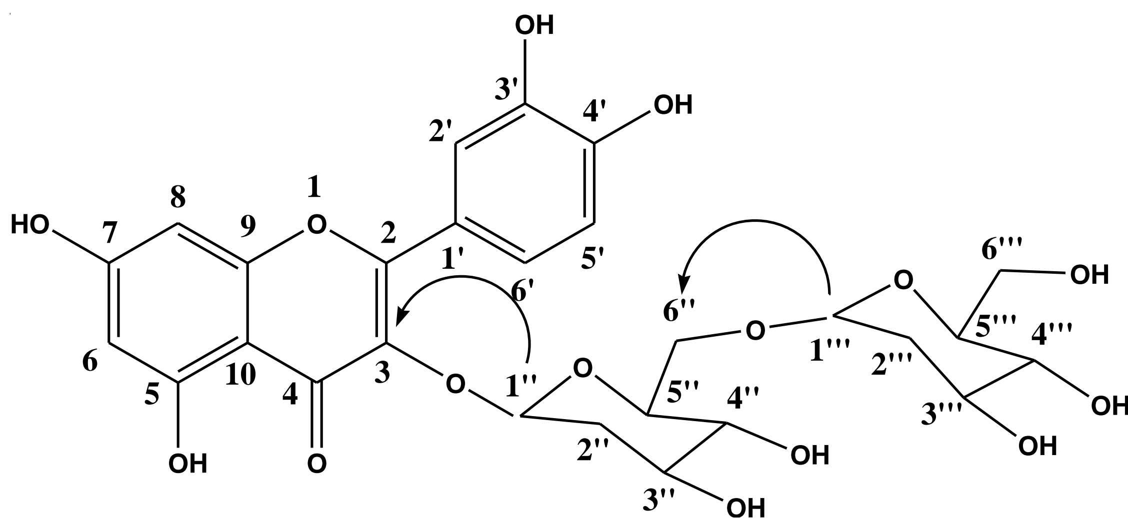


Figure 5.5: The key NMBC correlation of ML 2

The ^{1}H-NMR spectrum of ML 7displayed signals at 7.40 (d), 7.30 (d), 7.20 (t) and 7.00 (t) showing the presence of 1,4-disubstituted benzene ring.

The signal at 4.60 (m) assumed to be of an anomeric proton indicate the presence of sugar moiety. Signals centered at 2.50 and 2.40 in addition to the significant acetoxy signal observed at 2.07 suggested linkage of a CH_2-CH_2-CO-CH_3 moiety to the aromatic ring of disubstituted benzene ring. This hypothesis was confirmed by the ^1H-^1H correlation spectroscopy (COSY) spectrum revealing the connectivity of H_7 to H_8.The ^1H-NMR spectrum of ML 7 allowed the identification of the one terminal rhamnose methyl unit at δ0.9 and confirm by the corresponding carbon signals at δ=18.8 (Agrawal, 1992).The ^{13}C-NMR also shows two oxymethylene signals at δ72.33 and 60.36 in between the rhamnose moiety and the phenyl group.Comparison of these spectral data to those previously reported in literature (Hammami *et al.,* 2006),it is suggested that ML **7** is 1-*O*-β-D-rhamnopyranosyl- 4-ethylphenylbut-2-one.

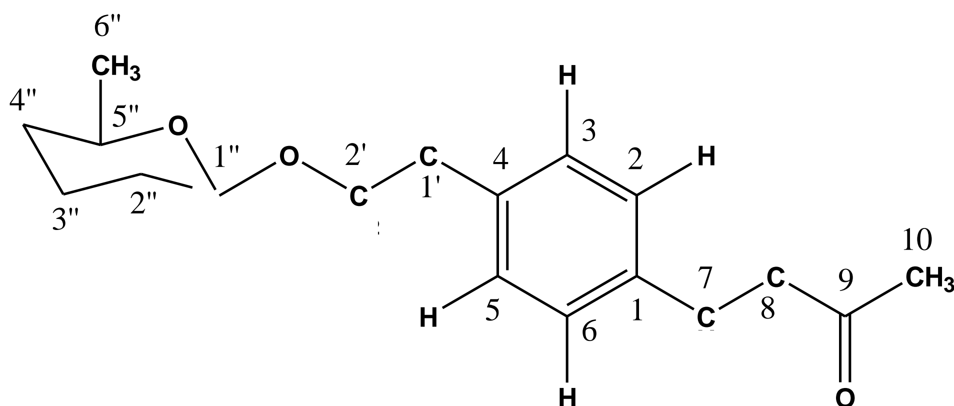


Figure 5.6: Structure of ML 7

5.2 Pharmacological Studies

The Preliminary acute toxicity studies suggest that, *intraperitoneally*, the crude methanol extract LD_{50} of 1265 mg/kg body weight (*i.p*) is slightly toxic according to classification of toxicity (Loomis and Hayes, 1996; Matsumura, 1975). It is therefore recommended that these drugs should be used with caution.

Acetic acid induced writhing in mice attributed to visceral pain finds much attention in screening analgesic drugs (Hasan *et al.*, 2010). Writhes can be described as a wave of constriction and elongation passing caudally along the abdominal wall with twisting of the trunk and extension of the hind limbs in mice. This is due to the nociceptive property of acetic acid (Surender and Mafumdar, 1995). The results showed that extract of the aerial parts of *Ampelocissus grantii* significantly ($P<0.001$) reduced the number of abdominal constriction induced by acetic acid in mice, and in a dose dependant manner since 300 mg/kg extract showed higher inhibition (95.19 %) relative to other doses. The percentage of inhibition, clearly indicated that the extract at all the doses tested (75, 150 and 300 mg/kg) produced a higher inhibition when compared with ketoprofen (20 mg/kg) a known standard analgesic drug.

Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting in the release of free arachidonic acid from tissue phospholipids (Ahmed *et al.*, 2006) via cyclooxygenase (COX), and prostaglandin biosynthesis (Duarte *et al.*, 1988). In other words, the acetic acid-induced writhing has been associated with increased level of PGE_2 and $PGF_{2\alpha}$ in peritoneal fluids as well as lipoxygenase products (Derardt *et al.*, 1980; Besra *et al.,* 1996). The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability (Zakaria and Abdul Ghani 2008). The acetic acid induced writhing method was found effective to evaluate mild analgesic and nosteroidal anti-inflamatory compounds (Berkenkoff and Weichman, 1988). The agent reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (Duarte *et al.*, 1988; Ferdous *et al.*, 2008). The significant pain reduction of the plant extract might be due to the presence of analgesic principles acting through the prostaglandin pathways. The acetic acid –induced writhing test is very sensitive and able to detect anti-nociceptive effects of compounds at dose levels that may appear inactive in other methods like tail flick test (Collier *et al.*, 1968; Bentley *et al.*, 1983). However, The abdominal writhing induced by acetic acid was also reported to be less selective (Collier *et al.*, 1968) and proposed to act indirectly by releasing endogenous mediators stimulating neurons that are sensitive to other drugs such as narcotics and centrally acting agents (Toma *et al.*, 2003).

Thermally-induced pain stimulus like hot plate test is used to selectively evaluate centrally acting analgesics (Chau, 1989) and predicts opioid-like analgesic compounds (Janssen *et al.*, 1963). The hot plat test measures the complex response to a non-inflammatory, acute nociceptive input and is one of the models normally used

for studying central nociceptive activity (Sabina *et al*., 2009). It is known that centrally acting analgesic drugs elevate the pain threshold of mice towards heat and pressure (Ibironke and Ajiboye, 2007). *Ampelocissus grantii* extract exhibited no significant increase in pain latency at a dose of 50mg/kg body weight, but at higher doses of 150 and 300 mg/kg body weight it increased significantly ($P < 0.05 – 0.01$) the pain threshold in dose related manner (Table 4.13). Therefore, the methanol extract of the plant have a central activity. Again, narcotic analgesics inhibit both peripheral and central mechanism of pain, while NSAIDs inhibit only peripheral pain (Elisabetsky *et al*., 1995; Pal *et al*., 1999). The analgesic effect of the aerial parts of *A. grantii* has not been reported. From the above findings, the extract inhibited both mechanisms of pain, suggesting that the plant extract may act as a narcotic analgesic. Such a mode of action is proposed for opioid analgesic such as morphine. The analgesic effect of the plant in both models suggests that the plant have been acting through central and peripheral mechanism (Sabina *et al*., 2009).

Variation in order of activity of themethanol extract of *A. grantii* in acetic acid-induced writhing, and hot plate tests indicated that the different constituents present in different extract may be responsible for central and peripheral analgesia.

The extract caused marked inhibition of carrageenan induced oedema in rats. Carrageenan induced inflammatory process is believed to be biphasic (Vinegar *et al*., 1969). The early phase seen at the first and second hours is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings (Crunkhon and Meacock, 1971). The late phase which occurs during the second to third hours is sustained by prostaglandins release and mediated by bradykinins, leukotrienes; polymorphonuclear cells and prostaglandins produced tissue macrophages (Brooks and Day, 1991).

From the results, it is clear that the extract have been able to control the increase in paw oedema in the early phase; however it has exhibited significant activity at the third hour and further time point, which support the fact that it is related to the inhibition of prostaglandins release which inhibits the second phase of inflammation. Hence, it can be said that the anti-inflammatory activity of methanol extract might be due to its action on the early and later phase of inflammation.

A. grantii (Baker) Planch aerial parts methanol extract contains tannins, flavonoids, terpenoids, saponins, and other phytochemical constituents (Musa*, et al.,* 2010). Analgesic and anti-inflammatory effects have been observed in flavonoids as well as tannins (Ahmadiani *et al*., 2000). Flavonoids have been shown to possess antinociceptive and anti-inflammatory mechanisms by targeting reactive oxygen species and prostaglandins which are involved in the late phase of acute inflammation and pain perception (Hesham and El-Seedi, 2007; Rajnarayana, *et al*., 2001; Rao *et al*., 1998).There are also reports on the role of terpenoids in anti-nociceptive and anti-inflammatory activities. Saponins are also well known for their ability to inhibit pain perception and inflammation (Choi *et al*., 2005; Sparg *et al*., 2004). At present, the compounds isolated could be responsible for the analgesic and anti-inflammatory activities of the extract of the aerial parts of *Ampelocissus grantii*. But it is difficult, at this stage, to draw any logical conclusion on the mechanism of action of such diverse mixture of phytochemical compounds contained in the extract of the aerial parts of *Ampelocissus grantii* examined in this study.

Although there is paucity of literature on the pharmacological actions of the extract of the aerial parts of *Ampelocissus grantii*, the compounds isolated and other bioactive constituents such as tannins, other flavonoids, saponins, and triterpenoids of the plant

are speculated to account for the analgesic and anti-inflammatory actions of the extract of the plant. Indeed, published biological effects of flavonoids, saponins, triterpenoids and tannins appear to justify some of the traditional, folkloric uses of the plant aerial parts in the control and/or management of painful and inflammatory conditions. Although the exact phytochemical constituents of the plant that is responsible for the analgesic and anti-inflammatory effects of the extract still remain speculative, a number of investigations have shown that a host of secondary plant metabolites with diverse chemical structures possesses analgesic and anti-inflammatory effects in various experimental animal models (Dongmo *et al.,* 2003; Ojewole, 2003; Onasanwo and Elegbe, 2006). Since the extract of the aerial parts of *Ampelocissus grantii* contains tannins, flavonoids, saponins, and triterpenoids (in addition to other phytochemical compounds), it is not unlikely that these phytochemical compounds might have contributed to the observed analgesic and anti-inflammatory effects of the plant's aerial parts extract. Several investigators have indeed attributed the analgesic and anti-inflammatory properties of plants to their tannins, triterpenoids, flavonoids, and saponins constituents (Gupta *et al.,* 1969; Ahmadiani *et al.,* 2000; Choi *et al.*, 2005). Since the extract is reported to contain tannins, flavonoids, saponins and triterpenoids, it may be reasonable to speculate that the analgesic and anti-inflammatory properties of the plant extract observed in this study could be due respectively, to the compounds isolated or one or more of the above phytochemical constituents of the plant.

5.3 Antimicrobial Studies

The indiscriminate use of commercial antimicrobial drugs has led to multiple drug resistant strains (Motamedi *et al*., 2010). Beyond the increasing prevalence of drug resistance, some synthetic antibiotics are demonstrating undesirable side effects. This

has highlighted the urgent need for new drugs particularly affordable for poorest countries (Raja *et al*., 2010). In many parts of the world, the extracts of medicinal plants are used for their antibacterial, antifungal and antiviral properties (Hassawi and Kharma, 2006).

The results have shown that the aerial parts of *A. grantii* possess a wide range of antimicrobial activity against both Gram positive (*Staphylococcus aureus, Streptococcuspyogenes*, *Bacillussubtilis*), Gram negative (*Escherichia coli, Klebsiellapneumonia, Pseudomonas aeruginosa*) and yeast (*Candidaalbicans).*This, therefore, shows that the extract contains substance(s) that can inhibit the growth of some microorganisms. Other workers have also shown that extracts of some plants inhibited the growth of various microorganisms (Akujobi *et al*., 2004; Nweze *et al*., 2004; Osadebe and Ukwueze, 2004). The observed antimicrobial effects on the isolates is believed to be due to the presence of the isolated compounds or other constituents like the saponinsand tannins which have been shown to possess antimicrobial properties (Ragasa *et al*., 2005; Draughon, 2004; Cuhnie and Lamb 2006; Soetan *et al*., 2006) and reportedly exert their effects by affecting the cell membrane integrity of the bacteria (Hendrich, 2006; Trombetta *et al*., 2005). Some workers have also attributed their observed antimicrobial effects of plant extracts to the presences of these secondary metabolites (Nweze *et al*., 2004).

The large zone of inhibition exhibited by the extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa*indicate that the extract can be used for the treatment of sores and open wounds. *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been implicated in cases of boils, sores and wounds. The extract has showed a good level of activity against *Escherichia coli* which is the commonest cause of urinary tract infection and accounts for approximately 90% of first urinary tract infection in

young women. Also the high growth inhibition against *Escherichia coli*shows that it can be used in the control of diarrhea and dysentery. *Escherichia coli*are the common cause of traveller's diarrhea and other diarrheagenic infections in humans (Brooks *et al*., 2002 and Usman *et al*., 2007).

In view of the fact that prevalence of *Staphylococcus aureus* resistant strains to conventional antibiotics has increased to high levels in some hospitals (Shalit *et al*., 1989 and Usman *et al*., 2007) and that *Staphylococcus aureus* is a pyogenic bacterium known to play significant role in invasive skin diseases including superficial and deep follicular lesion (Srinivan *et al*., 2001). The extract could serve as a remedy to such resistance folklorically. The sensitivity of *Candida albicans* to the methanol extract of the plant has increased the chemotherapeutic potentials of using the active compounds of *A. grantii* in the treatment of diseases such as oral and virginal candidiasis.

The inability of the extract to inhibit the growth of *Corynebacterium ulcerus, Salmonella typhi,* and *Neisseriagonorrhea*may be that they possess a mechanism for detoxifying the active principle in the extract. Some bacteria are known to possess mechanisms by which they convert substances that inhibit their growth to non-toxic compounds. For example *Staphylococcus aureus* produce the enzyme penicilinase which converts the antibiotic penicillin to penicillinoic acid which is no longer inhibitory to its growth (Singleton, 1999).

It is worthy of note that MBC values obtained for the extract against the pathogens are higher than MIC, indicating that the extracts are bacteriostatic at lower concentrations and bactericidal at higher concentrations. This suggests that the plant extract, when used traditionally as antimicrobials inhibit bacteria growth without necessarily killing the bacteria and since most of the traditional preparations lack

specific concentrations, this may thus account for the use of large quantity of the extract by traditional medical practitioners for the treatment of their patients.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Preliminary phytochemical screening of the methanol extract of *Ampelocissus grantii* revealed the presence of flavonoids, saponins, tannins, and steroids.

Extensive phytochemical investigation led to the isolation of flavonoids glycosides, Quercetin 3-O-[α-L- glucopyranosyl-(1'''→6'')-β- D- glucopyranosyl]- 4'-O- β- D-glucopyranoside, phenolic glycoside, 1-*O*-β-D-rhamnopyranosyl- 4-ethylphenylbut-2-one.

Pharmacological studies on the methanol extract showed that the extract exhibited analgesic and anti-inflammatory activity. The extract also showed antimicrobial activity against both Gram positive (*Staphylococcus aureus, Streptococcuspyogenes*, *Bacillus subtilis*), Gram negative (Escherichia *Coli, Klebsiela pneumonia, Pseudomonasaeruginosa*) and yeast (*Candidaalbicans).*

6.2 Conclusion

The methanol extract of *A. grantii* aerial parts was found to be promising antinociceptive and anti-inflammatory against pain and inflammation and also antimicrobial agent against human pathogens. Four compounds were isolated and identified from the acetone and n-butanol soluble fraction of the methanol extract that could be responsible for the observed biological activity, namely as 3', 4', 5, 7-tetrahydroxyflavone (Luteolin), isorhamnetin 3 - O – β – D – glucopyranosyl – (1''' - 6'') – β – D – glucopyranoside, quercetin 3 - O - α – L – glucopyranosyl – (1''' - 6'') – β – D –glucopyranoside and 4 – O – β – D – rhamnopyranosyl – 4 – ethyl phenyl but – 2 – one. Based on the findings in this work, it can be concluded that the use of

Ampelocissus grantii in the treatment of pain, inflammation and stomach disorder has scientific basis. To the best of our search this is the first time these activities are being reported. The analgesic and anti-inflammatory activities observed with the extract can be linked in part to the compounds isolated.

6.3 Recommendations

More detailed studies should be carried out on the analgesic, anti-inflammatory and antimicrobial properties of the portion of the methanol extract of the aerial parts of the plant.

Further work should also be carried out to isolate the bioactive compound(s) responsible for the observed analgesic and anti-inflammatory activities of the crude methanol extract of the aerial parts of *Ampelocissus grantii.*

There is need to carry out more toxicity studies on the plant so as to rule out the possibility of any side effect that may arise as a result of the prolong use of the plant.

REFERENCES

Aderbauer, B., Clausen, P. H., Kershaw, O., Melzig, M. F. (2008). *In vitro* and *in vivo* trypanocidal effects of lipophilic extracts of medicinal plants from Mali and Burkina Faso. *Journal of Ethnopharmacology*, 119:225-231.

Adjanohourn, E., Ahyi, M. R. A., Ake Assi, L., Dan Dicko, L., Daouda, H., (1980). *Contribution Aux Etudes Ethnobotaniques et Florisques au Niger* ACCT Paris ISBN 92-9028-009-3 pp.250.

Agrawal, P. K. (1992). NMR Spectroscopy in the structural elucidation of oligosaccharides and glycosides.*Phytoche*mistry. 31: 3307-3330.

Ahmadiani, A., Hosseiny, J., Semnanian, S., Javan, M., Saeedi, F., Kamalinejad, M., and Saremi, S. (2000). Antinociceptive and anti-inflammatory effects of *Elaeagnus angustifolia* fruit extract. *Journal of Ethnopharmacolog* 72: 287–292.

Ahmadu, A. A., Onanuga, A., and Aquino, R. (2010).Flavonoid glycosides from the Leaves of *Cissus ibuensis* Hook (Vitaceae), *African Journal of Traditional, Complementary and Alternative Medicine*, 7 (3): 225 - 230

Ahmed. F., Hossain, M. H., Rahman, A. A., Shahid, I. Z. (2006).Antinociceptive and sedative effects of the bark of *Cerbera odollam* Gaertn.*Oriental Pharmacy and Experimental Medicine*, **6:** 344-348.

Akujobi, C., Anyanwu, B. N., Onyeze, C., Ibekwe, V. I. (2004).Antibacterial activities and preliminary phytochemical screening of four medicinal plants. *Journal of Applied Sciences*, 7(3); 4328 – 4338.

Ansari, J.A. and Inamdar, N.N. (2010). The promise of traditional medicine, *International Journal of Pharmacology,* 6:808-812.

Aviram, M. and Fuhrman, B. (1998). LDL oxidation by arterial wall macrophages depends on the oxidative status in the lipoprotein and in the cells: role of prooxidants vs. antioxidants. *Molecular and Cellular Biochemistry,* 188, 1-2, 149-159.

Bah, S., Diallo, D., Dembels, S., Paulsen, B. S. (2006). Ethnophamcological survey of plants used for the treatment of schistosomiasis in Niano District, Mali,*Journal of Ethnopharmacology*, 105**:**387-399.

Balandrin, M. F., Kinghorn, A. D. and Farnsworth, N. R. (1993). Plant derived natural products in drug discovery and development: an overview. In: Human medicinal agents from plants. King horn, A. D. and Balandrin, M. F. (Eds).American Chemical Society, Washington DC.USA pp. 2-12.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M. (1966): Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology,* 45: 493-496.

Barnes, P. M. (2002). Complementary and alternative medicine use among adults, *United states Advance Data,*343:1-19.

Bentley, G.A., Newton, S.H., Starr, J. (1983). Studies on the anti-nociceptive action of α-agonist drugs and their Interaction with opioid mechanisms. *British Journal of Pharmacology,* 79:125-134.

Berkenkoff, J.W. and Weichmann, B.M. (1988). Production of prostacyclin in mice following peritoneal injection of acetic acid, phenylbenzoquine and zymosan: Its role in writhing response. *Prostaglandins*, 36: 693-709.

Besra, S.E., Sharma, R.M. and Gomes, A. (1996). Anti-inflammatory effect of petroleum ether extract of leaves of *Litchi chinensis* Gaertn (Sapindacea). *Journal of Ethnopharmacology*, 54: 1-6.

Besson, J. M. (1999). The neurobiology of pain. *The Lancet* 353: 1610-1615.

Bhurani, S.P., Chibber, S.S., Seshadri, T.R. (1969). *Components of the root of pueraria tuberose, Phytochemistry,* 8: 290 – 301.

Bitis, L., Kultar, S., Melikoglu, G., Ozsoy, N., Can, A. (2003). Flavonoids and antioxidant activity of *Rosa agrestis* leaves, *Natural Product Radiance*, 24: 580-589.

Bizimana, N., Tietjen, U., Zessin, K. H., Diallo, D., Djibril, C., Melzig, M. F., Clausen, P. H. (2006). Evaluation of medicinal plants from Mali for their *in vitro* and *in vivo* trypanocidal activity,*Journal of Ethnopharmacology*, 103:350-356.

Brain, K. R. and Turner, T. D. (1975).The practical evaluation of phytopharmaceuticals.Wright-Scientechnica, Bristol. UK Pp140- 144, 152-154.

Brooks, G. F., Butel, J. S. and Morse, S. A. (2002).Jawetz, Melnick and Adelberg's Medicinal Microbiology, (2nd Edn), McGraw-Hill, New Delhi, India, pps 197, 234, 550.

Brooks, P. M. and Day, R. O. (1991). Non steroidal anti-inflammatory drugs difference and similarities.*New England Journal of Medicine,*324**:**1716-1725.

Burkill, H. M. (2000) The useful plants of West Tropical Africa, 2nd Ed. Royal Botanical Garden, Kew London, UK. pp 969.

Calixto, J. B., Beirith, A., Ferreira, J., Santos, A. R. S., Filho, V. C., Yunes, R. A. (2000). Naturally occurring antinociceptive substances from plants. *Phytotherapy Research* 14: 401-418.

Castello, M. C., Phatak, A., Chandra, N., Sharon, M. (2002). Antimicrobial activity of crude extracts from plant parts and corresponding callis of *Bixa orellana* L. *Indian Journal of Experimental Biology*. 1378-1381.

Chau, T. (1989).Pharmacological methods in the control of inflammation. *In*: *Modern Methods in Pharmacology,* vol. 5, Alan, R. (Ed). Liss inc. New York, USA pp. 195-212.

Choi, J., Jung, H., Lee, K., Park, H. (2005). Antinociceptive and anti-inflammatory effects of saponins and sapogenin obtained from the stem of *Akebia quinata*. *Journal of Medicinal Food*, 8 (1): 78-85.

Claridge, T. (1999).*High-Resolution NMR Techniques in Organic Chemistry* (1st edition), Elsevier, ISBN 978-0080427997, UK.

Collier, H. O. J., Dinnean, L. C., Johnson, C. A. and Schenider, C. (1968). The abdominal constriction response and its suppression by analgesic drugs in the mouse *British Journal of Pharmacology*, 32:295-310.

Cordel, G. A., Quinn-Beattice, M. L. and Farnsworth, R. (2001).The potential of alkaloids in drug discovery. *PhytotherapyResearch,* 15:183-205.

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Review* 12: 564-582.

Cragg, G.M. and Newman, D.J. (2013). Natural products: A continuing source of novel drug leads.*Biochim.Biophys. Acta*, 1830, 3670–3695.

Croteau, R., Kutchan, T. M. and Lewis, N. G. (2000). Natural products (secondary metabolites).*In Biochemistry and Molecular Biology of Plants* (Edited by Buchanan, B., Gruissen, W., Jones, R.). American Society of Plant Physiology, Rockville Maryland USA pp.1250-1318.

Crunkhon, P. and Meacock, S. E. R. (1971). Mediators of inflammation induced in rats paw by carrageenan. *British Journal of Pharmacology,* 42:392-402

Cuhnie, T. P. and Lamb, A. J. (2006). Antimicrobial activity of flavonoids, *International Journal of Antimicrobial Agents,* 26:342-356.

Cushnie, T. P. T. and Lamb, A. J. (2005). Antimicrobial activity of flavonoids, *International Journal of Antimicrobial Agents*, 26: 343-356.

Daigle, D.J. and Conkerton, E. (1988). Analysis of flavonoids by HPLC: an update. *Journal of Liquid Chromatography,* 11: 309-325.

Dalziel, J. M. (1937). *The Useful plants of West Tropical Africa,* Crown Agents for Colonies, London, UK pp. 238-239.

David, B., Wolfender, J.L.and Dias, D.A. (2014). The pharmaceutical industry and natural products:Historical status and new trends. *Phytochemistry Review*, 1–17.

Derardt, R., Jougney, S., Delvalcee, F., Falhout, M. (1980).Release of prostaglandin's E and F in an algogenic reaction and its inhibition, *European Journal of Pharmacology,* 51:17-24.

Dongmo,A. B., Kamanyi, A., Dzikouk, G. (2003).Anti-inflammatory and analgesic properties of the stem-bark extract of *Mitragyna ciliate* (Rubiaceae) Aubrev, & Pellegr. *Journal of Ethnopharmacology,* 84:17-25.

Draughon, F. A. (2004). Use of Botanicals as biopreservatives in Foods. *Food Technology*, 58(2); 20-28.

Duarte, I. D. G., Nakamura, M., Ferreira, S. H. (1988).Participation of the sympathetic system in acetic acid-induced writhing in mice. *Brazilian Journal of Medicinal and BiologicalResearch*, 21:341–343.

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

Ekeanyanwu, C.R. (2011). Traditional medicine in Nigeria: Current status and the future, *Rearch Journal of Pharmacology,* 5(6): 90-94.

Elisabetsky, E., Amador, T. A., Albuquerque, R. R., Nunes, D. S., Cavalho, A. C. T. (1995).Analgesic activity of *Psychotria colorataJournal of Ethnopharmacology*, 48:77-83.

Etuk, E. U., Ugwah, M. O., Ajagbonna, O. P., Onyeyili, P. A. (2009). Ethnobotanical survey and preliminary evaluation of medicinal plants with antidiarrhoea properties in Sokoto state, *Nigerian Journal of Medicinal plants Research*, 3(10):763-766.

Exarchou, V., Troganis, A., Gerothanassis, I.P., Tsimidou, M., Boskou, D. (2002). Do strong intramolecular hydrogen bonds persist in aqueous solution? Variable temperature gradient ^{1}H, $^{1}H–^{13}C$ GE-HSQC and GE-HMBC NMR studies of flavonols and flavones in organic and aqueous mixtures. *Tetrahedron,* 58, 37, 7423-7429.

Farnsworth, N. R., and Morris, R. W. (1976). Higher plants: The sleeping giants of drug development. *American Journal of Pharmacy,* 147:46-52.

Felicio, J. D., Santos, R. da S. and Gonçalez, E. (2001). Chemical constituents from *Vitis vinifera* (Vitaceae)**,** *Arq. Inst. Biol.*, São Paulo, 68(1):47-50.

Ferdous, M., Rouf, R., Shilpi, J. A., Uddin, S. J. (2008). Antinociceptive activity of the ethanolic extract of *Ficus racemosa* Linn.(Moraceae).*Orient.Pharm. Expl. Med.*, **8:**93-96.

Ferreira, J. F. S., Luthria, D. L., Sasaki, T., Heyerick, A. (2010). Flavonoids from *Artemisia annua* as antioxidants and their potential synergism with Artemisinin against malaria and cancer, *Molecules* 2010; 15: 3135-3170.

Fossen, T. and Anderson, Q. M. (2005). Spectroscopic Technique Applied to Flavonoids, In: *Flavonoids – Chemistry, Biochemistry and Applications, Anderson, Q. M., Markham*, K. R. (37 – 42) Taylor and Francis, USA.

Fraga, C.G., Galleano, M., Verstraeten, S.V. & Oteiza, P.I. (2010).Basic biochemical mechanisms behind the health benefits of polyphenols.*Molecular Aspects of Medicine,* 31(6): 435-445.

Gessler, M. C., Nkunya, M. H. H., Mwsumbi, L. B., Heinrich, M., Tanner, M. (1994).Screening of Tanzanian medicinal plants for antimalarial activity.*Acta Tropics*56**:**65-77.

Gibbons, S. and Gray, A. I. (1998). ''*Isolation by planer chromatography*''. In: Cannell, R. J. P. (Ed) Natural products isolation; Humana press, Totowa New Jersey (USA), pp. 209-246.

Gomes, A., Fernandes, E., Lima, J.L.F.C., Mira, L. Corvo, M.L. (2008). Molecular mechanisms of anti-inflammatory activity mediated by flavonoids, *Current Medicinal Chemistry*, 15, 1586-1605.

González, M. P.,Terán,C.,Teijeira, M. (2007). Search for new antagonist ligands for adenosine receptors from QSAR point of view. How close are we? *Medicinal Research Reviews*, 28, 3, 329-371.

Gupta, M. B., Bhalla, T. N., Gupta, G. B. (1969). Anti-inflammatory activity of natural products I. Triterpenoids. *EuropeanJournal of Pharmacology,*6:67-70.

Hammami, S., Ciavatta, M. L., Ben Jannet, H., Cimino, G., Mighri, Z. (2006). Three phenolic and a sterol glycosides identified for the first time in *Matthiola longipetala* growing in Tunisia *Croatian Chemical Acta* 79(2): 215-218.

Harborne J. B. (1973) Phytochemical methods: Chapman and Hall publication, London, U.K. pp.52-56, 66-70, 212-213.

Harold, C. N. (1992). The crisis in antibiotic resistance. *Science* 257:1064-1072.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids, *Pharmacology and Therapeutics* 96: 67-202.

Hasler, A., Sticher, O. and Meier, B.J. (1990). HPLC Determination of five widespread flavonoid aglycones, *Journals of Chromatography*. 508: 236 – 240.

Hasler, A., Sticher, O., Meier, B.J. (1992). Identification and determination of the flavonoids from *Ginkgo biloba* by HPLC, *Journal of Chromatography A. 605:* 41 – 48.

Hassawi, D. and Kharma, A. (2006). Antimicrobial activity of some medicinal plants against *Candida albicans*, *Journal of Biological Sciences*, 6: 109-114.

Hassan, H. S., Ahmadu, A. A. and Hassan, A. S. (2010).Analgesic and anti-inflammatory activities of *Asparagus africanus* root extract, *African Journal of Traditional, Complementary and Alternative Medicine,* 5(1): 27-31

Heim, K. E., Tagliaferro, A. R. and Bobliya, D. J. (2002). Flavonoids antioxidants: Chemistry, metabolism and structure-activity relationships. *The Journal of Nutritional Biochemistry,* 13: 572-584.

Hendrich, A. B. (2006). Flavonoid-membrane interactions; possible consequences for biological effects of some polyphenolic compounds. *Act. Pharmacology*. Sinica 27 (1): 27-40.

Henry, C. M. (2000).Antibiotic resistance, Chemical engineering news. March 6:41-58.

Herz, W., Bhat, S.V., Crawford, H., Wagner, H., Maurev, G., Farkas, L. (1972).Bahifolin, a New Sesquiterpene Ketone and 5,7–dihydroxy–3, 3', 4', 6– tetra Methoxy Flavone from Bahis Oppositotia.*Phytochemistry.*11: 2825-2826.

Hesham R., El-Seedi, Shgeru N. (2007).Chemistry of bioflavonoids, *Indian Journal Pharmaceutical Education,* 39:172.

Higa T, Anka J, Kitamara A, Koyama T, Akshashi M, Uchida T (1994). Bioactive compounds from marine sponges. *Pure and Applied Chemistry* 66: 2227-2236.

Hostettman, K. (1998). The strategy for the biological and chemical evaluation of plant extracts. *Pure and Applied Chemistry,* Vol. 70 No. 11.

Hostettman, K., Marston, A. and Wolfendy, J. L. (1995). In: Phytochemistry of plants used in traditional medicines, Hostettman, K., Marston, A., Mainland, M., Hamburger, M. (Eds), Clareton Press, UK pp.17-45.

Hunt, S. P. and Mantyh, P. W. (2001). The molecular dynamics of pain control. Nature Reviews. *Neuroscience* 2(2): 83-91.

Ibironke, G. F. and Ajiboye, K. I. (2007). Studies on the anti-inflammatory and analgesic properties of *Chenopodiumambrosioides* leaf extract in rats. *International Journal Pharmacology*, 3:111-115.

Inngjerdingen, K., Nergard, C. S.,Diallo, D., Mounkoro, P. P., Paulsen, B. S. (2004). Ethnophamcological survey of plants used for wound healing in Dogoland, Mali, West Africa,*Journal of Ethnopharmacology*, 92:233-244.

Iwu, W. M., Duncan, A. R. and Okunji, C.O. (1999).New antimicrobials of plant origin.In Perspectives on new crops and new uses.Jannick J. (Ed) pp. 457-462 ASHS Press, Alexandra, VA.

Janssen, P.A.J., Niemegeers, C.J.E., Dony, J.G.H. (1963). The inhibitory effect of fentanyl and other morphine-like analgesics in the warm water-induced tail withdrawal reflex in rats. *Drug Research*, 6: 502-507.

Jansen, R. K., Kaittanis, C., Lee, S. B. , Saski, C., Tomkins, J., Alverson, A. J., Daniell, H. (2006). Phylogenetic analyses of *Vitis* (Vitaceae). *BMC Evolutionary Biology* 6: 32 [published online, 14 pp.].

Kaul, T. N., Middleton, F. Jr. and Ogra, P. L. (1985). Antiviral effects of flavonoids on human viruses, *Journal of Medical Virology,* 15:75-79.

Khan, M. A., Nabi, S. G., Prakash, S., Zaman, A. (1986).Pallidol, a resveratrol dimer from *Cissus pallida.Phytochemistry*, 25:1945-1948.

Kone, W. M., Kamanzi Atindehou, K., Terreaux, C., Hostettmann, K., Traore, D., Dosso, M. (2004).Traditional medicine in North Cote-d'Ivoire screening of 50 medicinal plants for antibacterial activity.*Journal of Ethnopharmacology,* 93: 43-49.

Koster, R., Anderson, M. and de Beer, E. J. (1959).Acetic acid for analgesic screening. Federation Proceedings 18**:**412.

Kozerski, L., Kamieński, B., Kawecki, R., Urbanczyk-Lipkowska, Z., Bocian, W., Bednarek, E., Sitkowski, J., Zakrzewska, K., Nielsen, K.T., Hansen, P.E. (2003). Solution and solid state ^{13}C NMR and X-ray studies of genistein complexes with amines. Potential biological function of the C-7, C-5, and C4'-OH groups. *Organic and Biomolecular Chemistry*, 1, 20, 3578-3585.

Krishnamurthy, V. (2000) ^{2}J, ^{3}J-HMBC: A New Long-Range Heteronuclear Shift Correlation Technique Capable of Differentiating ^{2}JCH from ^{3}JCH Correlations to Protonated Carbons. *Journal of Magnetic Resonance,* 146, 1, 232-239.

Kumar, M., Shete, A. and Akbar, Z. (2010).A Review on Analgesic: From Natural Sources*International Journal of Pharmaceutical and Biological Archives* 1(2): 95 – 100

Lambert, J.D. and Elias, R. J. (2010). The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention. *Archives of Biochemistry and Biophysics,* 501, 1, 65-72.

Lanhers, M. C., Fleurentin, J., Mortier, F., Vinche, A. and Younos, C. (1992).Anti-inflammatory and analgesic effects of an aqueous extract of *Harpagophytum procumbens. Plant medica,* 58:117-123.

Li, W. and Fitzloft, J.F. (2002). HPLC Determination of flavonoids and terpenes lactones in commercial *Ginkgo biloba* Products. *Journal of Liquid Chromatography and Related Technology.*25: 2501-2514.

Li, F., Li, Q., Gao, D., Peng, Y. (2009). The optimal extraction parameters and anti-diabetic activity of flavonoids from *Ipmoea batatas* leaf, *African Journal of Traditional, Complementary and Alternative Medicines* 6: 195-202.

Lin, C., Hwang, T., Lin, S., Huang, Y. (2012).Bioactive isocoumarins from *Cissus pteroclada, Journal of Chinese Medicine* 23(1): 41-49, 2012.

Loomis, T. A. and Hayes, A.W. (1996).Loomis's essentials of toxicology. 4th ed., California, *Academic press*: 208- 245.

López-Lázaro M. (2009).Distribution and biological activities of the flavonoid luteolin. Mini-Reviews in Medicinal Chemistry, 9(1): 31-59.

Lorke, D. (1983). Approach to Acute Toxicity Testing. Arch. Toxicology. 54:275-287.

Lucas, S. M., Rothwell, N.J., Gibson, R.M. (2006).The role of inflammation in CNS injury and disease.*British Journal Pharmacology, 147*, S232–S240.

Mabry T. J. (1970). The systematic identification of flavonoids, Springer Verlag: New York, USA

Malizos, K. N. (2009). Do steroids, conventional non-steroidal anti-inflammatory drugs and selectiveCox-2 inhibitors adversely effect fracture healing. J Musculoskelet Neuronal Interact.;9:44-52.

Markham, K. R. (1975). Isolation techniques for flavonoids. In: The Flavonoids. Harbone, J. B., Mabry, T. J., and Mabry, H. (Eds) Chapman and Hall, London, U.K. pp. 2-31.

Markham, K. and Geiger, H. (1994).1H nuclear magnetic resonance spectroscopy of flavonoids and their glycosides in *hexadeuterodimethylsulfoxide.*In *The Flavonoids*; Chapman & Hall: London, UK, pp. 441–498.

Matsumura, F. (1975). Toxicology of Inscticides. Plenum Press, New York and London Pp24-26

Medzhitov, R. (2008**)**. Origin and physiological roles of inflammation.*Nature.454*, 428-435.

Merfort I., Wray V., Barakat H. H., Hussein S. A. M., Nawwar M. A. M., and Willuhn G. (1997), Flavonol triglycosides from seeds of *Nigella sativa*.*Phytochemistry*46, 359-363.

Metcalfe, C. R., and Chalk ,L.(1950). Anatomy of Dicot Leaves, Stem, and Wood in Relation to Taxonomy, with Notes on Economic Uses, Vol.1. 413-419. Oxford Clarendon Press UK.

Middleton, E. (1984).The flavonoids. *Trends in pharmacological sciences,* 5:335-338

Middleton, E. J. R., Kandaswami, C. and Theoharides, T. C. (2000). The Effects of Plant Flavonoids on Mammalian cells: Implications for inflammation, heart disease, and cancer, *Pharmacological Reviews*, 52: 673-751.

Motamedi, H., Darabpour, E., Gholipour, M., Seyyed Nejad, S.M. (2010). Antibacterial effect of ethanolic and methanolic extracts of *Plantago ovata* and *Oliveria decumbens* endemic in iran against some pathogenic bacteria, *International Journal of Pharmacology*, 6: 117-122.

Muhammad, S., Amusa, N. A. (2005).The important food crops and medicinal plants of north western Nigeria.*Research Journal of BiologicalSciences*1 (13):254-260.

Mukherjee, P.K. (2008). Quality control of herbal drugs.Business Horizon Pharmaceutical Publishers,pp. 13.

Musa, Y. M., Sule, M. I., Pateh, U. U., Ilyas, M., Musa, A. M., Usman, H and Abdullahi, M.S. (2010). Preliminary phytochemical and antimicrobial studies of methanol extract of the aerial parts of *Ampelocissus grantii* (Baker) Planch. *International Journal of Chemical Sciences,* 3(2):167-174.

Newman, D.J. and Cragg, G.M. (2012).Natural products as sources of new drugs over the 30 years from1981 to 2010.*Journal of Natural Products,* 75: 311–335.

Nweze, E. T. Okafor, J. I., Njoku, O. (2004). Antimicrobial Activities of Methanolic extract of *Trumeguineesis* (Schumm and Thorn) and *Morinda Lucinda* Benth used in Nigerian Herb Medicinal Practice. *Journal of BiologicalResearch and Biotechnology,* 2 (1): 34-46.

Odabsoglu, F., Cakir, A., Suleyman, H., Aslan, A., Bayir, Y., Halici, M., Kazaz, C. (2006). Gastroprotective and antioxidant effects of usnic acid an indomethacin-induced gastric ulcer in rats.*Journal of Ethnopharmacology*, 103: 59-65.

Ojewole, J. A. O., (2003). Evaluation of the anti-inflammatory properties of *Sclerocarye birrea* (A. Rich) Hochst. (Family: Anacardiceae) stem-bark extracts in rats. *Journal of Ethnopharmacology*85:217-220.

Onasanwo, S. A. and Elegbe, R. A. (2006). Anti-nociceptive and anti-inflammatory properties of the leaf extracts of *Hedranthera barteri* in rats and mice. *African Journal of Biomedical Research,* 9(2): 109-118.

Osadebe, P. O. and Ukwueze, S. E. (2004). A Comparative Study of the Phytochemical and Antimicrobial Properties of the Eastern Nigerian Species of Mistletoe (*Loranthus micranthus*) Sourced from Different Host Trees. *Journal of Biological Research and Biotechnology,* 2(1); 18-23.

Pal, S., Sen, T., Chaudhuri, A. K. (1999). Neuropsychopharmacological profile of the methanolic fraction of *Bryophyllum pinnatum* leaf extract.*Journal of Pharmaceutical Pharmacology,* 51:313-318.

Pal, R. S., Ariharasivakumar, G., Girhepunjhe, K., Upadhay, A. (2009). In-vitro antioxidative activity of phenolic and flavonoids compounds extracted from seeds of *Abrus precatorius*, *International Journal of Pharmacy and Pharmaceutical Sciences* 1: 136-140.

Pande, P. C., Lalit Tiwari, H. C. Pande. (2007) *Indian Journal of Traditional Knowledge*, 6(3): 444-458.

Pandey, M. M. Rastogi, S. and Rawat. A.K. (2008).*The Internet Journal of Alternative Medicine*, 6(1): 1-10.

Parekh, J. and Chanda, V., (2007).In vitro antimicrobial activity and phytochemical analysis of some India medicinal plants.*Turkish Journal of Biology,* 31: 53–58.

Patra, J. C. and Chua, B. H. (2010). Artificial neutral network-based drug design for diabetes mellitus using flavonoids, *Journal of Computational Chemistry*, 32: 555-567.

Pettit, G. R., Mukku, V. J. R. V., Cragg, G., Herald, D. L., Knight, J. C., Herald, C. L., Chapuis, J. (2008). Antineoplastic Agents. 558. *Ampelocissus* sp. Cancer Cell Growth Inhibitory Constituents. *Journal of Natural Products*, 71(1): 130-133

Pietta, P., Maurie, P., Bruno, A. (1991). Identification of Flavonoids from *Ginkgo* Biloba, Anthermis Nobilis and Equsetun Unvense by HPLC with Diode-Arey UVm Detection.*Journal of Chromatography* A553: 223 – 231.

Raffauf, R.F., 1996. *Plant Alkaloids: A Guide to Their Discovery and Distribution*. Hawkworth Press, Inc., New York, ISBN: 1-56022-860-1.

Ragasa, C. Y., De Luna, R. D. and Hofilena, J. G. (2005). Antimicrobials terpenoids from *Pterocarpus indicus*.*Natural Products Research* 19 (4): 305-309.

Rajnarayana, K., Reddy, M. S., Chaluvadi, M. R., Krishna, D. R. (2001). Bioflavonoid classification, pharmacological, biochemical effects and therapeutic potential. *Indian Journal of Pharmacology,* 33:2-16.

Ramos, S. (2007). Effects of dietary flavonoids on apoptic pathways related to cancer chemoprevention, *Journal of Nutritional Biochemistry*, 18: 427-442.

Rao, M. R., Rao, Y. M., Rao, A. V., Prabhkar, M. C., Rao, C. S., Muralidhar, N. (1998). Antinociceptive and anti-inflammatory activity of a flavonoid isolated from *Caralluma attenuate*. *Journal of Ethnopharmacology,* 62:63-66.

Richard, J. P. C. (1998). Natural Product Isolation. Glaxo Welcome Research and Development Steven age Hertz U. K. Humana Press Totowa New Jersey USA Pp. 209 – 230, 243 – 261.

Robinson, T. (1967).The Organic Constituents of Higher Plants.*Gurgess Publications, U.S.A.* pp 178 – 205.

Rosch, D., Krumbein, A., Mugge, C., Kroh, L.W. (2004). Structural investigations of flavonol glycosides from sea buckthorn *(Hippopha rhamnoides)* pomace by NMR spectroscopy and HPLC-ESI-MS. *Journal of Agriculture and Food Chemistry* 13, 4039–4046.

Roux, D.G., Mails, E.A., Paulus, E. (1961).Condensed Tannins (IX).Distribution of Flavonoids Compounds in the Heart Wood and Barks of Some Inter-Related Waltles.*Journal of Chromatography,* 5:9-10.

Sabina, E. P., Chandel, S. and Rasool, M. K. (2009). Evaluation of analgesic, antipyretic and ulcerogenic effect of Withaferin A. *International Journal of Integrated Biology,* 6 (2):52-56.

Saifah,E., Kelly,C., Leary, J. D. (1983). Constituents of *Cissus rheifolia, Journal of Natural Products*, 46:353- 358.

Shalit, I., Berger,S. A. Gorea, A., Frimerman,H. (1989). Widespread Quinoline Resistance among Methicillin Resistant *Staphylococcus aureus* isolates in a General Hospital Antimicrobial. *Agents. Chemotherapy*, 33: 181-184.

Siebert, F. & Hildebrandt, P. (2007).*Vibrational Spectroscopy in Life Science* (1st edition), Wiley VCH, ISBN 978-3527405060, ISA.

Silva, L. G., Lee, L. S., Kinghorn, D. A. (1998).Special problem with extraction of plants. In: Natural products isolation Cornel, R. J. P. (Ed), Humana Press 999 Riverview Drive, Suite 208, Totowa, New Jersey, U. S. A. pp.343-364.

Singh G., Rawat, P., and Maurya, R (2007). Constituents of *Cissus quandrangularis Natural Products research*21 (6):522-528.

Singleton, P. (1999).Bacteria in Biology, Biotechnology and Medicine.4th edn. John Wiley and Sons Ltd, New York, USA

Soetan, K., Oyekunle, M. A., Aiyelaagbe, O. O., Fafunso, M. A. (2006). Evaluation of antimicrobial properties of saponins extract of Sorghum Bicolor L. Moench. *African Journal of Biotechnology,* 5(23): 2405-2407.

Sofowora, H. (1993). Screening Plants for Bioactive Agents.In: Medicinal Plants and Traditional Medicine in Africa, Spectrum Books Ltd., Sunshine House, Ibadan. Nigeria, 2ndEd.A Spectrum publication, Sunshine House Ibadan, Nigeria. pp. 81-93, 134-156.

Sparg, S., Light, M., Staten, J. (2004). Biological activities and distribution of plant saponins, *Journal of Ethnophamacology,* 94:219-243.

Spencer, J.P., Vauzour, D. and Rendeiro, C. (2009). Flavonoids and cognition: the molecular mechanisms underlying their behavioural effects. *Archives of Biochemistry and Biophyics,* 492, 1-2, 1-9.

Spencer, J.P. (2010).The Impact of fruit flavonoids on memory and cognition. *British Journal of Nutrition,* 31, 6, 546-557.

Spiegel, P. (1969). Chromatographic Separations of Phenolic Compounds .*Journal of Chromatography,* 39: 93-96.

Srinivasan, D., Nathan, S. Suresh, T. and Perumalsamy, PZ.(2001). Antimicrobial activities of certain Indian Medicinal plants in Folkloric Medicine. *Journal of Ethnopharmacology* 74:217-220.

Srinivasan, K., Muruganandan, S., Lal, J., Chandra, S., Tandan, S. K., Ravi Prakash, V. (2011). Evaluation of anti-inflammatory activity of *Pongamia pinnata* in rats, *Journal of Ethnopharmacology,*78:151-157.

Sriram, P. G. and Subramanian, S. (2011). Fisetin, a bioflavonoid ameliorates hyperglycaemia in streptozotocin-induced experimental diabetes in rats, *International Journal of Pharmaceutical Sciences Review and Research*, 6: 68-74.

Stein, A.-C., Sortino, M., Avancini, C., Zacchino, S., von Poser, G., (2005). Ethnoveterinary medicine in the search for antimicrobial agents: Antifungal activity of some species of *Pterocaulon* (Asteraceae).*Journal of Ethnopharmacology* 99: 211-214.

Sticher, O. (1993).Quality of *Ginkgo*Preparations. *Plant Medicines*, 59: 2 – 11.

Stuad, R. (2011). Peripheral pain mechanisms in chronic widespread pain, *Best practice Research Clinical Rheumatology,* 25:155-164.

Surender, S.andMafumdar, D. K. (1995).Analgesic activity of *Ocinum sanctum* and its possible mechanism of action. *International Journal of Pharmacognosy* 33(3): 188-192

Swain, T. (1965) Nature and Properties of Flavonoids.In Chemistry and Biochemistry of Plants Pigments, Goodwin, T.W. (Ed).Academic Press London, U.K. pp. 213-222.

Tanwar B and Modgil R (2012).Flavonoids: Dietary occurrence and health benefits. Spatula DD, 2(1): 59-68.

Tapas, A. R., Sakarkar, D. M. and Kakde, R. B. (2008). Flavonoids as nutraceuticals: A Review, *Tropical Journal of Pharmaceutical Research*, 7: 1089-1099.

Toma, W., Graciosa, J. S., Hiruma-Lima, C. A., Andrade, F. D. P., Vilegas, W., Souza Brita, A. R. M. (2003). Evaluation of the analgesic and antiedematogenic activities of *Quassia amara* bark extract. *Journal of Ethnopharmacology,* 85:19-23.

Trease, G.E. and Evans W.C. (2002).Pharmacology.15th Edn. Saunders Publishers, London, UK Pps 42-44, 221-229, 246-249, 303-306, 331-332, 391-393.

Tripoli, E., Guardia, M. L., Giammanco, S., Majo, D. D., Giammanco, M. (2007). Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chemistry* 104: 466-479.

Trombetta, D., Astell, F., Grazia-serpietro, M., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G., Bisignano, G (2005). Mechanism of antibacterial action of three monoterpenes.Antimicrob.*Agents Chemotherapy* 49:2474-2475.

Turner, R. A. (1965). Analgesics In: Turner, R. A. (Ed.) Screening Methods in Pharmacology. Academic Press, London, UK pp. 100.

Usman, H., Abdulrahman, F. I. and Ladan, A. H. (2007). Phytochemical and antimicrobial evaluation of *Tribulus terrestris* L. (*Zygophylaceae*) growing in Nigeria. *Research Journal of Biological Sciences.*

Vinegar, R., Schreiber, W. and Hugo, R. (1969).Biphasic development of Carrageenan oedema in rats.*Journal of Pharmacology and Experimental Therapeutics,*166:96-103.

Wagner, H. (1965). In: *Methods in Polyphenols Chemistry Including Qualitative Organic Analysis Longman Group, Ltd.*, London, UK. Pp. 303, 369.

Wang, H.K., Xia, Y., Yang, Z.Y., Natechke, S L., Lee, K.H. (1998). Recent advances in the discovery and development of flavonoids and their analogues as antitumor and anti-HIV agents. *Advances Experimental medicine and Biology,* 439: 191-225.

Wang, X., Traub, R. J. and Murphy, A. Z. (2006). Persistent pain model reveals sex difference in morphine potency. *American Journal of Physiology,* Regul Integr Comp Physiol 291, R300-R306.

WHO, (2000).Integration of traditional and complimentary medicines into National Health Care Systems, 23 *Journal of Manipulative and Physilogical Therapeutics* 139-140.

Winter, C. A., Riselay, E. A. and Nuss, G. W. (1962). Carrageenan induced oedema in the hind paw of the rats as an assay for anti – inflammatory drugs. Proceedings of the Society for *Experimental Biology and Medicine*111:544-547

Wong, E.J. and Taylor, A.O. (1962). Chromatography of Flavonoid Aglycones in the Solvent System–Benzene: Acetic Acid: Water. *Journal of Chromatography*, 9:449-452.

Zakaria, Z. A. and Abdul Gani, Z. D.F. (2008). Antinociceptive, anti-inflammatory, and antipyretic properties of an aqueous extract of Dicranopteris linearis leaves in experimental animal models. *Journal of Natural Medicine*, 62:179-187.

Zongo C, Akomo OE, Savadogo A, Obame LC, Koudou J, Traore SA. (2010). In vitro antibacterial properties of total alkaloids extract from Mitragyna inermis (Willd.) O. Kuntze, a West African traditional medicinal plant. *Asian Journal of Plant Science* 8:172-177.

Zou, Y.P.; Tan, C.H.; Wang, B.D.; Jiang, S.H.; Zhu, D.Y. (2007) Flavonoid Glycosides from *Ranunculus chinensis Bge*. *Helv. Chim. Acta*, *10*, 1940–1945.

APPENDICES

Appendix 1.0: Determination of LD50 of crude methanol extract of the aerial parts of *Ampelocissus grantii*

PHASE I

Group	Weight of Mice (g)	Dose (mg/kg body weight *i.p.)*	Mortality
1	27	1000	
	25	1000	0/3
	23	1ooo	
2	28	100	
	28	100	0/3
	27	100	
3	29	10	
	27	10	0/3
	24	10	

PHASE II

Group	Weight of Mice	Dose (mg/kg body weight *i.p.)*	Mortality
1.	20	5000	1/1
2.	24	2900	1/1
3.	25	1600	1/1

Appendix 2.0: Effect of methanol extract of *Ampelocissus grantii* on acetic acid-induced abdominal constriction in mice

Group 1 Normal saline treated group 1ml/kg BW (Negative control)

S. No	Weight	No of abdominal constrictions
1	24	30
2	23	23
3	17	28
4	24	18
5	21	25
6	24	22

Group 2 MeOH extract 300mg/kg BW

S. No	Weight	No of abdominal constrictions
1	24	0
2	23	1
3	17	1
4	24	0
5	21	4
6	24	0

Group 3 MeOH extract 150mg/kg BW

S. No	Weight	No of abdominal constrictions
1	24	2
2	23	1
3	17	0
4	24	4
5	21	0
6	24	5

Group 4 MeOH extract 75mg/kg BW

S. No	Weight	No of abdominal constrictions
1	24	4
2	23	3
3	17	9
4	24	10
5	21	4
6	24	5

Group 5 KETOPROFEN 20mg/kg BW

S. No	Weight	No of abdominal constrictions
1	24	9
2	23	19
3	17	5
4	24	2
5	21	1
6	24	6

Appendix 3.0: Effect of methanol extract of *Ampelocissus grantii* on pain reaction time in mice

Group 1 Normal saline 1ml/kg BW

S. No	Weight	0 Hr	30 min	60 min	90 min	120 min	150 min
1	27	1.38	1.45	2.96	3.07	1.29	1.29
2	17	1.35	1.47	2.67	2.88	1.87	1.25
3	23	1.11	1.20	1.50	1.38	1.23	1.29
4	29	1.32	1.85	1.88	1.02	1.36	1.05
5	22	1.19	1.46	1.92	1.04	1.73	1.15
6	27	1.15	1.39	1.60	1.49	1.25	0.98

Group 2 MeOH extract 300mg/kg BW

S. No	Weight	0 Hr	30 min	60 min	90 min	120 min	150 min
1	18	1.57	4.98	5.36	5.93	3.34	2.20
2	16	1.10	2.64	2.33	2.98	1.46	1.71
3	24	1.10	1.93	2.83	2.50	1.50	1.52
4	20	0.95	2.43	2.29	2.65	2.09	1.57
5	18	1.24	3.04	2.45	1.91	1.71	1.49
6	22	1.44	1.93	2.68	2.34	1.11	1.81

Group 3 MeOH extract 150mg/kg BW

S. No	Weight	0 Hr	30 min	60 min	90 min	120 min	150 min
1	19	1.48	3.12	2.70	2.06	2.24	1.60
2	23	1.16	2.90	2.86	2.99	1.23	1.35
3	26	1.03	2.31	1.61	1.34	1.09	1.29
4	28	1.40	3.30	3.99	2.78	1.62	1.28
5	22	1.40	2.11	4.00	1.52	1.07	1.49
6	20	1.23	2.59	2.64	1.54	1.54	2.29

Group 4 MeOH extract 75mg/kg BW

S. No	Weight	0 Hr	30 min	60 min	90 min	120 min	150 min
1	23	1.04	2.02	2.32	3.46	1.53	1.71
2	26	1.55	2.05	3.11	4.25	1.31	1.35
3	16	1.34	2.56	2.69	2.51	1.06	2.07
4	21	1.20	1.84	2.74	2.17	1.68	1.36
5	27	1.18	2.27	3.69	2.20	1.04	1.55
6	27	0.95	1.84	2.53	1.37	1.45	1.05

Group 5 Pentazocine 30mg/kg BW

S. No	Weight	0 Hr	30 min	60 min	90 min	120 min	150 min
1	24	1.68	4.45	4.44	1.48	2.21	1.15
2	22	1.69	5.11	3.96	1.35	2.23	1.90
3	25	1.23	3.46	5.33	1.64	3.08	1.73
4	25	1.19	4.11	6.41	2.34	3.27	1.96
5	24	1.13	3.67	2.89	1.50	3.24	1.71
6	21	1.07	3.60	4.42	1.93	2.98	1.65

Appendix 4.0: Effect of methanol extract of *Ampelocissus grantii* on Carrageenan induced paw oedema in rats.

Group 1 Normal saline

S. No	Weight	0 Hr	1 Hr	2 Hr	3 Hr	4Hr
1	213	.25	.34	.37	.39	.34
2	169	.24	.35	.42	.43	.38
3	152	.21	.35	.41	.41	.35
4	152	.23	.45	.44	.51	.39
5	151	.21	.39	.49	.51	.39
6	123	.24	.39	.40	.37	.39

Group 2 MeOH extract 75mg/kg BW

S. No	Weight	0 Hr	1 Hr	2 Hr	3 Hr	4Hr
1	120	.24	.26	.32	.32	.29
2	126	.22	.24	.27	.28	.28
3	122	.23	.26	.24	.29	.28
4	141	.23	.27	.35	.34	.28
5	130	.24	.25	.31	.27	.24
6	117	.22	.25	.31	.29	.26

Group 3 MeOH extract 150mg/kg BW

S. No	Weight	0 Hr	1 Hr	2 Hr	3 Hr	4Hr
1	148	.22	.26	.31	.26	.27
2	123	.23	.25	.24	.27	.24
3	124	.22	.24	.24	.24	.24
4	129	.21	.25	.25	.27	.27
5	152	.24	.24	.31	.28	.27
6	115	.23	.24	.26	.28	.26

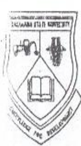
Group 4 MeOH extract 300mg/kg BW

S. No	Weight	0 Hr	1 Hr	2 Hr	3 Hr	4Hr
1	127	.19	.24	.32	.27	.26
2	120	.21	.22	.24	.24	.24
3	135	.21	.25	.24	.26	.26
4	128	.19	.24	.22	.27	.23
5	110	.21	.23	.25	.27	.26
6	124	.21	.25	.26	.24	.24

Group 5 Ketoprofen 20mg/kg BW

S. No	Weight	0 Hr	1 Hr	2 Hr	3 Hr	4Hr
1	180	.24	.32	.31	.32	.32
2	245	.25	.30	.28	.29	.26
3	205	.24	.41	.36	.35	.31
4	166	.25	.33	.31	.28	.31
5	221	.25	.31	.29	.26	.27
6	128	.20	.28	.25	.24	.24

Int. J. Chem.. Sci. Vol 3 No 2, ISSN: 2006-3350



PRELIMINARY PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF METHANOL EXTRACT OF AERIAL PARTS OF *AMPELOCISSUS GRANTII* (BAKER) PLANCH

Y. M. Musa[1]*, M. I. Sule[2], U. U. Pateh[2], M. Ilyas[2], A. M. Musa[2], H. Usman[3], and M.S. Abdullahi[4]

[1]Department of Basic Sciences, College of Agriculture, Lafia, Nasarawa State, Nigeria
[2]Department of Pharm. and Medicinal Chemistry, Ahmadu Bello University, Zaria, Nigeria
[3]Department of Chemistry, University of Maiduguri, P.M.B. 1069 Maiduguri, Nigeria
[4]Department of Microbiology National Research Institute of Chemical Technology, Zaria, Kaduna State, Nigeria
* Corresponding Author: muyahma@yahoo.com

Date Received: 18th Sept., 2010; Date Accepted: 2nd Dec., 2010

Abstract

Preliminary phytochemical screening on the aerial parts of Ampelocissus grantii revealed the presence of carbohydrates, tannins, flavonoids, cardiac glycosides, saponins, terpenes and steroids. Alkaloids were absent. The crude methanol extract was screened for antimicrobial activity using clinical isolates of Staphylococcus aureus, Streptococcus pyogenes Corynebacterium ulcerus, Bacillus subtilis, Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Pseudomonas aeriginosa, Neisseria gonorrhoea, and Candida albicans. The results showed that the methanol extract of the plant inhibited the growth of Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Candida albicans with zones of inhibition ranging from 17.0 to 27.0 mm. No activity was observed against Corynebacterum ulcerus, Salmonella typhi and Neisseria gonorrhoea. The minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of the methanol extract were 6.25 and 12.5 mm for the bacterial strains and 12.5 and 25.0 mm for the fungi strain respectively. The antimicrobial activities observed were attributed to the presence of some of the phytochemicals detected in the plant.

Key words: Ampelocissus grantii, Methanol extract, Antimicrobial activity.

INTRODUCTION

Antibiotic resistance has become a global concern (Westh *et al.*, 2004). There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. Therefore, there is a need for alternative antimicrobial drugs for the treatment of infectious diseases from various sources such as medicinal plants (Clark, 1996; Cordell, 2000). Medicinal plants represent a rich source from which novel antimicrobial chemotherapeutic agents may be obtained (Krishnaraju *et al.*, 2005). Substances derived from plants have recently become of great interest owing to their versatile applications (Baris *et al.*, 2006). Recent works revealed the potential of several herbs as sources of drugs (Iwu, 2002). The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants have a variety of chemical constituents which have the ability to inhibit the growth of pathogenic organisms representing a potential source for novel antibiotic prototypes (Afolayan, 2003).

In our search for plants with antimicrobial properties *Ampelocissus grantii* which belongs to the family-Vitaceae was investigated. A plant of herbaceous stems arising from a large, semi-succulent perennial underground root-stock, of the savannah, common in rocky sites across Senegal and Northern and Southern Nigeria.

Ampelocissus grantii (known in Hausa of northern Nigeria as Rogon daji) and other related species have been variously utilized in a number of folk medicine (Burkill, 2000; Dalziel, 1955). Ethnobotanical studies revealed that the plant is used alone or mixed with other medicinal plants to treat urinary schistosomiasis (Bah *et al.*, 2006), old wounds (Inngjerdingen *et al.*, 2004) and shigellosis and fever (Adjanohoun *et al.*, 1980), It is also claimed to be of use in the treatment of febrifuges, muscular pains, skin diseases, leprosy, menstrual cycle, stomach troubles, pulmonary troubles and

Int. J. Chem.. Sci. Vol 3 No 2, pp167-174, 2010

used as lactation stimulants (incl. veterinary) (Burkill, 2000; Dalziel, 1955). Ethnobotanical survey on some important drug plants of north-western Nigeria revealed that the root of *A. grantii* is claimed to be used to treat cancer (Muhammad and Amusa, 2005) and diarrhoea (Etuk *et al.*, 2009). The plant also showed marked antimalarial activity (Gessler *et al.*, 1994). The trypanocidal activity has been demonstrasted by *in vitro* and *in vivo* studies (Biziman *et al.*, 2006; Aderbauer *et al.*, 2008). In this paper we are reporting the result of our investigations on the phytochemical and antimicrobial studies of crude methanol extract of the aerial parts of the plant.

MATERIALS AND METHODS

Plant Material

The whole plant was collected from Gwoza Wakane village in Gwoza of, Borno State, Nigeria in October, 2008. The plant was confirmed and authenticated at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria.

Preparation of Plant Material and Extraction

The aerial parts of the plant were air dried under shade, and (finely) powdered using a porcelain mortar and pestle. 1.2 Kg of the powdered material was packed into a Soxhlet extractor and extracted exhaustively with methanol. The extract was concentrated *in vacuo* at 40°C using a rotary evaporator. The vacuum dried methanol extract (140 g) was washed with n-hexane to remove the non polar substances. The remnant was re-dissolved in methanol to obtain defatted methanol extract.

Preliminary Phytochemical Screening of Plant Extract

Preliminary phytochemical analysis of the defatted crude methanol extract was carried out according to the methods described by Sofowora (1993) and Trease and Evans (2002). This was conducted for determination of the presence of saponins, tannins, alkaloids, flavonoids, cardiac glycosides and steroids/terpenoids

Test Microorganisms

The bacterial and fungal strains were used in this study: *Staphylococcus aureus*, *Streptococcus pyrogenes*, *Cornebacterium pyogenes*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumonia*, *Pseudomonas aeriginosa*, *Neisseria gonorrhoea*, *Candida albicans* were clinical isolates obtained from the Department of Microbiology, Ahmadu Bello University, Teaching Hospital, Zaria, Nigeria. The isolates were purified and kept in slants of blood agar medium.

Antimicrobial Tests

Antimicrobial activities of the extract was determined using paper discs method of Bauer-Kirby (1966) using a stock concentration of 50 mg/ml, prepared by dissolving 0.5 g of the extract into 10 ml of sterilized distilled water. Blood agar base was the medium used as growth medium prepared according to manufacturers instructions and was sterilised at 121°C for 15 mins and cooled. 20 ml of the sterile medium was poured into sterile Petri dishes and were covered and allowed to solidify. The plates were then seeded with the test microbes by spread plate techniques and left to stabilise for half an hour. Filter paper discs were cut and sterilized at 160°C for 30 mins, then soaked into the solution of the extracts of the plant. The discs were dried at 45°C and planted on the surface of the medium previously seeded with the test microbes. Chloramphenicol (5 mg/ml), Erytromycin (5 mg/ml) and Sparfloxin (2 mg/ml) standard discs were placed on the agar plate. The plates were incubated at 37°C for 24 hours after which they were inspected for zones of inhibition of growth which were measured and recorded (in millimetres).

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration test was carried out on the microorganisms that were sensitive to the extract using the broth dilution technique according to the method described by Vollekovà *et al.* (2001). Nutrient broth agar was prepared according to manufacturer's instructions. 10ml each of the medium was dispensed into 5 screw cap test tubes and the medium was sterilised at 121°C for 15 minutes. MacFarland's turbidity standard scale No 0.5 was prepared by adding 9.95mls of 1% H_2SO_4 and 0.5ml of 1% $BaCl_2$ to give a turbid solution. 10 ml normal saline was used to make a turbid suspension of the microbes, dilution of the

microbes was carried out continuously using the normal saline until the turbidity marched that of the MacFarland's scale by visual comparison, the microbes' concentrations at that point was about 3×10^8 cfu/ml. Two fold serial dilution of the extract in the nutrient broth was carried out and the following concentrations were obtained, 50, 25, 12.5, 6.25 and 3.125mg/ml. Having obtained the different concentrations and dilutions, 3 drops of the microbes in the normal saline were inoculated into the dilution of the extracts in the broth and the culture was incubated at 37°C for 24 hours. The lowest concentration of the extract which inhibited the growth of the microbe was recorded as the minimum inhibitory concentration (MIC).

Minimum Bactericidal Concentration (MBC)

MBC were determined by using the broth dilution technique previously described by Volleková *et al.* (2001). The contents of the MIC tubes in the serial dilution were sub-cultured on the plates of the blood agar medium by dipping a sterile wire into each test tube and streaking the blood agar plates, the plates were then incubated at 37°C for 24 hours after which they were observed for growth. The MBC was the plate with lowest concentration of the extracts with out growth.

RESULTS AND DISCUSSION

The phytochemical screening of the aerial parts of *A. grantii* showed the presence of carbohydrates, tannins, flavonoids, cardiac glycosides, saponins, terpenes and steroids. Alkaloids were not detected in the extract (Table 1). It was reported that medicinal plants may contain many kinds of chemical components and their biological activities are not usually due to a single moiety (Cho *et al.*, 2003).

Flavonoids have attracted a great deal of attention in relation to their beneficial effects on health. Over the past few years, several experimental studies have demonstrated biological and pharmacological properties of many flavonoids, especially their antimicrobial activity (Narayana *et al.*, 2001), antioxidant (Robak and Mareinkiewiez, 1995) and anti-tumour (Castillo *et al.*, 1999; Inoue and Jackson, 1999) effects, which are associated with free radical-scavenging actions. Saponins are glycosides occurring widely in plants and they generally lower the surface tension and possess emulsifying activities. They tend to alter the permeability of the cell wall and hence exert a general toxicity on all organized tissues. They are known to possess some antibacterial activity. According to Birk and Petri (1980), saponins combine with cell membrane sterol to produce changes in cell morphology leading to lysis. Tannins are diverse organic substances with various compositions that have produced physiological astringent properties that hasten the healing of wounds and inflamed mucus membranes (Tyler *et al.*, 1988). Tannins also decrease bacterial cell proliferation by blocking key enzymes of microbial metabolism.

From this work, the results have shown that the aerial parts of *A. grantii* possessed a wide range of antimicrobial activity against both Gram positive (*Staph. aureus, Strpt. pyogenes, B. subtilis*); Gram negative (*E. coli, K. pneumonia, Ps. aeruginosa*) and yeast (*C. albicans*) (Table 2). The inhibition zones values for the bacterial strains and *C. albicans* isolates which were sensitive to the methanol extract were in the range of 17.0 to 27.0 mm. The results also showed that the methanol extract of the plant have wider spectrum of activity than that of Chloramphenicol which was not effective against *Staph. aureus, Strept. pyogenes and K. Pneumoniae.* Consequently, the zones of inhibition produced by the methanol extract on these organisms compared fairly well with the zones of inhibition produced by erytromycin and sparfloxin (Table 2), which are regular drugs used internationally for the treatment of diseases associated with these microbes.

The observed antimicrobial effects on the isolates is believed to be due to the presence of saponins, tannins and flavonoids in the crude extract which have been reported to possess antimicrobial properties (Ragasa *et al.*, 2005; Cuhnie and Lamb, 2006; Soetan *et al.*, 2006), and exert their effects by affecting the cell membrane integrity of the bacteria (Hendrich, 2006; Trombetta *et al.*, 2005). These active principles may have acted alone or in combination to inhibit the growth of the microbes.

In order to further substantiate the activity of the plant extract, we determined the minimum

Int. J. Chem.. Sci. Vol 3 No 2, pp167-174, 2010

inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the crude extract using a standard method. The results showed that methanol extract of the plants exerted good antibacterial activity on most of the strains of bacterial and fungi at the concentrations tested (Tables 3 and 4). It is worthy of note that MBC values obtained (12.5 mm and 25.0 mm for the

Table 1: Phytochemical results/composition/constituent of the crude methanolic extracts of the aerial part of *Ampelocissus grantii*

S/N	Constituents/Test	Results
1	ALKALOIDS	
	Dragendorffs Test	-
	Wagners Test	-
	Mayer Test	-
2	CARBOHYDRATES	
	Molischs Test	+
	Barfoeds Test	+
	Fehling (reducing sugar) Test	+
	Fehling (combine reducing sugar) Test	+
3	CARDIAC GLYCOSIDES	
	Legals Test	+
	Keller-Killianis Test	+
4	FLAVONOIDS	
	Shinodas Test	+
	Lead acetate Test	+
	NaOH Test	+
	$FeCl_3$	+
5	SAPONINS	
	Frothings test	+
6	STEROIDAL NUCLEUS	
	Salkwoskis Test	+
	Lieberman Burchards Test	+
7	TANNINS	
	$FeCl_3$	+
	Lead acetate	+

Key: + = present; - = absent

Table 2: Susceptibility results of the microorganisms against the crude methanolic extracts of the aerial parts of *A. grantii* and standard antibiotics

Test Organism	zone of inhibition of antibiotics against the microbes (mm)			
	Extract	Chloramphenicol	Erytomycin	Sparfloxin
Staphylococcus aureus	23.0	0.0	22.0	0.0
Streptococcus pyogenes	27.0	0.0	24.0	0.0
Corynebacterum ulcerus	0.0	0.0	19.0	30.0
Bacillus subtilis	26.0	20.0	30.0	34.0
Escherichia coli	21.0	24.0	20.0	20.0
Salmonella typhi	0.0	30.0	20.0	20.0
Klebsiella pneumonia	27.0	0.0	30.0	22.0
Pseudomonas aeruginosa	20.0	17.0	19.0	21.0
Neosseria gonorrhoeae	0.0	0.0	14.0	0.0
Candida albicans	17.0	NT	NT	NT

Key: NT = Not tested

Table 3: Minimum inhibitory concentration (MIC) results of the crude Methanolic extracts of the aerial part of *Ampelocissus grantii*

	Concentration (mg/ml)				
Test organism	50.0	25.0	12.5	6.25	3.125
Staphylococcus aureus	-	-	-	#	#
Streptococcus pyogenes	-	-	-	#	#
Bacillus subtilis	-	-	-	#	+
Escherichia coli	-	-	-	#	+
Klebsiella pneumonia	-	-	-	#	+
Pseudomonas aeruginosa	-	-	-	#	+
Candida albicans	-	-	#	+	++

Key: # = Clear solution (MIC); - = No growth; + = Cloudy solution (slight growth); ++ = Turbid solution (strong growth).

Table 4: Minimum bactericidal concentration (MBC) results of the crude Methanolic extracts of the aerial part of *Ampelocissus grantii.*

	Concentration (mg/ml)				
Test organism	50.0	25.0	12.5	6.25	3.125
Staphylococcus aureus	-	-	#	+	++
Streptococcus pyogenes	-	-	#	+	++
Bacillus subtilis	-	-	#	+	++
Escherichia coli	-	-	#	+	++
Klebsiella pneumonia	-	-	#	+	++
Pseudomonas aeruginosa	-	-	#	+	++
Candida albicans	-	#	+	++	+++

Key: # = MBC; - = no growth; + = scanty growth; ++ = moderate growth; +++ = heavy growth.

Int. J. Chem.. Sci. Vol 3 No 2, pp167-174, 2010

bacterial strains and fungi strain respectively) for the extract against the pathogens are higher than MIC (6.25 mm and 12.5 mm for the bacterial strains and fungi strain respectively), indicating that the extract are bacteriostatics at lower concentrations and bactericidal at higher concentrations.

The observed antimicrobial effects on the isolates corroborate with the use of the plant in traditional medicine. Ethnobotanical studies revealed that the plant is used alone or mixed with other medicinal plants to treat urinary schistosomiasis (Bah *et al.* 2006), old wounds (Inngjerdingen *et al.* 2004), claimed to be used to treat cancer (Muhammad and Amusa 2005), and stomach problems such as diarrhoea (Etuk *et al.* 2009).

The large zone of inhibition by the extract against *Staph. aureus* and *Ps. aeruginosa* indicated that the extract can be used for the treatment of sores, bores and open wounds. *S. aureus* and *Ps. aeruginosa* have been implicated in cases of boils, sores and wounds (Brande, 1982). The extract showed strong activity against *E. coli* which is the commonest cause of urinary tract infection and accounts for approximately 90% of first urinary tract infections in young women (Brooks *et al.*, 2002 and Usman *et al.*, 2007). Also the high growth inhibition against *E. coli* shows that it can be use in the control of diarrhoea and dysentery. *E. coli* is the common cause of traveller's diarrhoea and other diarrheagenic infections in humans (Adams and Moss, 1999).

In view of the fact that prevalence of *Staph. aureus* resistant strains to conventional antibiotics has increased to high levels in some hospitals (Shalit *et al.*, 1989; Usman *et al.*, 2007) and that *Staph. aureus* is a pyogenic bacterium known to play significant role in invasive skin diseases including superficial and deep follicular lesion (Srinivasan *et al.*, 2001), this extract could serve as a remedy to such resistances folklorically. The sensitivity of *C. albicans* to the methanol extract of the plant could elicit the chemotherapeutic potentials of using *A. grantii* in the treatment of diseases such as oral and vaginal candidiasis. The inability of the extract to inhibit the growth of *C. ulcerus, S. typhi,* and *N. gonorrhoea* may be that they possess a mechanism for detoxifying the active principle in the extract. Some bacteria are known to possess mechanisms by which they convert substances that inhibit their growth to non-toxic compounds. For example *Staph. aureus* produce the enzyme penicilinase which converts the antibiotic penicillin to penicillinoic acid which is no longer inhibitory to its growth (Singleton, 1999).

CONCLUSION

The results of the present study suggested that *A. grantii* methanol extract possesses compounds with antimicrobial properties that can be used in the therapy of infectious diseases caused by *C. albicans* and bacterial pathogens. Work is still in progress for the purification and characterization of the phytochemicals that would be obtained with a view to obtaining useful chemotherapeutic agent.

ACKNOWLEDGEMENT

The authors wish to acknowledge and thank the Department of Microbiology, Ahmadu Bello University, Teaching Hospital, Zaria, Nigeria for their assistance.

REFERENCES

Adams, M.R.; Moss, M.O. 1999. Food Microbiology. The Royal Society of Chemistry, Cambridge.

Adjanohoun, E.; Ahyi, M. R. A; Ake Assi, L; Dan Dicko, L; Daouda, H., *et al.* 1980. Contribution Aux Etudes Ethnobotaniques et Florisques au Niger ACCT Paris ISBN 92-9028-009-3 pp. 250

Afolayan, A.J. 2003. Extracts from the shoots of *Arctotis artotoides* inhibit the growth of bacteria and fungi, *Pharm. Biol.* **41**: 22-25.

Aderbauer, B.; Clausen, P. H; Kershaw, O; Melzig, M. F. 2008. In vitro and in vivo trypanocidal effects of lipophilic extracts of medicinal plants from Mali and Burkina Faso. J. Ethnopharmacol. 119: 225 - 231.

Bah, S.; Diallo, D; Dembels, S; Paulsen, B. S. 2006. Ethnopharcological survey of plants used for the treatment of schistosomiasis in Niano District, Mali. J. Ethnopharmacol. 105:387- 399.

Baris, O.; Gulluce, M; Sahin, F; Ozar, H; Killic, H; Ozkan,H; Sokmen, M; Ozbek, T. 2006. Biological activities of the essentials oil and methanol extract of *Achillea biebersteinii* Afan (Asteraceae). *Turk. J. Biol.* 30:65-73.

Bauer, A.W.; Kirby, W.M.M; Sherris, J.C; Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45:493-496.

Birk, Y.; Petri, I. 1980. Saponins, In: Toxic constituents of plant foodstuff, (2nd edn), Academic Press, New York, pp 161-182.

Bizimana, N.; Tietjen, U; Zessin, K. H; Diallo, D; Djibril, C; Melzig, M. F; Clausen, P. H. 2006. Evaluation of medicinal plants from Mali for their in vitro and in vivo trypanocidal activivty. J. Ethnopharmacol., 103:350 356.

Brande, A. I. 1982. Microbiology. W. B. Saunders Company, London.

Brooks, G.F.; Butel, J.S; Morse, S.A. 2002. Jawetz, Melnick and Adelberg's Medicinal Microbiology, (2nd edn). McGraw-Hill, New Delhi, India, pps 197, 234, 550.

Burkill, H.M. 2000. The Useful plants of the West Tropical Africa Vol 2. Royal Botanic Garden, Kew 1997; 969.

Castillo, M.H.; Perkins, E; Cambell, J.H; Doerr, R; Hassett, J.M; Kandaswani, C; Middleton Jr., E. 1989. The effects of bioflavonoids quercetin on squamous cell carcinoma of head and neck region, *Am. J. Surg.* 158:351-355.

Cho, E. J.; Yokozawa, T; Rhyu, D. Y; Kim, S. C; Shibahara, N; Park, J. C. 2003. Study on the inhibitory effects of Korean medicinal plants and their main compounds on the 1,1-diphenyl-2-picrylhydrazyl radical *Phytomed.* 10:544-551

Clark, A.M. 1996. Natural products as resource for new drugs. *Pharm. Res.* 13:1132-1141.

Cordell, G.A. 2000. Biodiversity and drug discovering a symbiotic relationship, *Phytochemistry* 55:463-480.

Cuhnie, T.P.; Lamb, A.J. 2006. Antimicrobial Activity of Flavonoids, *Int. J. Antimicrob. Agents.* 26:342-356.

Dalziel J.M. 1955. The useful plants of West Tropical Africa, Crown Agents for Colonies, London, pp 238-239.

Etuk, E. U.; Ugwah, M. O; Ajagbonna, O. P; Onyeyili, P. A. 2009. Ethnobotanical survey and preliminary evaluation of medicinal plants with antidiarrhoea properties in Sokoto state, Nigeria *Journal of Medicinal Plants Research.* 3(10), 763-766,

Gessler, M.C.; Nkunya, M.H.H; Mwsumbi, L.B; Heinrich, M; Tanner, M. 1994. Screening of Tanzanian medinal plants for antimalarial activity. *Acta, Tropics* 56:65-77

Hendrich, A.B. 2006. Flavonoid-membrane interactions; possible consequences for biological effects of some polyphenolic compounds. *Act. Pharmacol.* Sinica 27 (1): 27-40.

Inngjerdingen, K.; Nergard, C. S; Diallo, D; Mounkoro, P. P; Paulsen, B. S. 2004. An ethnopharmacological survey of plants used for wound healing in Dogonland, Mali, West Africa. J. Ethnopharmacol. 92: 233-244.

Inoue, T.; Jackson, E.K. 1999. Strong antiproliferative effects of baicalein in cultured rat hepatic stallate cells. *Eur. J. Pharmacol.*, 378:129-135

Int. J. Chem.. Sci. Vol 3 No 2, pp167-174, 2010

Iwu, M.M. 2002: In: Threapeutic Agents from Ehtnomedicine. and Drug Discovery. Iwu M.M; Wootton J.C. (eds) Elsevier Science, Amsterdam.

Krishnaraju, A.V.; Rao, T.V.N; Sundararaju, 2005. Assessment of bioactivity of Indian medicinal plants using Brine shrimp (*Altenaria salania*) Lethality Assay. *Int. J. Appl. Sci. Engg.*, **2:** 125-134.

Muhammad, S.; Amusa, N.A. 2005. The important food crops and medicinal plants of North-western Nigeria. *Res. J. Agric. Biol. Sci.* **1(3):** 254-260.

Narayana, K.R.; Reddy, M.S; Chaluvadi, M.R; Krishna, D.R. 2001. Bioflavonoids; classification, pharmacology, biochemical effects and therapeutic potential. *Indian J. Pharmacol.*, **33:**2-16

Ragasa, C.Y; De Luna, R.D; Hofilena, J.G. 2005. Antimicrobials terpenoids from *Pterocarpus indicus*. *Nat. Prod. Res.* **19(4):** 305-309.

Robak, J.; Mareinkiewiez, E. 1995. Scavenging of oxygen species as the mechanism of drug action. *Pol. J. Pharmacol.* **47:**89-98.

Shalit, I.; Berger, S. A; Gorea, A; Frimerman, H. 1989. Widespread Quinoline Resistance among Methicillin Resistant *Staphylococcus aureus* isolates in a General Hospital Antimicrob. *Agents Chemother.*, **33:** 181-184.

Singleton, P. 1999. Bacteria in Biology, Biotechnology and Medicine. (4th edn), John Wiley and Sons Ltd, New York.

Soetan, K.; Oyekunle, M.A; Aiyelaagbe, O.O; Fafunso, M.A 2006. Evaluation of antimicrobial properties of saponins extract of Sorghum Bicolor L. Moench. *Afr. J. Biotech.* 5**(23):** 2405-02407.

Sofowora, H. 1993. Screening Plants for Bioactive Agents In: Medicinal Plants and Traditional Medicine in Africa, Spectrum Books Ltd., Sunshine House, Ibadan. Nigeria, (2nd edn), pp. 134-156.

Srinivasan, D.; Nathan, S; Suresh, T; Perumalsamy, P.Z. 2001. Antimicrobial activities of certain Indian Medicinal plants in Folkloric Medicine. *J. Ethnopharmacol.* **74:** 217-220.

Trease, G.E.; Evans W.C. 2002. Pharmacology. (15th edn), Saunders Publishers, London. pp. 42-44, 221-229, 246-249, 303-306, 331-332, 391-393.

Trombetta, D.; Astell, F; Grazia-serpietro, M; Venuti, V; Cristani, M; Daniele, C; Saija, A; Mazzanti, G; Bisignano, G. 2005. Mechanism of antibacterial action of three monoterpenes. Antimicrob. *Agents Chemoth.* **49:** 2474-2475.

Tyler, V.E.; Braddy, L.R; Roberts, J.E. 1988. Pharmacognosy. Lea and Febriger, Philadelpia pp, 85-909.

Usman, H.; Abdulrahman, F.I; Ladan, A.A. 2007. Phytochemical and Antimicrobial Evaluation of *Tribulus terrestris* L. (*Zygophylaceae*). Growing in Nigeria. *Res. J. Bio. Sci. Medwell Journals.* **2(3):** 244-247.

Vollekovà, A..; Kòst'àlovà, D; Sochorovà, R. 2001. Isoquinoline Alkaloids from *Mahonia aquifolium* Stem bark is Active against *Malassezia spp.* Folia Microbiol. **46:**107-111.

Westh, H.; Zinn, C.S; Rosdahl, V.T; Sarisa Study Group 2004. An international multicenter study of antimicrobial consumption and resistance in *Staphylococcus aureus* isolates from 15 hospitals in 14 countries. *Microbial Drug Resistance* **10:** 169-176.