PHYTOCHEMICAL AND ANTIVENOM STUDIES ON THE STEM BARK OF *ALBIZIA CHEVALIERI* HAMS (MIMOSACEAE) ON *NAJA NIGRICOLLIS* BROADLEY ENVENOMATED MICE

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FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY, ZARIA

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FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

DECEMBER, 2016

DECLARATION

I declare that the work in the thesis entitled '**Phytochemical and Antivenom Studies on the Stem bark of *Albizia chevalieri* Hams (Mimosaceae) on *Naja Nigricollis* Broadley Envenomated Mice**' has been performed by me in the Department of Pharmacognosy and Drug Development under the supervision of Prof. M.S. Abubakar, Prof. K.Y. Musa, and Dr. A. Ahmed. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other university.

Sylvester Nefai MATHIAS	....	....
Name of student	Signature	Date

CERTIFICATION

This thesis entitled "**Phytochemical and Antivenom Studies on the Stem bark of *Albizia chevalieri* Hams (Mimosaceae) on *Naja Nigricollis* Broadley Envenomated Mice**'' by Sylvester Nefai MATHIAS meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To my dear wife, Leah; sons, Shealtiel and Shimei, and daughter, Shiphrah.
…Also to all who read and obtain insight from this thesis.

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ABSTRACT

Albizia chevalieri Hams (Mimosaceae), mostly found in the Northern Sahel Savannah region of Nigeria as well as in Nigér and Senegal is a tree of the acacia type with a long list of folklore therapeutic claims which include its use as purgative, taenicidal, cough remedy, dysentery, cancer, diabetes mellitus, tuberculosis and snake bite remedy. The stem bark was collected, dried and powdered. The powder was firstly extracted with hexane, and subsequently with methanol. The methanolic extract was then fractionated into ethyl acetate (EtOAc) and n-butanol soluble parts. Chromatographic separation of the EtOAc and hexane fractions led to the isolation of four pure compounds—β-sitosterol (EAC_1) and three pentacyclic triterpenoids (Friedelin, Friedelinol and Lupeol, labelled as HXC_1, HXC_2, and HXC_3 respectively), which were characterized using spectroscopic techniques (HREIMS, NMR, IR and UV). Two of the four isolated compounds (HXC_1 and EAC_1), were investigated for antivenom activity, including the extract and fractions in experimental albino mice using standard methods. The results showed that the isolated compound, EAC_1 offered the highest protection to the animals by prolonging time of death of animal in the treated group at a dose dependant concentration of 8mgkg^{-1} with a minimum survival rate of 60%, followed by compound HXC_1 which recorded 20% survival rate at same doses; compared to the group administered with the minimum lethal dose (MLD) of venom only, where 80% of the animals died within an average time of 17minutes. The fractions of n-butanol and hexane recoded antivenom activity at 200 mgkg^{-1} while those of methanol and EtOAc recorded no activity. Gross histopathological changes in liver and kidney tissues of envenomated mice treated with two of the isolated compounds were observed to be less obvious than seen in the tissues of envenomated mice treated with the candidate plant extracts and fractions. This study went to show the likely antivenom potentials in the isolated compounds evaluated as well as those of the fractions of n-butanol and hexane, which is being reported for the first

time in the plant *A. chevalieri*. The results obtained also provided scientific basis for the use of *A. chevalieri* in folklore medicine in the treatment of snake bites.

TABLE OF CONTENTS

CONTENT	PAGE
Cover page	i
Title page	ii
Declaration	iii
Certification	iv
Dedication	v
Acknowledgements	vi
Abstract	vii
Table of Contents	viii
List of Tables	ix
List of Figures	x
List of Schemes	xi
Lists of Plates	xii
List of Appendices	xiii
List of Abbreviations	xiv
CHAPTER ONE	1
1.0 INTRODUCTION	1
1.1 The Consciousness of Phytochemistry	1
1.1.2 Drug discovery process and sources	2
1.1.3 Isolation of lead molecules	3
1.1.4 Historically important isolated lead molecules	5
1.2 Animal Venom	8
1.2.1 Snake bite and venom	9

1.2.2 Potentials of plants used in ethnomedicine for the treatment of poisonous snake bites 10

1.2.3 Worldwide ethnomedical use of plants in the treatment of poisonous snake bites 10

1.2.4 Reported plant derived compounds and their biochemical action as antivenom agents of poisonous snake-bites 12

1.2.5 Classification and identification of snakes 15

1.2.6 Naja species (Family Elapidae) 15

1.3 Research Problem Statement 17

1.4 Justification of Research 18

1.5 Aim 20

1.5.1 Objectives 20

1.6 Hypothesis 20

CHAPTER TWO 21

2.0 REVIEW OF RELATED LITERATURE 21

2.1 The Plant Family Fabaceae 21

2.1.1 Description of Fabaceae 23

2.1.2 Growth habit of Fabaceae 23

2.1.3 Distribution and habitat of Fabaceae 23

2.1.4 Economic and cultural importance of Fabaceae 23

2.1.5 Use of Fabaceae as food and forage 24

2.1.6 Chemical composition of Fabaceae 24

2.2.1 The Family Mimosaceae 25

2.2.2 Distribution of Mimosaceae 26

2.2.3 Floral structure of Mimosaceae 26

2.3.1 The genus Albizia 26

2.3.2 Phytochemical constituents of genus Albizia 26
2.3.3 Some Bioactive compounds isolated from the genus Albizia 33
2.3.4 Biological activity and ethnophamacology report of the genus Albizia 39
2.3.5 Reported ethnobotanical and ethnomedicinal value of the genus Albizia 44
2.4.1 The plant *Albizia chevalieri* 45
2.4.2 Biological evaluations of *Albizia chevalieri* 47
2.4.3 Ethnobotany information of *A. chevalieri* 48
CHAPTER THREE 51
3.0 MATERIALS AND METHODS 51
3.1 Materials 51
3.1.1 Chemicals and solvent 51
3.1.2 Equipment/materials 51
3.1.3 Animals 51
3.2 Methods 51
3.2.1 Collection and identification of plant material 52
3.2.2 Drying and preservation of plant material 52
3.3 Extraction and preliminary phytochemical screening of *A. chevalieri* stem bark 52
3.3.1 Extraction of plant material 52
3.3.2 Solvent-solvent fractionation 52
3.3.3. Preliminary phytochemical screening 53
3.3.4 Thin layer chromatography of gradient extracts 55
3.3.5 Column chromatography of hexane extracts 55
3.3.6 Column chromatography of ethyl acetate extract 57
3.4 Structure Elucidation of Isolated Compounds 57
3.4.1 Melting point (MP) determination 57

3.4.2 Ultra violet light spectroscopy (UV) 58

3.4.3 Infrared Spectroscopy 58

3.4.4 Nuclear magnetic resonance spectroscopy (NMR) 59

3.4.5 Mass spectrometry analysis 59

3.5 Animals and Venom 60

3.5.1 Lethality assay of the crude venoms of *Naja nigricollis*. 60

3.5.2 Median lethal dose determination (LD_{50}) of extract of *Albizia chevalieri* 60

3.5.3 Evaluation of the effect of crude extracts/fraction of *A. chevalieri* on lethal dose of the crude venom. 61

3.5.4 Evaluation of the use of *Albizia chevalieri* as prophylaxis of *Naja nigricollis* envenomation. 61

3.5.5 Studies of in-vivo anti-venom effect of pure isolated compound(s) of *A. chevalieri* on *Naja nigricollis* envenomation. 62

3.5.6 Histological studies 62

CHAPTER FOUR 63

4.0 RESULTS 63

4.1 Extraction of Plant Material 63

4.2 Preliminary Phytochemical Screening of the Stem Bark of *A. chevalieri* 64

4.3 Thin Layer Chromatography 65

4.4 Column Chromatography of Hexane and Ethyl acetate Extracts 67

4.5 Structure Determination of Compound HXC_1 69

4.4.1 Infra red (IR) spectroscopy of compound HXC_1 69

4.4.2 Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) of Compound HXC_1 70

4.4.3 ^{13}CNMR spectrum of compound HXC_1 72

4.4.4 Distortionless enhancement by polarisation transfer (DEPT-135) spectrum of compound HXC_1 73

4.4.5 Mass spectrometry of compound HXC_1 75

4.4.6 Suggested structure of compound HXC_1 78

4.5 Structure Determination of Compound HXC_2 79

4.5.1 Infra-red (IR) spectroscopy of compound HXC_2 79

4.5.2 Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) of compound HXC_2 80

4.5.3 ^{13}CNMR spectrum of compound HXC_2 81

4.5.4 Distortionless enhancement by polarisation transfer (DEPT)spectrun of compound HXC_2 84

4.5.5 Mass spectrometry of compound HXC_2 86

4.5.6 Suggested structure of compound HXC_2 89

4.6 Structure Determination of Compound HXC_3 90

4.6.1 Ultra-visible light (UV) spectroscopy of compound HXC_3 90

4.6.2 Infra red (IR) spectroscopy of compound HXC_3 91

4.6.3 Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) of compound HXC_3 91

4.6.4 ^{13}CNMR spectrum of compound HXC_3 94

4.6.5 Mass spectrometry of compound HXC_3 95

4.6.6 Suggested structure of compound HXC_3 98

4.7 Structure Determination of Compound EAC_1 99

4.7.1 Ultra-visible light (UV) spectroscopy of compound EAC_1 99

4.7.2 Infra red (IR) spectroscopy of compound EAC_1 100

4.7.3 Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) of compound EAC_1 101

4.7.4 ^{13}CNMR spectrum of compound EAC_1 103

4.7.5 Distortionless enhancement by polarisation transfer (DEPT-135) spectrum of compound EAC_1 106

4.7.6 Mass spectrometry of compound EAC_1 108

4.7.7 Suggested structure of compound EAC_1 111

4.8 Determination of Biological Activity 112

4. 8.1 Lethality assay of the crude venoms of *Naja nigricollis*. 112

4.8.2 Determination of LD_{50} of extracts and fractions of *A. chevalieri* by Lorke's method 113

4.8.3 Evaluation of the effect of crude extracts/fraction of *A. chevalieri* on lethal dose of the crude venom. 115

4.8.4 Evaluation of *Albizia chevalieri* as prophylaxis of *Naja nigricollis* envenomation. 116

4.8.5 Studies of *in vivo* antivenom effect of pure isolated compound from hexane and ethyl acetate extract fractions of *A. chevalieri* on *Naja nigricollis* envenomation. 117

4.8.6 Histopathological effect of venom of *Naja nigricollis* on some mice organs treated with MLD of venom, HXC_1, EAC_1 and extracts of *A. chevalieri* 119

CHAPTER FIVE 121

5.0 DISCUSSIONS 121

CHAPTER SIX 135

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS 135

6.1 Summary 135

6.2 Conclusions 136

6.3 Recommendations 137

REFERENCES 138

APPENDICES 152

LIST OF TABLES

TABLE	TITLE	PAGE
1.1	Plants used as antidotes of snake bites worldwide	12
2.2	Taxonomic Classification of *Albizia chevalieri*	46
4.1	Mass and Percentage Yield of Extracts	63
4.2	Preliminary Phytochemical Screening of the stem bark of *Albizia chevalieri*	64
4.3	Masses, Melting Points and Physical Appearances of Compounds Isolated	68
4.4	^{1}H and ^{13}C-NMR Chemical Shifts of Compound HXC_1 in $CDCl_3$,400 MHz	71
4.5	DEPT 135 Spectrum analysis of C- atoms present in Compound HXC_1	73
4.6	Summary of Spectra data of HXC_1	78
4.7	^{1}H and ^{13}C-NMR Chemical Shifts of Compound HXC_2 in $CDCl_3$, 400MHz	82
4.8	Summary of Spectra data of HXC_2	89
4.9	^{1}H and ^{13}C-NMR Chemical Shifts of Compound HXC_3 in $CDCl_3$, 400MHz	93
4.10	Summary of Spectra data of HXC_3	98
4.11	^{1}H and ^{13}C-NMR Chemical Shifts of Compound EAC_1 in $CDCl_3$, 400MHz	105
4.12	Summary of Spectra data of EAC_1	111
4.13	Determination of Minimum Lethal Dose (MLD) of venom of *Naja nigricollis* on mice	113
4.14	Determination of LD_{50} of extracts and fractions of *A. chevalieri* by Lorke's method	113
4.15	Evaluation of extract fractions of *A. chevalieri* at 200 $mgkg^{-1}$	115
4.16	Evaluation of hexane and n-butanol extract fraction of *A. chevalieri* at100 $mgkg^{-1}$ on MLD of venom	116
4.17	Evaluation of compound HXC1 of *A. chevalieri* on MLD venom	117
4.18	Evaluation of compound EAC_1 of *A. chevalieri* on MLD of venom	117
5.1	^{1}H and ^{13}C-NMR chemical shifts of compound HXC_1 in $CDCl_3$, 400 MHz as compared with Abreu et al., (2013).	124

5.2	^{1}H and ^{13}C-NMR chemical shifts of compound HXC_2 in $CDCl_3$, 400 MHz as compared with Boonyaratavej and Petsom (1991).	126
5.3	^{1}H and ^{13}C-NMR chemical shifts of compound HXC_3 in $CDCl_3$, 400 MHz as compared with Ahmadu *et al*., (2013).	129
5.4	^{1}H and ^{13}C-NMR chemical shifts of compound EAC_1 in $CDCl_3$, 400 MHz as compared with Ahmed *et al*., (2013).	131

LIST OF FIGURES

FIGURES	TITLE	PAGE
2.1	Structure of some saponins (IX-XX) isolated from genus Albizia	28
2.2	Structure of some terpenes (XXI-XXIV) isolated from Genus Albizia	29
2.3	Structure of some nitrogenous compounds (XXV-XXX) isolated from Genus Albizia	31
2.4	Structure of some flavonoids (XXXI-XXXII) isolated from Genus Albizia	31
2.5	Structure of some phenolic compounds (XXXI-XXXII) isolated from the genus Albizia	32
2.6	Some Saponins bioactive compound (XXXVIII-XLVIII) of Genus Albizia	35
2.7	Some bioactive nitrogenous compounds (LX-XLIX) of Albizia	36
2.8	Some bioactive flavonoid compounds (LXI-LXVI) of Albizia	38
2.9	Chemical structure of bioactive phenolic compounds (LXVII-LXVIII) from the genus Albizia	39
4.1	IR Spectrum of Compound HXC_1	69
4.2	^{1}HNMR Spectrum of compound HXC_1 in $CDCl_3$, 400 MHz	70
4.3	^{13}CNMR Spectrum of compound HXC_1 in $CDCl_3$, 400	72
4.4	DEPT 135 spectrum of compound HXC_1 in $CDCl_3$, 400 MHz	74
4.5	Mass Spectra of Compound HXC_1	76
4.6	Suggested structure of compound HXC_1	78
4.7	IR Spectrum of Compound HXC_2 (KBr)	79
4.8	^{1}HNMR spectrum of compound HXC_2 in $CDCl_3$, 400 MHz	80
4.9	^{13}CNMR spectrum of compound HXC_2 in $CDCl_3$, 400 MHz	83
4.10	DEPT 135 spectrum of compound HXC_2 in $CDCl_3$, 400 MHz	85
4.11	Mass spectra of compound HXC_2	87

4.12	Suggested structure of compound HXC_2	89
4.13	UV Spectra of Compound HXC_3[1]	90
4.14	IR Spectra of Compound HXC_3	91
4.15	HNMR Spectrum of Compound HXC_3 in $CDCl_3$, 400 MHz	92
4.16	^{13}CNMR Spectrum of Compound HXC_3 in $CDCl_3$, 400 MHz	94
4.17	Mass Spectrum of Compound HXC_3	96
4.18	Suggested structure of compound HXC_3	98
4.19	UV Spectra of Compound EAC_1	99
4.20	FTIR Sprctrum of Compound EAC_1	100
4.21	^{1}HNMR Spectrum of compound EAC_1 in $CDCl_3$ 400 MHz	102
4.22	^{13}CNMR Spectrum of Compound EAC_1 in $CDCl_3$ 400 MHz	104
4.23	DEPT 135 Spectrum of Compound EAC_1 in $CDCl_3$ 400 MHz	107
4.24	Mass Spectrum of Compound EAC_1	109
4.25	Suggested structure of compound EAC_1	111

LIST OF SCHEMES

SCHEMES	TITLE	PAGE
4.1	Fragmentation patterns of compound HXC_1	77
4.2	Possible fragmentation pattern of compound HXC_2	88
4.3	Possible fragmentation pattern of compound HXC_3	97
4.4	Possible fragmentation pattern of compound EAC_1	110

LIST OF PLATES

PLATES	TITLE	PAGE
I	Morphological parts of *A. chevalieri*	49
II	*A. chevalieri* in its natural habitat	50
III	TLC profile of hexane extract	66
IV	TLC profile of ethyl acetate extract	66
V	Histological changes in the Liver of envenomated mice and envenomated mice treated with EAC_1, HXC_1 and plant extracts.	119
VI	Histological changes in the Kidney of envenomated mice and envenomated mice treated with EAC_1, HXC_1 and plant extracts.	120
VII	Histological changes observed in Liver and Kidney of envenomated mice treated with the various extracts of *A. chevalieri*	121

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
I	Column Fraction TLC Profile	153
1a	The TLC profile of some fractions of *A. chevalieri* Hexane extract	153
1b	The TLC profile of some fractions of *A. chevalieri* Ethyl acetate extract	155
II	2D NMR spectra of compounds HXC1 and EAC1	156
IIa	The ^{1}H-^{1}H COSY spectrum of compound HXC_1	156
IIb	The ^{1}H-^{13}C HSQC spectrum of compound HXC_1	157
IIc	The ^{1}H-^{13}C HMBC spectrum of compound HXC_1	158
IId	The ^{1}H-^{1}H COSY spectrum of compound EAC_1	159
IIe	The ^{1}H-^{13}C HSQC spectrum of compound EAC_1	160
IIf	The ^{1}H-^{13}C HMBC spectrum of compound EAC_1	161
III	Probit Analysis determination of MLD (LD_{99}) of *Naga nigricollis* venom	162
III	Probit Transform Responses	164

LIST OF ABREVIATIONS

Acronyms	Meaning
COSY	Correlation Spectroscopy
^{13}CNMR	Carbon-13 Nuclear Magnetic Resonance
$CDCl_3$	Deuterated Chloroform
DEPT	Distortionless Enhancement by Polarisation Transfer
DMSO	Dimethyl sulphoxide
EAC_1	Isolated Compound 1 from Ethyl acetate
EISMS	Electron Impact Mass Spectrometry
EtOAc	Ethyl acetate
GC-MS	Gas Chromatography/ Mass Spectrometry
HMQC	Heteronuclear Multiple-Quantum Correlation
^{1}HNMR	Proton Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
HREIMS	High Resolution Electron-spray Ionisation Mass Spectrometry
HSQC	Heteronuclear Single Quantum Correlation
HXC_1	Isolated Compound 1 from Hexane Extract
HXC_2	Isolated Compound 2 from Hexane Extract
HXC_3	Isolated Compound 3 from Hexane Extract
LD_{99}	Lethal dose that is capable of killing the entire Population
LD_{50}	Lethal Dose that can Kill 50% of the Population
IR	Infrared
MLD	Minimum Lethal Dose
NMR	Nuclear Magnetic Resonance

NOESY	Nuclear Overhauser Effect Spectroscopy
NNBV	*Naja nigricollis* Broadley Venom
PMA	Phosphomolybdic acid
PTT	Pentacyclic Triterpenoids
TLC	Thin Layer Chromatography
UV	Ultraviolet
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 The Consciousness of Phytochemistry

In its entirety Phytochemistry or plant chemistry is ever now than before more activity conscious in the search for active pure plant compounds; as put by Harborne (1998, cited in Beattie, 2009), it is that aspect of science that ‘‘*concerned itself with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, their biosynthesis, turnover and metabolism, their natural distribution and their biological function’’.* The sessile nature and lack of a sophisticated immune system in plants has necessitated the need for the development of these complex chemical systems. Consequently, the compounds produce by plants have been categorized into primary and secondary metabolites. Compounds contributing to fundamental metabolism are termed primary metabolites while in contrast, secondary metabolites are limited in their distribution; both throughout the plant families and between different species (Raven *et al.,* 2005).

Secondary metabolites were once thought to be waste compounds; however, our understanding of their importance in-plant and the role they play has expanded. It is been revealed that many of these secondary metabolites are potent bactericidal, repellent, or even toxic agents to pests and herbivores (Dewick, 1997, cited in Beattie, 2009). Some plant chemicals are relied on as a means of defence against pathogens and predators, as attractants to lure mobile creatures for fertilization and dissemination and also for aerial allelopathy (interplant communication) (Beattie, 2009). Volatile organic compounds and pigments are revealed to be attractive to insects that help with fertilization, or warning colours to defend against predators (Dewick, 1997), while other plant pigments can provide protection against

environmental damage such as free radicals and UV radiation (Raven *et al.,* 2005). However, some secondary products perform signalling functions as plant hormones and pheromones. Consequently, natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Chen *et al.,* 2010).

Plants produce an array of secondary metabolites and many of these have been extracted, isolated and developed into economically important products including; oils, gums, resins, tannins, rubber, waxes, pigments, flavours, fragrances, surfactants, preservatives, pesticides and pharmaceuticals (Raven *et al.,* 2005). Thus, plant secondary metabolites represent a tremendous resource for commerce. Phytochemists would therefore continue to play a fundamental role in the chemical investigation of these plants. Phytochemical studies may be directed towards characterizing the chemical composition of complex essential oils or plant extracts. Phytochemical screening can assist taxonomic classification while bioassay guided studies can target and identify biologically active compounds in complex plant extracts (Harborne, 1998). Such is the task of this research; to explore and isolate bioactive compounds from *Albizia chevalieri* on the basis of ethnobotanical information and earlier empirical research on same, which recorded a significant hypoglycaemic effect (Saidu *et al.,* 2007a) as well as antioxidant activity (Aliyu *et al.,* 2009) on the leave extracts of the plant.

1.1.2. Drug discovery process and sources

Drug discovery is the process by which new candidate medications are discovered. Drugs from natural sources may fall into one of three categories of compounds: those that were isolated from biological organisms, those that are modified versions of natural products, and those that are completely synthetic but yet, based upon models of natural origin (Cragg

2002). Today, natural products are responsible for about half of the approved drugs that are currently available. For example, 18 of the 42 new drugs discovered in 1992 are either natural products or synthetic analogs of natural products (Davis, 1992). Obviously, nature has had quite an effect on the science of drug discovery, and the role of the chemist has become important for work in isolation, structure determination, and synthesis of bioactive compounds (Newman *et al.,* 2000). The questions for natural products chemists may pose as follows: which biological species produce these compounds, what is the structure of the molecules, and how potent are they as therapeutic agents (Yoder, 2005).

Drug discovery from natural products has reclaimed the attention of the pharmaceutical industry and is on the verge of a comeback due to new technological inputs that promise better returns on investment (Bhutani, 2007). This is because natural products, particularly, microbial and plant products in their native form have been associated with mankind since ancient times. These have played a vital role in the discovery of New Chemical substance in a range of time referred to as 'the golden period (1981-2002) of drug discovery'. An important aspect of it all is that drugs derived from natural sources served as drug leads suitable for optimisation by synthetic means (Bhutani, 2007).

1.1.3. Isolation of lead-molecules from plants

Drug Discovery in ancient times was by chance and was based on clinical practices (Arun, 2015). Understanding therapeutic benefits deepens Natural Product (NP) and increase previously serendipitous discoveries, evolving into active researches for new medicines (Wang *et al.*, 2007). Progress in Life Sciences has not only revealed many pathological processes of diseases but also the molecular mechanisms. These has then led to the development of molecular and cellular assays in conjunction with 'Ultra-High-Throughput Screening' (HTS) of NP. Consequently, the transition from traditional to empirical and

molecular screening will increase the probability of discovering new leads and drug candidate from natural products (Arun, 2009).

NP isolation and obtaining pure material in large quantities is generally difficult if the active molecule is present in small amounts. Many NP are difficult to isolate, purify and many a times difficult to synthesize (Arun, 2015). Marston *et al*., (1997) reaffirmed earlier that thin-layer chromatography (TLC) remains the simplest, cheapest and fastest method of detecting plant constituents, owing to its ease of operation, reproducibility and equipment. However, efficient separation of metabolites will require a more sensitive equipment with good selectivity and detection techniques, together with the capability of providing online structural information. Hence, most modern separation equipment are designed as hyphenated systems that performs isolation of pure NP and their detection in a most efficient and rapid manner.

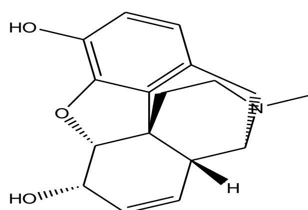
Earlier than now, most pharmaceutical companies find NP unattractive owing to the difficulties concerning isolation and structure elucidation of bioactive molecules. However, more rapid methodologies by which purification can be enhanced, have made people to relook into NP resources. Identify 'hits' (novel active chemical compounds), that can be developed into a potential drug lead is often easier with modern techniques such as using High Performance Liquid Chromatography (HPLC) and subsequent fractionation analysis by Liquid Chromatography / Mass Spectrometry (LC / MS) (Rollinger *et al*., 2006); by combining MS data with those from libraries of known compounds, net novel molecules can be distinguished–a process called de-replication. This then helps to terminate projects without novel compounds in the beginning while focusing on those of interest. Automated sample injection and fraction collection enables multiple samples to be studied at one time (Rollinger *et al*., 2006).

These advancement in separation science are now applied in fields such as combinatorial chemistry, where large and structurally diverse chemical libraries can be generated, including parallel synthesis which help in producing several analogs faster using NP scaffolds (Arun, 2009). Generally, innovations in computer applications, automation technologies, micro-fluid management, and software design, have all been incorporated into separation science techniques to offer enhanced speed of screening of large number of compounds for the establishment of bioactive lead molecules (Rollinger *et al*., 2006).

1.1.4. Historically important isolated lead-molecules from plants

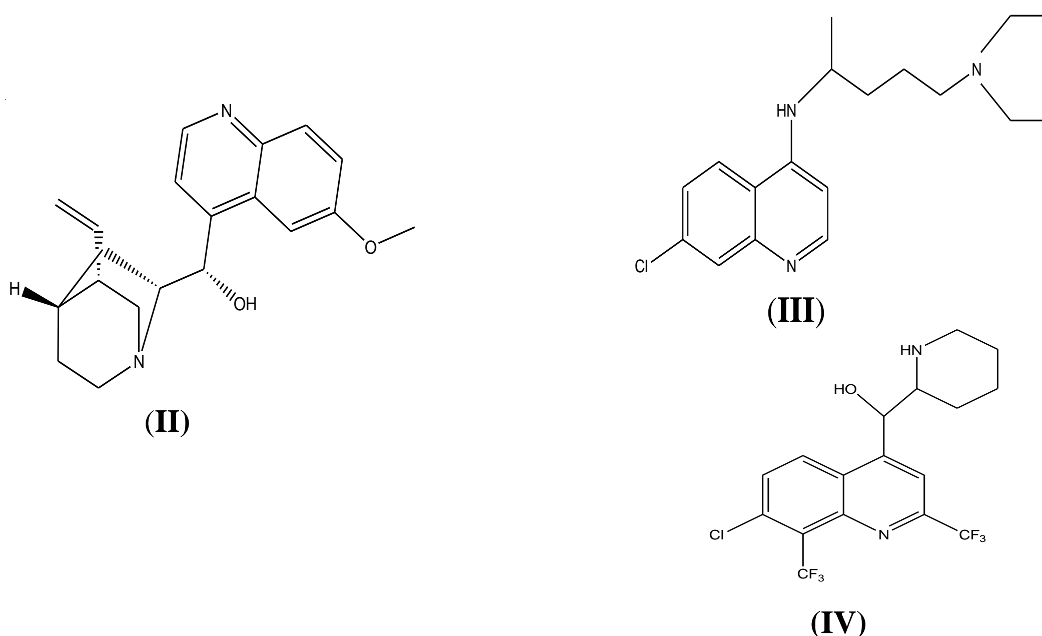
Some well-known and important drugs have originated from natural sources. A few examples are given to indicate what an important impact these drugs have had on medical treatment and disease control. It can be noted that these drugs were isolated from natural sources many of which have been used by various cultures throughout history (Guza, 2004).

Morphine (**I**) was first isolated from the opium poppy (*Papaver somniferum*) between 1803 and 1805 (Courtwright, 2009), just like in ancient Mesopotamia the oils of *P. somniferum* were used as an analgesic; this discovery and isolation of morphine led to an increased interest in alkaloid chemistry and resulted in the development of other analgesic agents (Cragg and Newman, 2001). Morphine was the first commercially available pure natural product, marketed in 1826 (Newman *et al*., 2000).



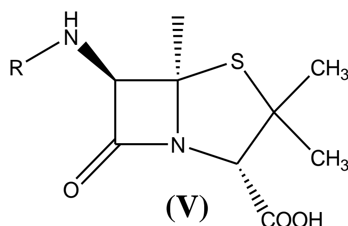
I

Quinine is another historic drug that has earlier served as malaria therapy. Malaria has been and continues to be a problem in many areas around the world. The native Amerindians of the Amazon region used the bark of the Cinchona tree to treat malaria (Clark, 1996). Quinine (**II**), the active component of Cinchona bark, was isolated in 1820 from *C. officinalis* (Cragg, 2002). Quinine was the first effective anti-malarial drug to be isolated (Philipson, 2001). Other anti-malarial drugs such as chloroquine (**III**) and mefloquine (**IV**) were synthesized based on the structure of quinine (Cragg and Newman, 2001a).



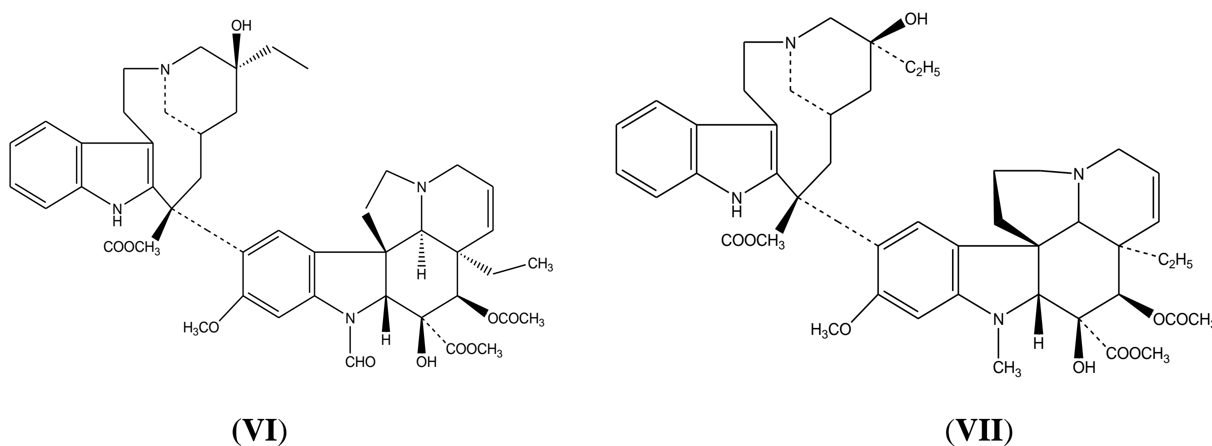
(II) **(III)** **(IV)**

Penicillin (**V**) was first discovered in 1929 by Fleming from the fungus, *Penicillium notatum* (Cragg and Newman, 2001b). This discovery was important to the development of antibiotics and changed medicine forever (Clark, 1996). The discovery of this revolutionary drug from a natural source prompted the investigation of nature for other novel compounds.



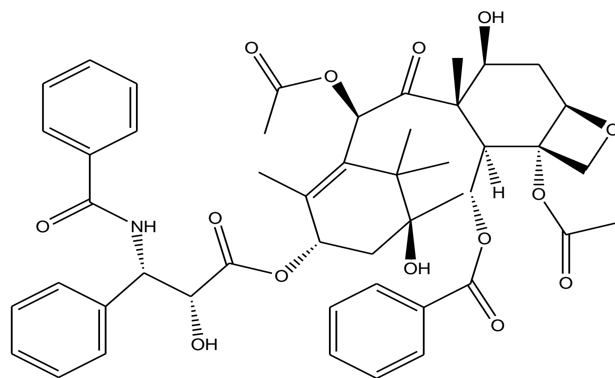
(V)

The plant *Catharanthus roseus*, commonly known as the Madagascar periwinkle, was used in some cultures as a folk remedy to treat diabetes (Cragg and Newman, 2001b). However, when *C. roseus* was evaluated for hypoglycemic activity, no compounds with such activity were found (Clark, 1996). Instead, the results led to the hypothesis that the plant extract contained a compound potentially useful for the treatment of cancer (Clark, 1996). Upon further investigation of *C. roseus* for anti-cancer agents, vinblastine (**VI**) and vincristine (**VII**) were isolated in 1954 (Noble, 1990). These drugs were developed by Eli Lily and are important agents in the treatment of cancer.



(VI) **(VII)**

A random collection of plants by the United States Department of Agriculture (USDA) for the National Cancer Institute (NCI) yielded an extract with anti-cancer activity (Cragg and Newman, 2001). The extract was from the Pacific yew tree (*Taxus brevifolia*) and the active compound, paclitaxel (**VIII**), was isolated in 1969 (Cragg and Newman 2001b). Cragg and Newman (2001b), also reported that several Native American tribes use various parts of Taxus trees for the treatment of a wide range of non-cancerous ailments. Paclitaxel is an important anti-cancer drug in use today (Guza, 2004).



(VIII)

1.2 Animal Venom

Animal venoms are complex mixtures of proteins, peptides, carbohydrates, nucleosides, biogenic amines, metallic cations (such as sodium, calcium and zinc) and very low levels of free amino acids and lipids. Proteins and peptides comprise 90–95% of the dry weight of venom (Abubakar, 2004).

Venom is produced by an apparatus composed of modified exocrine glands and is excreted and injected into the prey or victim through bristles, sticks or fangs. Toxic animals include insects, arachnids (spiders and scorpions), snakes, marine animals etc., which uses venoms for catching prey and as defence mechanisms (Coppola and Hogan, 1992).

Animal venoms are an extremely dangerous mixture of different substances whose effects vary from negligible or moderate local symptoms to severe systemic reactions, death sometimes being the outcome. Clinical evaluation of the patients and experimental work has permitted the elucidation of a number of biochemical, pharmacological and pathophysiological mechanisms that are involved in the mode of action of animal venoms. The most common animal used for analysis is the albino mouse, into which the venom or isolated components are intravenously, intraperitoneally or subcutaneously administered.

Despite the fact that animal venoms especially those from snakes are deleterious to the body, they may be important sources of drugs and biomolecules (Stocker, 1990).

1.2.1. Snake bite and venom

Information from literature has informed that there are over 420 species of snake in Africa, 100 of which occur in Nigeria of which 40 are venomous, 10 being very deadly (Pugh and Theakston, 1980). Both poisonous and non-poisonous snakes occur throughout Africa (Warrell, 1995). However, statistics on snake bites are hard to come by especially in Nigeria (Pugh and Theakston, 1980).

In the Sahel it could be estimated that 23,000 people are killed each year by snakes. It will be easy to verify that those who die are the rural poor, who walk at night without lights, have no proper shoes, cultivate the soil using short-handle tools and have little or no access to medical care (Abubakar, 2004). Hence, Warrell (1995) had earlier opined that in more remote areas of Africa, snakebite can be a significant medical risk. *Naja nigricollis* also called the black spitting cobra or black necked cobra is among the common spitting cobras found in Nigeria with quite a number of species. This deadly snake is occasionally found in the semi-desert areas of Northern Nigeria particularly in Sokoto (Russell, 1980). In a similar behaviour of treatment remedy by folks of unlettered society, Newman *et al* (1997) had noted that most victims usually prefer traditional treatment when bitten by venomous snakes and cases of such are not reported to hospitals unless conditions have reached critical stages. Plants with reputations for use against poisonous snake venoms are found worldwide, especially in areas inhabited by poisonous snakes (Abubakar *et. al.*, 2006). Consequently, the use of plants in poisonous snake bites is practiced worldwide (Nuno *et al.,* 1994, Walter *et al.,* 1994 and Selvanayagam *et al.,* 1995).

1.2.2. Potentials of plants used in ethnomedicine for the treatment of poisonous snake bites

Quite a good record of plant anti venom agent used among the various ethnic groups and regions of Nigeria has been established through ethnobotanical and ethnomedical records. Notably among the survey data are plants with therapeutic claims on cancer and venoms in the predominant Hausa-Fulani tribe of Northern Nigeria (Abubakar *et. al.*, 2006 and Abubakar *et al.*, 2007). Records has also showed plants used in the treatment of poisonous snake bites as spanning genera and species of plants, their uses usually depending on the type of poisonous snake found within the locality (Abubakar *et. al.*, 2006). Some of the methods of therapy use in traditional medicine have been pointed to have scientific basis (Selvanayagam *et al* 1995), hence to this end, quite a number of anti- venom compounds have been isolated from some of these plants (Haruna and Choudry, 1995 and Tsai *et al* 1980).

1.2.3. Worldwide ethnomedical use of plants in the treatment of poisonous snake bites

The use of plants in poisonous snake bites is practiced worldwide (Nuno, *et al* 1994 and Selvanayagam *et al* 1995). The plants used in the treatment of poisonous snake bites span genera and species of plants, their uses usually depending on the type of poisonous snake found within the locality. Table one below, adopted from Abubakar (2004) shows the different uses of plants in ethnomedicine for treatment and prophylaxis of poisonous snake bites.

Table 1.1. Plants reported as antidotes for snake bites worldwide*

Plant family	Common name (if any)	Locality	Plant part used
ACANTHACEAE *Blepheris capensis*		South Africa	Herbs used by Natives
APOCYNACEAE			
Tabernaemotana sralensis		Indochina	Root
Urechites suberecta		Tropical America	Whole Plant
Wrghtia tometosa		India and Pakistan	Bark
ARACEAE		Himalayas	Root
Arisaema speciosum	Black pepper	India and Indonesia	The whole plant
Rhaphiodora pertusa			
ARALIACEAE			
Aralia spinosa	Angelica tree	Southern united states; used for Rattle snakes bites	Root bark decoction
ARISTOLCHIACEAE			
Aristolochia bartbata		Brazil	Rhizome
Aristolochia longa		Mediterranean to Iran	Root
Aristolochia maxima		Yucatan to Venezuela	Root
Aristolochia taliscana		Sinaloa, Mexico	Root
Aristolochia theriaca		America	Root
Bragantia corymbosa		Java	Stem and leaves
ASTERACEAE			
Antennaria plataginifolia	Everlasting pussy toes	Eastern North America for rattles snakes bites	Whole plant
Echinacea angustifolia	Purple cone flower	North American plains for rattles snakes bites	Whole plant also used for other bites and stings
BIGNONACEAE			
Bignonia unguis-cati		Mexico	Whole Plant
CARYOPHYLLACEAE			
Polycaea corymbosa		India	Leaves and Flowers
CONVOLVULACEAE			
Ipomoea arborescens		Sinaloa, Mexico for rattle snake bites	Bark
EUPHOBIACAE			
Cluytia similis		South Africa	Root
FABACEAE			
Alysicarpus zeyhri		Tropical Africa	Root
Cassia alata	Ring worm senna	Pantropic	Juice from leaves
Uraria picta		Eastern India	Leaves
GENTIACEAE			
Gentiana andrewsii	Closed gentian	Mostkwakis, Eastern North America	Root
Sebaca crassulaefolia		south Africa for puff adder bites	Whole plant
LAMIACEAE			
Leonotis leonorus	Drug lionsear	South Africa	Whole plant
Leucas aspera		Tropical Asia	Leaves

MELIACEAE		Mozambique	Root
Trichilia capitata			
MELANTHACEAE			
Meliantus comosum		South Africa	Leaves and root bark
MENISPERMACEAE			
Cissampelos capensis		South Africa	Leaves
Cooculus filipendula		Brazil	Fruit
PIPERACEAE			
Piper medium		Costa Rica	Whole plant
POLYGALACEAE			
Polygala senega	Seneca snake root	Eastern north America; Seneca	Root
RUBIACEAE			
Chiococa alba		Tropical America	Whole plant
Psychotria jackii		Malaysia	
SIMAROUBEACEAE			
Simarouba versicolor		Brazil	Bark
TACCACEAE			
Tacca fatsiifolia		Phillipines and Indonesia	Whole plant
Tacca palmata		Phillipines and Indonesia	Whole plant

*(Houghton and Osibogun; Walter and Lewis, 1977)

1.2.4. Plant derived compounds and their biochemical action as anti-venom agents of poisonous snake bites

Plants used in folkloric treatment of snakebites have furnished compounds with widespread activity against pathological action of poisonous snake bites (Tsai *et al* 1980, Mors *et al.,* 2000). Many plant species used against snake venoms contain compounds with antiinflammatory properties such as flavonoids (rutin, hesperidin, and quercetin), coumarins (coumarin, umbelliferone, bergapten etc), triterpenes, sterols (Pereira *et al,* 1994, Hutt and Houghton, 1998). Many plant-derived compounds have beneficial effect on poisonous snakebites due to their immunostimulants action usually achieved at very low doses. This action is exhibited by some alkaloids, quinones, isobutylamides, phenolcarboxylic acid esters and aromatic acid derivatives (example found in resins and balsams) and terpenoids (Wagner, 1993). Several other plant compounds with immunostimulatory effects, which may be beneficial in snake bites, include cepharanthine, tylophorine and sesquiterpene lactones (Houghton and Osibogun, 1993).

Many polysaccharides, tannins (such as euphobin A and B) and glycoproteins enhance the unspecific immune system by activating the phagocytotic activity of granulocytes and macrophages, or by inducing cytokine production or influencing complement factors (Wagner, 1993). Some compounds from plants used for general inflammation also inhibit enzymes (like phospholipase A_2) in snake and scorpion venoms (Houghton and Osibogun, 1993, Hutt and Houghton, 1998). Some of these plant compounds are hypolaetin-8-glucoside and related flavanoids. Stimulation of the immune system might also contribute to reducing the effects of snakebites and improvement in recovery from envemomization by contributing to a more rapid removal of the venom (Hutt and Houghton, 1998).

A more direct antivenom activity involves complexation (binding) of the compounds with venom constituents thus preventing their action on the receptors (preventing the competitive blockade of receptors). This binding may also inhibit the venom-mediated release of catecholamines thereby preventing the consequent action of catecholamines (Hutt and Houghton, 1998). Typically, chlorogenic acid and related polyphenolic compounds act as snake venom antidote by binding to proteins through hydrophobic interactions and hydrogen bonds, leading to an inhibitory action on the classical complement system (Ejzemberg *et al.*, 1999). Analgesic properties like those provided by tropane alkaloids would also lessen the pain of the bite, as would compounds that act as sedatives and tranquilisers (Houghton and Osibogun, 1993).

Several pharmacological properties of plants reputed to be snakebite antidotes include antimyotoxic, antihaemorrhagic, analgesic, and antiedematogenic, blockage of cutaneous and intraperitoneal capillary permeability activity caused by the venom (Pereira *et al.,* 1994). Extracts as those of *Mucuna pruriens* var. *utilis* produce a dose-related increase in the

clotting time of blood, this may be useful in venom containing procoagulant proteins like the carpet viper venom *(Echis carinatu*s), *Bothrops* species and many other vipers (Houghton and Osibogun, 1993). Antivenom compounds so far isolated from plants include protocatechuic acid, a catechin, caffeic acid derivatives (chlorogenic acid, cynarin) coumarins (bergapten), flavonoids (e.g. quercetin, primetin, rutin,), α-turmerone, aristolochic acids, coumestans (wedelolactone), sterols and triterpenes and triterpenoid glycoside (gymnenic acid), gymnagenin acid as well as the alkaloids (allantoin, shumaniofioside) and lignoflavonoids (silymarin) (Martz, 1992, Houghton and Osibogun, 1993, Reyes-Chilpa, *et al*, 1994).

Two prenylated pterocarpans, cabenegrins A-I and A-II potent antidotes against snake venom, were isolated and identified from a well-known anti-snake bite medicine named "Específico Pessoa", manufactured and sold in the north and northeast of Brazil and available to plantation workers as an oral antidote (Nakagawa, *et al* 1982). The plant, commonly called "cabeça de negro", which furnishes the extract used in the preparation of this remedy has not been identified so far, being kept secret by the manufacturers. There are about ten plants with the name "cabeça de negro" in South America, two plants reputed as anti-snake bite medicines occur in the Ibiapaba region in Northeast Brazil, these are *Bredemeyera floribunda* Willd (Polygalaceae) (the active principle was identified as a saponin known as bredemeyeroside) and *Harpalyce brasiliana* "pacari" (Papilionaceae). Many other relevant antivenom compounds are widely distributed; these are usually nitrogen-free, low molecular weight compounds (except aristolochic acid, an untypical non-basic, nitro-derivative), (Pereira *et al,* 1994). Structurally similar compounds are ubiquitously found in plants and may be useful for snakebites. The later have an isoflavone skeleton, are acidic in nature and have a dioxy- functional group (Reyes-Chilpa, *et al*, 1994).

Evaluation of the inhibitory effects of these compounds were carried out by several groups, one study showed a total inhibition of *Bothrops asper* venom induced haemorrhage by the ethanolic, ethyl acetate and aqueous extracts of plants containing catechines, flavones, anthocyanines and condensed tannins. The inhibition was shown to be due to their ability to chelate the zinc ion required for the catalytic activity of venom's haemorrhagic metalloproteinases (Castro *et al.*, 1998). Other groups have shown that inhibition and reduction of envenomation could have been achieved by a direct neutralisation of venom peptides, polypeptides, proteins and enzymes (Pereira *et al,* 1994, Hutt and Houghton, 1998). Other plant compounds inhibit the proteolytic activities of the venom thus antagonising the venom-induced haemolysis, myotoxicity and haemorrhagic activities of venoms (Melo *et al.,* 1994, Melo and Ownby, 1999).

1.2.5. Classification and identification of snakes

Snakes are cold-blooded (ectothermic) reptile vertebrates (Reptilia), Order Squamata, which also includes lizards, crocodiles and alligators, tortoises and turtles (Smith, *et al,* 1977). There are at least 3,000-snake species worldwide, most of which are non-venomous (Swaroop and Grab, 1954). Some snakes have evolved specialised glands that produce venom, mostly derived from salivary glands. Venom may have several functions, including rapid immobilisation and predigesting of prey (Coppola and Hogan, 1992). Snakes arose during the Cretaceous era, approximately 100–120 million years ago (Smith, *et al,* 1977, Swaroop and Grab, 1954). The poisonous forms, however, are more recent, evolving possibly during the Miocene, 30 million years ago. The snake systematic has not yet been solved, different names apply to the same forms, the same name to different forms, and there is a lack of agreement in the recognition of subspecies, these are just some of the factors complicating their classification. The features commonly used in snake systematic are maxillary dentition, the

characteristics of the hemipenis, the presence or extension of the anterior trunk vertebrae bearing ventral processes known as hypapophyses, the form and disposition of scales and the presence of pits and tubercles. Snakes can be divided into families and are grouped according to the presence or absence of fangs (Smith *et al.*, 1977, Kochva, 1987).

1.2.6. Naja species (Family Elapidae)

There are many species of this snake in Africa, all with variable colours (Reid and Thekaston, 1983). The common *Naja* species found in Nigeria includes *Naja nigricollis, Naja melanoleuca* Rodel & Mahsberg., *Naja goldii* Dumeril Bibron and less commonly *Naja haje* Dumeril Bibron. *Naja melanoleuca* (the black and white cobra) has similar appearance to the spitting cobra (*Naja nigricollis*) but usually has white or yellowish markings on the back. *Naja goldii,* which is usually found in trees and low shrubs, is rather not hooded and attacks without provocation. *Naja haje* (Egyptian cobra), is occasionally found in the semi-desert areas of Northern Nigeria particularly in Sokoto (Russell, 1980). It is hooded, brown or olive coloured with a dark band on the neck. Common spitting cobras found in Nigeria are *Naja nigricollis* Broadley, *Naja nigricollis katiensis* and *Naja mossambica*. *Naja katiensis* is rare being exclusively found in Northern Nigeria especially around the Katsina and Malumfashi axis, (Abubakar, 2004).

1.2.6.1. Naja nigricollis Broadley

Naja nigricollis also called the black spitting cobra or black necked cobra is synonymous to the following: *Naja nigricollis* Reinhardt, *Naja nigricollis var. crawshayi* Gunther, *Naja nigricollis var. occidentalis* Bocage, *Naja nigricollis* Schmidt fide Broadley, *Naja nigricollis atriceps* Laurent, *Naja nigricollis occidentalis* Laurent, *Naja nigricollis* Broadley & Howell, *Naja nigricollis nigricollis* Welch, *Naja nigricollis* Broadley.

Naja nigricollis (black spitting cobra or black necked cobra) is predominantly black with dull metallic sheen, the under surface of the neck and front part of the body has salmon pink cross bars of varying width, alternating with black. Cobras are relatively fast moving, mostly active by night, may have grooved fangs which when provoked will spit, squirting a stream of extremely poisonous venom from their fangs to a distance of 2m or slightly more in the eyes (Warrell *et al*., 1976). Being secretive and common, spitting cobras are often found in and around quite large towns. The venom is unusually toxic, and a bite can cause a lot of tissue destruction if untreated (Abubakar 2004). The spitting cobra is not usually aggressive, but when molested it will attack viciously and persistently, always rearing up the forepart of the body and spreading a small hood at the base of the neck (Tamiya, 1985). This snake is usually called "Baki" in Hausa the local language in Northern Nigeria.

1.3. Statement of Research Problem

The unmatched availability of chemical diversity present in plants brings to bare the so many active constituents still unknown in most species, especially in the tropical world where facilities for modern scientific research is still a challenge. Consequently, literature search has shown that empirical research leading to the investigations of pure active compounds of *Albizia chevalieri* was not available, despite its ethnobotanical exploits.

Although the hypoglycaemic and anti-oxidant effects of the plant has been evaluated in line with folklore (Saidu *et al.,* 2007a and Aliyu *et al.,* 2009), no work was done on isolation of bioactive target molecules to determine and validate therapeutic folklore claims. For instance, ethnomedical information on the use of same plant within its phytogeographical Sahel Savannah habitat revealed versatility of traditional application; one notably is the application of its portion as an antivenom agent. Statistics on snakebites are hard to come by especially in

Nigeria (Pugh and Theakston, 1980a). Most victims usually prefer traditional treatment and cases of bites are not reported to hospitals unless conditions have reached critical stages (Newman *et al* 1997). In the more remote areas of Africa, however, snakebite can be a significant medical risk (Warrell, 1995). In the Sahel it is reliably estimated that 23,000 people are killed each year by snakes. Earlier study on snake bites in the Savanah region of Northern Nigeria had reported incidence of 497/100,000 and a mortality of 12.2% (Pugh and Theakston, 1980b). But those who die are the rural poor, who walk at night without lights, have no proper shoes, used primitive tools in farming and have little or no access to medical care.

The fabaceae family (with a long list of species being mentioned) are among those used as antidotes of snake bites worldwide (Houghton and Osibogun 1993; Walter and Lewis, 1977 as cited in Abubakar, 2004). Consequently, *A. chevalieri* remains to be evaluated for these properties of it phytoconstituents.

1.4. Justification of the study

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity they contain (Cos *et al.,* 2006). Botanicals and herbal preparations for medicinal usage contain various types of bioactive compounds (Chen *et al.,* 2011).

Tropical plants have long been a major source of drugs that we use to treat many diseases. A partial list of important medicinal compounds from tropical plants includes oubain, picrotoxin, emetine, tubocurarine, quinine, quinidine, vincristine, vinblastine, pilocarpine, physostigmine, ibogaine, strychnine, resperine, theobromine, rotenone; all made possible as a result of comparatively little investment in phytochemical screening efforts with these plants

as in contrast with plants from the temperate regions, which have been studied much more intensively because they are accessible to the major pharmaceutical laboratories of Europe and North America (Rodriguez and West, 1995).

Comparative phytochemical researches in various laboratories have indicated that plants found in the tropics/tropical rain forests of the world have three to four times more biologically active compounds than their temperate counterparts. Consequently, it has been observed that the primary sources of chemical compounds that exhibit novel pharmacological activities essential for new drug development are likely to come from marine invertebrates, insects and vascular plants from the tropics/ tropical rain forest (Rodriguez and West 1995).
It is therefore the overwhelming consensus of Phytochemists that tropical plants are understudied and contain many undiscovered drugs. These facts necessitated the need to investigate *Albizia chevalieri* (a tropical plant) of its phytoconstituents for bioactivity.

Credibility for the choice of *A. chevalieri* is also supported from it ethnobotany; owing to the fact that researchers have found that chances of success in finding useful drugs can be increased threefold if the search for a medicinal plant is concentrated on plants used for medicinal purposes by indigenous peoples of regions who have preserved their traditional culture (Rodriguez and West 1995). Achieving the objectives of this research will potentiate the use the compounds from the plant in formulations. Isolation is a part of natural product chemistry through which it is possible to separate different components and biologically active ones which can be incorporated as ingredients in the modern system of medicine. This research has therefore, the potentials of identifying biologically active pure compounds.

1.5. Aim

- This study aim at isolation and identification of biologically active compounds from the stem bark of *Albizia chevalieri* Hams

1.5.1 Objectives

I. To extract constituents of *A. chevalieri*, using hexane, ethyl acetate, n-butanol and methanol solvents

II. To isolate pure compounds from the extracts fractions of *A. chevalieri* through the means of gravity column chromatography and other chromatographic processes.

III. To elucidate the structure of the isolated compounds of *A. chevalieri* using UV, IR, NMR spectroscopy and MS spectrometry spectra data.

IV. To evaluate the inhibitory effect of the extract fractions/pure compounds of *A. chevalieri* on the crude venom of *Naja nigricollis*

V. To evaluate the possible effect of the extract fractions/isolated pure compounds of *A. chevalieri* as prophylaxis of bite against *Naja nigricollis* envenomation

1.6. Research Hypothesis

The stem bark of *Albizia chevalieri* possesses *in vivo* antivenom activity.

CHAPTER TWO

2.0. REVIEW OF RELATED LITERATURE

2.1. The Plant Family Fabaceae

The name 'Fabaceae' comes from the defunct genus *faba*, now included in *vicia*. The term "faba" comes from Latin, and appears to simply mean "bean". Leguminosae is an older name still considered valid, and refers to the fruit of these plants, which are called legumes. The legume family (Fabaceae) is the third largest family of flowering plants with more than 18,000 described species (Judd *et al*., 2002; Stevens, 2006). It is surpassed in size only by the orchid family (Orchidaceae) with about 20,000 species and the sunflower family (Asteraceae) with about 24,000 species (Parrotta, 2002). The family includes herbs, shrubs, trees and vines distributed throughout the world, especially the tropical rain forest. The fruit is technically called a legume or pod. It is composed of a single seed-bearing carpel that splits open along two seams. Legume fruits come in an enormous variety of shapes and sizes, including indehiscent pods that do not split open (Parrotta, 2002).

The leaves are usually alternate and compound. Most often they are even or odd-pinnately compound (e.g. *Caragana* and *Robinia* respectively), often trifoliate (e.g. *Trifolium*, *Medicago*) and rarely palmately compound (e.g. *Lupinus*), in the Mimosoideae and in the commonly bipinnate Caesalpinioideae (e.g. Acacia, Mimosa). They always have stipules, which can be leaf-like (e.g. *Pisum*), thorn-like (e.g. *Robinia*) or be rather inconspicuous. Leaf margins are entire or, occasionally, serrate. Both the leaves and the leaflets often have wrinkled pulvini to permit nastic movements. In some species, leaflets have evolved into tendrils (e.g. *Vicia*). Many species have leaves with structures that attract ants to protect the plant from herbivore insects (a form of mutualism). Extrafloral nectaries are common among the Mimosoideae and the Caesalpinioideae, and are also found in some Faboideae (e.g. *Vicia*

sativa). In some Acacia, the modified hollow stipules are inhabited by ants (Hélène *et al*., 2006).

The flowers always have five generally fused sepals and five free petals. They are generally hermaphrodite, and have a short hypanthium, usually cup shaped. There are normally ten stamens and one elongated superior ovary, with a curved style. They are usually arranged in indeterminate inflorescences. Fabaceae are typically entomophilous plants (i.e. they are pollinated by insects), and the flowers are usually showy to attract pollinators (Hélène *et al*., 2006). Fruits of legume family have ovaries which most typically develop into legumes. A legume is a simple dry fruit that usually dehisces (opens along a seam) on two sides. A common name for this type of fruit is a "pod", although that can also be applied to a few other fruit types. A few species have evolved samarae, loments, follicles, indehiscent legumes, achenes, drupes, and berries from the basic legume fruit (Gurcharan, 2004).

Many Fabaceae host bacteria in their roots within structures called root nodules. These bacteria, known as rhizobia, have the ability to take nitrogen gas (N_2) out of the air and convert it to a form of nitrogen that is usable to the host plant (NO_3^- or NH_3). This process is called nitrogen fixation. The legume, acting as a host, and rhizobia, acting as a provider of usable nitrate, form a symbiotic relationship (Gurcharan, 2004). There are three subfamilies of the legume family which are Subfamily Papilionoideae, Subfamily Caesalpinioideae and Subfamily Mimosoideae. Members of the subfamily Mimosoideae have flowers with radial symmetry, small, inconspicuous corollas and numerous, showy stamens. The flowers are typically in many-flowered heads or spikes. This subfamily includes *Acacia* (wattle), *Albizia* (silk tree), *Samanea* (monkeypod), *Prosopis* (mesquite) and *Calliandra* (powder puff) (Parrotta, 2002).

2.1.1. Description of Fabaceae

Fabaceae range in habit from giant trees (like *Koompassia excelsa*) to small annual herbs, with the majority being herbaceous perennials. Plants have indeterminate inflorescences, which are sometimes reduced to a single flower. The flowers have a short hypanthium and a single carpel with a short gynophore, and after fertilization produce fruits that are legumes.

2.1.2. Growth habit of Fabaceae

The Leguminosae have a wide variety of growth forms including trees, shrubs or herbaceous plants or even vines or lianas. The herbaceous plants can be annuals, biennials or perennials, without basal or terminal leaf aggregations. They are upright plants, epiphytes or vines. The latter support themselves by means of shoots that twist around a support or through cauline or foliar tendrils. Plants can be heliophytes, mesophytes or xerophytes (Watson and Dallwitz 2007, Judd *et al.*, 2002).

2.1.3. Distribution and habitat of Fabaceae

The Fabaceae have an essentially worldwide distribution, being found everywhere except Antarctica and the high arctic (Stevens 2006). The trees are often found in tropical regions, while the herbaceous plants and shrubs are predominant in extra tropical regions (Watson and Dallwitz 2007).

2.1.4. Economic and cultural importance of Fabaceae

Legumes are economically and culturally important plants due to their extraordinary diversity and abundance, the wide variety of edible vegetables they represent and due to the variety of uses they can be put to: in horticulture and agriculture, as a food, for the compounds they

contain that have medicinal uses and for the oil and fats they contain that have a variety of uses (Allen and Allen, 1981; Duke, 1992; Graham and Vance, 2003; Wojciechowski, 2006).

2.1.5. Use of Fabaceae as food and forage

The history of legumes is tied in closely with that of human civilization, appearing early in Asia, the Americans (the common bean, several varieties) and Europe(broad beans) by 6,000 BCE, where they became a staple, essential as a source of protein. Their ability to fix atmospheric nitrogen reduces fertilizer costs for farmers and gardeners who grow legumes, and means that legumes can be used in a crop rotation to replenish soil that has been depleted of nitrogen. Legume seeds and foliage have comparatively higher protein content than non-legume materials, due to the additional nitrogen that legumes receive through the process. Some legume species perform hydraulic lift, which makes them ideal for intercropping (Sprent, 2009). Farmed legumes can belong to numerous classes, including forage, grain, blooms, pharmaceutical/industrial, fallow/green manure and timber species, with most commercially farmed species filling two or more roles simultaneously. Other uses are as natural gum, dye stuffs and ornamental (Britannica 2009).

2.1.6. Chemical composition of Fabaceae

The Leguminosae are rarely cyanogenic however, where they are, the cyanogenic compounds are derived from tyrosine, phenylalanine or leucine. They frequently contain alkaloids. Proanthocyanidins can be present either as cyanidin or delphinidine or both at the same time. Flavonoids such as kaempferol, quercitin and myricetin are often present. Ellagic acid has never been found in any of the genera or species analysed. Sugars are transported within the plants in the form of sucrose. C3 photosynthesis has been found in a wide variety of genera (Watson and Dallwitz, 2007). The family has also evolved a unique

chemistry. Pterocarpans are a class of molecules (derivatives of isoflavonoids) found only in the Fabaceae (Britannica, 2009).

Discovery of cyclotides in the fabaceae family provides new insights into the cyclization, evolution, and distribution of circular proteins. Cyclotides are plant proteins whose defining structural features are a head-to-tail cyclized backbone and three interlocking disulfide bonds, which in combination are known as a cyclic cystine knot. This unique structural motif confers cyclotides with exceptional resistance to proteolysis. Their endogenous function is thought to be as plant defence agents, associated with their insecticidal and larval growth-inhibitory properties. However, in addition, an array of pharmaceutically relevant biological activities has been ascribed to cyclotides, including anti-HIV, anthelmintic, uterotonic, and antimicrobial effects. Moreover, their study provides impetus for the examination of other economically and agriculturally significant species within Fabaceae, now the largest plant family from which cyclotides have been described (Poth *et al*., 2011).

2.2.1. The family Mimosaceae

Some classification systems, for example the 'Cronquist System', treat the Fabaceae in a narrow sense, raising the Mimosoideae to the rank of family as Mimosaceae. The Angiosperm Phylogeny Group (APG) however still treats Fabaceae in the broad sense. The Mimosaceae as a family includes 82 genera and more than 3,200 species. Like Caesalpinioideae, Mimosoideae legumes are primarily woody plants of the tropics, and the few species native to temperate parts of the world are mostly herbaceous. The Mimosaceae, as mentioned earlier are characterized by flowers with small petals and numerous prominent stamens. This family is further subdivided into four tribes: Acacieae, Ingeae, Mimoseae, and Mimozygantheae (Mimosoideae, 2009).

2.2.2. Distribution of Mimosaceae

Their distribution is worldwide, but mostly tropical and subtropical with a focus on dry regions, as indicated by the large and typical genus *Acacia*. Flora records in Texas shows documentation of 12 genera and 59 species including the 'mimosa tree' (*Albizia*) that grows on campus and sometimes escapes cultivation; as well as mesquite (*Prosopis*), plus 'sensitive' herbs (leaves move when touched) of the genera *Mimosa* and *Schrankia* (Orwa *et al.*, 2009).

2.2.3. Floral structure of Mimosaceae

Significant features shows that leaves of this family tend to be bipinnately compound, while the actinomorphic flowers are distinctive relative to other beans, they are usually quite small. The most distinctive floral feature of the Mimosaceae is the inflorescence, which is usually a spherical head of small, regular flowers with the stamens well exerted from the (often) sympetalous corolla and the filaments taking on an attractive function (Orwa *et al.*, 2009).

2.3.1 The genus *Albizia*

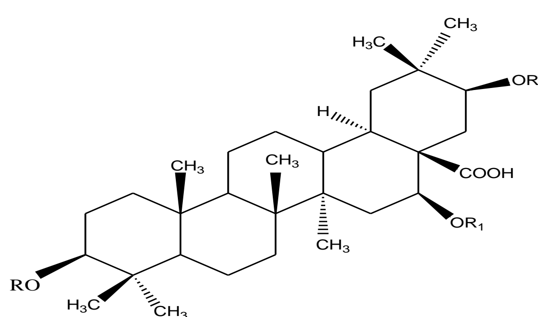
The genus *Albizia* comprises approximately 150 species, mostly trees and shrubs native to tropical and subtropical regions of Asia and Africa. Leaves are bipinnate with leaflets in numerous pairs or larger in fewer pairs. Petiolar glands are conspicuous. Flowers are in globose heads or spikes. Stamens elongate, usually white. Corolla is funnel-shaped, connate beyond the middle. Fruit is broadly linear indehiscent or 2- valved, valves not twisted (Migahid, 1989). The species of the genus Albizia are listed as in Table 2.1 below.

2.3.2. Phytochemical constituents of the genus Albizia

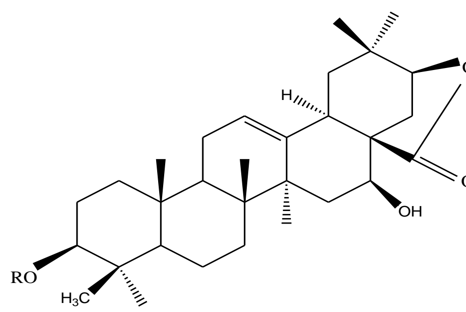
Phytochemical investigation of different species belonging to genus *Albizia* afforded different classes of secondary metabolites such as saponins, terpenes, alkaloids and flavonoids.

2.3.2.1. Saponins reported from the genus Albizia

Genus *Albizia* has been known to contain substantial amounts of saponins. Lebbekanin E (**IX**) was isolated from *A. lebbeck* (Varshney *et al,* 1976). Three saponins also isolated from the seeds of *A. lucida* were established as 3-*O*-[β-D-xylopyranosyl (1→2)- α-L-arabinopyranosyl (1→6)] [β-D-glucopyranosyl (1→2)]-β-D-glucopyranosyl echinocystic acid,(**X**) 3-*O*-[α-L-arabinopyranosyl (1→6)][β-D-glucopyranosyl (1→2)]- β-D-glucopyranosyl echinocystic acid (**XI**) and 3-*O*-[β-Dxylopyranosyl (1→2)-β-D-fucopyranosyl (1→6)-2- acetamido-2-deoxy-β-Dglucopyranosyl echinocystic acid (**XII**). In addition, three main saponins were isolated from the bark of *A. lebbeck* and named albiziasaponins A, B and C (**XIII-XV**) (fig 2.1) (Bikas *et al.*, 1995). The stem bark of *A. gummifera* yields oleanane saponins: vitalboside-A (**XVI)** and vitalboside-A 2'-methylglucuronate (**XVII**). Moreso, albiziahexoside (**XVIII**), a hexaglycosylated saponin, was isolated from the leaves of *A. lebbeck* (Minoru *et al.*, 2003). Two oleanane-type triterpene saponins, adianthifoliosides A (**XIX**) and B (**XX**) were also isolated from an ethanolic extract of roots of *A. adianthifolia.* The two compounds were characterized as glycosides of acacic acid acylated by an *O*-hydroxybenzoyl unit (Haddad *et al.*, 2003).



(IX) R and/or R1 = glucose, arabinose, xylose and rhamnose



(**XIII**) R=β-glucopyranosyl-α-arabinosyl-β-xylopyranosyl
(**XIV**) R=β-glucopyranosyl-α-arabinosyl-β-glucopyranosyl
(**XV**) R=β-xylopyranosyl-α-arabinosyl-β-glucopyranosyl-β- glucopyranosyl

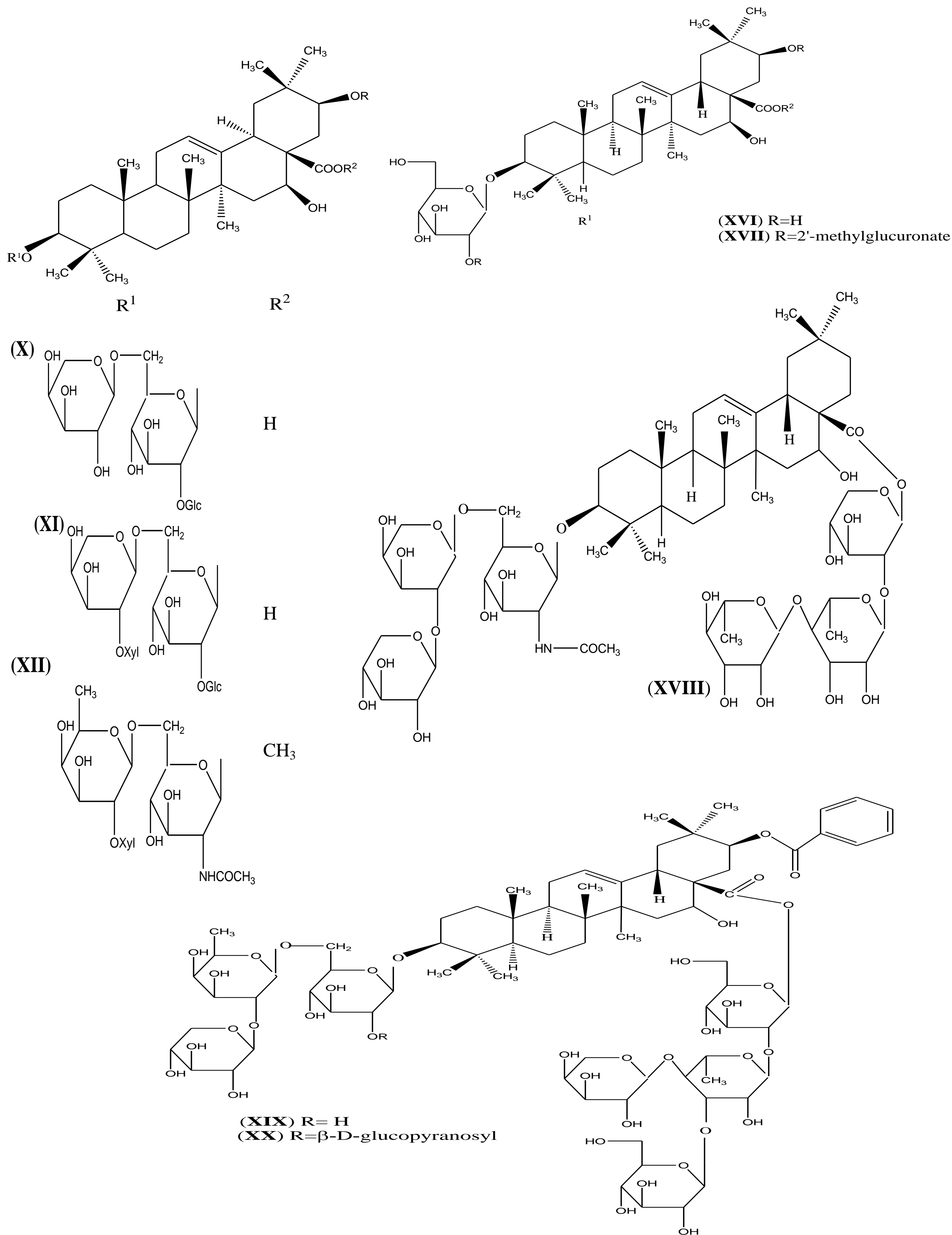


Fig.2.1. Structures of some saponins (IX-XX) isolated from genus Albizia

2.3.2.2. Terpenes reported from the genus Albizia

Occurrence of triterpene aglycones was reported in some species of *Albizia.* A monoterpene conjugated triterpene from the stem bark of *A. julibrissin* was isolated and identified as 21-[4-(ethylidene)-2- tetrahydrofuranmethacryloyl] mechaerinic acid **(XXI)** [Woo and Kang, 1984]. In addition, lupeol **(XXII)** and acacic acid lactone **(XXIII)** were isolated from *A. versicolor* (Rukunga and Waterman, 2001). The stem bark of *A. gummifera* has also yielded three triterpenes, lupeol**,** lupenone **(XXIV)** and vitalboside-A **(XVI) (**Rukunga and Waterman, 2001).

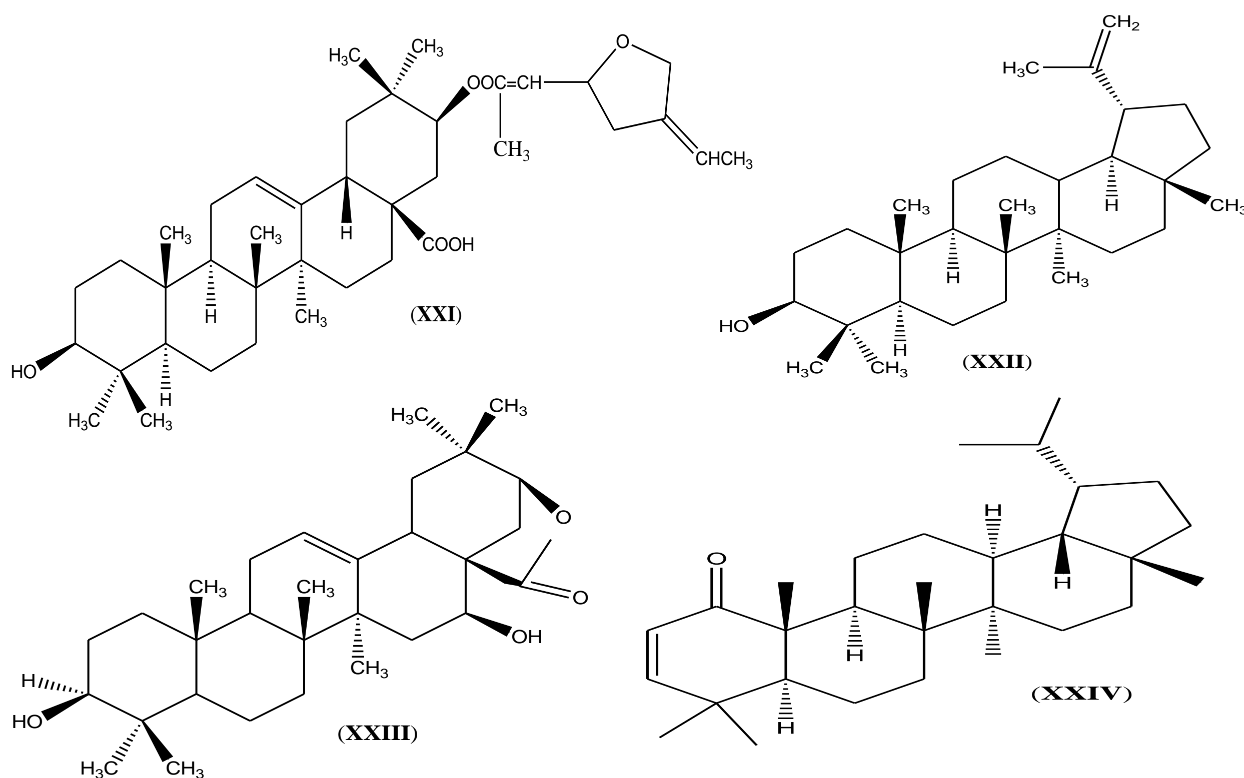
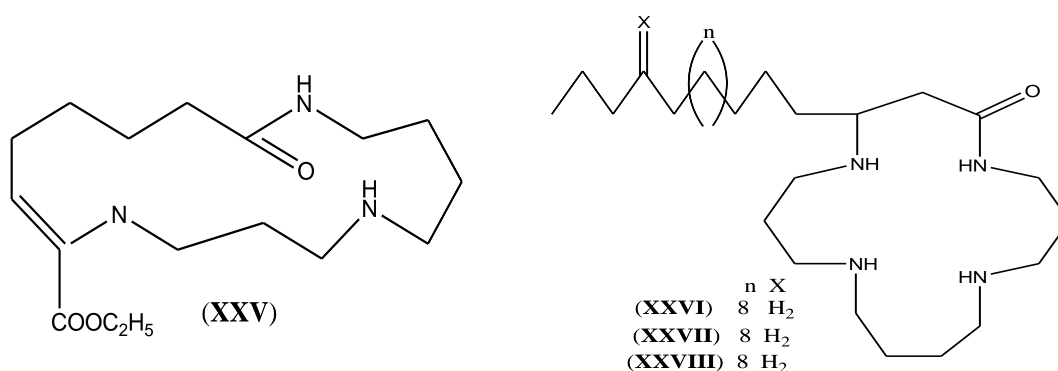


Fig.2.2. Structures of some terpenes (XXI-XXIV) isolated from genus Albizia

2.3.2.3. Alkaloids and nitrogenous compounds reported from the genus Albizia

Spermine-type alkaloids are characteristic for genus *Albizia.* They are characterized by the presence of a macrocyclic lactam ring, formed by combination of the base with long-chain fatty acids or cinnamic acid and its derivatives. The natural polyamines putrescine, spermidine and spermine are common bases reported to have several important functions in animals, plants and microorganisms. In plants their involvement in organ development, flowering, fruit ripening, senescence and stress responses is reported (Hesse *et al.*, 2001). A macrocyclic spermidine alkaloid, albizzine A **(XXV)** was isolated from stem bark of *A. myriophylla* (Ito *et al.*, 1994). Three macrocyclic spermine alkaloids, budmunchiamines L1-L3 **(XXVI-XXVIII)** (Fig. 2.3) were isolated from the methanol extract of seeds of *A. lebbeck* (Misra, *et al.*, 1995). In addition, a ceramide and its glycoside were isolated from the flower of *A. julibrissin*. Their structures were established as (2S, 3S, 4R, 8E)-2-[(2'R)-hydroxyhexadecanoylamino-8-tetra-cosene-1, 3, 4-triol **(XXIX)** and 1-*O*-β-D-glucopyranosyl-(2S, 3S, 4R, 8E)-2-[(2'R)-hydroxyhexadecanoylamino-8-tetra- cosene-1, 3, 4-triol **(XXX)** on basis of chemical and spectroscopic studies (Kang *et al.*, 2007).



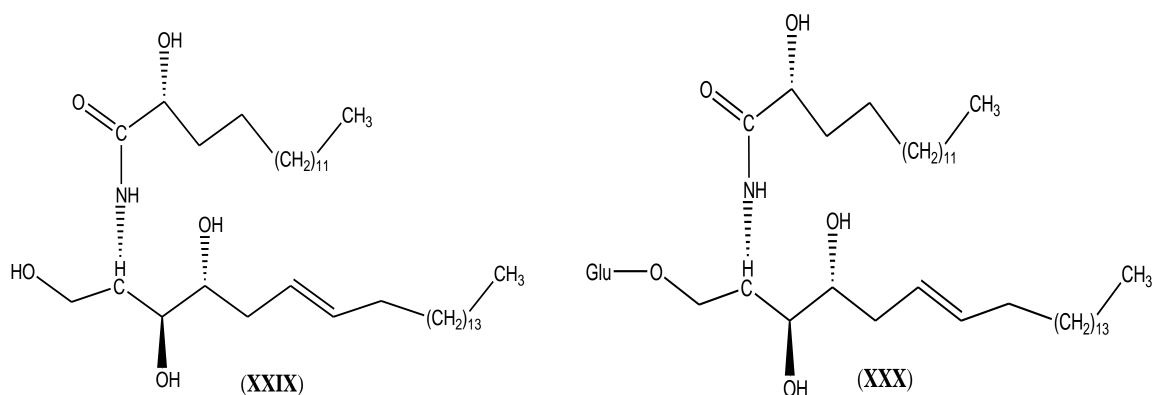


Fig.2.3. Structures of some nitrogenous compounds (XXV-XXX) isolated from genus *Albizia*

2.3.2.4 Flavonoids reported from the genus Albizia

Two tri-*O*-glycoside flavonols, quercetin and kaempferol 3-*O*-α- rhamnopyranosyl (1→6)-β-glucopyranosyl (1→6)-β-galactopyranosides (**XXXI & XXXII**), were identified from the leaves of *A. lebbeck* (El-Mousallamy, 1998).

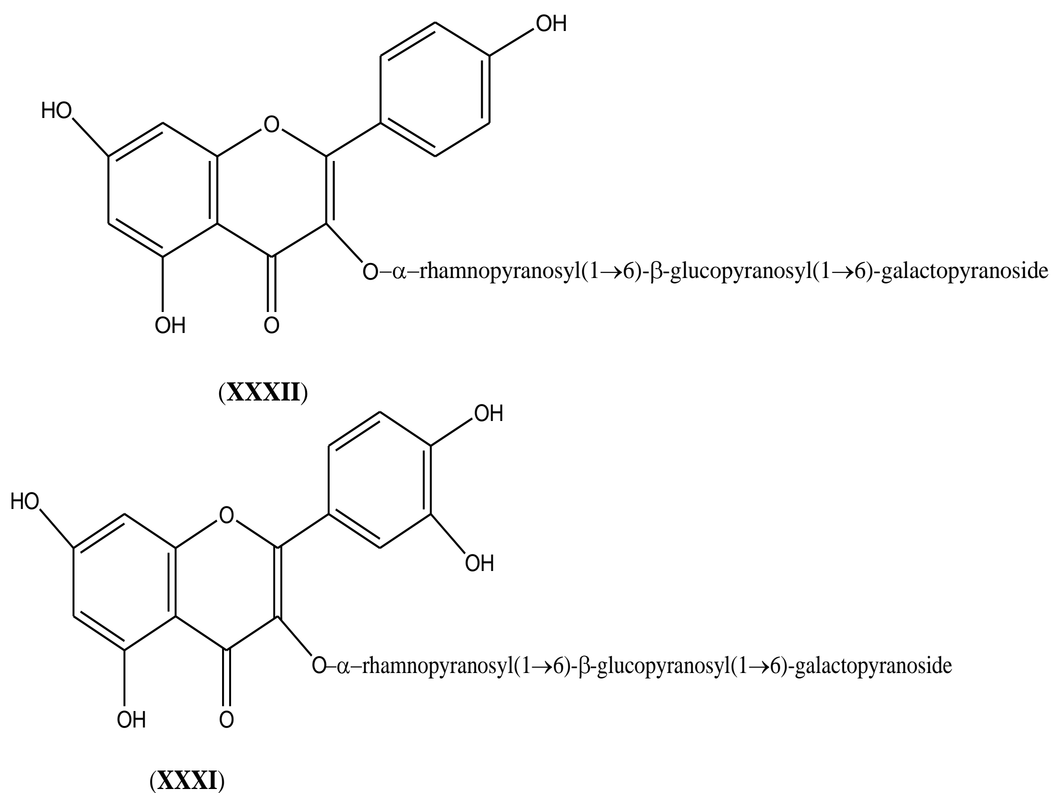


Fig.2.4. Structures of some flavanoids (XXXI-XXXII) isolated from genu *Albizia*

2.3.2.5. Phenolic compounds reported from the genus Albizia

Four phenolic glycosides (**XXXIII-XXXVI**) and icariside E5 (**XXXVII**) (Fig. 2.5), were isolated from the dried stem bark of *A. julibrissin.* These were determined to be 3,4,5-trimethoxyphenol 1-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside (**XXXIII**), vomifoliol 3'-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (**XXXIV**), (+)-lyoniresinol 9'-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (**XXXV**), (+) – lyoniresinol 4, 9'- di-*O*-β-D- glucopyranoside **(XXXVI)** (Higuchi *et al.*, 1992).

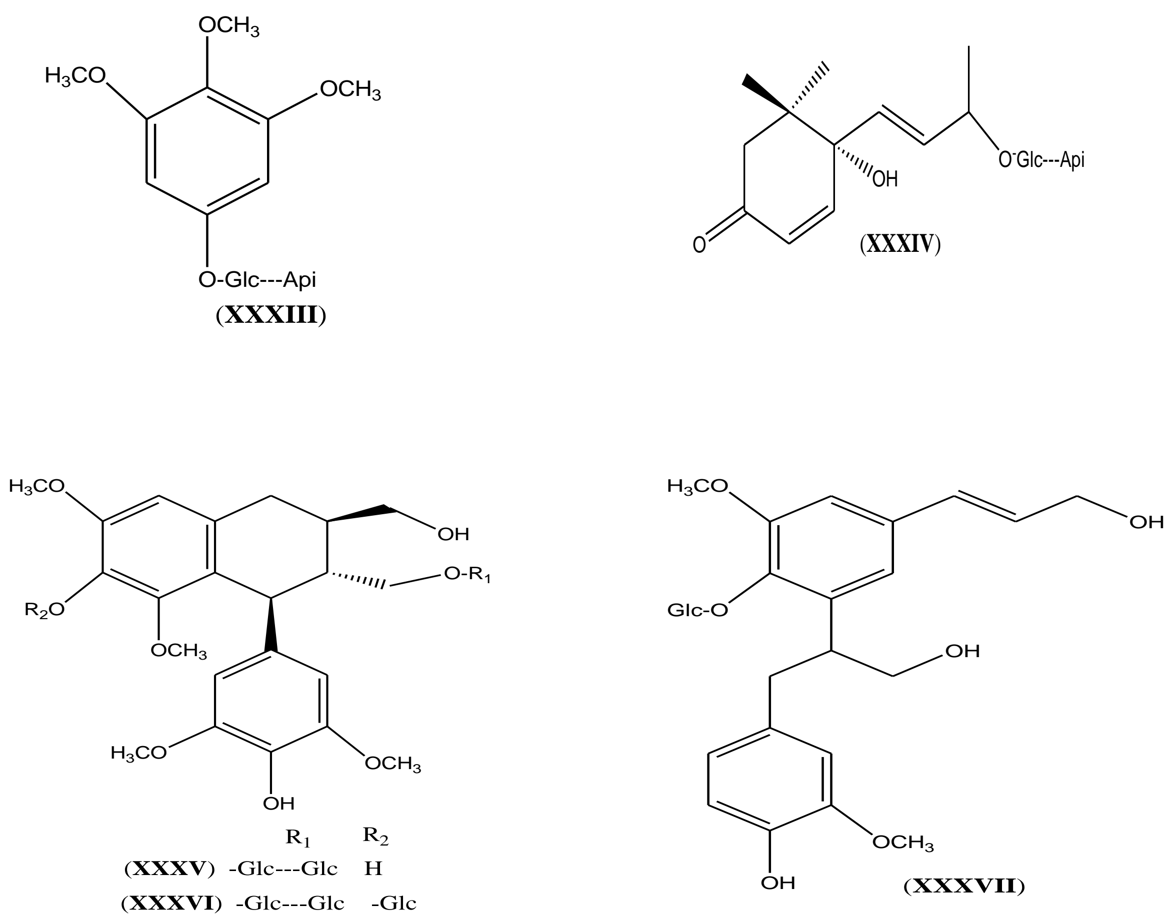


Fig.2.5. Structures of some phenolic compounds (XXXI-XXXII) isolated from the genus *Albizia*

2.3.3. Some bioactive compounds reported from the genus *Albizia*

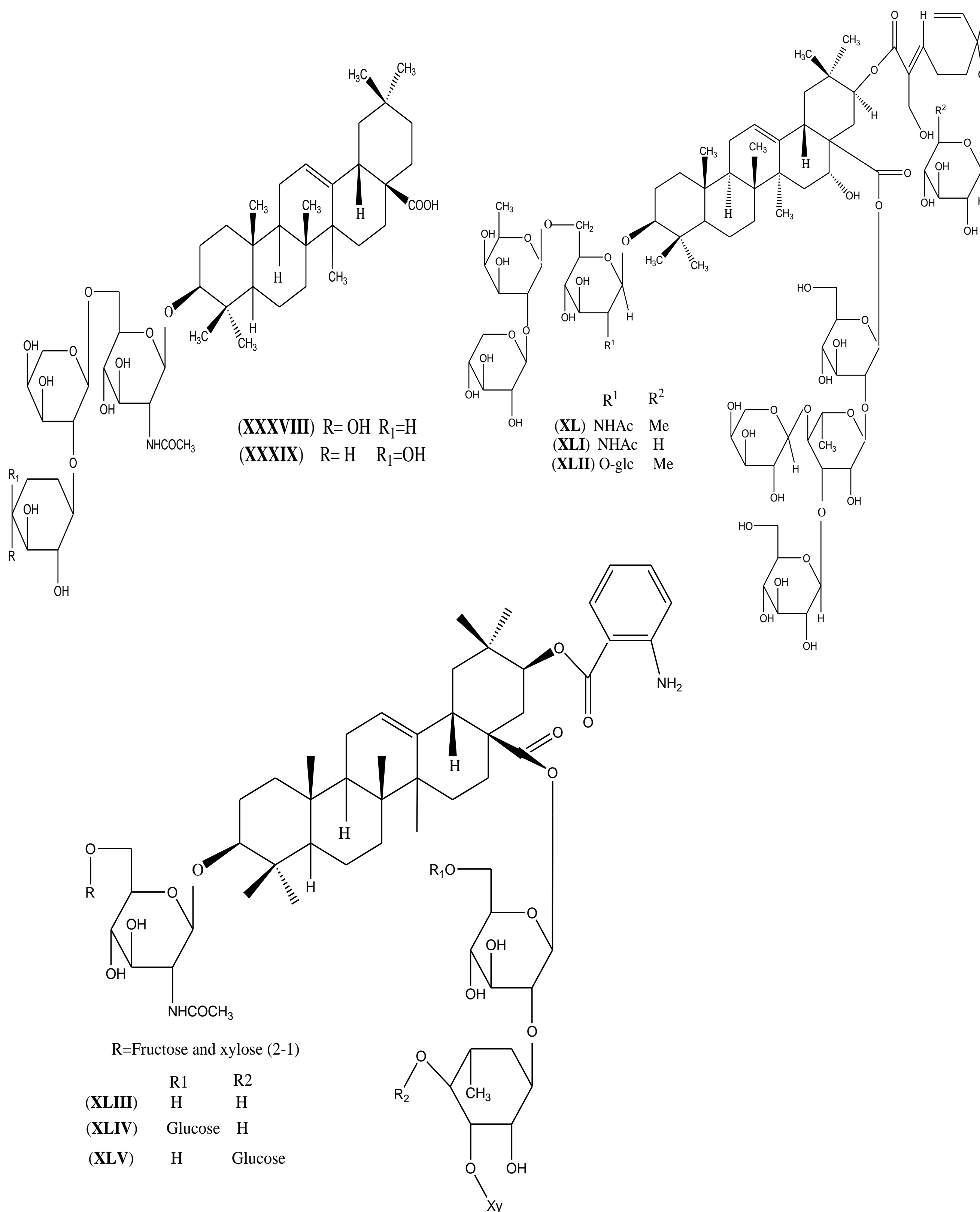
Some bioactive compounds isolated and identified from genus *Albizia* include those of saponins, alkaloids, flavonoid and phenolic componds.

2.3.3.1. Bioactive saponin compounds reported from Albizia

Two active saponins, Albiziatrioside A and B **(XXXVIII & XXXIX)** were isolated using bioassay- guided fractionation of methanolic extract of *A. subdimidiata* in which both isolated compounds showed significant cytotoxicity against A2780 cell line (Abdel-Kader *et al.*, 2001).

Three anti-tumor triterpenoid saponins (julibroside J29-31 (**XL-XLII**) were isolated from the bark of *A. julibrssin* (Zheng *et al.*, 2006); three oleanane-type triterpene saponins (**XLIII-XLV**) (grandibrateosides A-C) isolated from the methanolic extract of leaves of *A. grandibracteata* showed significant inhibition against KB and MCF7 tumor cell lines in vitro (Sabrina *et al.*, 2005); three saponin glycosides (3-O-[β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-2-acetamido -2-deoxy -β-D-gluco-pyranosyl] echinocystic acid) (**XLVI**), 3-O-[α-L-arabinopyranosyl-(1→2)-β-D-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-→D-glucopyranosyl] echinocystic acid (**XLVII**) and 3-O-[β-D-xylopyranosyl-(1 → 2)-α-L- arabinopyranosyl-(1 → 6)-2-acetamido-2-deoxy-β-D-glucopyranosyl] acacic acid lactone (**XLVIII**) isolated from the bark of *A. procera* exhibited cytotoxicity of saponin (**XLVI**) against HEPG2 cell line (Melek *et al.*, 2007); three oleanane type triterpene saponins (albizosides A-C 12-14) isolated from the stem bark of *A. chinensis* showed cytotoxic activity against a small panel of human tumor cell lines as well as hemolytic activity against rabbit erythrocytes (Rui *et al.*, 2009); an oleanane-type saponin (coriariosides A 15) along with other known saponin isolated from the roots of *A. coriaria*

tested for cytotoxicity against two colorectal human cancer cells and showed activity against the HCT 116 (IC_{50} 4.2 μM) and HT-29 (IC_{50} 6.7 μM) cell lines (Not *et al.*, 2009).



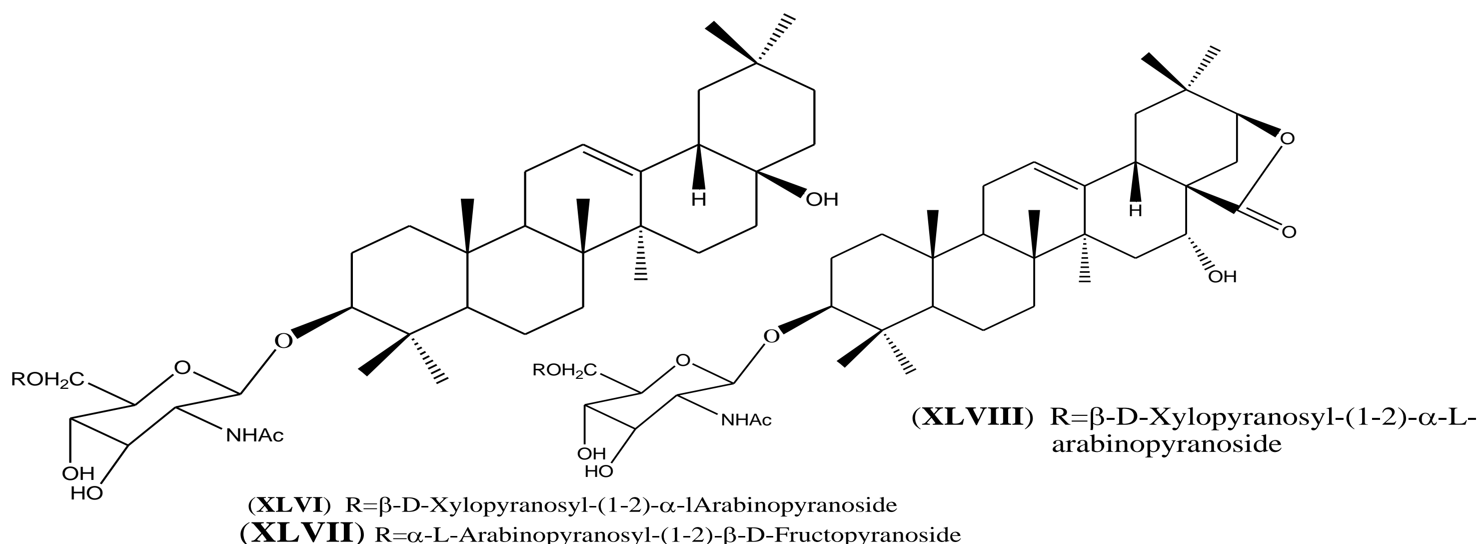
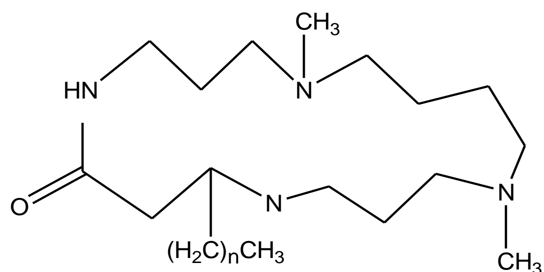


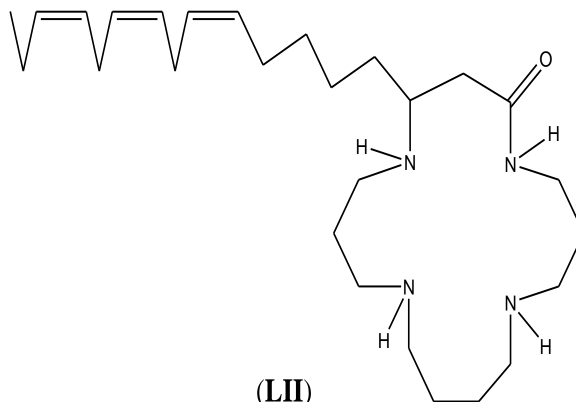
Fig. 2.6. Structures of some saponins bioactive compounds (XXXVIII-XLVIII) from the genus Albizia

2.3.3.2. Bioactive alkaloidal compounds of Albizia

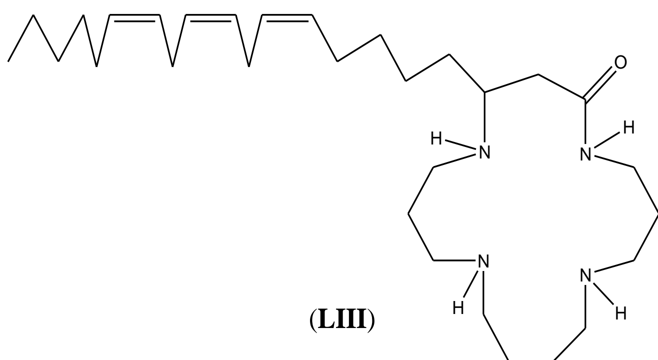
Macrocyclic alkaloids (budmunchiamines A, B and C **(XLIX-LI)**, isolated from *A. amara* showed antiplatelets aggregation and bactericidal activity (Mar *et al.*, 1991); two macrocyclic spermine alkaloids **(LII & LIII)** isolated as a mixture from the leaves of *A. inopinata* preliminarily indicated compounds that showed a possible pharmacological depressor activity on the central nervous system (De Assis *et al.*, 1999); bioassay guided fractionation of the crude methanol extracts of stem bark and leaves of *A. adinocephala* led to the isolation of two bioactive spermine alkaloids (budmunchiamines L4 **(LIV)** and L5 **(LV)** which were found to inhibit the malarial enzyme plasmepsin II (Ovenden *et al.*, 2002); the alkaloidal fraction of methanolic extract of *A. gummifera* in which five known spermine alkaloids **(LVI-LX)** were isolated exhibited strong activity against chloroquine sensitive (NF54) and resistant (ENT30) strains of *Plasmodium falciparum* with IC_{50} of 0.16 ± 0.05 and 0.99 ± 0.06 µg/ml, respectively (Rukunga *et al.*, 2007).



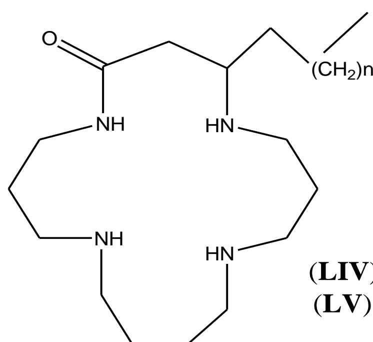
(**XLIX**) n=10
(**L**) n=8
(**LI**) n=12



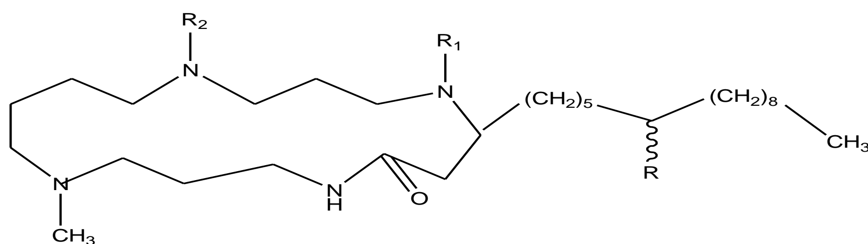
(**LII**)



(**LIII**)



(**LIV**) n= 11
(**LV**) n= 13



	R	R_1	R_2
(**LVI**)	H	CH_3	CH_3
(**LVII**)	OH	CH_3	CH_3
(**LVIII**)	H	H	CH_3
(**LIX**)	OH	H	CH_3
(**LX**)	H	CH	H

Fig. 2.7. Structures of some bioactive nitrogenous compounds (LX-XLIX) from the genus Albizia

2.3.3.3. Bioactive flavonoid compounds of *Albizia*

A flavonol glycoside (identified as 5,2', 4'-trihydroxy-3,7,5'- trimethoxyflavonol-2'-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopyranoside **(LXI)** isolated from the acetone soluble fraction of ethanolic extract of stem of *A. procera* showed moderate anti-inflammatory activity on albino rats by using non-imunological carrageenan induced hind paw edema method (Yadava and Tripathi, 2000). Two flavonol glycosides (quercitrin **(LXII)** and isoquercitrin **(LXIII)** isolated from the flowers of *A. julibrissin* showed sedative activity; however, both compounds **(LXI)** and **(LXII)** showed increasing pentobarbital-induced sleeping time in dose-dependent manner in mice—the results which goes to support the use of the flowers of this plant as a sedative agent in oriental traditional medicine (Kang *et al*., 2000). A biologically active flavonol glycoside (3, 5, 4'-trihydroxy, 7, 3'- dimethoxy-3-*O*-β-D-glucopyranosyl-(1→4)-α-L-xylopyranoside **(LXIV)** isolated from the chloroform-soluble fraction of the seeds of *A. julibrissin* showed antibacterial activity of the chloroform-soluble fraction from the methanolic extract as fairly active against gram positive and gram negative bacteria (Yadava and Reddy, 2001). A quercetin derivative (hyperoside (quercetin-3-*O*-galactoside) **(LXV),** and quercitrin (quercetin-3-*O*-rhamnoside) **(LXVI)** isolated from *A. julibrissin* showed a significant oxygen radical absorbance capacity (ORAC) values in the methanolic extracts. Hence, this display of the plant foliage antioxidant activity goes to confirm the presence of three compounds in *A. julibrissin* foliage (Lau *et al*., 2007).

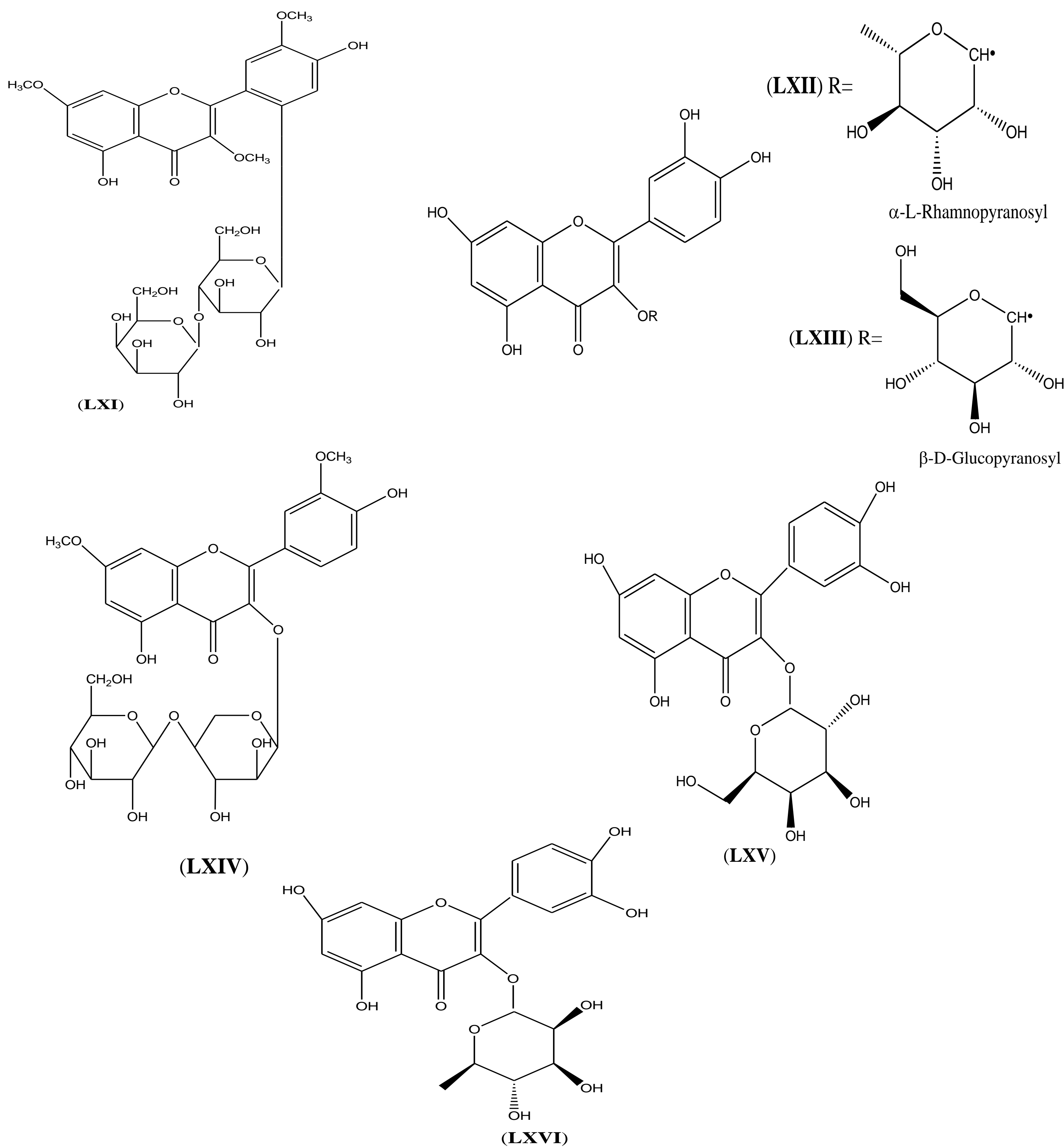


Fig. 2.8. Structures of some bioactive flavonoids compounds (LXI-LXVI) reported from the genus Albizia

2.3.3.4. Bioactive phenolic compounds from the genus *Albizia*

Two phenolic glycosides (albibrissinosides A **(LXVII)** and B **(LXVIII)**) isolated from the stem bark of *A. julibrissin* showed albibrissinoside B to be a radical scavenging action on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical (Jung *et al.* 2004).

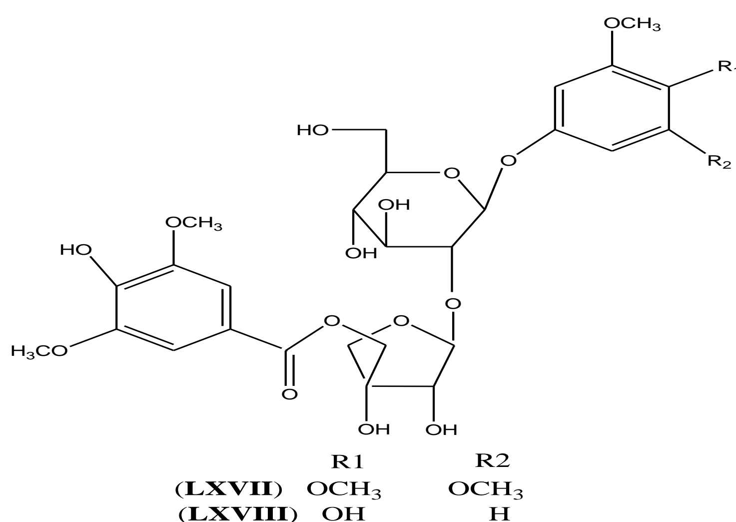


Fig. 2.9. Chemical structure of bioactive phenolic compounds (LXVII-LXVIII) from the genus Albizia

2.3.4. Biological activity and ethnopharmacolgy report of the genus Albizia

Albizia lebbeck is used in Indian traditional system and folk medicine to treat several inflammatory pathologies such as asthma, arthritis and burns (Ayurvedic Pharmacopoeia of India, 2001). It is also reported in Indian folk medicine that *A. lebbeck* has antiseptic, antidysentric, and anti-tubercular activities. The bark and flowers of *Albizia julibrissin* tree are used in China as medicine. Bark extract is applied to bruises, ulcers, abscesses, boils, hemorrhoids and fractures, and has displayed cytotoxic activity (Higuchi *et al.*, 1992). *Albizia saman* and *Albizia inundata* were found to have good anti-plasmodial and anti-candida activity (Gupta *et al.*, 2005). *Albizia odoratissima* is used in the treatment of leprosy, ulcers

and cough, while *Albizia mollis* is well known for its sedative and sleeping pill properties (Zou *et al.*, 2000).

The bark and leaves of *Albizia procera* were extensively used for the treatment of variety of wounds and considered useful in pregnancy and stomachache. Lipophilic extracts of *Albizia gummifera* revealed very promising anti-trypanosomal activity (Rukunga *et al.*, 2007); also it is used in the indigenous medical system for various ailments, bacterial infections, skin diseases, malaria and stomach pains. The seeds of *Albizia amara* are used as an astringent, also for the treatments of piles, diarrhoea, gonorrhoea, leprosy, leucoderma, erysipelas and abscesses. The leaves and flowers have been applied to boils, eruptions, and swellings and is also regarded as an emetic and as a remedy for coughs, ulcer, dandruff and malaria (Yadava *et al.*, 2001). *Albizia schimperiana* is used as a traditional medicine for the treatment of bacterial and parasitic infections, notably pneumonia and malaria, respectively. The alcoholic extract of *A. lebbeck* has antihistaminic property, by neutralizing histamine directly or due to corticotrophic action as evidenced by raising cortisol levels in plasma (Babu *et al.*, 2009). *Albizia zygia* showed high anti-malarial activity (Abdalla and Hartmut 2012).

2.3.4.1. Antioxidant properties

There are many reports on the antioxidant property for *Albizia* species. *A. julibrissin* foliage produced a quercetin derivative, hyperoside (quercetin-3-*O*-galactoside) and quercitrin (quercetin-3-*O*-rhamnoside) that showed excellent antioxidant activity (Lau *et al.*, 2007). Two phenolic glycosides (albibrissinosides A and B) were isolated from the stem bark of *A. julibrissin*. The albibrissinoside B was found to be a radical scavenger on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical (Jung *et al.*, 2004). Khatoon *et al.*, (2013) studied the antioxidant activity of *Albizia procera* leaves through DPPH reducing power and total antioxidant capacity. Their leaf extract exhibited an IC_{50} value of about 90%. The aqueous

ethanol extract of *Albizia anthelmintica* showed significance analgesic and antioxidant activities. Isolation from *A. anthelmintica* produced quercetin-3-O-β-D-glucopyranoside, kaempferol-3-O-β-D-glucopyranoside, kaempferol-3-O-(6β-O-galloyl-β-D-glucopyranoside) and quercetin-3-O-(6β-O-galloyl-β-D-glucopyranoside), which exhibited potent antioxidant scavenging activity towards DPPH. *Albizia myriophylla* showed the highest antioxidant activity on DPPH radical assay and lipid peroxidation assay (Steinrut *et al.*, 2011).

Aurantiamide acetate was the most active compound isolated from the stem bark of *A. adianthifolia* through antioxidant activity (DPPH); trolox equivalent antioxidant capacity (TEAC) assays were used to detect the antioxidant activity. The bark extracts of *Albizia lebbeck* possess free radical scavenging activity against DPPH and reducing power assays. Ethanolic extract of *Albizia procera* showed strong scavenging activity against free radicals compared to various standards. These *in-vitro* assays indicate that these plant extracts are better sources of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. Aliyu *et al.*, (2009) studied the antioxidant activity of *Albizia chevalieri* leaves through DPPH. *Albizia amara* leaves extracts showed good antioxidant activity, which were studied by three different methods: 2, 2-diphenyl-1-picrylhydrazyl radical assay, nitric oxide free radical scavenging assay and reducing power assay. These were compared to standard samples.

2.3.4.2. Anticancer properties

Three triterpenoid saponins (julibroside J29-31) from *Albizia julibrissin* bark, served as anti-tumorals by the induction of apoptosis in certain cell types (human acute leukemia junket T-cells) and butanol extract from the bark of *Albizia julibrissin* (Zheng *et al.*, 2006). A cytotoxic compound, Echinocystic acid 3, 16-O-bisglycosides from the bark of *Albizia*

procera is worth mentioning. In contrast to other cytotoxic echinocystic acid glycosides with N-acetyl glucosamine unit, the new glycosides were found inactive when assayed by MTT method for their cytotoxicities against the different cancer cell lines (Miyase *et al*., 2010). Three oleanane-type triterpene saponins named grandibracteosides A−C were isolated from the methanolic extract of leaves of *A. grandibracteata*, showed significant inhibitory activity against tumor cell lines *in vitro* (Sabrina *et al*., 2005). Three saponins from the bark of *A. procera*, characterized as 3-*O*-[β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6) -2-acetamido-2-deoxy-β-D-glucopyranosyl] echinocystic acid exhibited cytotoxicity against HEPG2 cell line (Melek *et al*., 2007). Three oleanane type triterpene saponins, albizosides A-C were isolated from the stem bark of *A. chinensis*. These compounds showed cytotoxic activity against a small panel of human tumor cell lines as well as hemolytic activity against rabbit erythrocytes (Rui *et al*., 2009).

An oleanane-type saponin coriariosides A, along with other saponin were isolated from the roots of *A. coriaria*. These compounds when tested for cytotoxicity against two colorectal human cancer cells, showed excellent activities (Not *et al*., 2009). Two diastereomeric saponins, julibrosides J1 (1) and J9 (2) obtained from the stem bark of *A. julibrissin*, both show cytotoxic activity. A triterpenoidal saponin (Julibroside) with a xylopyranosyl moiety located at its C-21 side chain was isolated from *A. julibrissin*. This Julibroside showed marked inhibitory action against Bel-7402 cancer cell line (Zou *et al*., 2006). Two active cytotoxic saponins viz. Albiziatrioside A and B from methanolic extract of *Albizia subdimidiata* showed significant effects against tumor cell line (Lau *et al*., 2007). *A. gummifera* led to the isolation of three cytotoxic oleanane-type triterpenoid saponins, gummiferaosides, which showed cytotoxicity against human ovarian cancer cell line.

2.3.4.3. Antidiabetic properties

Two flavonol glycosides, quercitrin and isoquercitrin from the flowers of *A. julibrissin* showed diabetic activity (Kang *et al.*, 2000).

2.3.4.4. Anti-inflammatory properties

A novel flavonol glycoside of *A. procera* stem showed moderate anti-inflammatory action on albino rats by using non-immunological carrageen and induced hind paw edema method. Ethanolic extract *of A. lebbeck* seed at 200 mg/kg dose showed highest anti-inflammatory activity. The aqueous ethanol extract of *Albizia anthelmintica* showed moderate anti-inflammatory activity (Sujatha *et al*., 2013).

2.3.4.5. Antibacterial properties

The bark of *Albizia lebbeck* has acrid taste and its extract showed antimicrobial activity. Macrocyclic alkaloids (budmunchiamines A, B and C) were isolated from *A. amara.* They were also found to have antiplatelets aggregation and bactericidal activity (Yadava &Tripathi, 2001). A biologically active flavonol glycoside 3, 5, 4'-trihydroxy, 7, 3'-dimethoxy-3-*O*-β-D-glucopyranosyl-(1→4)-α-L-xylopyranoside from the seeds of *A. julibrissin* was fairly active against gram positive and gram negative bacteria. The extracts of *Albizia ferruginea* were also reported to have significant anti-microbial activity on selected micro-organisms. Three flavonoids: 4', 7-dihydroxyflavanone, 3', 4', 7-trihydroxyflavone, and 3-*O*-methylfisetin (3', 4', 7-trihydroxy-3-methoxyflavone, isolated for the first time from the Sudanese medicinal plant, *Albizia zygia*, tested against *Plasmodium falciparum*. However, hepatoprotective effect of *Albizia procera, Albizia lebbeck, Albizia inopinata* and *Albizia amara* seem to exhibit potent hepatoprotective activity along with various pharmacological activities such as CNS

activity, cardiotonic activity, lipid-lowering activity, antioxidant activity, hypoglycemic activity etc. (Correia da Silva *et al*., 1962 and Mar *et al*., 1991).

2.3.5. Reported ethnobotanical and ethnnomedicinal value of the genus Albizia

The flowers of many Albizia species are being commonly used to treat anxiety, depression and insomnia in traditional Chinese medicine. The Indian species *Albizia thomsonii* are classified as vulnerable. *Albizia* species are socially significant for producing high quality timber and as a valuable resource for gum yield. *A. julibrissin, A. lebbeck, A. procera* and *A. amara* are some importantly considered species in Ayruvedic medicine. *A. lebbeck* is an astringent, also used by some cultures to treat boils, cough, to treat the eye, flu, gingivitis, lung problems, pectoral problems, is used as a tonic, and is used to treat abdominal tumors (Sujatha *et al*., 2013). In ancient Tamil culture, the flowers of *A. lebbeck* decorated as a crown were used to welcome victorious soldiers. The leaves are boiled to make a drink, and the bark is cooked with food in Madagascar. Its sweet-smelling gum or resin is used in cosmetics in some African countries. The root bark and young shoots are widely used in traditional medicine. The bark is poisonous but is used medicinally by the Zulus of South Africa who also sometimes make a love charm from the plant. They also prepare an infusion (hot or cold) from the bark and roots to treat skin diseases such as scabies, inflamed eyes and bronchitis. The seeds of *Albizia amara* are regarded as astringent, and used in the treatment of piles, diarrhoea and gonorrhoea. Some *Albizia* species are regarded as potential fodder resource. Species like *A. lebbeck* and *A.procera* have shown high potential in soil redevelopment process during early phase of mine spoil restoration in dry tropical environment (Sujatha *et al*., 2013).

2.4.1. The plant *Albizia chevalieri*

Albizia chevalieri Harms (Mimosaceae) is a tree of acacia type native to tropical and subtropical regions including Nigeria and Niger Republic, with loose balls of whitish fragrant flowers and flat brown pods.

2.4.1.1. Morphological description of A. chevalieri

Shrub or small tree, up to 6 m, occasionally 12 m, with an open and rounded or umbrella shaped canopy, bark pale-greyish, corky and deeply creviced, dark brown slash. Twigs pubescent with white lenticels, leaves with 8-12 pairs of pinnae, with 20-40 pairs of leaflets each. Leaflets 1 cm long x 2-3 mm wide, sometimes slightly curved, greyish-pubescent on both sides and apiculate. Rachis also pubescent, with a large gland at the base of the petiole. Flowers are small and borne in globose pinkish heads (or finger-shaped clusters) on ca 5 cm long axillary petioles. The fruit is a large, strap-shaped pod, the pods 10-15 cm long x 2-2.5 cm wide, flat, containing 7-10 seeds (Aubréville, 1950; Geerling, 1988; Maydell 1986).

2.4.1.2. Habit/habitat and distribution of A. chevalieri

A. chevalieri is a tree of the dry deciduous forest. Found in well watered places, sandy terraces, not gregarious, nor common. It could be a shrub under harsher conditions of the dry Southern Sahel and Northern Sudanian Savannahs from Chad, Senegal, Nigér and Northern Nigeria; replaced eastward by *A. amara* (Aubréville, 1950; Brenan, 1957; Berhaut, 1975).

2.4.1.3. Cultivation and propagation of A. chevalieri

A. chevalieri is a plant of the semi-arid tropics, where it is found at elevations up to 1,000 metres. It grows best in areas where annual daytime temperatures are within the range of 24 - 30°C, but can tolerate 18 - 35°C. It prefers a mean annual rainfall in the range 600 – 900 mm,

but tolerates 500 - 1,000 mm. The plant requires a sunny position, prefers a well-drained light soil of moderate fertility, prefers a pH in the range 5.5-6.5, tolerating 4.5-7.5. This species has a symbiotic relationship with certain soil bacteria, where these bacteria form nodules on the roots and fix atmospheric nitrogen. Some of these nitrogen are utilized by the growing plant but some can also be used by other plants growing nearby (Huxley, 1992). *A. chevalieri* seed has a hard coat and may benefit from scarification before sowing to speed up germination. This is usually done by pouring a small amount of nearly boiling water on the seeds (being careful not to cook them!) and then soak for 12 - 24 hours in warm water, by which time the seeds should have imbibed moisture and are swollen; if they have not, then carefully a nick in the seed coat is made (being careful not to damage the embryo) and soak for a further 12 hours before sowing. Pollinators are bees and insect (Fern, 2014).

2.4.1.4. Documented products and uses of A. chevalieri

Leaves and fruits are good forage, pale wood of medium density used for making furniture, occasionally for construction. Bark used for tanning, young shoots are cooked and edible to humans, fine roots used as threads to repair calabashes (Maydell, 1990).

Table 2.1. Taxonomic Classification of *Albizia chevalieri*

Angiosperm Phylogeny Group (APG)		*Cronquist System*	
Reign:	Plantae	Reign:	Plantae
Sub-Kingdom:	Tracheobiota	Sub-Kingdom:	Tracheobiota
Division:	Magnoliophyta	Division:	Magnoliophyta
Class:	Magnoliopsida	Class:	Magnoliopsida
Sub-Class:	Rosidae	Sub-Class:	Rosidae
Order:	Fabales	Order:	Fabales
Family:	Fabeceae	Family:	Mimosaceae
Sub-family	Mimosoideae	Genus:	*Albizia*
Genus:	*Albizia*	Species:	*Chevalieri* Hams.
Species:	*Chevalieri* Hams.		

2.4.2. Biological evaluations of *Albizia chevalieri*

Reported literature revealed scientific work on *A. chevalieri,* recording great medicinal values and thereby validating some therapeutic folklore claims. The leaves of *A. chevalieri* are widely used for the management of diabetes mellitus by traditional medicinal practitioners in some part of Nigeria and Niger Republic. Consequently, hypoglycaemic effects of the leaves (Saidu *et al.,* 2007a) and root (Saidu *et al.,* 2010) have been reported for which the aqueous leaf extract showed some significant hypoglycemic and hypolipidaemic effect in alloxan induced diabetic rats.

Furthermore, reports on *in vivo* study *of* acute and sub chronic effects of crude aqueous leaf extract of the plant showed no significant ($p > 0.05$) effect on haemoglobin concentration, red blood cell count, packed cell volume, white blood cell and differential counts, and platelets count in the acute toxicity test, (Saidu *et al.,* 2007b). On the bases of the above hematotoxicity study, it was concluded that the aqueous leaf extract of *A. chevalieri,* (previously reported to have significant hypoglycemic effect in alloxan diabetic rats) might be considered relatively safe in the tested doses. The antioxidant evaluation of the methanolic leave extract of *A. chevalieri* using free radical scavenging activity of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), total phenolics content and reducing power assay showed a promising DPPH scavenging activity of the extract, with no significant difference ($p < 0.05$) in the antioxidant activity between the extract and those of standard ascorbic and gallic acids at 50, 125 and 250μgml^{-1}concentrations.

2.4.3. Ethnobotany information of *A. chevalieri*

A. chevalieri is used in Borno, North-Eastern Nigeria as purgative, taenicide and also as a remedy for coughs (Aliyu *et al.,* 2009). A decoction of the leaves is used in Northern Nigeria as remedy for dysentery (Burkill, 1995; Le Houèrou, 2009 as cited in Aliyu *et al.,* 2009). There are also reports on the local use of the leaves extract for cancer treatment in Zaria, Kaduna State (Abubakar *et al*., 2007), while in Northern Sahel Savannah of Sokoto-Nigeria and Niger Republic, the leaf extract is used either as cold water decoction or the dried ground sieved leaf powder is mixed with pap and consume for the management of diabetes mellitus (Saidu *et al.,* 2007a). Meanwhile, ethnobotanical survey conducted in the cause of this research revealed the use of the root and stem-barks of *A. chevalieri* for therapy against tuberculosis, toothache, inflammations and snake bite remedy among the Zuru people of Kebbi State, Nigeria. The plant *A. chevalieri,* a tree often found on the hills and mountainous vegetation of the Zuru area is shown in plate I and II below.

Leaves

Stem bark

Fruits & Seeds

Herbarium specimen

Plate I. Morphological parts of *A. chevalieri*

Plate II. ***A. chevalieri*** **in its natural habitat**

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and solvents

(All through, laboratory and analar grade (AR) solvents were used)

Hexane, Ethyl acetate (Merck-Germany); Chloroform, N-Butanol, Methanol, Phosphomolybdic acid (PMA), Anisaldehyde, 5% H_2SO_4, Tween 80 (Sigma-Aldrich-St. Louis, MO, USA); Chloroform, Dichloromethane, Chloroform ($CDCl_3$), Merck- Germany (A.R.); Normal Saline water (Sterile mixture, 500 ml), Distilled water, Lyophilized *Naja nigrocollis* Broadley venom (NNBV).

3.1.2. Equipment/materials

TLC silica gel pre-coated aluminium plates, Silica gel-60 for column (0.063-0.200 mm; 70-230 mesh), (Merck-Germany); HR-ESIMS: SYNAPT G1 HDMS QTOF (4 kDa) Mass Spectrometer, Perkin Elmer Spectrum RX IFT-IR System, NMR Top spin 300 Hz and 400 Hz Bruker-(Germany); Perking Elmer Precisely Lambda 25 UV/Vis spectrophotometer, Melting point apparatus (Gallencamp, USA); Heat gun (for heating TLC plates), Rotary evaporator (Buchi water-bath B-480 Rotavapor RII, UV-Lamp, Buchi Rotavapor EL130 (CAMAG (Switzerland); Vacuum pump (Labotech South Africa).

3.1.3. Animals

Albino mice of both sexes (18-30g)

3.2. Methods

3.2.1. Collection and identification of plant material

Albizia chevalieri stem bark were collected in the month of July 2013 in Zuru Local Government Area of Kebbi State North-West Nigeria, was identified and authenticated by Mal. M. Musa of Herbarium Section, Department of Biological Sciences, Ahmadu Bello University, Zaria-Nigeria by comparison with voucher specimen number 900247.

3.2.2. Drying and preservation of plant material

The stem bark of the plant was shed dried in a well ventilated room until a constant weight was obtained. The plant material was ground to coarse powder using pestle and mortar. The powder was stored in an appropriate container until required for use.

3.3. Extraction and Preliminary Phytochemical Screening of *A. chevalieri* Stem bark

3.3.1. Extraction of plant material

Air-dried and powdered stem bark (2kg) were extracted exhaustively with hexane by maceration in 4L hexane for 24 hrs at room temperature, after which the extract was filtered and separated from the marc. The filtrate was concentrated *in vacou* to obtain the hexane extract (HE). The marc was air-dried after this, and was subsequently extracted by maceration in absolute methanol (5L) (using a ten litres glass jar) at room temperature for 48 hrs to give the methanolic extract (ME).

3.3.2. Solvent-solvent fractionation

The total alcoholic extract (ME) obtained above was evaporated to dryness under reduced pressure to give a dark brown mass residue (0.207kg). A portion of the later (0.15kg) was suspended in distilled water and successively partitioned (ratio 20:80) with each of ethyl acetate (EtOAc) and n-Butanol (n-BuOH) (five times for each with 150ml) to give EtOAc

portion (EA) and n-BuOH (portion) (NB). Consequently, four samples, labelled: EA, NB, ME and HE, were obtained. These were (at each stage) concentrated at reduced pressure using rotary evaporator and were kept under dry condition until needed.

3.3.3 Preliminary phytochemical screening

Portions of the extract and fractions were subjected to preliminary phytochemical screening through the test tube (wet test) method as well as by praying developed TLC plates with specific detecting reagents for the presence or absence of metabolites such as flavonoids, saponins, tannins, alkaloids, anthraquinones, carbohydrates and/or glycoside, cardiac glycoside and triterpenes and/or sterols. The procedures used were according to standard methods (Evans, 2005; Onwukaeme *et al*., 2007; Harborne, 1991 and Sofowora 1993).

3.3.3.1 Froth test for saponins: The dried extract/fractions was dissolved in water, transferred into a test tube and shaken vigorously, then left to stand for 10 minutes, appearance of a thick persistent froth would indicate the presence of saponins (Sofowora, 1993*)*.

3.3.3.2 Test for alkaloids: 50 mg of extract/fractions was dissolved in 50 ml of methanol in 5% sulphuric acid in a water bath for 20 minutes; the extract was filtered off and allowed to cool. Two ml of the extract was poured into test tubes. Dragendorff's, Mayer's or Hager's reagent was added to the tubes. An orange or orange-yellow, white and brownish precipitates respectively, was an indication for the presence of alkaloids (Evans, 2005).

3.3.3.1 Test for Flavonoids: Each fraction was treated with dil. NaOH, followed by the addition of diluted HCl. Solubility and colour were noted. A yellow solution with NaOH, which turns colourless with dil HCl was an indication of the presence of flavonoids (Onwukaeme *et al*., 2007).

3.3.3.1 Test for tannins (Ferric chloride test solution): aqueous extracts/fractions was treated with a 15% ferric chloride reagent. A blue colour indicates condensed tannins; a green colour indicates hydrolysable tannins (Evans, 2005).

3.3.3.1 Borntrager's test for anthraquinone derivatives: To 2 ml of the extracts/fractions, 1ml of dilute (10 %) ammonia was added and the mixture was shaken. A pink-red colour in the ammoniacal (lower) layer shows anthracene derivatives (Onwukaeme *et al.*, 2007*)*.

3.3.3.1 Test for Carbohydrates and/or glycosides (Molisch's test): 1 ml of the extract solution was pipetted into a test tube; 3 drops of Molisch reagent were added to the extract solution. After mixing, 1ml of concentrated sulfuric acid was added to the wall of test tube. A positive test for carbohydrates is indicated by a violet ring forming at the interface between the denser sulfuric acid and the less dense test solution above (Harborne, 1991).

3.3.3.1 Test for sterols and/or triterpenes (Liebermann-Burchard test): 1 ml of glacial acetic acid was added to 1 ml chloroform and cooled to 0°C, then one drop of concentrated sulphuric acid was added to the cooled mixture followed by the extract. The solution was observed for a blue, green, red or orange colour that changes with time (Harborne, 1991).

3.3.3.1 Test for cardiac glycosides (Keller-Killiani test): The extracts/fractions were extracted with chloroform and evaporated to dryness. Some 0.4 ml glacial acetic acid containing trace amount of ferric chloride were added. The later was transfer to a small test tube and carefully, 0.5 ml sulphuric acid was added by the sides. A reddish brown layer formed, which turns bluish (bluish-green) on standing was an indication of the presences of (cardiac glycoside) digitoxose (Harborne, 1991).

3.3.4. Thin Layer chromatography of the samples

The hexane extract was profiled using solvent systems, Hexane / EtOAc (49:1) and Hexane / EtOAc (9:1), after which the chromatograms were viewed under UV at 254 and 365nm before spraying with phosphomolybdic acid (PMA) or 5% H_2SO_4 and heated in an oven for 5 minutes at 105 ^oC.

Similarly, the ethyl acetate fraction was resolved by using EtOAc/ Acetone (3:1), viewed under UV (254 and 365 nm) and sprayed with PMA followed by heating in an oven for 5 minutes at 105 ^oC.

3.3.4. Column chromatography of hexane extract

Silica-gel (150 g) was wet packed in glass column (3.0 x 60 cm) using 100% Hexane. The extract (3 g) was mixed with equal quantity of silica gel (pre-absorbed with silica in DCM and concentrated to obtain a fine powder) and loaded onto the packed silica-gel and allowed to stabilize for 2 hours before elution commenced. The column was gradiently eluted with 100% Hexane, Hexane/EtOAc mixtures (19:1; 9:1; 4:1; 2:1; 1:1; 1:2; 1:4; 1:9), and ethyl acetate (100%). Finally, 10% methanol in ethyl acetate was used to wash the column. In all, a total of 151 fractions were collected at 5ml each.

The fractions were monitored on TLC and similar ones from the column were pooled to give eight (8) fractions designated HA to HH. Appendix I (a) gives the TLC profile of some fractions from *A. chevalieri* hexane extract. The TLC chromatogram of fraction HF (58-80) and HG (81-94) eluted with 19:1 and 9:1 hexane/ethyl acetate gradient respectively, indicated these as mixtures of few components (Appendix Ia). On concentration of each of the two, a yellowish sticky mass was obtained. On addition of a 100% hexane to each of HF and HG in a 50ml beaker at room temperature (20^0C), a white precipitate was produced in each case.

These were decanted for each to obtain the sediments (the precipitates) into a separate beaker, after which the precipitates were dried by evaporating out the solvent. The solubility of both precipitates was tested and found readily soluble in acetone and dichloromethane but more in chloroform. Their TLC analysis revealed a chromatogram of single spots of different R_f with some impurity (Appendix Ia- HF & HG). The precipitates were then further purify by recrystallizing several times in hexane at an elevated temperature to yield white flakes crystalline substance from HF, labelled HXC_1 (46mg) and a white amorphous powder from HG, labelled HXC_2 (27mg). Both HXC_1 and HXC_2 formed a red and greenish spots on TLC when treated with Salkowski and Libermann-Burchard reagents respectively, indicating the likelihood of a steroid/triterpenoid compounds. However, digitonide formation reaction was not carried out prior to their spectroscopic analysis.

Column fraction HH (95-151, ≈1g) gave a brown solid mixture on concentration *in vacou*, and was subjected to re-column chromatography on 50g silica packed in a smaller column (1.5cm x 65cm) of 50ml capacity. An eluent with the same solvent mixture above was utilised for a 100 collections at 5ml. The fractions were monitored on TLC and similar ones from the column were pooled to give five (5) fractions designated Ha to He (Appendix Ia). The fractions were left to stand overnight in the fume cabinet. On TLC analysis of all the fractions (using increasing polar mixtures of hexane/ethyl acetate), fractions Hd (61-80) showed a chromatogram with single spots of same R_f (Appendix Ia, Hd). The later gave a positive Liebermann-Burchard test and also showed absorption of UV at both 254 and 365nm and was readily soluble in chloroform. On completely concentrating fraction Hd, a dull white amorphous powder was observed and was labelled HXC_3 (9mg). No compounds were isolated from the other fractions. All the column fractions and compounds isolated were concentrated using the fume cabinet.

3.3.5. Column chromatography of ethyl acetate fraction

In a similar manner to the hexane column above, a portion of the EtOAc fraction (3g) was loaded onto a 150 silica gel parked in glass column (60 x 3cm) and eluted with 100% hexane, hexane/acetone gradient mixtures (19:1; 9:1; 4:1; 2:1; 1:1; 1:2; 1:4; 1:9), acetone (100%) and finally 10% methanol in acetone was used to wash the column. In all, a total of 100 fractions were collected at 5 ml each. The fractions were monitored on TLC (using dichloromethane, ethyl acetate and ethyl acetate/methanol mixture as the developing solvent for early eluting fractions, intermediate and last fractions) and similar ones from the column were pooled to give five (5) fractions designated E_1 to E_5 (Appendix Ib). The fractions were left to stand overnight in the fume cabinet. Fraction E_3 (45-75) showed a yellowish sticky mass on concentration. An addition of 100% hexane to the later in the collecting beaker yielded a white precipitate, which were decanted and separated from the soluble portion. Pure hexane was added to the precipitates and heated on a water bath to dissolve, after which it was cooled to form back the white crystals. The recrystallization was done several times by heating and cooling repeatedly in pure hexane to obtained pure white crystalline solids. The TLC chromatograms of the later developed with hexane/ acetone (3:1) indicated a single spot (absorbing at both 254 and 365nm UV light) of same R_f, (Appendix Ib E_3); which also gave a positive Salkowski and Libermann-Burchard test. Consequently, these was labelled EAC_1 (25mg), after further concentrating in the fume cabinet.

3.4. Structure Elucidation of Isolated Compounds

3.4.1. Melting point (MP) determination

The melting points of the pure compounds were determined using Melting point apparatus (Gallencamp, USA) at the Department of Chemistry, University of Pretoria.

3.4.5.1 MP sample preparation

About 3 mg of the samples to be investigated were transferred in to different capillary tubes and inserted one after the other, into the melting point apparatus. The melting points were determined as the temperature raises.

3.4.2. Ultra-violet spectroscopy (UV)

The UV-Vis analysis was carried on the isolated (pure) compounds using Perking Elmer Precisely Lambda 25 UV/Vis spectrophotometer at the Department of Chemistry, University of Pretoria, Hatfield, South Africa.

3.4.4.1. UV sample preparation

The solid compound (2 mg) was dissolved in 1ml of chloroform to obtain a homogenous solution. This was transferred into a glass cuvette as the sample, while a second cuvette containing the blank (chloroform) was also made. The sample and the blank cuvettes were then place in their appropriate compartment in the instrument and (run) subjected to analysis. These were scanned within 800-200nm.

3.4.3. Infrared spectroscopy (IR)

The IR analysis was carried on the compounds using the Perkin Elmer Spectrum RX IFT-IR System at the Department of Chemistry, University of Pretoria, Hatfield, South Africa.

3.3.3.1 IR sample preparation

The powdered compounds (0.5 mg) was placed in the sample compartment of the instrument and subjected to analysis. This was scanned within 400-4000 cm^{-1} (wave number).

3.4.4. Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopic experiments carried out include 1D (^{1}H, ^{13}C, DEPT-135,) and 2D (^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC). NMR spectra were obtained at 300 K on Top Spin 300 Hz and 400 Hz NMR spectrometers (Bruker, Germany) at University of Pretoria, Hatfield, South Africa. Chemical shifts (δ-values) in (ppm) relative to TMS (internal standard), coupling constant (J-values) and multiplicities were recorded.

3.4.2.1 NMR sample preparation

The isolated compounds were weighed (10 mg) and dissolved in 5 mL of deuterated solvents (chloroform). These solvent were used as solvent of choice because the compounds were soluble in them. The solutions were pipette in NMR tubes (5 mm in diameter) using a clean Pasteur pipette and then subjected to 1D / 2D NMR analyses.

3.4.5. Mass spectrometry analysis

The compounds isolated were analysed using an electrospray ionisation-quadrupole time-of-flight mass spectrometry (ESI-QTOF MS) with a scanning range of 100 to 1200 Da kDa), and was operated in both the positive and negative mode, Synapt G1 facility, Department of Chemistry University of Pretoria, Hatfield, South Africa.

3.3.1.1 ESI-MS sample preparation

The isolated compounds were weighed (1 mg) and dissolved in 200 µL of dichloromethane in a glass vial, transferred into an injection bottle before subjecting same to ESI-MS analysis.

3.5. Animals and Venom

Albino mice of both sexes weighing between 18–30g were used for the lethal effect (MLD) and inhibition/prophylaxis activity assays, respectively. Lyophilized *Naja nigrocollis* Broadley (NNBV) venom was provided by Prof M.S. Abubakar of the Department of Pharmacognosy and Drug Development, ABU Zaria-Nigeria.

Animal care and handling was conducted in compliance with standard and humane procedures, as spelt out in the International Animal Welfare Guidelines (Rollin and Kesel, 1990).

3.5.1. Lethality assay of the crude venoms of *Naja nigricollis*.

The venom was reconstituted with normal saline to obtain concentrations ranging from 5 and 10 $mgml^{-1}$. The method of Broad *et al.,* (1979), Theakson and Reid (1983) as cited in Abubakar, (2004) was adopted with slide modification. This entails having five groups of mice (n = 6) which were injected intraperitoneally (i.p.) with 0.2 ml of different doses of the reconstituted venom. The control group (6 mice) received only normal saline (0.2 ml, each i.p.). For each of these groups the average time of death and number of deaths was recorded within 24 hours. The LD_{99} value (minimum lethal dose, MLD) of venom was determined by probit analysis (Appendix III). Mice that survive the experiment were euthanatized at the end of the study and similarly examine.

3.5.2. Median lethal dose determination (LD_{50}) of extract of *Albizia chevalieri*

Four fractions of hexane, ethyl acetate, n-butanol and methanol were evaluated for acute toxicity using Lorkes (1983) method. The method involved the use of 13 mice in two phases for each fraction. In the first phase, three groups of mice (n=3), were giving an i.p. graded doses of the extract/fractions, while following the outcome of the phase one, the second phase involved the use of four groups of mice (n=1), replicated for each of the extract/fraction. In

both phases, the animals were observed for a maximum of 24 hours for any death. The LD_{50} was then calculated using the formula: $LD_{50} = \sqrt{D^0 * D^{100}}$; where D^0 implies the highest concentration in phase two for which no death occurred and D^{100} denotes the lowest concentration in phase two for which death was recorded.

3.5.3. Evaluation of the effect of crude extracts/fraction of *A. chevalieri* on lethal dose of the crude venom.

A maximum tolerated dose of 200 $mgkg^{-1}$ was used to determine the effect of various samples of *A. chevalier*i using the method described in Abubakar *et al*., (2000). This was by pre-treating five groups of mice (n = 6) with the various samples (ME, NB, EA and HE), including the n-saline group at 200$mgkg^{-1}$, followed 1 hour later with MLD of venom. The average time of death and number of deaths was recorded within 24 hrs.

3.5.4. Evaluation of the use of *Albizia chevalieri* as prophylaxis of *Naja nigricollis* envenomation.

This was done using the method described in Abubakar *et al*., (2000). In this experiment, the effect of the extract on lethal doses of venom was determined by grouping the albino mice into three groups of six animals (n=6); group 1 were given an i.p. dose of MLD venom only, group 2 were given an i.p. dose of 100 mg kg^{-1} of extract in normal saline and was followed 1hour later by MLD of venom. Group three were given normal saline and followed 1 hour later by an i.p dose of 100 mg kg^{-1} of extracts. The average time of death and mortality after 24 hours was determined.

3.5.5. Studies of *in-vivo* anti-venom effect of pure isolated compound(s) of *A. chevalieri* on *Naja nigricollis* envenomation.

Three groups of mice (n=6) were used in this protocol. The first group (group1, control) received 0.2 ml MLD ($LD_{99;}$ 5mgkg^{-1}) of *Naja nigricollis* venom only. The second group (group 2) receive 4 mgkg^{-1} of pure isolated compound (HXC_1); third group (group 3) received 8mgkg^{-1} HXC_1. Mortality and average time of death was determined over a period of 24 hours. Mice that survived the experiment were euthanatized at the end of the study and examined (Abubakar, *et. al.,* 2000). The same procedure was repeated to evaluate the anti-venom activity of the isolated compound, EAC_1 obtained from ethyl acetate fraction.

3.5.6. Histological studies

3.5.6.1. Preparation of samples for histopathology

The liver and kidney were quickly removed from sacrificed mice after which the kidney was decapsulated. They were cleansed of blood using 0.25M sucrose solution. They were then fixed separately in 10% formalin solution before taken to Chemical Pathology Department, Usmanu Danfodiyo University Teaching Hospital, Sokoto (UDUTHS), where the histopathology was carried out.

3.5.6.2. Histological procedures

Fixed tissues were dehydrated in an ascending series of alcohol, cleansed in xylene, and embedded in paraffin wax melting at 60^0C. Serial sections (5-µm thick) were mounted on 3-aminopropyltriethsilane-coated slides and dried for 24 hours at 37^0C (Baravalled *et al*, 2006). The sections on the slides were deparaffinised, hydrated, and stained with Mayer's hematoxylin and eosin dyes, dried and mounted. The resulting slides were then viewed under the light microscope. The photomicrographs were printed at a total magnification of x200.

CHAPTER FOUR

4.0 RESULTS

4.1. Extraction of Plant Material

The weight of the extracts and fractions obtained and their percentage yields are presented in Table 4.1.

Table 4.1. Mass and percentage yield of extracts

Extracts Fractions	Hexane	MeOH	EtOAc	n-Butanol
Yield (g)	3.80	207.23	14.50	47.45
% yield (g)	0.20	10.40	0.80	2.60

4.2 Preliminary phytochemical screening of the stem bark of *A. chevalieri*

The metabolites screened for their presence in the extracts and fractions of *A. chevalieri* are as presented in Table 4.2.

Table 4.2. Preliminary phytochemical screening of the stem bark of *A. chevalieri*

Constituents Tested	Reagent used	Fractions tested			
		HX	ME	EA	NB
Saponins	Froth test	-	+	-	+
Alkaloids and nitrogenous Compound	Drangendorff's	-	+	+	+
	Hager's	-	+		+
	Mayer's	-	+		+
Flavonoids	NaOH	-	+	+	+
Tannins	$FeCl_3$	-	+	-	+
Carbohydrates and glycosides	Molisch's test	+	+	+	+
Sterol and or terpenoids	Liebermann-Burchard's	+	+	+	+
	Salkowkis's		+	+	+
Anthraquinones/Coumarines	Borntrager's test	+	-	+	-
Cardiac glycosides	Killer-killaini' test	+	-	-	-

Key: + = present
 = not detected

4.2. Thin Layer Chromatography

The separation profiles of hexane extract and ethyl acetate fraction are presented on plates III and IV respectively. The TLC profile of the hexane extract showed eight major spots. The colours observed for the various spots include, purple, red, yellow and pink. The R_f values and the colour of the various spots are as indicated on plate III. Similarly, the TLC profile of the ethyl acetate fraction showed five major spots. The colours of spots observed are generally greenish black. The R_f values of the various spots are as presented on plate IV.

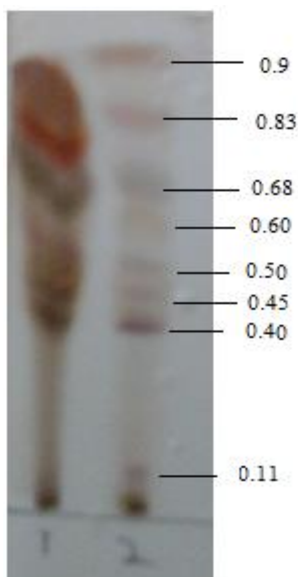


Plate III: Sample TLC plate of hexane extract. Solvent system: Hexane/EtOAc (49:1) sprayed with 5 % H_2SO_4, heated at 105^oC for 5 min.

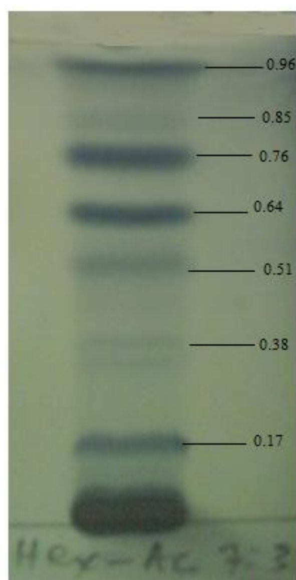


Plate IV: Sample TLC plate of ethyl acetate extract. Solvent system: EtOAc/Acetone (7:3), sprayed with PMA and heated at 105^oC for 5 min.

4.3. Column Chromatography of Hexane and Ethyl acetate Extracts

A total of four compounds were isolated from column chromatography of the hexane extract and ethyl acetate fraction of *A. chevalieri*. Compounds HXC_1 – HXC_3 were isolated from hexane extract while EAC_1 was isolated from the ethyl acetate fraction of the methanol extract. The column fractions from which the compounds were obtained, solvent systems of elution and TLC, Rf values, colour of spots in day light and under UV as well as before and after spray, weights, melting points and physical (state) appearances on concentration of the isolated compounds are all presented in Table 4.3.

Table 4.3. Masses, melting points and physical appearances of compounds isolated

S/No.	Compd Label	Column Fraction	Solvent Syst for Elution &	Rf Values	Quantity (mg)	M.P. (°C)	Appearances			
							Colour of Spot			State
							Day light		UV_{365}	
							Before Spray	*After spray/ heating*	(nm)	
1.	HXC_1	58-80	Hex:EtOAc 19:1	0.73	46	264±2*	No colour	Yellow-brown	Dark, (inactive)	White flakes
2.	HXC_2	81-94	Hex:EtOAc 9:1	0.50	27	282±2*	No colour	Yellow-brown	Dark, (inactive)	White amorphous
3.	HXC_3	$^{+}$61-80	Hex:EtOAc 9:1	0.72	9	ND	No colour	Dark brown	Sky bluish 365	Cream-white Powder
4.	EAC_1	45-75	Hex:Ac 9:1; tlc 3:1	0.57	25	140±2	No colour	Dark blue (PMA)	Sky bluish 365	White crystalline

ND = Not Determined; $^{+}$Re-column separation of previous column fractions 95-151; *Corrected

4.4. Structure Determination of Compound HXC_1

4.4.1. Infra-red (IR) spectroscopy of compound HXC_1

The FTIR spectrum of compound HXC_1 showed significantly IRυ_{max} cm^{-1}:1700.9 (C=O stretching), also observed are saturated C-H stretching due to the signal at 2918 and 2852.8 and 1461-1373 (C-H bending and rocking respectively). The spectrum is presented in Figure 4.1.

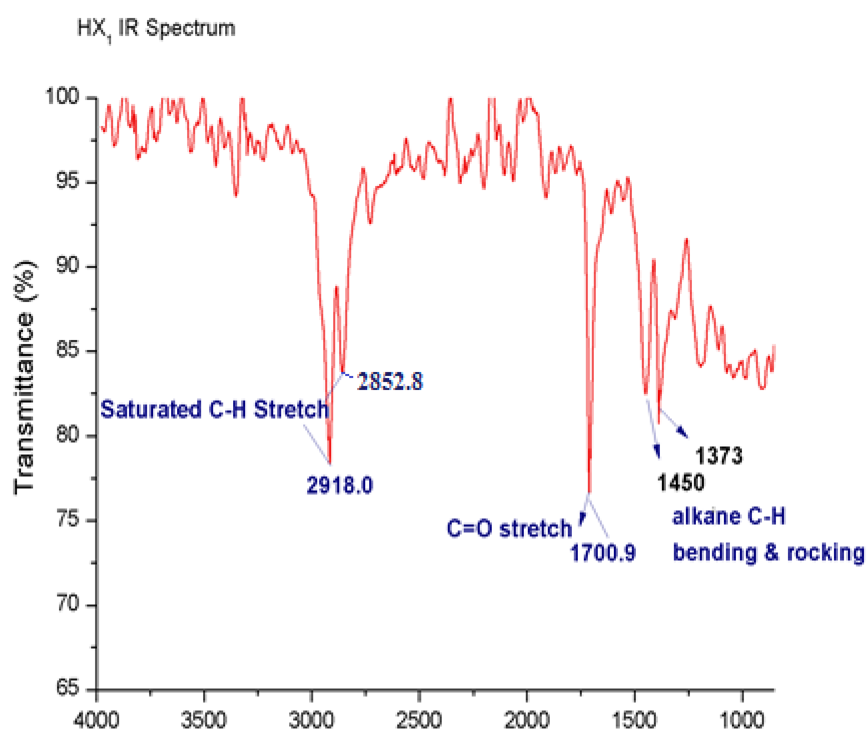


Figure 4.1. IR spectrum of compound HXC_1

4.4.2. Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) of compound HXC_1

The ^{1}H NMR (Fig. 4.2) revealed over lap signals for eight singlets of methyl proton at δ 0.93 (H-28), 0.85 (H-27), 0.85 (H-26), 1.00 (H-30), 1.00(H-29), 0.85(H-25) and 0.70 (H-24), a doublet of methyl at δ 1.18 (d, J = 7.7 Hz, 1H), a methine proton (CH) at δ 2.25 (q, J = 6.7 Hz, H-4) and methylene protons (CH_2) at δ 0.85-1.23(m, 23H). The peak around δ 7.24 arises due to isotopic solvent impurity of $CDCl_3$. This is presented in Fig.4.2. Table 4.4 present the chemical shift assignments for both ^{1}H and ^{13}C NMR spectra.

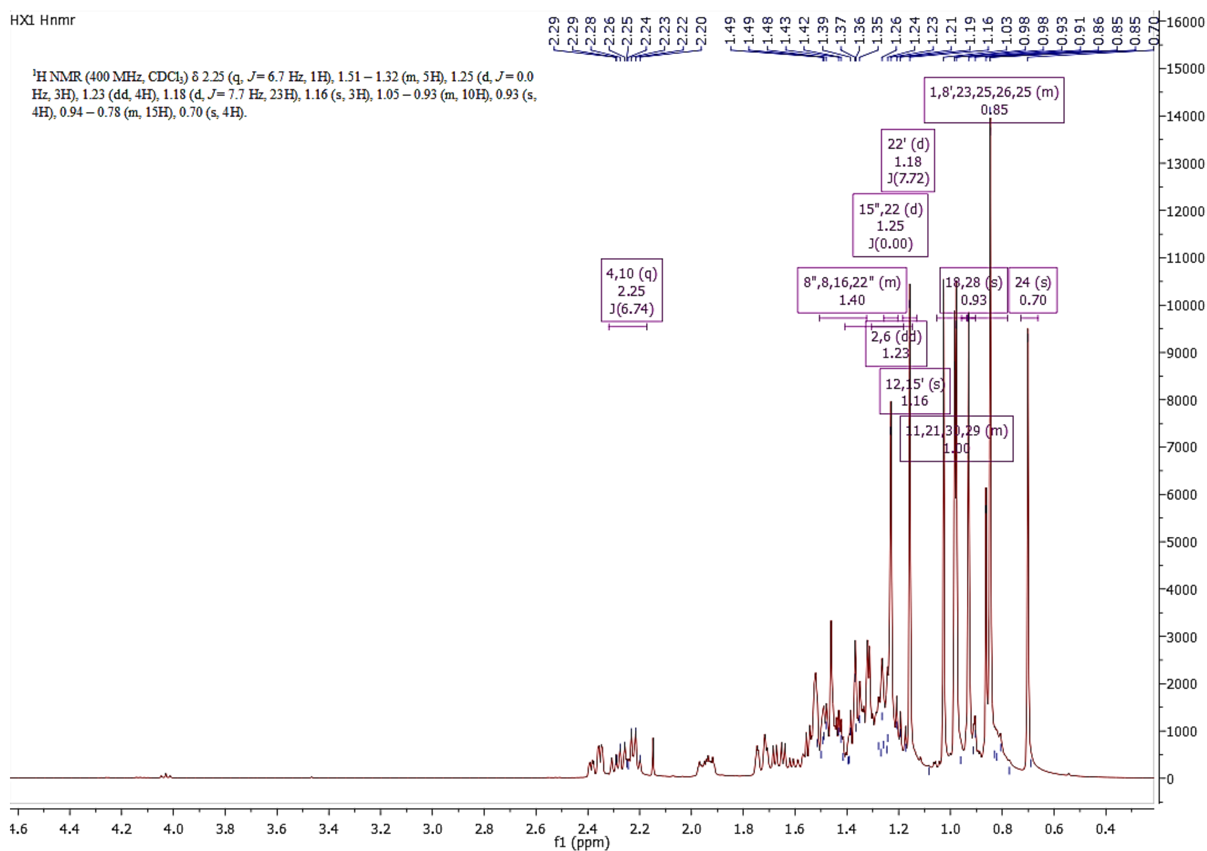


Figure 4.2. ^{1}**HNMR spectrum of compound** HXC_1 **in** $CDCl_3$**, 400 MHz**

Table 4.4. ^{1}H and ^{13}C-NMR chemical shifts of compound HXC_1 in $CDCl_3$, 400 MHz

H/C-Position	δ_C (ppm)	δ_H (ppm)	No. of H, Multiplicity, J(Hz)
1	22.28	1.70, 1.45	1H, m, each
2	41.74	2.25, 1.29	1H, m, 1H, qd
3	213.45	-	-
4	58.14	2.25	1H, q
5	41.88	-	-
6	35.83	1.25, 1.37	1H, m, each
7	18.16	1.40, 1.93	1H, m, each
8	52.26	1.40	1H, m, each
9	36.22	-	-
10	59.54	1.56	1H, m, each
11	35.24	1.45, 1.17	1H, m, each
12	29.88	1.20; 1.16	1H, m, each
13	39.20	-	-
14	38.51	-	-
15	30.21	1.16; 1.25	1H, m, each
16	35.55	1.40; 1.20	1H, m, each
17	30.03	-	-
18	42.36	1.15	1H, m, each
19	32.63	1.18, 1.40	1H, m, 1H, dd
20	28.18	-	-
21	32.00	1.45; 1.19	1H, m, each
22	39.01	1.25; 1.23	1H, m, each
23	7.04	0.85	3H, d
24	14.41	0.70	3H, s
25	18.04	0.85	3H, s
26	18.88	0.85	3H, s
27	19.45	0.93	3H, s
28	29.92	1.00	3H, s
29	32.30	1.00	3H, s,
30	32.30	1.00	3H, s

Coupling constant (J), singlet (s), double doublet (dd), multiplet (m), quartet of doublet (qd)

4.4.3. ^{13}CNMR spectrum of compound HXC_1

The ^{13}C NMR showed a total of thirty carbons, including the significantly diagnostic ketonic (carbonyl) C-3 carbon at δ = 213.45ppm. The other 29 carbons were fully accounted for by the DEPT 135 spectrum, indicating the presence of 8 methyl groups along with CH_2, 4 of CH and 6 of quaternary carbons. This is presented in Figure 4.3.

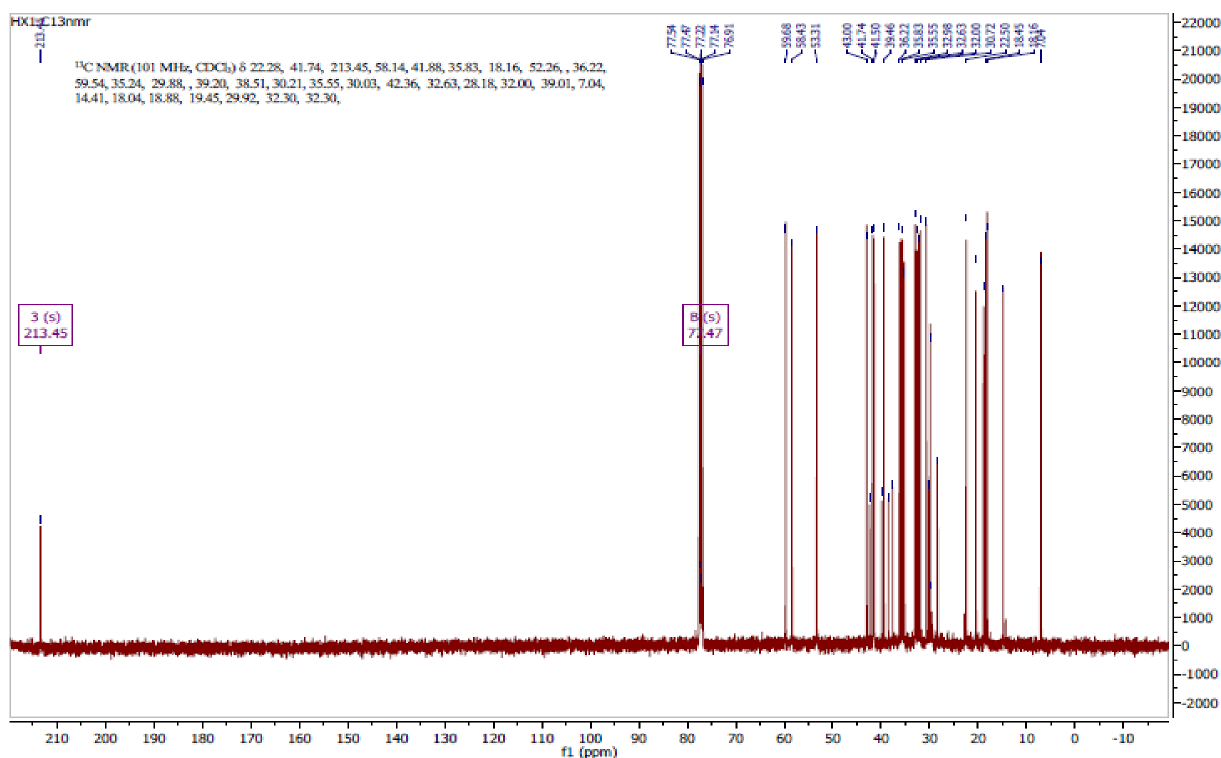


Figure 4.3. ^{13}**CNMR spectrum of compound** HXC_1 **in** $CDCl_3$**, 400 MHz**

4.4.4. Distortionless enhancement by polarisation transfer (DEPT-135) spectrum of compound HXC_1

The DEPT 135 spectrum contain only signals arising from protonated cabon. Consequently, a total of twenty nine signals are seen in the spectrum of figure 4.4. These include a total of twelve methyl (CH_3) and methine (CH) signals (those with an odd number of attached protons, and appeared oppositely phased up or positive) and a total of eleven methelene (CH_2) signals (those with even number of attached protons and appeared oppositely phased downward or negative). The resulting DEPT 135 spectrum does not cotain signal arising from non-protonated carbon, hence seven quartenary carbons can be easily recognised at δ213.46, δ43.00, δ36.22, δ39.46, δ38.51, δ30.72 and δ28.38 as not bieng part of the signals observered in the specrum. This is presented in Table 4.5 and Figure 4.4.

Table 4.5. DEPT 135 spectrum analysis of C- atoms present in compound HXC_1

δ-values for C- atoms (ppm)			
Quaternary	Methine	Methylene	Methyl
213.46	58.43	22.50	7.04
43.00	59.63	41.74	14.87
36.22	53.31	35.83	18.45
39.46	42.36	18.16	18.88
38.51		35.24	20.47
30.72		29.88	29.92
28.38		30.21	32.30
		35.55	32.30
		32.63	
		32.00	
		41.50	

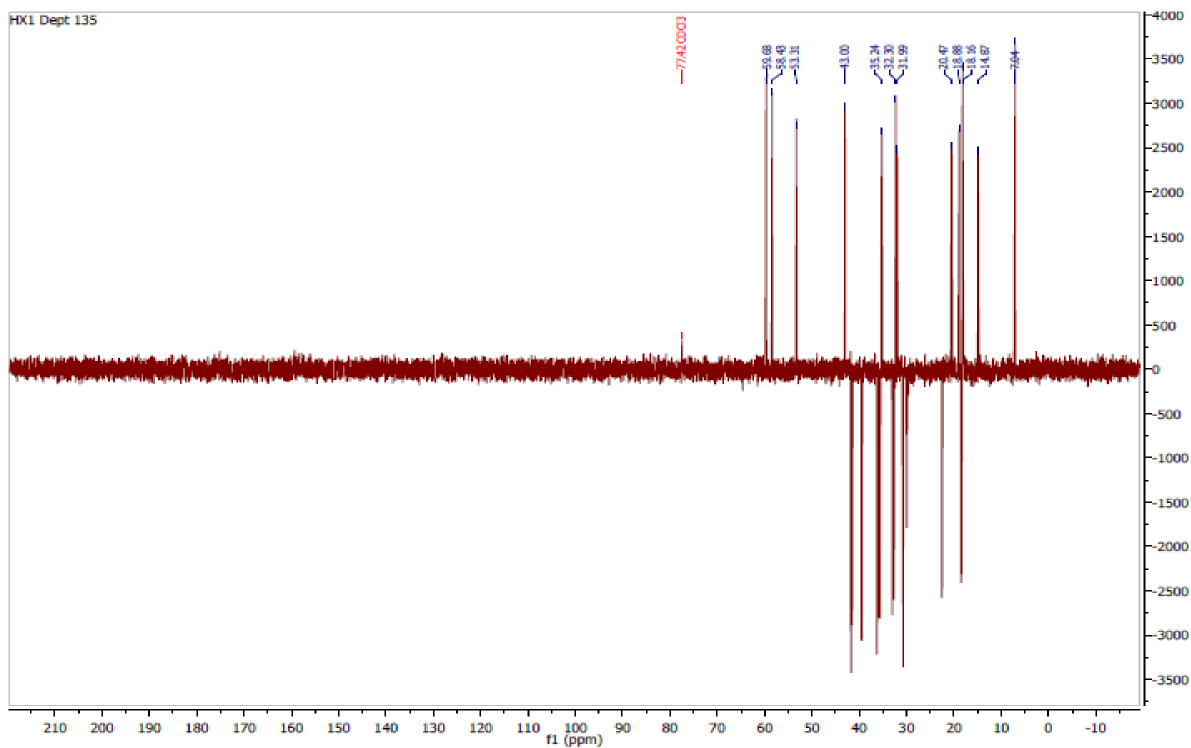


Figure 4.4. DEPT 135 spectrum of compound HXC_1 in $CDCl_3$, 400 MHz

4.4.5. Mass spectrometry of compound HXC_1

The electro-spray ionisation mass spectroscopy (ESI-MS) spectrum showed a molecular ion (*m/z*) peak at 427.15 in the positive ion mode $[M+H]^+$, which corresponds to a compound with molecular formula $C_{30}H_{50}O$. The fragment ion at *m/z* 425.14 may corresponds to a loss of two protons from the molecular ion while *m/z* 411.53 corresponds to the loss of one methyl group from the molecular ion. Scheme 4.1 gives a proposed fragmentation of compound HXC_1. The base peak at m/z 187.04 and other significant ions at *m/z* 341.19, 273.14, 206.90 and 122.98 can be attributed to fragmentations of rings A, B, C and D, respectively. This is represented in scheme 4.1 and figure 4.5.

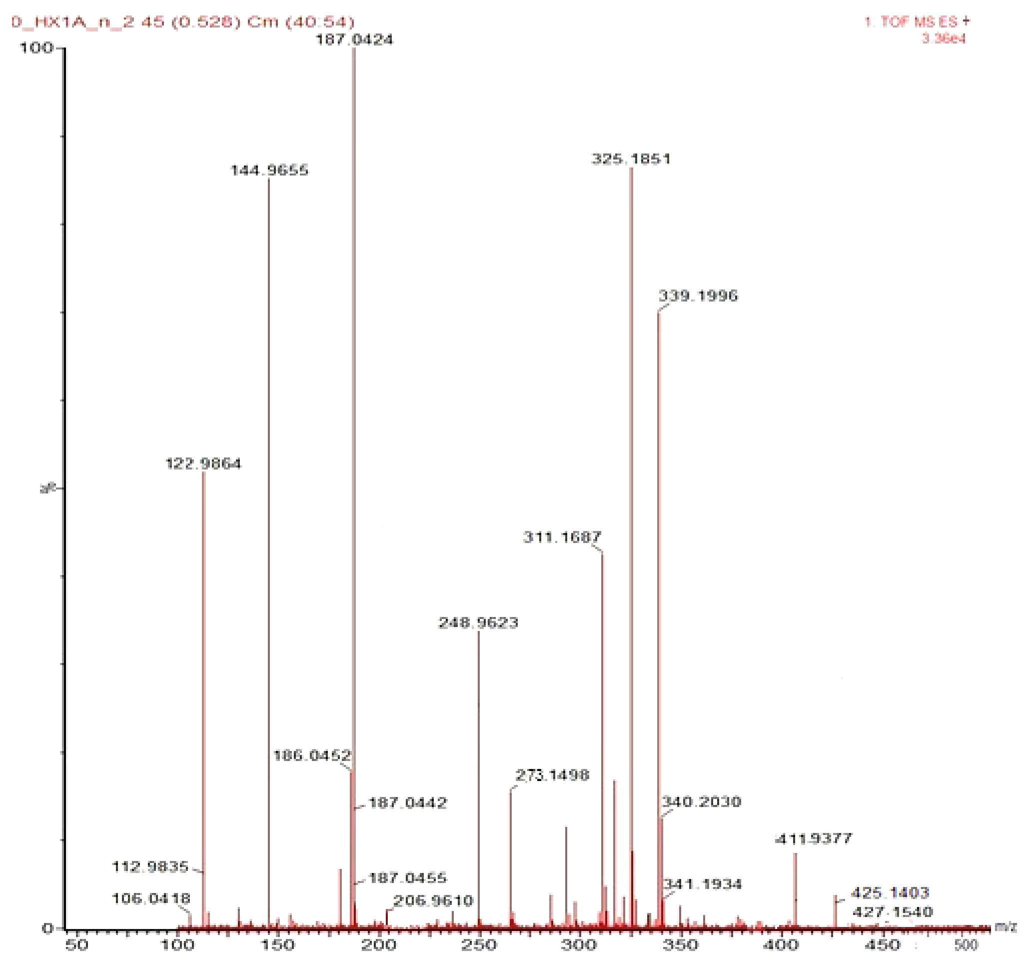
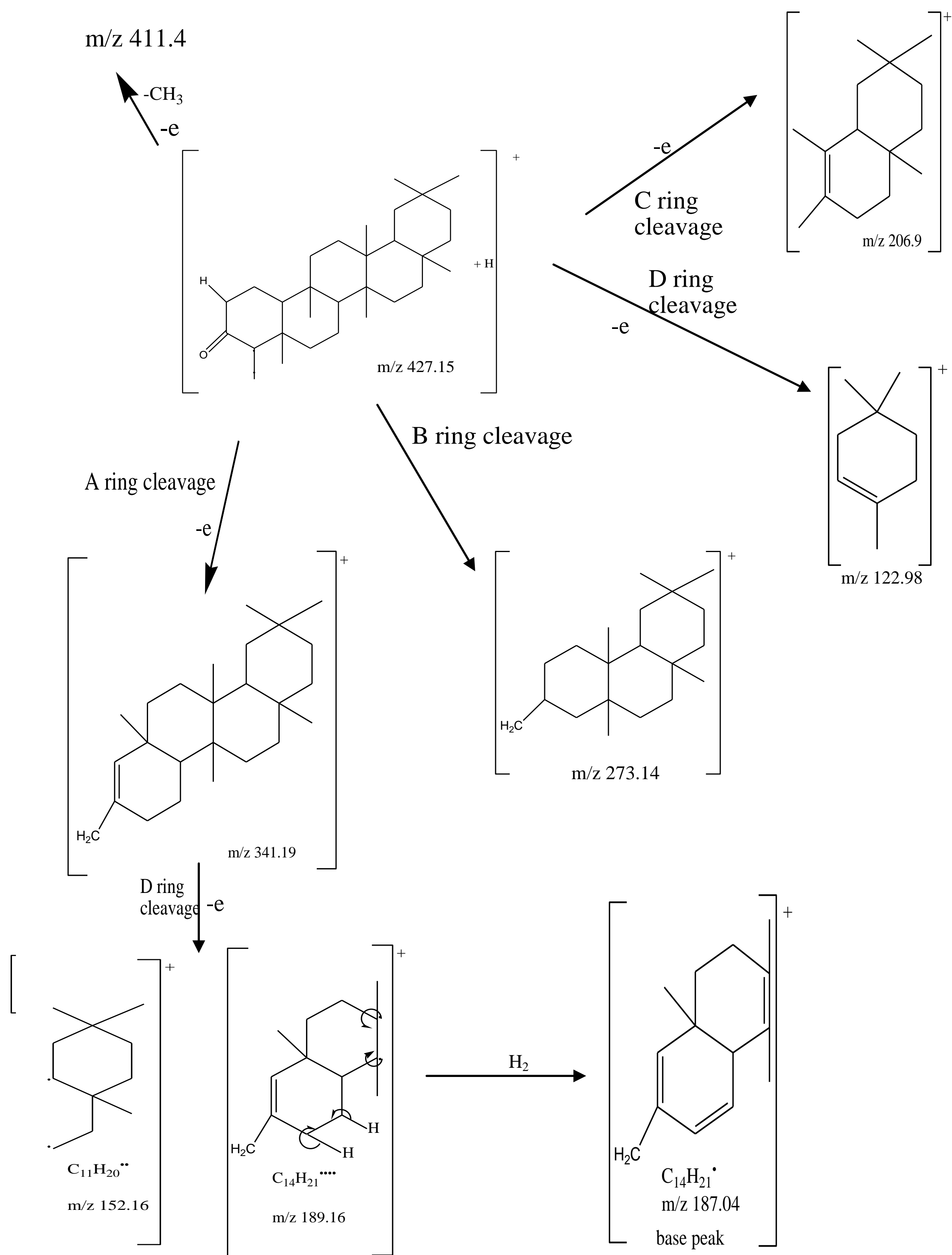


Figure 4.5. Mass spectra of compound HXC_1



Scheme 4.1. Fragmentation patterns of compound HXC_1

Table 4.6. Summary of spectra data of HXC_1

Spectroscopic technique	Data								*References (Compare with)
UV λmax:	No absorption								Sousa *et al*., 2012
IRυ_{max} cm^{-1} [FTIR]	2918.0 - (C-H str)	2844.0	1700.9 (C=O str)	1461.90	1158.20	1022.04			
^{1}HNMR ($CDCl_3$) [400 MHz]	1.77–1.32: Signals overlap	δ 2.25 (q, *J* = 6.7 Hz, 1H), 1.51 – 1.32 (m, 5H), 1.25 (d, *J* = 0.0 Hz, 3H), 1.23 (dd, 4H), 1.18 (d, *J* = 7.7 Hz, 23H), 1.16 (s, 3H), 1.05 – 0.93 (m, 10H), 0.93 (s, 4H), 0.94 – 0.78 (m, 15H), 0.70 (s, 4H).							Putra *et al.,* 2004
^{13}CNMR ($CDCl_3$) [101 MHz]	δ22.28	δ41.74	**δ 213.46**	δ 58.14	δ 41.88	δ 35.83	δ 18.16	δ 52.26	
	δ36.22	δ59.54	δ 35.24	δ 29.88	δ 39.20	δ 38.51	δ 30.21	δ 35.55	
	δ 30.03	δ 42.36	δ 32.63	δ 28.18	δ 32.00	δ 39.01	δ 7.04	δ 14.41	Abreu *et al*., 2013
	δ 18.04	δ18.88	δ 19.45	δ 29.92	δ 32.30	δ 32.30			
HREIMS: m/z (rel. % abundance)	427.1540 $[M+H]^+$; (2)		411.94 (4)	341.19 (3)	273.15 (15)	206.96 (27)	122.99 (54)	187.04 (100)	

*as discused in chapter 5

4.4.6. Suggested structure of compound HXC_1

The 2-D spectra of cosy and HSQC (Appendix IIa-c) also showed adequate correlation of all the ^1H-^1H as well as ^1H-^{13}C respectively. Hence, the HSQC showed no proton attached to C-3 at δ213.46. The spectroscopic data above for compound HXC_1 agree with those from literature (Table 5.1), and was identified as Friedelin (octadecahydro-4,4a,6b8a,11,11,12b,14a-octamethylepicen-3-(4H,6bh,14bH)-one) as presented in figure 4.6 below.

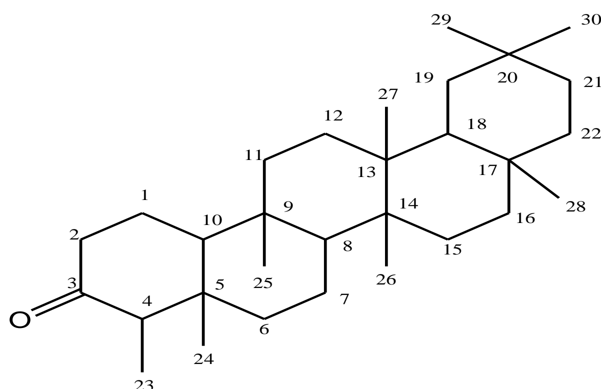


Figure 4.6. Suggested structure of compound HXC_1

4.5. Structure Determination of Compound HXC_2

4.5.1. Infra red (IR) spectroscopy of compound HXC_2

The FT-IR spectrum of compound HXC_2 showed $IR\upsilon_{max}$ cm^{-1}:3342 (a broad peak due to aliphatic O-H stretching), 2920 (alkyl C-H stretching), 1600 (C-C stretching in ring) and 1450-1355 (C-H bending and rocking). This is presented in Figure 4.7

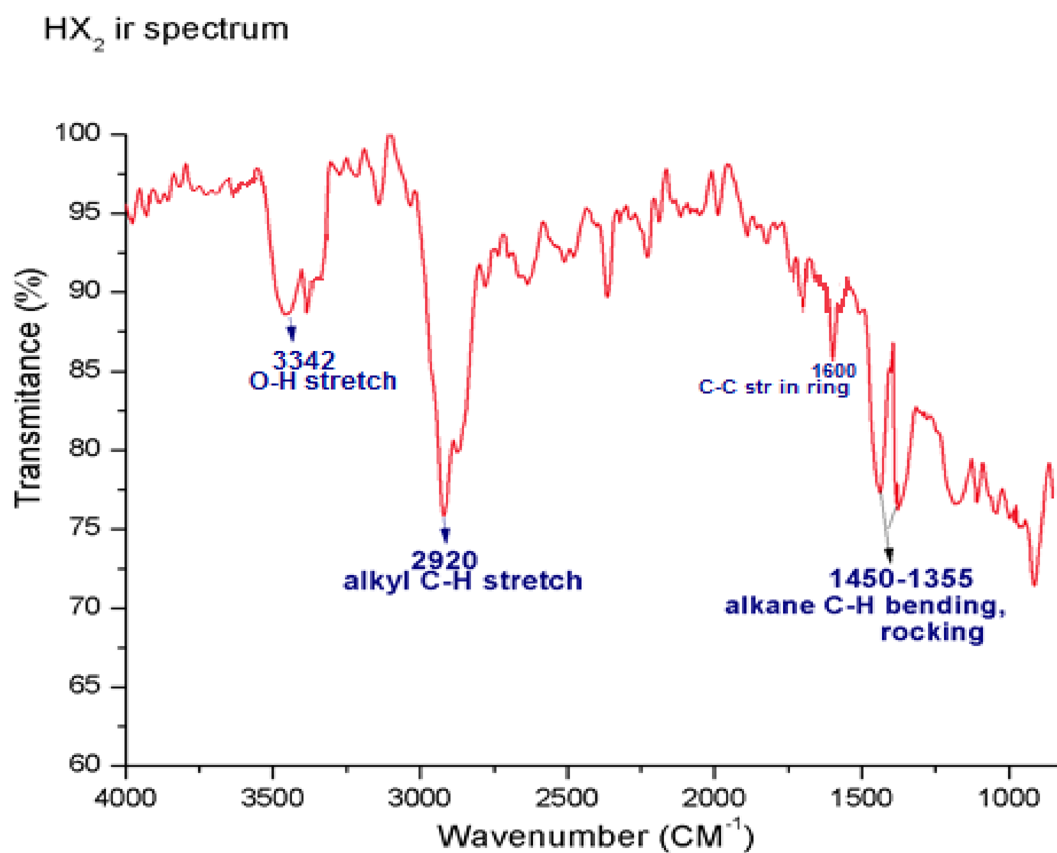


Figure 4.7. IR spectrum of compound HXC_2 (KBr)

4.5.2. Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) of compound HXC_2

The ^{1}H NMR of HXC_2 displayed singlet signal for eight tertiary methyl proton at δ_H 0.69-1.16 (s, CH_3, 24H)) of a pentacyclic triterpene and also presented a double doublet at δ_H 4.25 (J=7.1 and 4.9Hz 1H), which is typical of hydrogen bounded to an oxygenated carbon, suggesting the presence of a downfield hydroxyl (OH) group in the structure. The other methylene and methine protons with δ_H 1.25-1.74 (m, CH_2, CH, 27H) are in agreement and consistent with data found in literature (see Table 5.2). The ^{1}H NMR spectrum with the region of detected chemical shift values for compound HXC_1 is as shown in Figure 4.8.

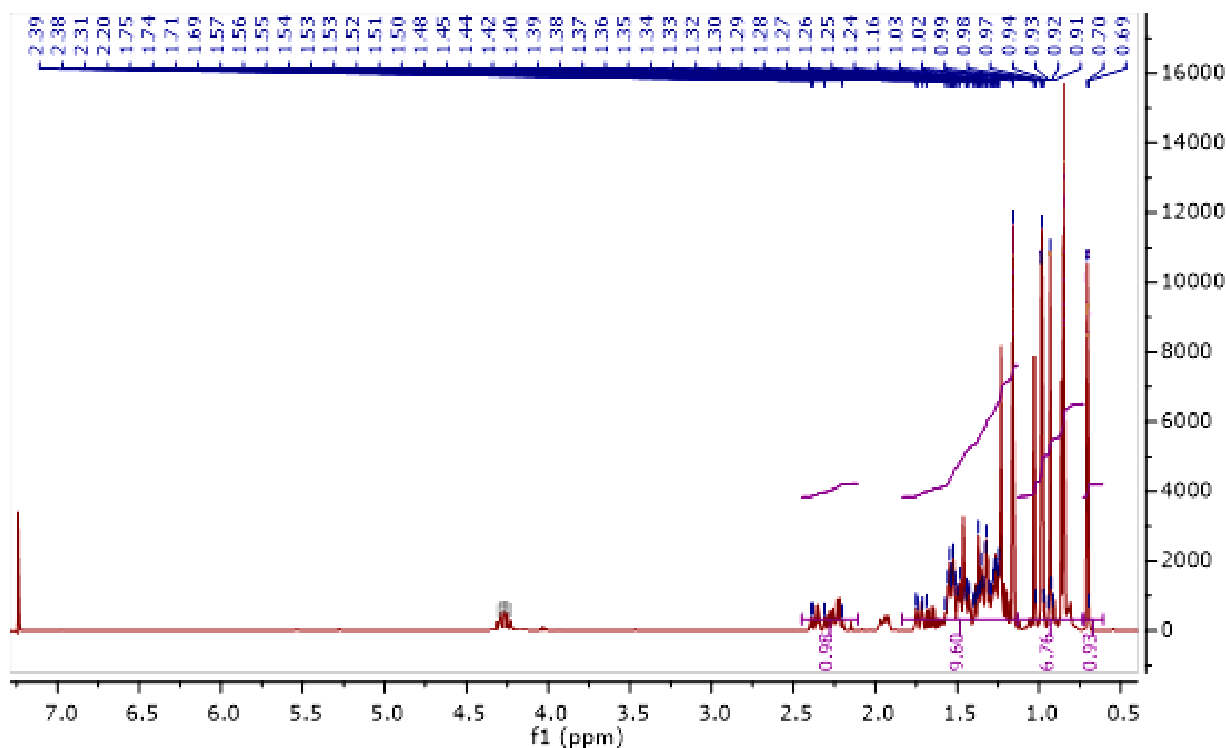


Figure 4.8. ^{1}HNMR spectrum of compound HXC_2 in $CDCl_3$, 400 MHz

4.5.3. ^{13}CNMR spectrum of compound HXC_2

The ^{13}C NMR showed distinctive signal at δ 72.89, indicative of a carbon bearing hydroxyl group. The ^{13}CNMR spectrum in fig 4.8 generally accounted for 30 carbons with chemical shift at δ 72.89 attributed to C-3 due to a hydroxyl group. Chemical shift for other carbons were assigned by comparing with δ_C values of friedelan-3β-ol given in literature (see Table 5.2). Table 4.7 gives the chemical shifts assignments for carbon atoms as well as hydrogen atoms of compound $HXC_{2,}$ while Figure 4.9 present the ^{13}CNMR spectrum.

Table 4.7. ^1H and ^{13}C-NMR chemical shifts of compound HXC_2 in $CDCl_3$, 400 MHz

H/C-Position	δ_c (ppm)	δ_H (ppm)	No. of H, Multiplicity, J(Hz)
1	17.92	1.36, 1.54	3H, d,
2	36.06	1.38, 1.74	1H, d, 5.14
3	**72.89**	4.34, 1.37	1H, s
4	50.08	1.10	
5	38.22	-	-
6	42.06	1.10, 1.74,	1H, m, each
7	17.19	1.10, 1.75	1H, m, each
8	53.26	1.10	1H, m, each
9	37.68	-	-
10	61.47	1.20	1H, m, each
11	35.66	138, 1.17	1H, m, each
12	30.50	1.49, 1.22;	1H, m, each
13	39.20	-	-
14	39.01	-	-
15	32.12	1.37, 1.19	1H, m, each
16	37.03	1.40, 1.04;	1H, m, each
17	30.03	-	-
18	42.65	1.25	1H, m, each
19	35.32	1.38	1H, d, 10.8
20	28.18	-	-
21	32.78	1.09, 1.51	1H, m, each
22	39.01	1.11, 1.38	1H, m, each
23	11.80	1.48, 0.89, 0.89	3H, d
24	16.44	0.88, 0.93, 0.93	3H, s
25	17.37	0.99	3H, s
26	19.41	0.89	3H, s
27	19.45	0.86	3H, s
28	32.11	0.97	3H, s
29	33.35	0.93	3H, s,
30	33.35	0.79	3H, s

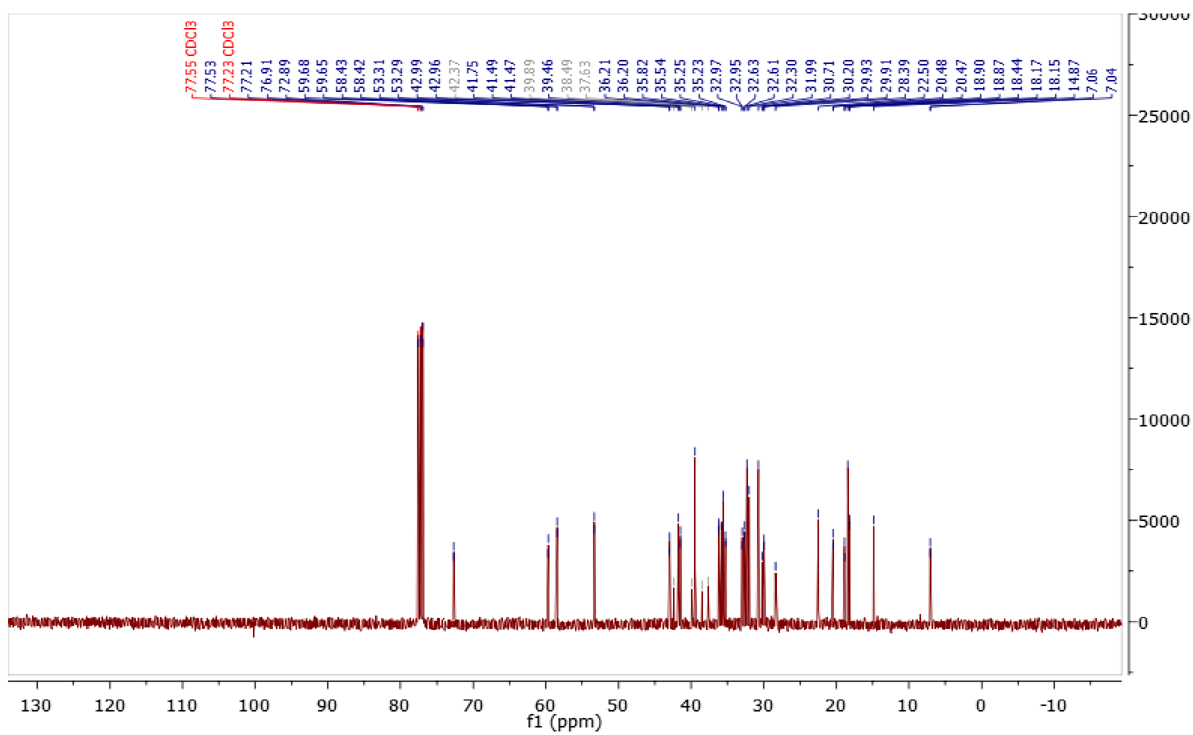


Figure 4.9. ^{13}CNMR spectrum of compound HXC_2 in $CDCl_3$, 400 MHz

4.5.4. Distortionless enhancement by polarisation transfer (DEPT) spectrun of compound HXC_2

The DEPT 135 of compound HXC_2 showed 12 positive signals due to methine and methyl at δ_c (ppm) 7.03, 14.86, 18.15, 18.87, 20.46, 31.98, 32.97, 35.54, 41.74, 53.30, 58.43 and 59.67. Eleven negative signals due methylene were also seen at δ_c (ppm) 18.07, 22.18, 29.59, 30.62, 32.77, 35.20, 35.52, 35.87, 39.41, 41.48 and 42.65. On comparing the DEPT 135 with the ^{13}CNMR, six quarternary carbon atoms were observed at δ_c (ppm) 38.22, 37.68, 39.20, 39.01, 30.03, and 28.18. DEPT 135 spectrum of compound HXC_2 is represented in Figure 4.10.

Figure 4.10. DEPT 135 spectrum of compound HXC_2 in $CDCl_3$, 400 MHz

4.5.5. Mass spectrometry of compound HXC_2

The ESI-MS spectrum showed a molecular ion (*m/z*) peak at 429.785 in the positive ion mode $[M+H]^+$ (>10% relative intensity), with important fragment ions observed at m/z 413, 409 (base peak), 342, 273, 205, and 123. M/Z 409 is likely due to a loss of water and molecular hydrogen; m/z 413 could be a $-CH_3$ lost. Other fragment ions also occur and are presented in Figure 4.11. Scheme 4.2 gives a proposed fragmentation of compound HXC_2. The proposed fragmentation pattern as described in literature for fridelanes is as shown is scheme 4.2.

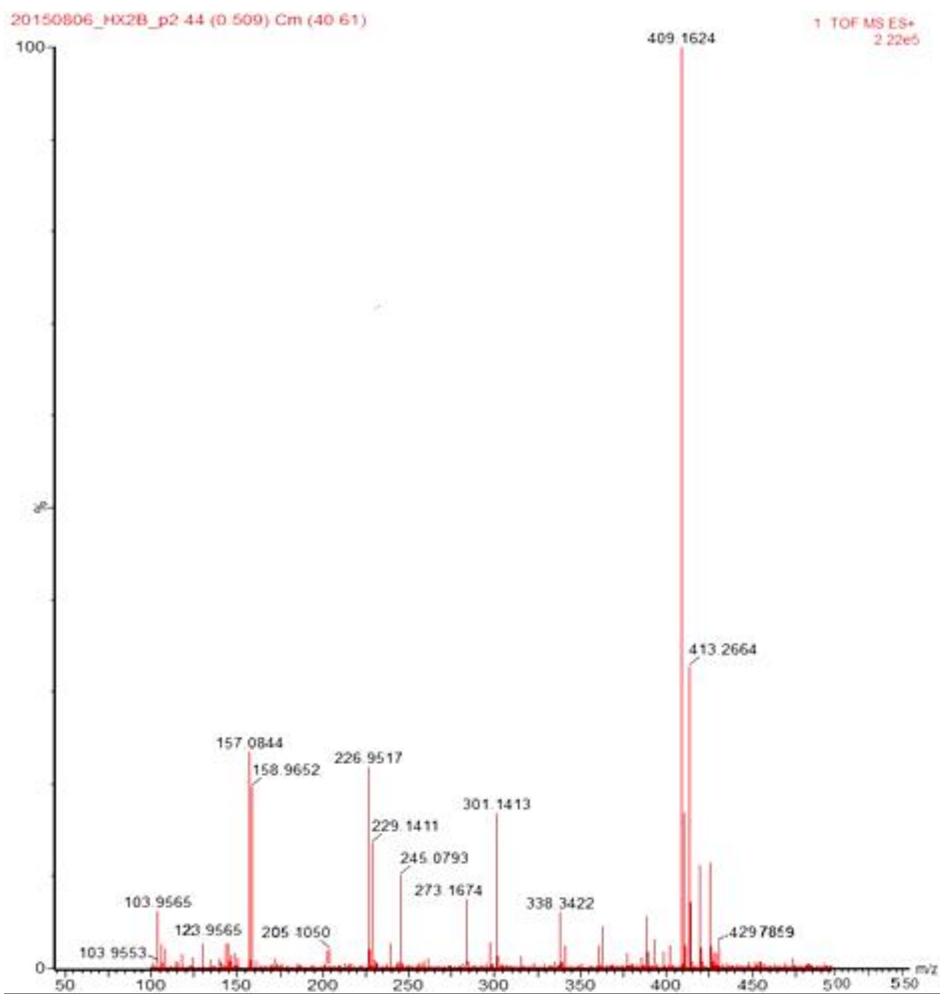
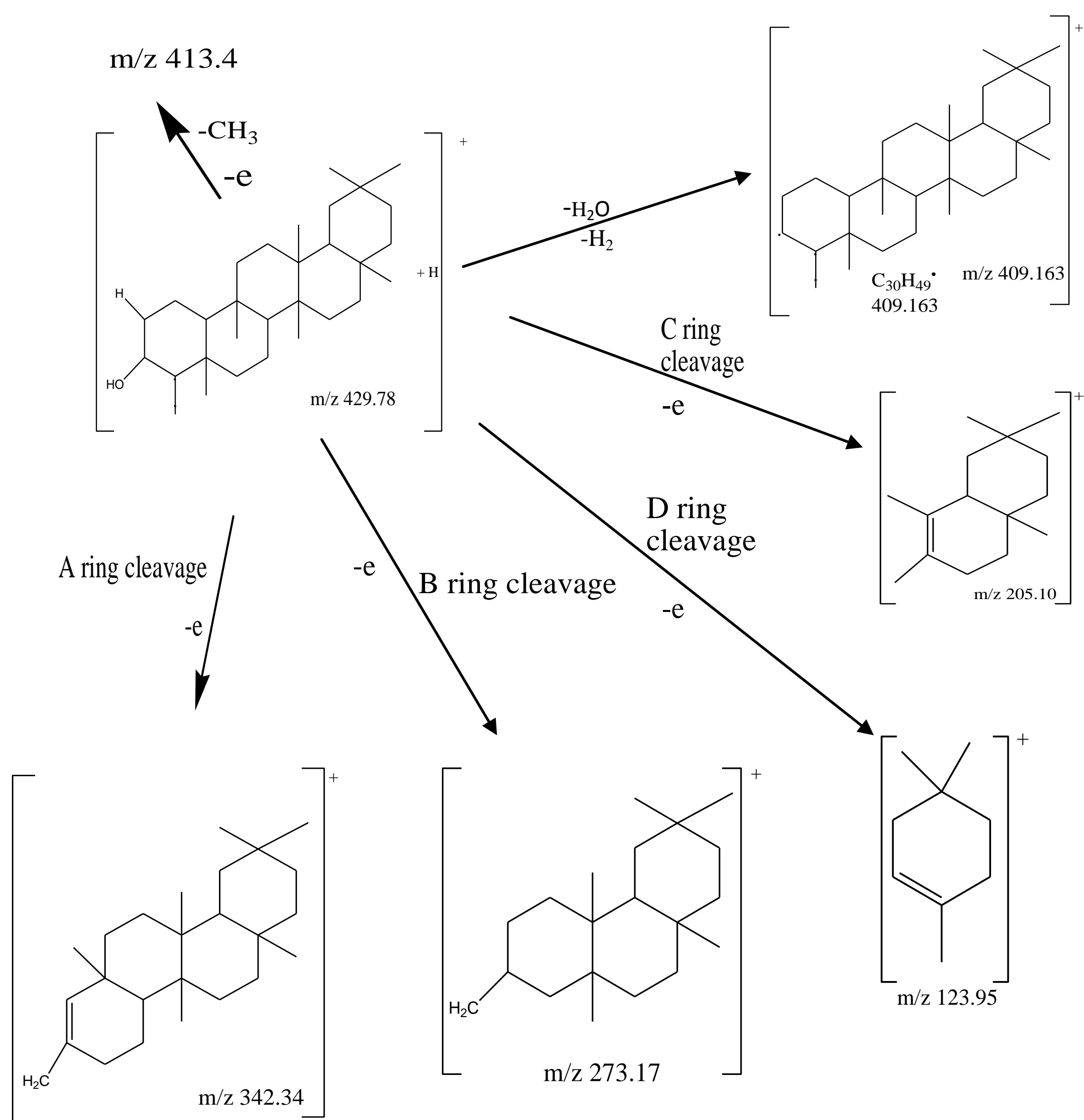


Figure 4.11. Mass spectra of compound HXC_2



In line with Shiojima *et al*., (1992).

Scheme 4.2. Possible fragmentation pattern of compound HXC_2

Table 4.8. Summary of spectra data of HXC_2

Spectroscopic technique	Data								References (Compare with)
UV λmax:	No absorption								Sousa *et al.*, 2012;
IRυ_{max} cm^{-1} [FTIR]	3342.3 (O-H str),	2937.5 (Aliphatic str),	2844.0	1461.9	1382.96	1158.2	1022.04		Kamboj & Saluja, 2011
^{1}HNMR ($CDCl_3$) [400 MHz]	Signals overlap	δ 2.32 – 2.17 (m, 2H), 1.51 – 1.32 (m, 11H), 1.16 – 1.30 (m, 20H), 1.18 (d, *J* = 7.7 Hz, 1H), 1.03 – 0.94 (m, 15H), (m, 15H), 0.69-1.16 (s, 24H).							Duarte *et al.*, 2009
^{13}CNMR ($CDCl_3$) [101 MHz]	δ17.92 δ37.68 δ30.03 δ17.37	δ36.06 δ61.47 δ42.65 δ19.41	δ 72.16 δ 35.66 δ 35.32 δ19.45	δ 50.08 δ 30.50 δ 28.18 δ 32.11	δ 38.22 δ 39.20 δ 32.78 δ33.35	δ42.06 δ39.01 δ39.01 δ33.35	δ17.19 δ11.80 δ33.35	δ 53.26 δ16.44 δ33.35	Boonyaratavej & Petsom, 1991
HREIMS: m/z (rel. % abundance)	429.7; $[M+H]^+$	411.3 $[M+H-18]^+$	413.2 (30)	409.12 (100)	273 (11)	205 (6)	123 (7)		

4.5.6. Suggested structure of compound HXC_2

Elucidation of the spectra information from the MS, NMR and IR data, which compared well with available literatures (see Table 5.2) leads to the suggested structure and name of compound HXC_2 as 3β-friedelinol (docosahydro-4,4a,6b,8a,11,11,12b,14a-octamethylpicen-3-ol) (Figure 4.12).

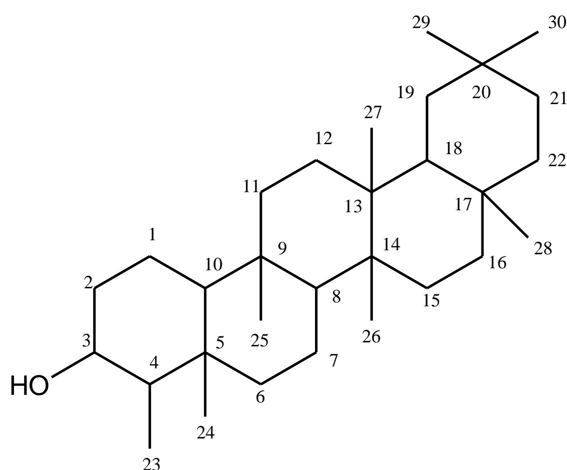


Figure 4.12. Suggested structure of compound HXC_2

4.6. Structural Determination of Compound HXC_3

4.6.1. Ultra-visible light (UV) spectroscopy of compound HXC_3

The UV spectrum observed for compound HXC_3 showed the presence of a chromophoric group in the structure, displaying absortion of UVλ_{max} at 370 nm. The UV spectrum is presented in Figure 4.13 .

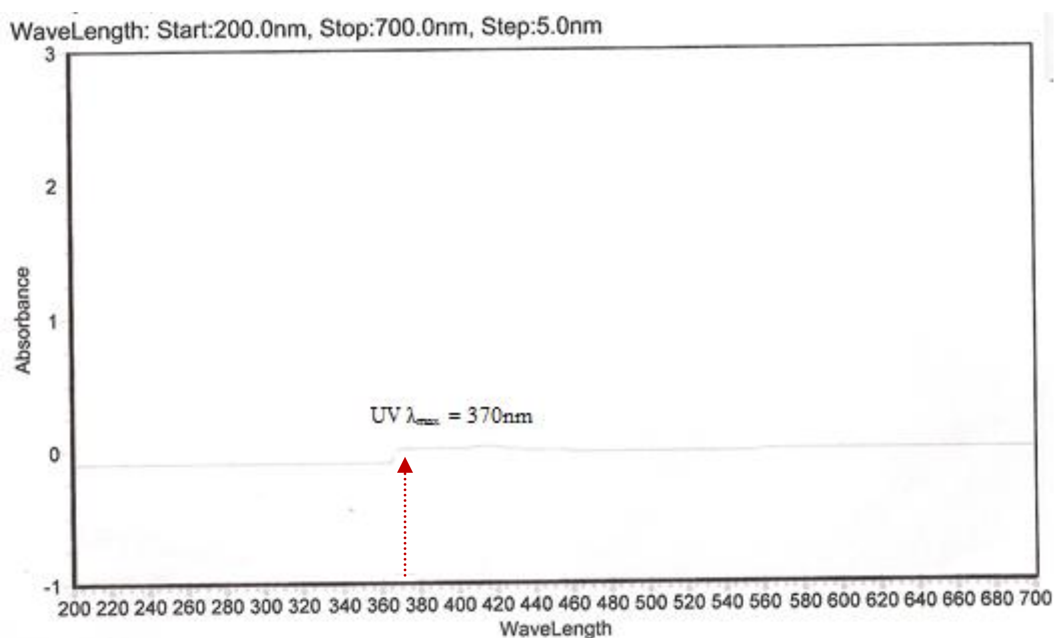


Figure 4.13. UV spectra of compound HXC_3

4.6.2. Infra red (IR) spectroscopy of compound HXC_3

The presence of hydroxyl group was confirmed by the FT-IR spectrum which showed a broad peak at IRυ_{max} 3253cm^{-1} while a week peak at 1680 cm^{-1} (C=C str) can be attributed to a vinylic (double bond) group. This is presented in Figure 4.14.

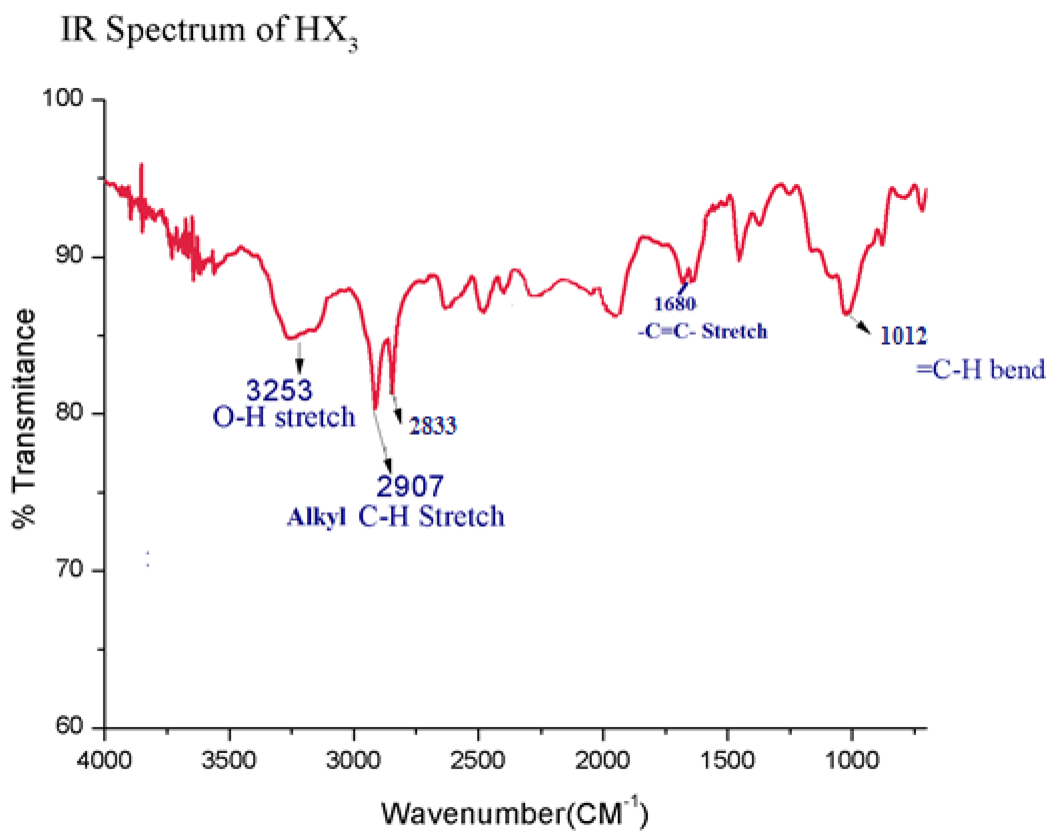


Figure 4.14. IR spectra of compound HXC_3

4.6.3. Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) of compound HXC_3

The proton NMR of compound HXC_3 revealed the presence of seven singlet tertiary methyl protons at δ 0.75, 0.78, 0.83, 0.86, 0.92, 0.97 and 1.23ppm (3H each, s, CH_3). Two protons appeared at δ 4.66 and 4.69 as singlet representing exocyclic double bond protons assigned to H-29a and H-29b respectively. A down field signal observed at δ 3.47 may be due to a 3-hydroxy substitution, with H-3 proton appearing as a triplet of a doublet (tdd) (J = 7.0, 5.0 Hz). This is presented in Figure 4.15, while Table 4.9 gives the chemical shift signal assignments for hydrogen and carbon atoms in the ^{1}H and ^{13}C NMR spectra.

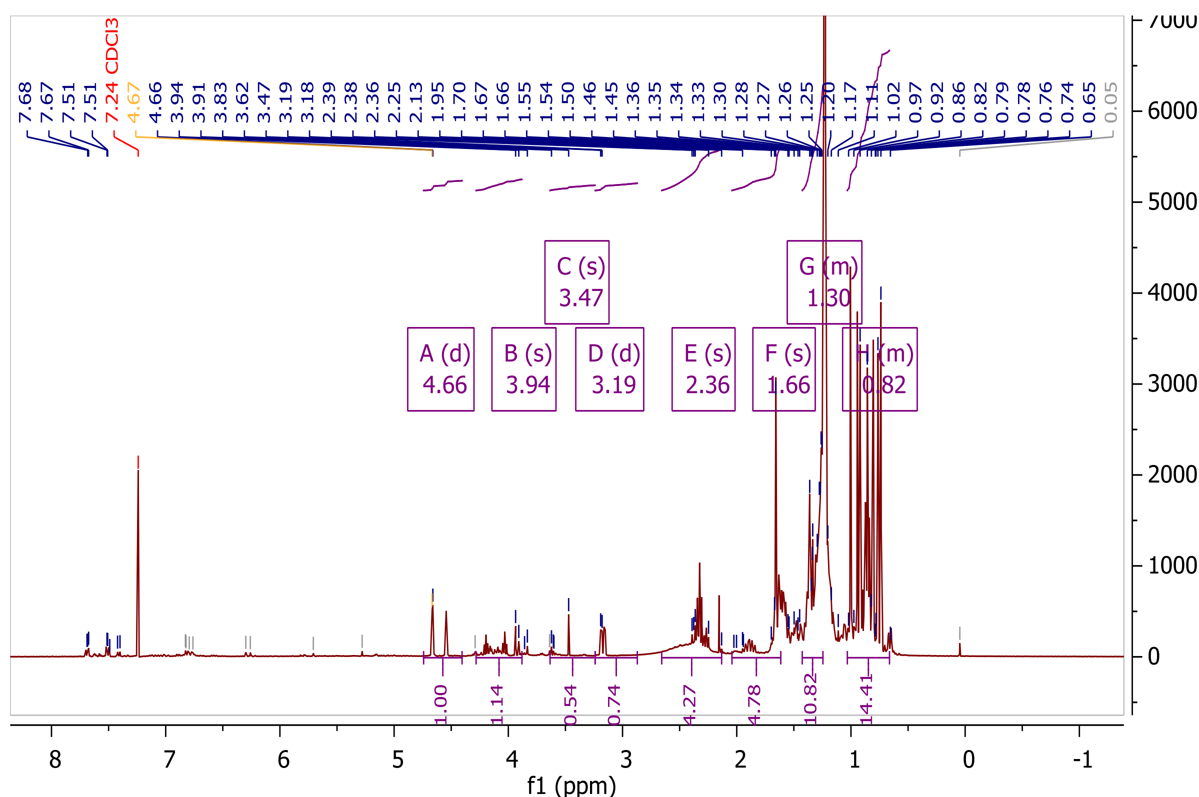


Figure 4.15. ^{1}HNMR spectrum of compound HXC_3 in $CDCl_3$, 400 MHz

Table 4.9. ^{1}H and ^{13}C-NMR chemical shifts of compound HXC_3 in $CDCl_3$, 400 MHz

H/C-Position	δ_c (ppm)	δ_H (ppm)	No. of H, Multiplicity, J(Hz)
1	38.01	1.22	1H, m, each
2	29.81	2.13	3H, s, 3H, s
3	79.02	3.44	-
4	38.83	-	-
5	55.25	0.65	1H, d
6	17.98	0.86, 1.10	1H, q
7	34.24	1.23, 1.10	1H, m, each
8	40.79	-	-
9	50.39	0.89	1H, s
10	37.14	-	-
11	20.90	1.25	1H, d
12	25.09	1.17	1H, q
13	38.66	1.36	1H, t
14	42.80	-	
15	29.65	1.13	1H, d
16	35.55	1.23	1H, d
17	42.98	-	-
18	47.97	0.98	1H, t
19	48.26	2.02	1H, m
20	150.98	-	-
21	31.92	1.36;	1H, s
22	39.98	1.23; 1.25	1H, m, each
23	29.70	0.92	1H, s, 3H, s
24	29.70	0.97	3H, s
25	16.11	0.86	3H, s
26	15.95	0.78	3H, s
27	14.52	0.78	1H, s, 3H, s
28	18.29	0.82	3H, s
29	109.30	4.66, 4.69	1H, s, 1H, s
30	19.28	1.70	3H, s

Coupling constant (J), singlet (s), double doublet (dd), multiplet (m), quartet of doublet (qd), triplet (t), quartet (q)

4.6.4. ^{13}CNMR spectrum of compound HXC_3

^{13}C NMR showed 30 signals for triterpenoids of a typical lupane skeleton, represented by seven methyl carbon signal in the range 14.5-22.6. The carbon bonded to the hydroxyl group C-3 appeared at δ 79.02, while the vinylic carbon signals ($CH{=}CH_2$) at C-20 and C-29 appeared at δ 150.98 and 109.30 ppm respectively.

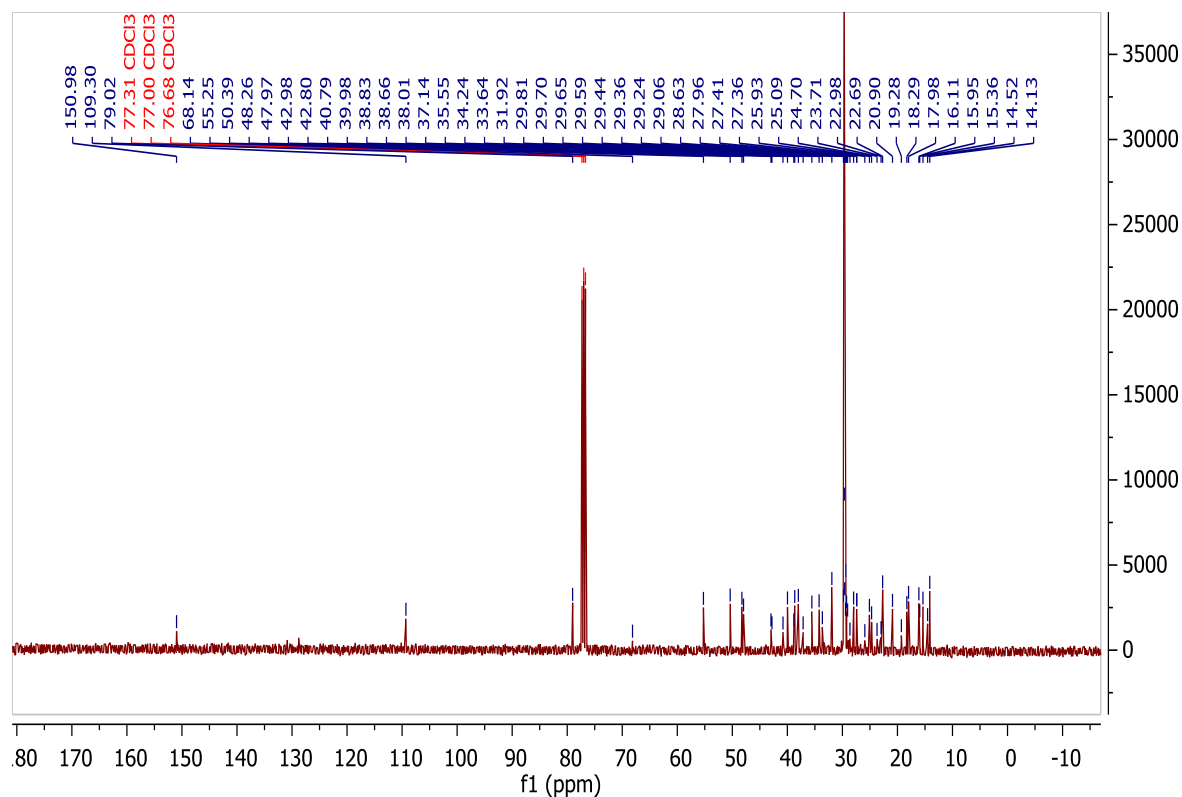


Figure 4.16. ^{13}**CNMR spectrum of compound** HXC_3 **in** $CDCl_3$**, 400 MHz**

4.6.5. Mass spectrometry of compound HXC_3

The ESI-MS spectrum showed a molecular ion (*m/z)* peak at 246.33 in the positive ion mode $[M+H]^+$ which coresponds to the fomular $C_{30}H_{50}O$. The spectrum also displayed some feature ions such as the fragments ions with m/z 189 and 207, which is characteristic for fragmentation of triterpenes with a lupane skeleton bearing a hydroxyl group at position 3 (Branco *et al*., 2004). Scheme 4.3 gives a proposed fragmentation of compound HXC_3. These information are in good comparism with other spectra data from litrature (see Table 5.3).

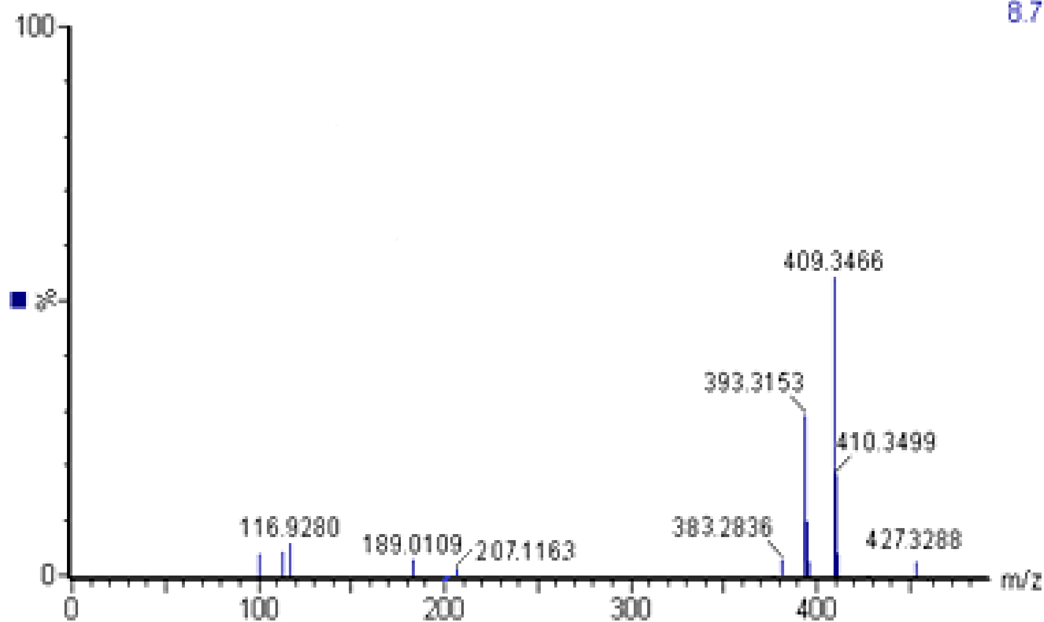
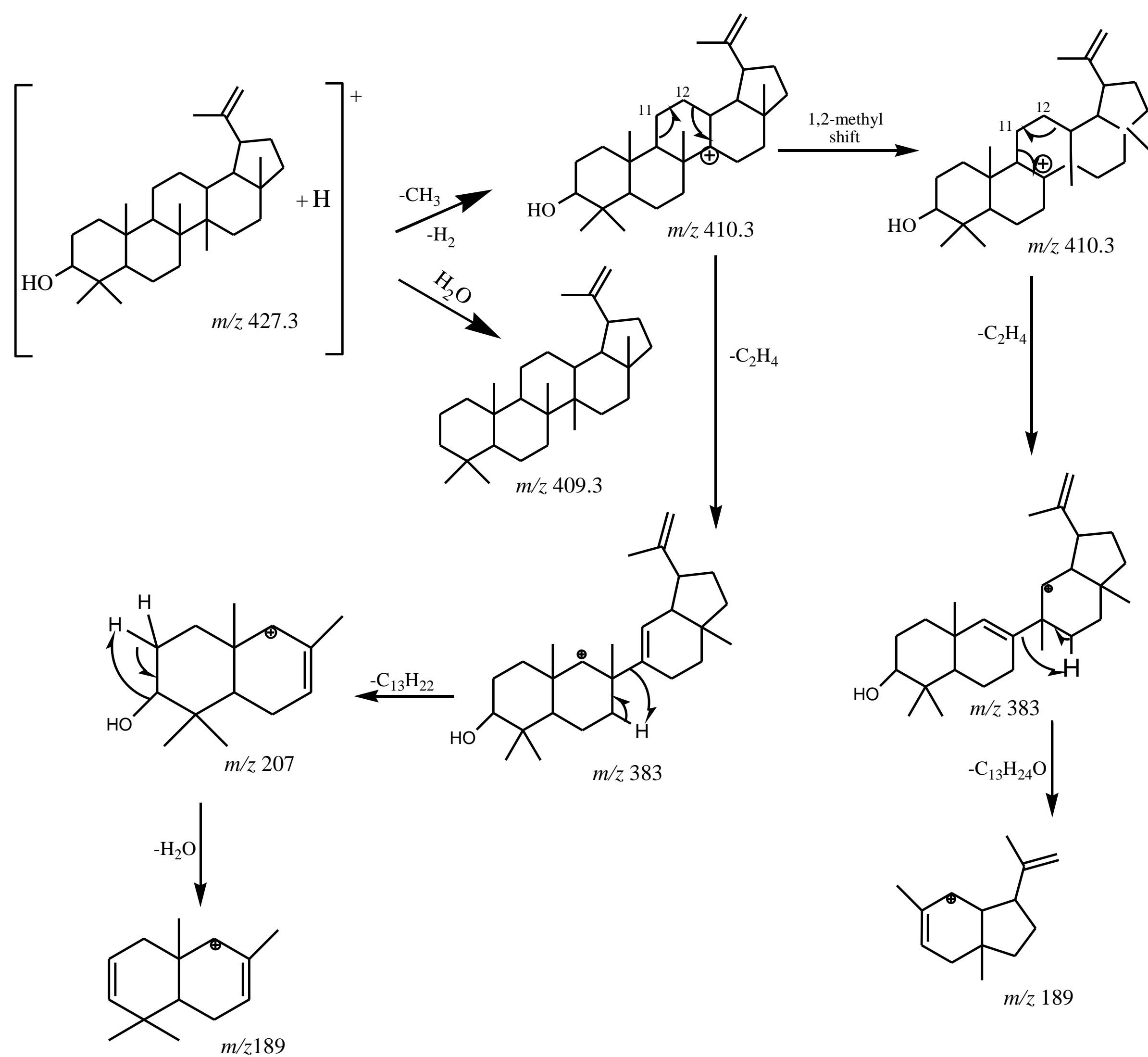


Figure 4.17. Mass spectrum of compound HXC_3



Scheme 4.3. Possible fragmentation pattern of compound HXC_3

Table 4.10. Summary of spectra data for HXC_3

Spectroscopic technique	Data								References (Compare with)
UV λmax:	370								Saratha *et al.*, 2011;
IRυ_{max} cm^{-1} [FTIR]	3253.4 (O-H str),	2907.5 - (C-H Alkyl str)	2851.4 (C-H str)	1680 (C=C str)	1012 - =CH bend	880			
^{1}HNMR ($CDCl_3$) [400 MHz]	δ 4.66 (1H, dq, J=0.4, 0.5 Hz, Hb-29), 4.69 (1H, d, J=0.4 Hz, Ha- 29), 3.48 (1H, dd, J=7.0, 5.0 Hz, Ha-3), 2.13 – 1.95 (m, 1H), 1.82 – 1.70 (m, 1H), 1.67 (3H, brd, J=0.5 Hz, Me-30), 0.75, 0.78, 0.83, 0.86, 0.92, 0.97, 1.23 (3H each, s, CH_3).								
^{13}CNMR ($CDCl_3$) [101 MHz]	δ38.01, δ50.39, δ42.98, δ16.11,	δ29.81, δ37.14, δ47.97, δ15.95,	δ79.02, δ20.90, δ48.26, δ14.52,	δ38.83, δ25.09, **δ150.98**, δ18.29,	δ55.25, δ38.66, δ31.92, **δ109.30**,	δ17.98, δ42.80, δ39.98. δ19.28	δ34.24, δ29.65, δ29.70	δ40.79, δ35.55, δ29.70,	Puebla *et al.*, 2010 Ahmadu *et al.*, 2013
HREIMS: m/z (rel. % abundance)	427.3; $[M+H]^+$	410 (25)	409 (60)	383 (5)	207 (3)	189 (5)			

4.6.6. Suggested structure of compound HXC_3

Elucidation from the spectra information of the MS, NMR, IR and UV data, which compared well with available literatures (Table 5.3), implies the suggested structure of compound HXC_3 to be lupeol (icosahydro-3a,5a,5b,8,8,11a-hexamethyl-1-1-(prop-1-en-2-yl)-1H-cyclopenta[α]chrysen-3-ol).

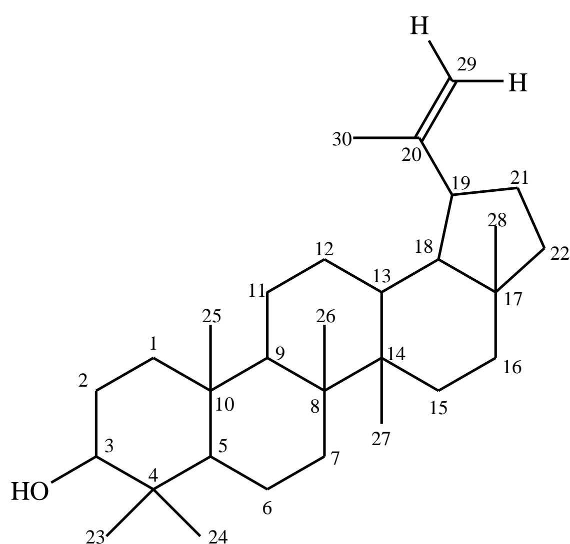
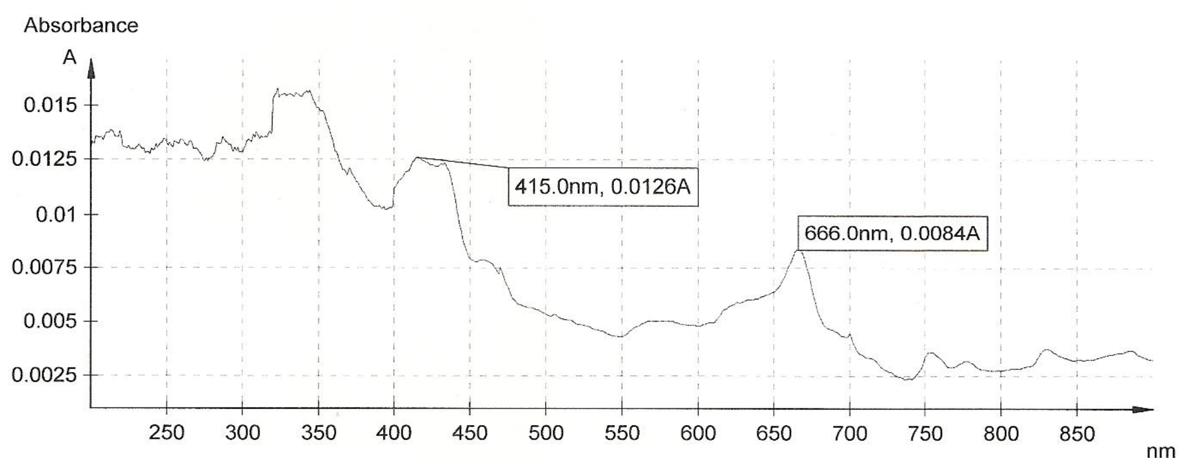


Figure 4.18. Suggested structure of compound HXC_3

4.7. Structure Determination of Compound EAC_1

4.7.1. Ultra-visible light (UV) spectroscopy of compound EAC_1

The UV spectrum observed for compound EAC_1 showed the presence of a chromophoric group in the structure, displaying absorption of UVλ_{max} at 323nm. The UV spectrum is presented in Figure 4.19.



Peaklist

Sample 1

	X [nm]	Y [A]
Maximum	323.0	0.0158
Maximum	415.0	0.0126
Maximum	666.0	0.0084

Figure 4.19. UV spectrum of compound EAC_1

4.7.2. Infra Red (IR) spectroscopy of compound EAC_1

The FT-IR spectrum of compound EAC_1 showed IRυ_{max}: a broad peak at 3358.8 cm^{-1} due to aliphatic OH; 2929.2 and 2852.2 cm^{-1} (CH_3 stretching), 1648.11 cm^{-1} (C=C stretching), 1000-650 cm^{-1}(due to C-H bending). This is presented in Figure 4.20.

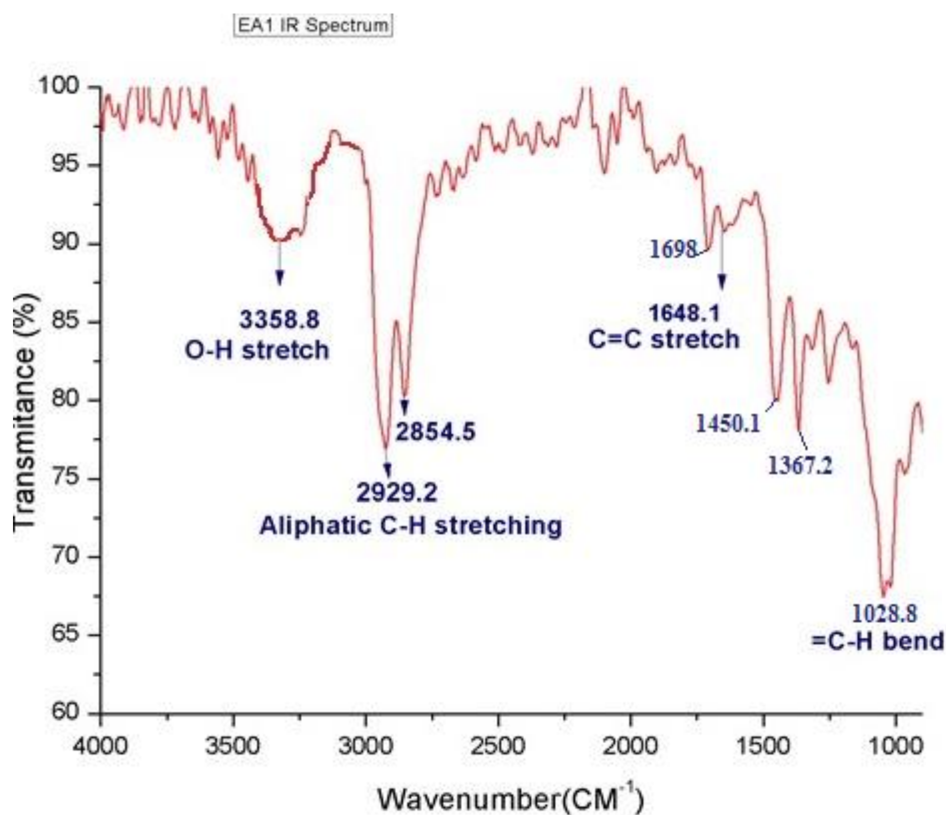


Figure 4.20. FTIR spectrum of compound EAC_1

4.7.3. Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) of compound EAC_1

The proton NMR spectrum of EAC_1 showed one proton multiplets at δ 3.53 for H-3 and a doublet at δ 5.34 for H-6 downfield vinylic proton, which is typical of a steroidal nucleus. The spectrum also displayed two of a three proton singlets at δ 1.00 and 0.67 assignable for H-19 and H-18 respectively, which is also characteristic for a steroidal compound. In addition, two doublets at δ 0.82 (3H, d, 7.2 Hz) and 0.80 (3H, d, 7.2 Hz) could be ascribed to the two methyl groups at H-26 and H-27 and another three-proton doublet at δ 0.91 (3H, d, 6.8 Hz) for H-21. On the other hand, one three-proton triplet at δ 0.85 (3H, t, 7.2 Hz) could be assigned to the primary methyl group attached for H29. All the assignments hence, compare very well with those found in literature (Table 5.4). The ^{1}HNMR spectrum and chemical shift values are presented in Figure 4.21 and Table 4.11.

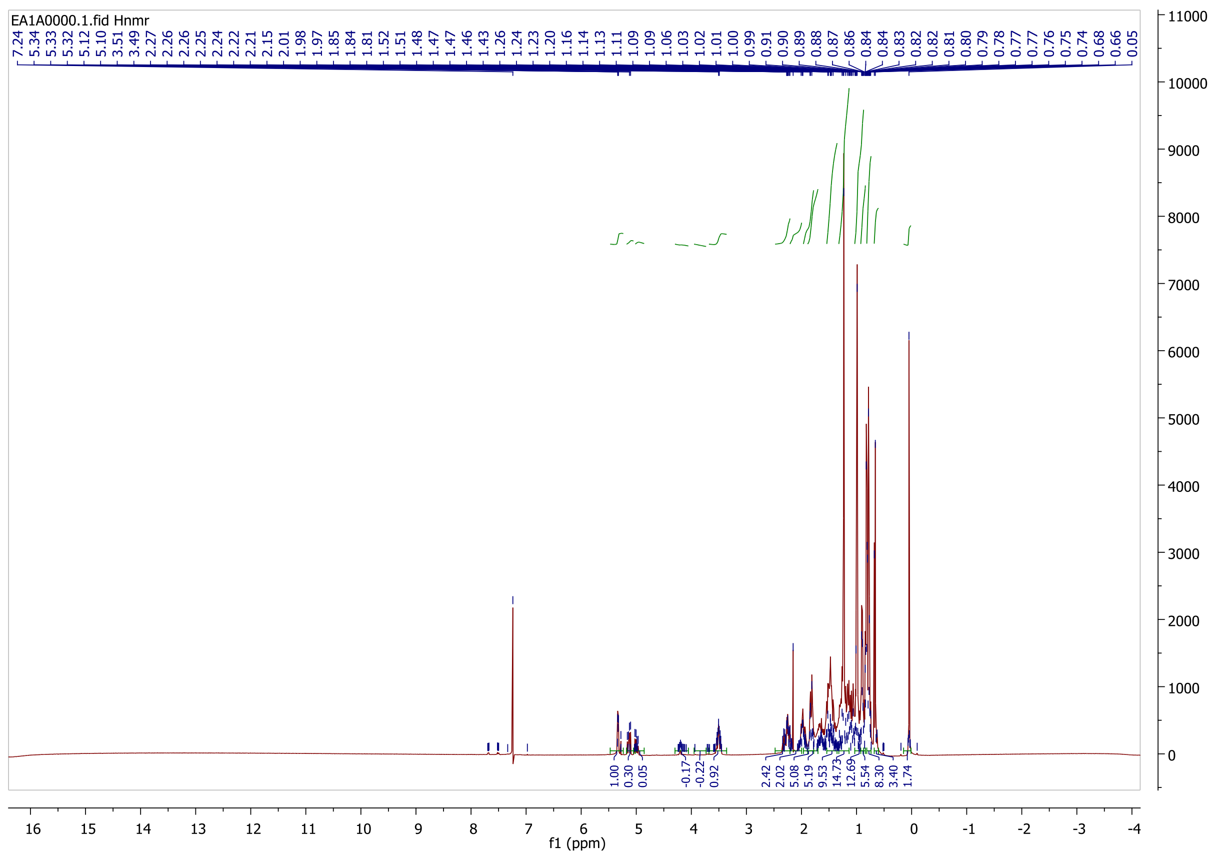


Figure 4.21. ^{1}HNMR Spectrum of compound EAC_1 in $CDCl_3$ 400 MHz

4.7.4. ^{13}CNMR spectrum of compound EAC_1

The ^{13}C NMR spectrum showed 29 carbons including an oxymethine carbon signal at δ 72·04 and two olefinic carbons at δ 140·96 and δ 121·94 assigned for C-5 and C-6, which is typically indicative of the presence of double bonded unsaturation and characteristics of spirostene (Agarwal *et al.*, 1985). Two methylene carbon signals were observed at δ34.16 and δ26.29, assigned for C-22 and C-23 respectively and conversely, no olefinic carbons were observed at these positions, hence ruling out a stigmasterol nucleus. The signals observed agree strongly with those from literature (Table 5.4). The ^{13}C NMR chemical shift of EAC_1 is presented in Figure 4.22 and Table 4.11.

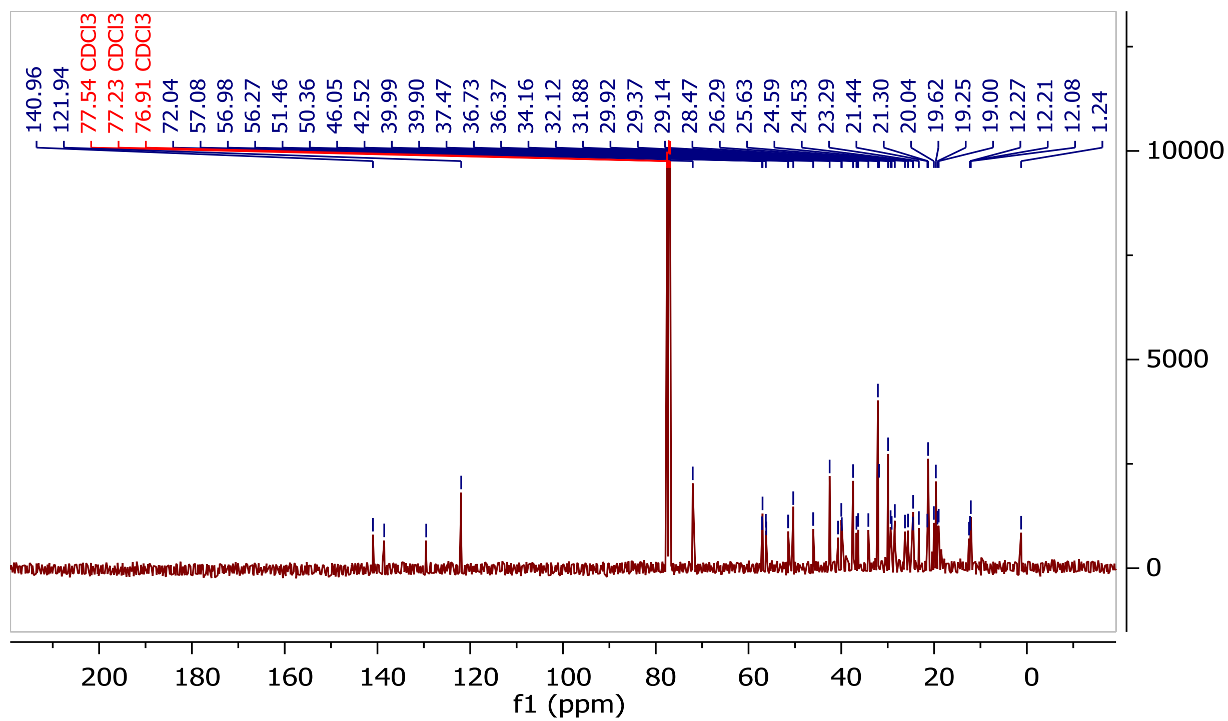


Figure 4.22. ^{13}**CNMR spectrum of compound EAC$_1$ in CDCl$_3$ 400 MHz**

Table 4.11. ^1H and ^{13}C-NMR chemical shifts of compound EAC_1 in $CDCl_3$, 400 MHz

H/C-Position	δ_c (ppm)	δ_H (ppm)	No. of H, Multiplicity, J(Hz)
1	37.32	1.13, 1.39	1H, m
2	31.71	1.36, 1.72	1H, s
3	72.04	3.85	1H, tt (4.60, 4.60, 11.11, 11.11)
4	42.3	1.96, 2.44	2H, m
5	140.96	-	-
6	121.56	5.13, 5.38	1H, d, (5.2)
7	32.14	1.91, 2.16	1H, m
8	31.83	1.63	-
9	50.07	0.90	IH, s
10	36.53	-	-
11	21.06	1.27, 1.26	3H, d (7.34)
12	39.35	1.38, 1.46	2H, m
13	42.28	1.27, 1.39	-
14	56.43	1.42, 1.65	4H, s
15	24.47	1.28, 1.73	3H, d (7.34)
16	28.42	0.91	3H, dddd (2.61, 6.27, 12.89, 16.85)
17	55.91	1.32	1H, m
18	11.90	0.81	3H, d (8.68, 15.14)
19	19.30	0.93	3H, s
20	35.52	1.31, 1.39	2H, m
21	18.78	0.85	3H, d, (6.0)
22	34.16	1.02	1H, s
23	26.29	1.60	4H, m
24	46.04	1.07, 1.84	1H, m
25	29.05	0.87	1H, m
26	19.42	0.81	3H, d, (7.2)
27	19.42	0.86	3H, d, (7.2)
28	23.19	1.14	3H, m
29	12.09	0.86	3H, d, (8.0)

coupling constant (J), singlet (s), multiplet (m), doublet (d)

4.7.5. Distortionless enhancement by polarisation transfer (DEPT-135) spectrum of compound EAC_1

The DEPT 135 of compound EAC_1 showed 15 positive signals due to methine and methyl at δ_c (ppm) 12.27, 19.25, 19.62, 20.04, 21.44, 24.59, 28.47, 29.93, 31.88, 32.14, 37.47, 39.99, 42.52, 72.04 and 121.95; and eleven negative signals due methylene at δ_c (ppm) 21.14, 23.29, 24.59, 25.63, 26.29, 29.14, 29.92, 31.88, 37.47, 39.90, and 42.52. On comparing the DEPT 135 with the ^{13}CNMR, the quarternary carbon atoms were observed at δ_c (ppm) 140.96, 42.28 and 36.53. The number and type of carbon atoms present is consistent with the structure of β-sitosterol. The DEPT 135 of compound EAC_1 is represented in Figure 4.23.

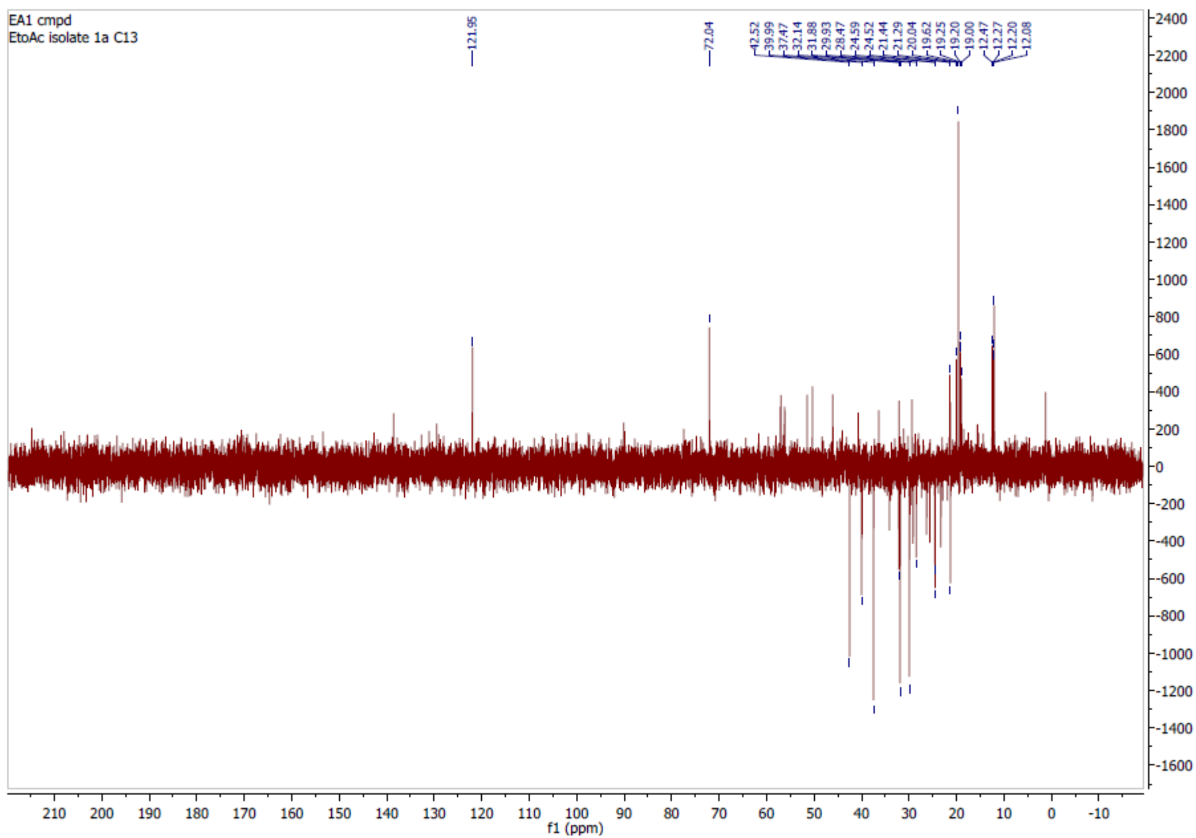


Figure 4.23. DEPT 135 spectrum of compound EAC_1 in $CDCl_3$ 400 MHz

4.7.6. Mass spectrometry of compound EAC_1

The ESI-MS spectrum of compound EAC_1 showed a characteristic molecular ion $[M]^+$ peak at m/z 414.38. A fragment ion peaks occurred at m/z 395 which may be due to dehydration (loss of water molecule) after the lost a hydrogen ion (H^+) from the fragment ion m/z 413.4. A loss of methyl group from the fragment ion m/z 395 gave rise to the fragment ion at m/z 383.4. Other observed fragment are m/z 134.1 and 81. The fragmentation pattern is presented in Scheme 4.4 and Figure 4.24.

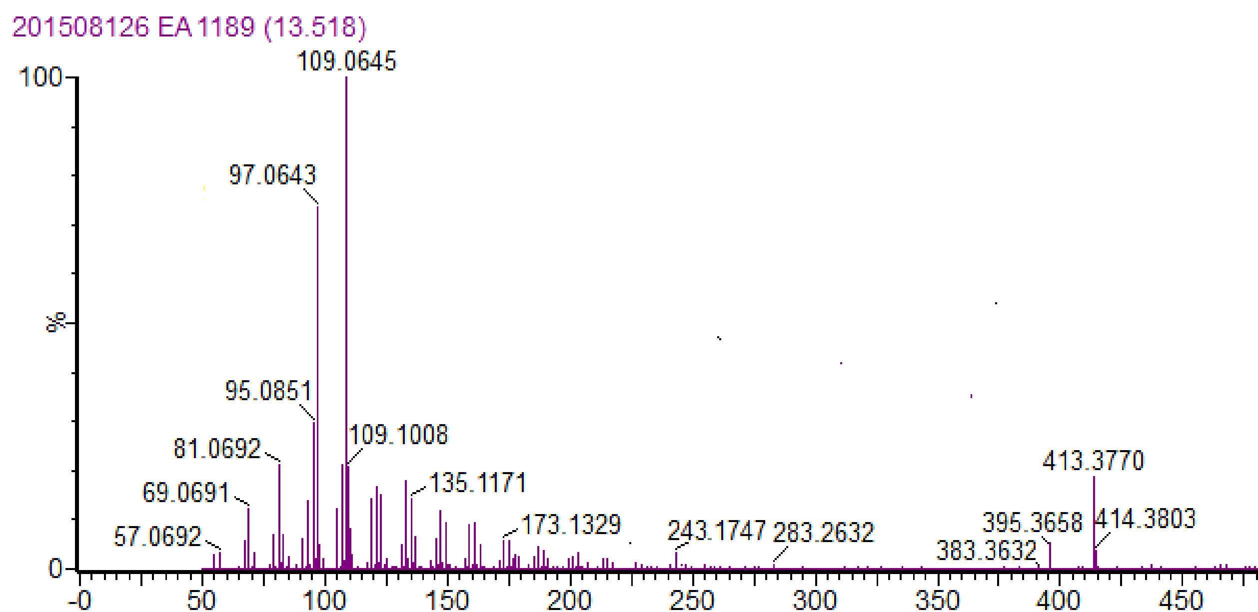
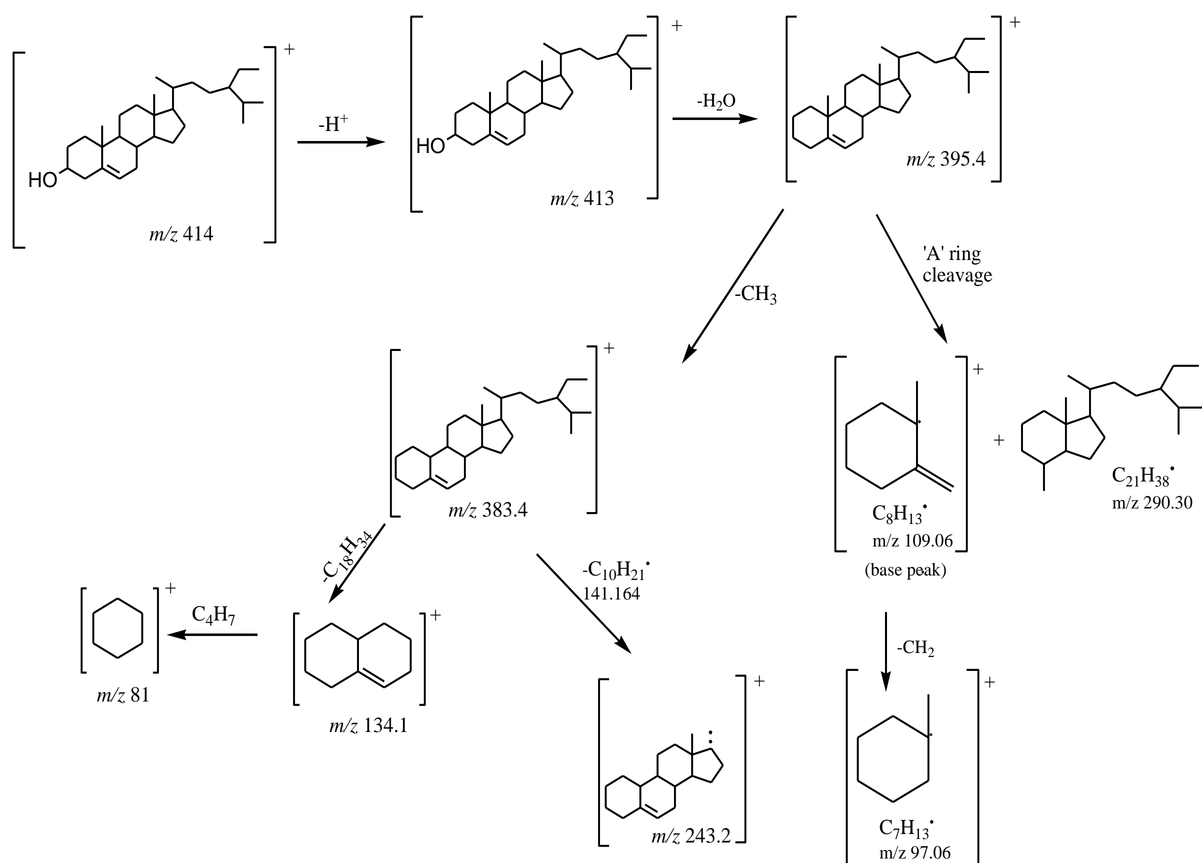


Figure 4.24. Mass spectrum of compound EAC$_1$



Scheme 4.4. Possible fragmentation pattern of compound EAC_1

Table 4.12. Summary of spectra data of EAC_1

Spectroscopic technique	Data							References (Compares)
UV λmax:	323	415						
IR ($CHCl_3$)	3358.8 (O-H str),	2929.2 (C-H Alkyl str)	2854.5	1648.1 (C=C str)	1461.9	1158.2	1022.04	Kamboj & Saluja, 2011
^{1}HNMR ($CDCl_3$) [400 MHz]	δ 5.37 – 5.29 (m, 1H), 4.99 (dd, *J* = 15.1, 8.7 Hz, H), 3.50 (tt, *J* = 11.1, 4.6 Hz, 1H), 2.37 – 2.14 (m, 2H), 1.98 (dddd, *J* = 16.9, 12.9, 6.3, 2.6 Hz, 3H), 1.90 – 1.74 (m, 2H), 1.81 (s, 1H), 1.75 – 1.32 (m, 5H), 1.36 – 1.18 (m, 1H), 1.24 (s, 1H), 1.23 (s, H), 1.24 – 0.92 (m, 5H), 0.99 (s, H), 0.97 – 0.84 (m, 3H), 0.89 – 0.70 (m, 12H), 0.67 (s, 3H).							
13CNMR(CDCl) [101 MHz]	δ 37.32, δ31.83, δ24.47, δ33.94, δ12.09	δ31.71, δ50.07, δ28.42, δ26.35,	δ71.33, δ36.53, δ55.91, δ46.04	δ42.36, δ21.06 δ 11.90, δ29.05,	δ140.56, δ39.35, δ19.30, δ19.42,	δ121.56, δ42.28, δ35.52, δ19.42,	δ32.14 δ56.43, δ18.78, δ23.12,	Ahmed *et al.*, 2013
HREIMS: m/z (rel. % abundance)	415.40 $[M+H]^+$	calc. 414.70	413.4 (30)	395.4 (10)	383.4 (3)	134.1 (21)	81 (28)	

4.7.7. Suggested structure of compound EAC_1

From the MS, NMR, IR and UV data which compared well with available literatures (Table 5.4), in addition with further information obtained from correlation spectroscopic data of 2DNMR spectra of ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC and HMBC (spectra shown in appendix IId-f), the suggested structure of compound EAC_1 is β-sitosterol (Figure 4.25).

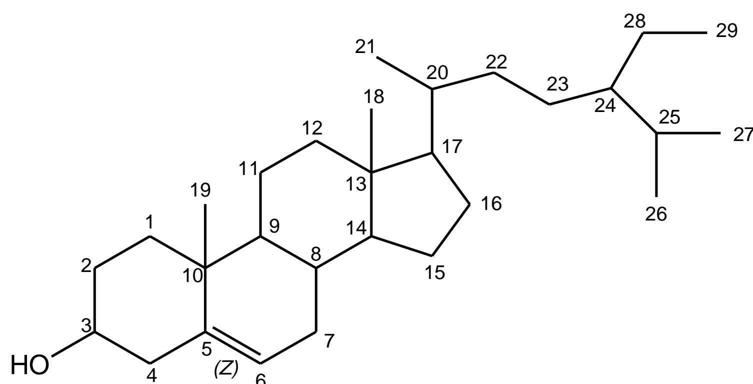


Figure 4.25. Suggested structure of compound EAC_1

4.8. Determination of Biological Activity

4.8.1. Lethality assay of the crude venoms of *Naja nigricollis*

The lethality assay shows the LD_{99} of crude venoms of *Naja nigricollis* to be 5.0 mg kg^{-1}. The mice treated with MLD of *Naja nigricollis* venom showed principally neurotoxic symptoms, these usually begin with transient excitement followed by depression and paralysis beginning with the hind limbs followed by forelimbs, difficulty in breathing, stupor and finally death. The average time of death recorded was 15minutes. Table 4.13 present results for the determination of MLD of Naja nigricollis using probit analysis.

Table 4.13. Determination of minimum lethal dose (MLD) of venom of *Naja nigricollis* on mice

Group	Treatment ($mgkg^{-1}$)	$\frac{\text{No. of deaths}}{\text{No. of mice used}}$	Survival % (within 24 hr)	Average time of death (min)
1	5	4/6	33.3	527
2	7	6/6	0	20
3	9	6/6	0	15
4	11	6/6	0	10
5	N-Saline	0/6	100	>1440

MLD of Venom = 5.0mgkg^{-1} (using probit analysis, Appendix III)

4.8.2. Median lethal dose determination (LD_{50}) of extract of *Albizia chevalieri* using the Lorke's method

The estimated LD_{50} of *A. chevalieri* for the extracts of hexane and methanol are 2900 and 565.7 ($mgkg^{-1}$) respectively, while those of n-butanol and ethyl acetate fractions are 288.5 and 5000 ($mgkg^{-1}$) respectively. These shows them to be well tolerated (at a venom protection dose of 200 mg/kg). Using the Lorke's method, this is presented in Table 4.14.

Table 4.14. Determination of LD_{50} of extracts and fractions of *A. chevalieri* by Lorke's method:
[Phase One]

Extract/ Fractions	Groups/Conc ($mgkg^{-1}$)	Ave. weight (g)	Ave. Doses (ml)	No. of death / No. of mice used	Survival % (within 24 h)	Ave. time of death (h)
Methanol	10	21.30	0.02	0/3	100	No dead
	100	14.00	0.02	0/3	100	No dead
	1000	10.33	0.02	2/3	33.3	6
n-Butanol	10	13.67	0.02	0/3	100	No dead
	100	13.00	0.13	0/3	100	,,
	1000	14.67	1.03	3/3	0	12
Ethyl acetate	10	14.30	0.02	0/3	100	No dead
	100	13.00	0.13	0/3	100	,,
	1000	14.67	0.93	0/3	100	,,
Hexane	10	17.00	0.02	0/3	100	No dead
	100	19.00	0.19	0/3	100	,,
	1000	20.00	1.00	0/3	100	,,

[Phase Two]

Fraction	Groups/Conc ($mgkg^{-1}$)	Weight (g)	Doses (ml)	No. of deaths / No. of mice used	Survival % (within 24 h)	Ave. Time of death (h)
Methanol	200	36.0	0.10	0/1	100	No dead
	400	38.0	0.20	0/1	100	No dead
	800	40.0	0.40	1/1	0	5
	1600	20.0	0.40	1/1	0	6
n-Butanol	140	24	0.15	0/1	100	No dead
	225	24	0.24	0/1	100	,,
	370	22	0.36	1/1	0	12
	600	28	0.75	1/1	0	12
Ethyl acetate	1,600	24	0.32	0/1	100	No dead
	2,900	26	0.63	0/1	100	,,
	5000	23	1.00	0/1	100	,,
Hexane	600	31	0.35	0/1	100	No dead
	1000	26	0.50	0/1	100	
	1,600	24	0.70	0/1	100	
	2,900	23	1.25	0/1	100	

LD_{50} *of* methanol extract = $\sqrt{D^0 * D^{100}} = \sqrt{400 * 800}$ = ***565.7mgkg^{-1}***

LD_{50} of n-butanol fraction = $\sqrt{D^0 * D^{100}} = \sqrt{225 * 370}$ = ***288.5mgkg^{-1}***

4.8.3. Evaluation of the effect of crude extracts/fraction of *A. chevalieri* on lethal dose of the crude venom.

The Table 4.14 shows the effect of 200 $mgkg^{-1}$ extracts and fractions challenged with MLD envenomated mice. The dose was deduced preliminarily based on the results of the LD_{50} of *A. chevalieri* determined using Lorkes method as a guide for the dose levels to be administered as a treatment on envenomated mice. Survival % (protection) was calculated after 24 hrs. Extract of hexane and n-butanol fractions offered the highest protection (both showing 16.7%) as compared to those of methanol and ethyl acetate (both showing 0%). This is presented in Table 4.15 below.

Table 4.15. Evaluation of extract fractions of *A. chevalieri* at 200 $mgkg^{-1}$

Groups/Conc (200 $mgkg^{-1}$)	Ave. weight (g)	Ave. doses/vol.	$\frac{\text{No. of deaths}}{\text{No. of mice used}}$	Survival % (within 24 h)	Ave. Time of death (min)
MeOH	17.30	3.5mg/0.2	6/6	0	60
n-Butanol	17.50	3.6mg/0.2	5/6	16.7	253.5
EtOAc	18.17	3.7mg/0.2	6/6	0	13.3
Hexane	18.00	3.8mg/0.2	5/6	16.7	250.1
N-Saline	27.70	NS-mg/0.2	6/6	0	35.5

4.8.4. Evaluation of *Albizia chevalieri* as prophylaxis of *Naja nigricollis* envenomation.

The n-butanol fraction and hexane extract of *A. chevalieri* which earlier showed 16.7% protection at 200mgkg^{-1} were investigated at a lower dose of 100mgkg^{-1} (in mice challenged with MLD of venom) to determine the possible use of *A. chevalieri* as prophylaxis of *Naja nigricollis* envenomation. The results are presented in Table 4.16.

Table 4.16. Evaluation of hexane extract and n-butanol fraction of *A. chevalieri* at 100mgkg^{-1} on MLD of venom

Groups/Conc	Ave. weight(g)	Ave. doses/vol.	$\frac{\text{No. of deaths}}{\text{No. of mice used}}$	Survival % (within 24 h)	Ave. time of death (min)
Venom only (MLD)	28.20	0.2	4/5	20	17.5
Hexane 100 mgKg^{-1} + MLD	28.20	0.3	5/5	0	2.6
n-But 100 mgKg^{-1} + MLD	27.20	0.3	5/5	0	10.2
N/S + 100 mgkg^{-1} Hex	26.20	0.3	0/5	100	>24h
N/S + 100 mgkg^{-1} n-But	24.80	0.3	1/5	80	1170 (19.5h)

4.8.5. Studies of *in vivo* antivenom effect of pure isolated compound from hexane extract and ethyl acetate fractions of *A. chevalieri* on *Naja nigricollis* envenomation.

The Tables 4.16 and 4.17 showed the *in vivo* activity of the isolated compounds HXC_1 and EAC_1 against the venom of *Naja nigricollis*. It was seen that both the animals in the first group (injected with MLD only) and the second group (injected with MLD and 4 $mgkg^{-1}$ of pure compounds HXC_1 and EAC_1) died, but the average time of death in group 2 was lengthened presumably as a result of some protection offered by 4$mgkg^{-1}$ of HXC_1 and EAC_1. However, at an increased administered dose of 8$mgkg^{-1}$ of EAC_1 and HXC_1, 60% of animals survived the lethal injection (Table 4.17). These results are presented in Table 4.17 and Table 4.18 below.

Table 4.17. Evaluation of compound HXC_1 of *A. chevalieri* on MLD of venom

Groups/Conc	Ave. weight(g)	Ave. doses/vol.	No. of deaths / No. of mice used	Survival % (within 24 h)	Ave. time of death (min)
Venom only (MLD)	28.20	0.2	4/5	20	17.5
HXC_1 4$mgKg^{-1}$ + MLD	16.80	0.1	5/5	0	21.2
HXC_1 8$mgKg^{-1}$ + MLD	17.30	0.2	5/5	0	38.4

Table 4.18. Evaluation of compound EAC_1 of *A. chevalieri* on MLD of venom

Groups/Conc	Ave. weight.(g)	Ave. doses/vol.	No. of deaths / No. of mice used	Survival % (within 24 h)	Ave. time of death (min)
Venom only (MLD)	28.20	0.2	4/5	20	17.5
EAC_1 4$mgKg^{-1}$ + MLD	27.60	0.1	4/5	20	541.4 (9h)
EAC_1 8$mgKg^{-1}$ + MLD	24.80	0.2	2/5	60	732.4min (15hrs)

4.8.6. Histopathological effect of venom of *Naja nigricollis* on some mice organs treated with MLD of venom, HXC_1, EAC_1 and extracts of *A. chevalieri*

Histological studies carried out on kidney and liver tissues of envenomated mice treated with the extracts, fractions and isolated compounds from *A. chevalieri* are shown on the images of Plate V-VII. The magnification was kept at x200 and x400, haematoxylin and eosin (H and E) stained.

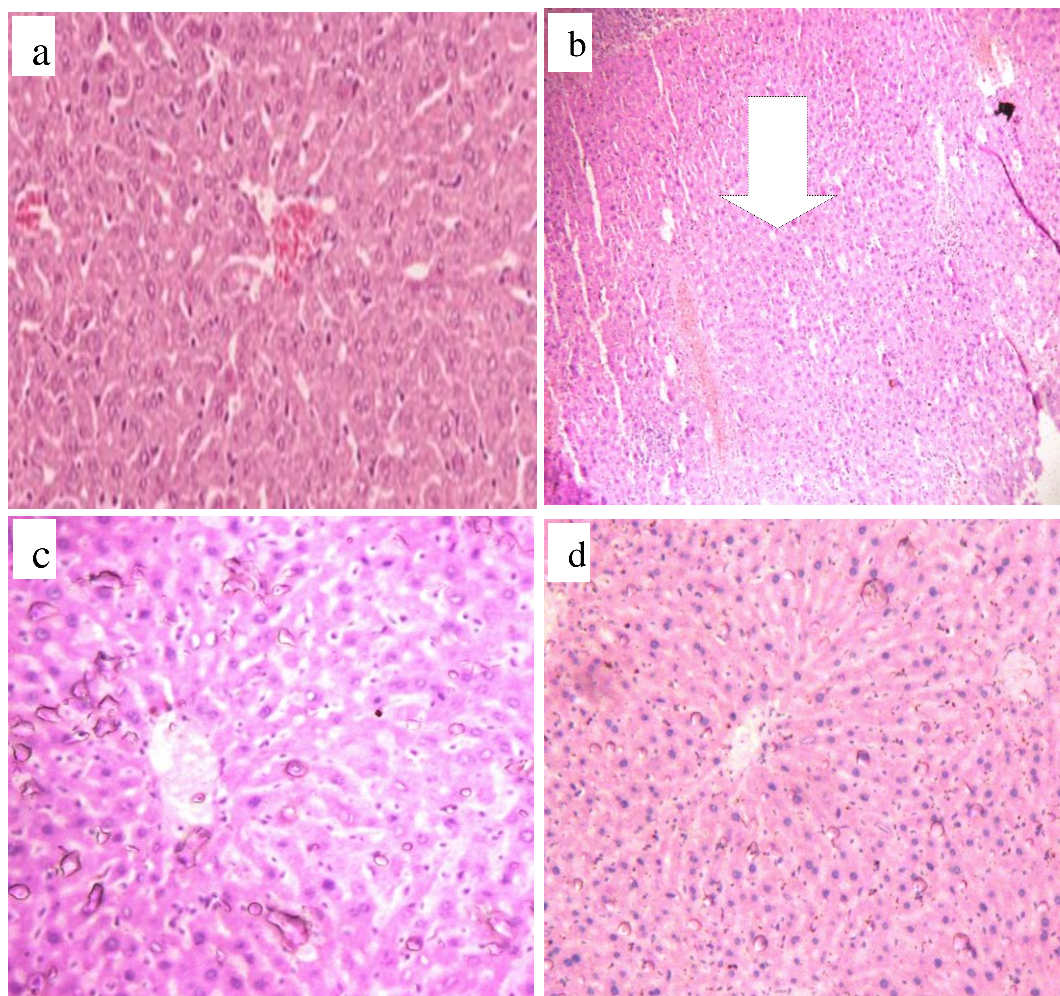


Plate V. Histological changes in the liver of envenomated mice and envenomated mice treated with EAC_1, HXC_1 and plant extracts.

(a) Normal mice; **(b)** envenomated mice treated with MeOH extract at 200mg/ml (shows severe hepatic necrosis around regions denoted by arrows); **(c)** envenomated mice treated with EAC_1 isolated from EtOAc extract; **(d)** envenomated mice treated with HXC_1 from hexane extract. [H and E Stain; Mgn x 200]

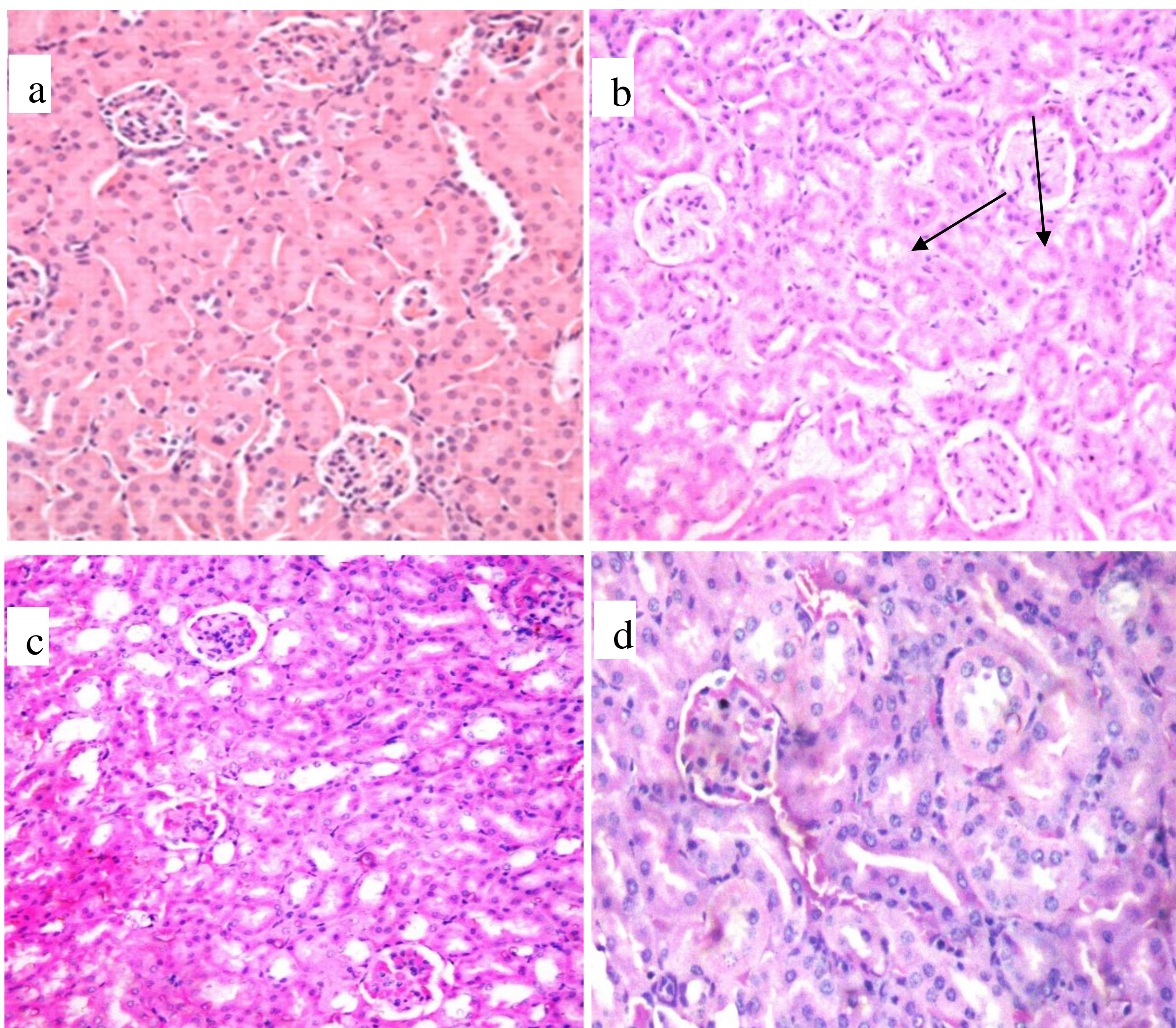


Plate VI. Histological changes in the kidney of envenomated mice and envenomated mice treated with EAC_1, HXC_1 and plant extracts.

(a) Normal mice; **(b)** envenomated mice treated with n-butanol extract at 200mg/ml (severe tubular necrosis around regions are denoted by arrows); **(c)** envenomated mice treated with EAC_1 isolated from EtOAc extract; **(d)** envenomated mice treated with HXC_1 isolated from hexane extract. [H and E Stain; Mgn x 200]

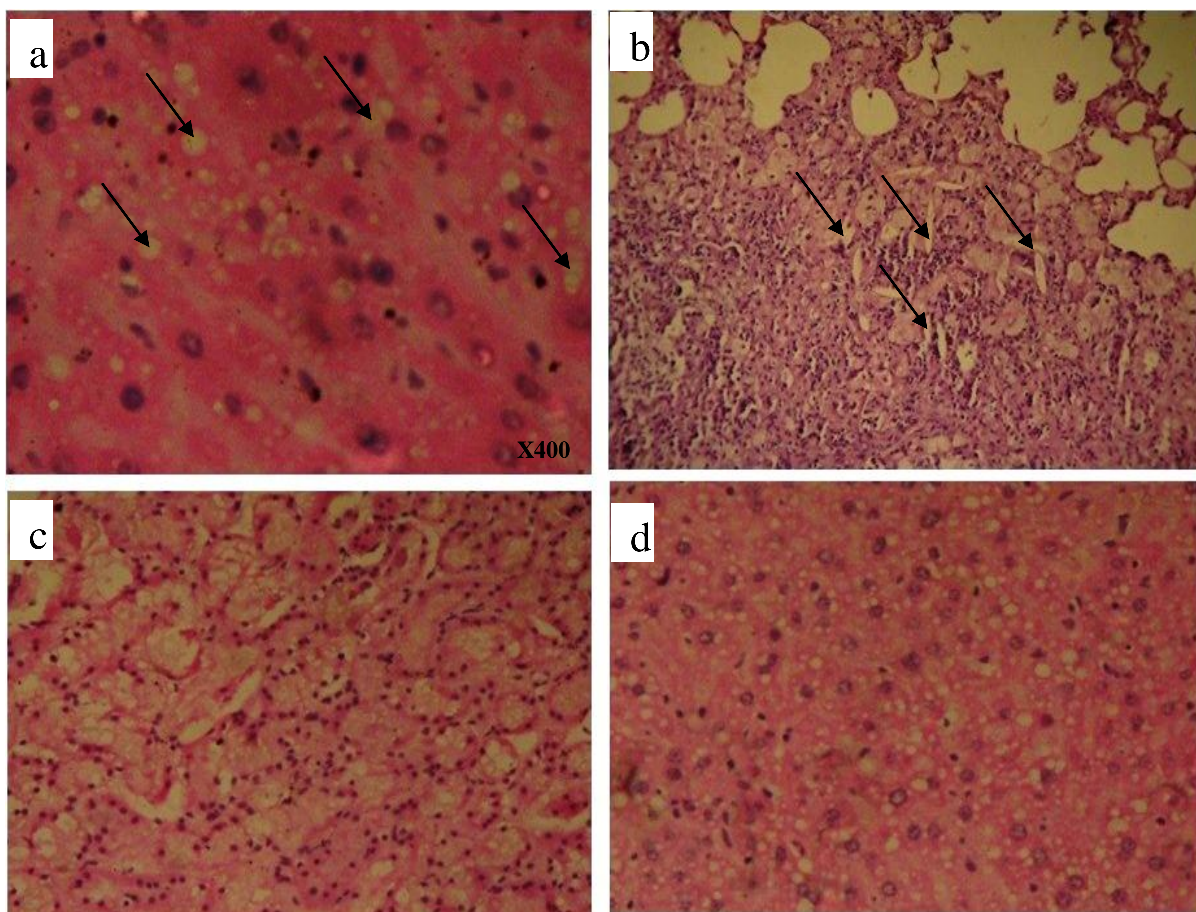


Plate VII. Histological changes observed in liver and kidney of envenomated mice treated with the various plant extracts of *A. chevalieri*

(a) envenomated mice treated with ethyl acetate extract at 200mg/ml (arrows shows micro vesicular steatosis of liver cells); **(b)** envenomated mice treated with hexane extract at 200mg/ml (arrows shows effect in liver cells resulting from formation of fatty chain, cholesterol granuloma, fat embolism or cholesterol cleft); **(c)** envenomated mice kidney treated with MeOH extract 200mg/ml (shows acute tubular necrosis) **(d)** envenomated mice liver treated with n-butanol extract 200mg/ml (shows micro vesicular steatosis). [H and E Stain; Mgn x 200]

CHAPTER FIVE

5.0. DISCUSSIONS

The best resolution of the Hexane extract was achieved with hexane/ ethyl acetate (49:1) and spraying with 5 % H_2SO_4. This can be seen from the number of spots on the plate (Plate III). Observation of red/ pink colours could be attributed to the presence of steroids/ triterpenoids (Wagner and Bladt, 1996). The ethyl acetate extract was best resolved by ethyl acetate/ acetone (7:3). The chromatograms under UV light at 365nm showed the presence of fluorescence spots, is an indication of chromophore which is likely to be phenolic or unsaturation in the compound(s). On spraying the plate with PMA and heating for a minute, dark-blue spots (Plate IV) were prominently observed.

The phytochemicals detected in the preliminary phytochemical screening of the extracts and fractions of *A. chevalieri* (Table 4.2) revealed typically, constituents often present in the genus Albizia as reviewed from literatures in section 2.3.2 of chapter two. The plant *A. chevalieri* has a high saponins content as evident from the polar n-butanol fraction when screened. This also explains the sterol and terpenoid compounds isolated from the hexane extract and ethyl acetate fraction, since both earlier showed a positive Lieberman-Burchard and Salkowkis's test. Steroid and terpenoid often shared the same biosynthetic pathways in plant (the mevalonic acid pathways), yielding steroidal (C27) or terpenoidal (C30) sapogenin compounds.

Compound HXC_1, isolated from the hexane extract was seen as a white (crystalline) flakes, soluble in chloroform, with melting point of 264- 268 ^oC. The ^1H-NMR (Figure 4.2) revealed signals for seven siglets of methyl at δ 0.93 (H-28), 0.85 (H-27), 0.85 (H-26), 1.00 (H-30), 1.00(H-29), 0.85(H-25) and 0.70 (H-24) and a doublet of methyl at δ 1.18 (d, J = 7.7 Hz, 1H), suggesting that there are eight methyl groups present in the compound. The signals at δ1.25 and 1.2, δ1.70 and 1.45, δ 1.25 and 1.4, δ 1.25 and1.23 were due to unequivalent methylene protons (CH_2) attached to carbon C-2, C-1, C-6 and C-22 respectively. The presence of a cross correlation between a quartet methine proton (CH) at δ 2.25 (q, J = 6.7 Hz, H-4) and a doublet methyl signal at δ 0.81 (at C-23) in the COSY spectrum (appendix I) as well as the presence of signals due to one secondary and seven quaternary methyl in the ^{1}HNMR specrum suggested the friedelane skeleton of pentacyclic triterpenoid (PCTT) (Susidarti *et al*., 2014). These data are supported by the ^{13}C-NMR spectrum (Figure 4.3). The ^{13}C-NMR showed the presence of thirty carbon resonance and also gave signal at δ_c (ppm) 213.45 (C-3) which is characteristic of a ketone carbonyl or a carbon bearing an oxygen in a triterpenoidal structure. Thus, Table 5.1 compares agreeably ^{1}HNMR and ^{13}CNMR signal values of compound HXC_1 with those of Abreu *et al*., (2003). The DEPT spectrum (Figure 4.4) further reaffirmed the later and also showed eight methyls, eleven sp^3 methylenes, four sp^3 methines and six quartenary sp^3 carbons. The FTIR spectrum (Figure 4.1) showed an intense band at $IR\upsilon_{max}$ 1700.9 cm^{-1} consistent with a six membered ring ketone for C=O stretching. There were no absorption observed in the UV spectrum, suggesting the absence of unsaturation in the compound. The mass spectral data showed a positive mode molecular ion peak $[M+H]^+$ with M/Z 427, with characteristic peaks of other fragment ions at M/Z 411, 341, 273 and 206 (Figure 4.5 and Scheme 4.1). The molecular ion peak 427 represented by $[M+H]^+$ or simply MH^+ is the intact molecule having an m/z value one amu greater than that of the molecular ion (called quasimolecular ion). The chemical ionisation (CI) MH^+ ions are

generally prominent and undergo less fragmentation, and thus provide a method to locate the molecular ion (Kalsi, 2004). The fragment ion at m/z 425 is attributed to a loss of proton before the further loss of a methyl group from the later to give the fragment ion at m/z 411. Other significant ions at m/z 273, 205 and 123 could be attributed to the fragmentation of A, B, C and D rings respectively. It indicates that the compound is composed of rings. Generally, retro Diels-Alders (RDA) cleavage have been regarded to be the characteristic fragmentation pathways for most PCTT compounds and their analogues (Xia *et al*., 2015), consequently, the ring fragmentations might be through the RDA pathways. Therefore, from all the assignments made (Figure 4.5, Scheme 4.1) suggested that the isolated compound HXC_1 may be a triterpenoid. These information derived from the MS, NMR and IR are in agreement with those reported in literature (Abreu *et al*., 2013; Sousa *et al.*, 2012; Putra *et al.,* 2004; Boonyaratavej and Petsom, 1991) for Friedelin, a PCTT. Friedelin is a triterpenoid common in the plant Kingdom and have been isolated by many other researchers as cited above. Its distribution in the plant Kingdom has been mainly in the plant families of Celastraceae, Guttiferae and Flacourtiaceae, as reported by Gunatilaka, (1984). In addition, friedelin has been reported in many species of the genus *Calophyllum*: *Calophyllum innophyllun* (Ali *et al*., 1999; Yimdjo, *et al*. 2004), *C. gracilipes* (Cao *et al*., 1997), *C. lankaensis*, *C. thwaitesii* (Dhamaratne, *et al*., 1984), *C. calaba* (Gunatilaka *et al*., 1984), *C. amoenum* (Banerji, *et al*., 1977) and *C. cuneifolium* (Gunasekera, *et al*., 1977), *C. soulattri* (Susidarti *et al*., 2014); others are *Dombeya torrida*, family Sterculiaceae (Ndwigah, 2013), *Bridelia tomentosa*, family Euphorbiaceae (Boonyaratavej & Petsom, 1991), but only for the first time in *Albizia chevalieri*, family Mimosaceae.

Table 5.1. ^1H and ^{13}C-NMR chemical shifts of compound HXC_1 in $CDCl_3$, 400 MHz as compared with Abreu et al., (2013).

H/C-Position	δ_C (ppm)	δ_H (ppm)	δ_C*(ppm)	δ_H*(ppm)	No. of H, Multiplicity, J(Hz)
1	22.28	1.70, 1.45	22.3	1.90; 1.65	1H, m, each
2	41.74	2.25, 1.29	41.2	2.22; 2.32	1H, m, 1H, qd
3	213.45	-	213.2	-	-
4	58.14	2.25	58.2	2.25	1H, q
5	41.88	-	42.1	-	-
6	35.83	1.25, 1.4,	41.2	1.21, 1.66	1H, m, each
7	18.16	1.40, 0.93	18.2	1.45; 1.35	1H, m, each
8	52.26	1.40	53.0	1.35	1H, m, each
9	36.22	-	37.4	-	-
10	59.54	1.56	59.4	1.48	1H, m, each
11	35.24	1.45, 1.17	35.6	1.38; 1.19	1H, m, each
12	29.88	1.20; 1.16	30.4	1.31; 1.24	1H, m, each
13	39.20	-	39.6	-	-
14	38.51	-	38.2	-	-
15	30.21	1.16, 1.25	32.4	1.14, 1.27	1H, m, each
16	35.55	1.40; 1.20	35.9	1.50; 1.28	1H, m, each
17	30.03	-	29..9	-	-
18	42.36	1.15	42.7	1.51	1H, m, each
19	32.63	1.18, 1.40	35.3	1.31, 1.14	1H, m, 1H, dd
20	28.18	-	28.1	-	-
21	32.00	1.45; 1.19	32.7	1.42; 1.17	1H, m, each
22	39.01	1.25; 1.23	39.2	1.41, 0.90,	1H, m, each
23	7.04	0.85	6.8	0.88	3H, d
24	14.41	0.70	14.6	0.73	3H, s
25	18.04	0.85	17.9	0.87	3H, s
26	18.88	0.85	20.2	0.98	3H, s
27	19.45	0.93	18.6	0.94	3H, s
28	29.92	1.00	32.0	1.11	3H, s
29	32.30	1.00	35.0	0.96	3H, s,
30	32.30	1.00	31.7	1.00	3H, s

(Abreu *et al*., (2013)* coupling constant (J), singlet (s), double doublet (dd), multiplet (m), quartet of doublet (qd)

Compound HXC_2 was isolated as white amorphous powder, highly soluble in chloroform, with melting point of 287-290°C. The ^{1}H-NMR (Figure 4.8) displayed signal for eight tertiary methyl proton singlet at δ_H (ppm) 0.89 (23H), 0.93 (24H), 0.99 (25H), 0.89 (26H), 0.86 (27H), 0.97 (28H), 0.93 (29H) and 0.79 (30H). This suggests that there are eight methyl groups present in the compound. The presence of down field signals at δ_H (ppm) 4.34 (1H, br.) suggest the presence of hydroxyl group. The appearance of the signal showing a double doublet at 4.34 δ_H (J=7.1 and 4.9Hz 1H) is typical of a hydrogen bonded to an oxygenated carbon, further suggesting the existence of a hydroxyl group in the structure (Duarte *et al*., 2009). ^{13}C-NMR spectrum (Figure 4.9) showed the presence of thirty carbon resonance and gave important signal peaks at δ_c (ppm) 72.89 (C-3), is typically indicative of a carbon bearing hydroxyl group in a triterpenoidal structure. Table 5.2 gives a comparison of the chemical shifts for carbon atoms of compound HXC_2 with those of Boonyaratavej and Petsom, (1991) as well as Duarte *et al*., (2009), which compares agreeably. The DEPT 135 spectrum (Figure 4.10) further reaffirmed the later by showing eight methyls, eleven sp^3 methylenes, four sp^3 methines and six quartenary sp^3 carbons. The FTIR spectrum in Figure 4.7 showed a strong broad peak at IRυ_{max} 3342 cm^{-1} affirming to an OH group (at C-3) which is characteristic of an aliphatic OH stretching. Other signals at 2920cm^{-1} (alkyl C-H stretching) and 1600cm^{-1} (C-C stretching in ring) further help to indicate the absence of unsaturation in the structure. There were no absorption observed in the UV spectrum, hence. The mass spectral data showed a molecular ion peak $[M+H]^+$ in the positive mode with m/z 429, indicating the quasimolecular ion. Other characteristic fragment ions peaks were seen at m/z 413, 409, 342, 273, 205 and 123 (Figure 4.11 and Scheme 4.2). The molecular ion peak MH^+ at m/z 429 represent the intact molecule having an m/z value one amu greater than the molecular ion. The m/z 409 can be associated with loss in mass of 18 $[M^{\cdot +} – 18]$, which is likely to be equivalent to a loss of water (H_2O) molecule. The latter is thus an indication of

the presence of a hydroxyl (HO^-) group in compound HXC_2. The m/z 413, is associated with the loss in mass of 15 from m/z 429, which in essence can be associated to the loss of a methyl (CH_3) group (M- CH_3). Other fragmented m/z ion involved the ring A, B, C and D cleavages in line with fragmentation pattern for fridelanes described by Shiojima *et al*., (1992), which might have been characterised by the RDA cleaving pattern for PCTT (Scheme 4.2 and Figure 4.11). Thus, these information derived from the MS, NMR and IR are in agreement with those reported in literature (Duarte *et al.,* 2009 and Boonyaratavej & Petsom, 1991 as well as Sousa *et al*., 2012 and Kamboj & Saluja, 2011) for 3β-friedelinol

Table 5.2. ^{1}H and ^{13}C-NMR chemical shifts of compound HXC_2 in $CDCl_3$, 400 MHz as compared with Boonyaratavej and Petsom (1991).

H/C-Position	δ_c (ppm)	δ_c*(ppm)	δ_H (ppm)	No. of H, Multiplicity, J(Hz)
1	17.92	16.14	1.36, 1.54	3H, d,
2	36.06	35.21	1.38, 1.74	1H, d, 5.14
3	**72.89**	72.76	4.34, 1.37	1H, s
4	50.08	49.19	1.10	
5	38.22	37.87	-	-
6	42.06	41.77	1.10, 1.74,	1H, m, each
7	17.19	17.55	1.10, 1.75	1H, m, each
8	53.26	53.25	1.10	1H, m, each
9	37.68	37.16	-	-
10	61.47	61.38	1.20	1H, m, each
11	35.66	35.38	138, 1.17	1H, m, each
12	30.50	30.66	1.49, 1.22;	1H, m, each
13	39.20	38.41	-	-
14	39.01	39.71	-	-
15	32.12	32.34	1.37, 1.19	1H, m, each
16	37.03	36.13	1.40, 1.04;	1H, m, each
17	30.03	30.07	-	-
18	42.65	42.85	1.25	1H, m, each
19	35.32	35.59	1.38	1H, d, 10.8
20	28.18	28.17	-	-
21	32.78	32.83	1.09, 1.51	1H, m, each
22	39.01	39.28	1.11, 1.38	1H, m, each
23	11.80	11.59	1.48, 0.89, 0.89	3H, d
24	16.44	15.82	0.88, 0.93, 0.93	3H, s
25	17.37	18.26	0.99	3H, s
26	19.41	18.64	0.89	3H, s
27	19.45	20.09	0.86	3H, s
28	32.11	32.13	0.97	3H, s
29	33.35	35.05	0.93	3H, s,
30	33.35	31.80	0.79	3H, s

**Boonyaratavej and Petsom (1991)*

Compound HXC_3 was isolated as creamy white powder, highly soluble in chloroform. The ^1H-NMR (Figure 4.15 and Table 4.9) showed seven tertiary methyl (CH_3) singlet signals at δ_H (ppm) 0.75, 0.78, 0.83, 0.86, 0.92, 0.97 and 1.23 (3H each, s, CH_3). This suggests that there are seven methyl groups present in the compound. Two protons appeared at δ 4.66 and 4.69 as singlet representing an exocyclic double bond protons assigned to H-29a and H-29b respectively. The signal at δ 3.48 is typical for a triterpenoids with a 3-hydroxy substitution (Cavie *et al.*, 1968), with H-3 proton appearing as a triplet of a doublet (tdd) (J = 7.0, 5.0 Hz). The assignments are in good agreement for elucidated structure of lupenol in literature (cavie *et al*., 1968; Reynolds *et al*, 1986; Imam *et al*., 2007; Jain and Bari 2010 and Ahmadu *et al*., 2013). The ^{13}CNMR gave 30 signals (Figure 4.16), suggesting that the compound consists of 30 carbon atoms. The appearance of signal at δ_C (ppm) 79.02 gave information about the carbon bonded to a hydroxyl functional group at (C-3), which has a downfield signal in the ^{1}HNMR. Diagnostically appearing is the signals at δ_C 150.98 and 109.30 ppm assigned for C-20 and C-29, is characteristic of a vinylic carbon signals for triterpenoid of the lupane (skeleton) series (Cavie *et al*., 1968). Hence, Table 5.3 gives an agreeable comparative ^{1}H and ^{13}C NMR chemical shifts values with those of Ahmadu *et al*., (2013). The UV spectrum (Figure 4.13) however, is a further proof, revealed a chromoporic absorbtion at $UV\lambda_{max}$ 370nm which can be associated with an olefinic mioety carbon signal observed at δ150 and δ 109 in the ^{13}CNMR. The FT-IR spectra of the compound (Figure 4.14) showed a broad peak at $IR\upsilon_{max}$ 3253 cm^{-1} (OH Stretching) and a peak at 1680 cm^{-1} accounting for a vinylic carbon-carbon double bond (C=C stretching). The mass spectral data showed molecular ion (quasimolecular ion) peak $[MH]^+$ with m/z 427, with other peaks of fragment ions at m/z 410, 409, 383, 207 and 189 (Figure 4.17 and Scheme 4.3). The molecular ion peak 427 represents the intact molecule having an m/z value one amu greater than the molecular ion. The m/z 410, is associated with the loss in mass of 15 from the molecular ion. This can be

associated to the loss of methyl (CH_3) group ($M^{\cdot +}$ - CH_3). In another possibility, the m/z 409 may arise from the loss in mass of 18 from the molecular ion. This is thus, associated with the loss of water (H_2O). Through a 1, 2-methyl shift at ring C, with a loss in mass of 28, two possibilities resulted, giving two fragment ions with m/z 383 from m/z 410. The latter is associated with a loss of ethylene (-C_2H_4). The m/z 207 resulted from loss in mass of 175 ($C_{13}H_{22}$) from the fragment ion m/z 383, cleaving ring C. The loss of mass 18 from m/z 207 resulted in the fragment ion m/z 189. This can easily be associated to a loss of water (-H_2O). However, the fragment ions m/z 207 and 189 observed, have been proposed to form via two competitive pathways, often used to diagnose the presence of substituents in A, B, C, D or E rings (Branco *et al*., 2004 and Budzikiewicz *et al*., (1963). The fragmentation pattern (Scheme 4.3 and fig. 4.17) was in line with the fragmentation pathways of pentacyclic triterpenes using electron ionisation mass spectrometry (EI-MS) reported by Budzikiewicz *et al*., (1963). The data are also consistent published works of Puebla *et al*., (2010) and Ahmadu *et al.,* (2013) for the elucidation of lupeol.

Table 5.3. ^{1}H and ^{13}C-NMR chemical shifts of compound HXC_3 in $CDCl_3$, 400 MHz as compared with Ahmadu *et al*., (2013).

H/C-Position	δ_c (ppm)	δ_H (ppm)	δ_c*(ppm)	δ_H*(ppm)	No. of H, Multiplicity, J(Hz)
1	38.01	1.22	38.9	1.24	1H, m, each
2	29.81	2.13	27.5	2.05	3H, s, 3H, s
3	79.02	3.47	79.2	3.21	-
4	38.83	-	38.8	-	-
5	55.25	0.65	55.1	0.69	1H, d
6	17.98	1.36,	18.1	1.39	1H, q
7	34.24	0.86, 1.10	34.3	0.84	1H, m, each
8	40.79	-	41.2	-	-
9	50.39	0.89	50.5	0.90	1H, s
10	37.14	-	37.2	-	-
11	20.90	1.25	20.9	1.28	1H, d
12	25.09	1.17	25.3	1.19	1H, q
13	38.66	1.36	37.2	1.38	1H, t
14	42.80	-	42.8		
15	29.65	1.13	27.6	1.10	1H, d
16	35.55	1.23	35.6	1.30	1H, d
17	42.98	-	43.2	-	-
18	47.97	0.98	47.9	0.91	1H, t
19	48.26	2.02	48.1	2.37	1H, m
20	150.98	-	150.9	-	-
21	31.92	1.36;	32.7	1.33	1H, s
22	39.98	1.23; 1.25	40.2		1H, m, each
23	29.70	0.92	28.0	0.94	1H, s, 3H, s
24	29.70	0.84	16.0	0.82	3H, s
25	16.11	0.86	16.1	0.84	3H, s
26	15.95	0.78	15.9	0.83	3H, s
27	14.52	0.92	14.8	0.97	1H, s, 3H, s
28	18.29	0.82	18.0	0.83	3H, s
29	109.30	4.66, 4.69	109.4	4.61, 4.57	1H, s, 1H, s
30	19.28	1.70	19.2	1.71	3H, s

Ahmadu *et al*., (2013)* Coupling constant (J), singlet (s), double doublet (dd), multiplet (m), quartet of doublet (qd), triplet (t), quartet (q)

Compound EAC_1 was isolated as white crystalline powder, highly soluble in chloroform, with melting point of 140 –142 ^{o}C. The ^{1}H-NMR (Figure 4.21) showed six singlet methyl signals at δ_H (ppm) 1.38(H-18), 0.93(H-19), 1.27(H-21), 0.81(H-26), 0.86(H-27) and 0.86(H-29). This suggests that there are six methyl groups present in the compound. The presence of down field signals at δ_H (ppm) 5.34 (1H, br.) suggest the presence of vinylic proton typical of a steroidal nucleus (Ahmed *et al.*, 2013). The ^{13}CNMR gave 29 signals (Figure 4.22 and Table 4.11). This suggests that the compound consists of 29 carbon atoms. The appearance of signal at δ_c (ppm) 121 confirms the presence of the carbon (C-5) bearing the olefinic proton at δ_H (ppm) 5.34 (1H, br.). The signal at δ_c (ppm) 140 was due to a quaternary carbon (C-6) atom. The carbon at δ_c (ppm) 72 confirms the presence of carbon (C-3) bearing a hydroxyl group. This data is consistent with earlier published works of Pateh *et al.*, (2009), Kamboj and Saluja (2011) as well as Ahmed *et al.*, (2013) characteristic for a β-sitosterol. Hence, Table 5.4 compares very closely and agreeably with Ahmed *et al.*, (2013). Furthermore, the UV spectrum (Figure 4.19) gave absorption at λ_{max} 323nm, (confirming the unsaturation at C-5 and C-6 observed in the ^{1}H and ^{13}CNMR spectra). The FT-IR spectra of the compound also attestested to the later by showing IRυ_{max} 3358.8 cm^{-1} (broad peak) due to aliphatic OH stretching, and 2929.2 cm^{-1}, 2852.2 cm^{-1} (CH_3 stretching) , 1648.11 cm^{-1} (C=C stretching), and a bending C-H stretching at 1000-650 cm^{-1} (Figure 4.20). The mass spectral data showed molecular ion peak $[M]^+$ at m/z 414, with other fragment ions peaks observed at m/z 395, 383, and 134 (Figure 4.24 and Scheme 4.4). The molecular ion peak 414 represents the intact molecule and also gives the exact molecular weight of the compound. The peak at m/z 395 of the fragment ion is associated with loss in mass of 18 [M^+ – 18]. The loss in mass of 18 may be attributed to loss of water (H_2O) (Wretensjö, 2004), which by extension is indicative of the presence of hydroxyl (OH) group in the compound EAC_1 .The peak at M/Z 383 is associated with the loss in mass of 15 from m/z 395, signifying an associated loss of a methyl (CH_3)

group (M^+- CH_3) (Pateh *et al*., 2009). The peak at m/z 243, results from the loss in mass of 141 ($C_{10}H_{21}$) from the fragment ion m/z 383. This suggests the presence of a long external side chain. The presence of rings in the compound is confirmed by the presence of peaks at m/z 134 and 81 (Scheme 4.4 and Figure 4.24). The molecular ion peak 414 confirms the suspected compound of β-sitosterol. β-sitosterol has been isolated from many plant sources including *Stylochiton lancifolius* (Pateh *et al*., 2009).

Table 4.10. ^{1}H and ^{13}C-NMR chemical shifts of compound EAC_1 in $CDCl_3$, 400 MHz as compared with Ahmed *et al*., (2013).

H/C-Position	δ_c (ppm)	δ_H (ppm)	δ_c*(ppm)	δ_H*(ppm)	No. of H, Multiplicity, J(Hz)
1	37.32	1.13, 1.39	37.29		1H, m
2	31.71	1.36, 1.72	31.95		1H, s
3	72.04	3.85	71.84	3.51	1H, tt (4.60, 4.60, 11.11,
4	42.3	1.96, 2.44	42.36		2H, m
5	140.96	-	140.80		-
6	121.56	5.13, 5.38	121.73	5.34	1H, d, (5.2)
7	32.14	1.91, 2.16	31.71		1H, m
8	31.83	1.63	31.95		-
9	50.07	0.90	51.0		IH, s
10	36.53	-	36.18		-
11	21.06	1.27, 1.26	21.12		3H, d (7.34)
12	39.35	1.38, 1.46	39.82		2H, m
13	42.28	1.27, 1.39	42.36		-
14	56.43	1.42, 1.65	56.81		4H, s
15	24.47	1.28, 1.73	24.33		3H, d (7.34)
16	28.42	0.91	28.26		3H, dddd (2.61, 6.27, 12.89,
17	55.91	1.32	56.11		1H, m
18	11.90	0.81	11.88	0.67	3H, d (8.68, 15.14)
19	19.30	1.23	19.41	1.00	3H, s
20	35.52	1.31, 1.39	36.54		2H, m
21	18.78	0.85	19.07	0.92	3H, d, (6.0)
22	34.16	1.02	34.00		1H, s
23	26.29	1.60	26.16		4H, m
24	46.04	1.07, 1.84	45.89		1H, m
25	29.05	0.87	29.23		1H, m
26	19.42	0.81	19.83	0.83	3H, d, (7.2)
27	19.42	0.86	18.81	0.79	3H, d, (7.2)
28	23.19	1.14	23.12		3H, m
29	12.09	0.86	12.01	0.85	3H, d, (8.0)

Ahmed *et al*., (2013)* coupling constant (J), singlet (s), multiplet (m), doublet (d)

The biological activity exhibited by plant extracts are related to the type of phytochemicals the plant is composed of (Kaefer and Milner, 2008). Hence, drug discovery process from plant resources involve targeting bioactive molecules from medicinal plant which most often demonstrate physiological activity, which could consequently serve as a template for the synthesis of more potent drugs with reduce toxicity. In this work the antivenom studies of *A. chevalieri* is reported based on the evaluations (antivenom assay) carried out on the plant's extracts and fractions of hexane and methanol, n-butanol and ethyl acetate, respectively, as well as on two isolated compounds (HXC_1 and EAC_1) from the hexane extract and ethyl acetate fractions respectively. Generally, a known sterol and three pentacyclic triterpenoid have been successfully isolated from this work in which both classes of metabolites have been representatively evaluated, pharmacologically.

Gomes *et al.,* (2007); Santhosh *et al.*, (2013) and Venkatesan *et al*., (2014) and are among the recent researchers who have demonstrated that triterpenoid and phytosterol isolated from plants showed effective antivenom agent of *Naja nigricollis* and other snake venoms. In line with literature, this work has shown that β-sitosterol and Friedelin has an effect on both gross and histological changes induced by the venom of *Naja nigricollis*. The results of the assay in Table 4.16 showed that the dose of 100mg/kg could not protect the animal against lethal MLD dose of venom, as they died earlier than those injected with MLD only. The hexane extract and n-butanol fraction earlier showed protection at $200mgkg^{-1}$ (Table 4.15) as against those of ethyl acetate fractions at same dose. This informed their selection for investigation at a much lower dose of $100mgkg^{-1}$.

However, in Table 4.18 antivenom evaluation of the compound EAC_1 (β-sitosterol) isolated from ethyl acetate fraction showed a good activity as against the crude ethyl acetate fraction which had earlier showed no-activity. A possible explanation to the foregoing could mean

that the plant constituent antivenom is not synergistic or more so, could mean that some phytoconstituents in the crude ethyl acetate extract are antagonist of the plant constituent antivenom agent (β-sitosterol). The LD_{50} (of > 5000mgkg^{-1}) ethyl acetate fraction determined (Table 4.14) also showed that the dose administered were relatively save. The findings in Table 4.17 and 4.18 showed that the compounds isolated, HXC_1 and EAC_1 (Friedelin and β-sitosterol) were more potent than all the extracts and fractions of *A. chevalieri* evaluated, which recorded some activity at 200mgkg^{-1} and almost non at 100 mgkg^{-1}. However, as earlier observed, the result of the activity exhibited by compound EAC1 contradicts the ethyl acetate fraction, which recorded no activity.

It is also possible that the bioactive anti-venom molecule in the plant may be dose dependant. Thus, as seen in Table 4.17 through Table 4.18, a higher protection is recorded (at a dose of 8mgkg^{-1}) in terms of delaying the time of dead of the animals due to the venom's lethal effect (yielded 60% surviving animals within the maximum observed time of 24hour), than seen for the group treated with a dose of 4mgkg^{-1}. In the overall, a 60% survival rate of animals treated with the isolated compound EAC_1 (offered as prophylaxis) can be attributed to its potency as an anti-venom bioactive agent. The seeming alleviation of toxic symptoms and survival of laboratory animals (within 24 hours) after being challenged with lethal venom doses is in agreement with the finding of Okonogi *et al*., (1979), Haruna *et al*., (1995), Mors, *et al* (2000), Abubakar *et al*., (2000), Gomes *et al.,* (2007), Santhosh *et al.*, (2013), Venkatesan *et al*., (2014) and Kadiyala *et al*., (2014), where different classes of plant constituents have been shown to possess *in vivo* activity against some snakes venom; the antivenom plant constituents including sterols, triterpenes, phenolic compounds (flavonoids and tannins), aristolochic acids etc. The presence of some of these active classes of

compounds with antivenon effect and their mechanism of actions has also been suggested (Tsai *et al*., 1980).

Histological changes observed for two vital organ tissues of liver and kidney (as indicated in Plate V, VI and VII) demonstrated some changes in the cell of the tissues of envenomated mice. However, histopathological changes in the kidney and liver of envenomated mice treated with the isolated compounds HXC_1 and EAC_1 were minimal compared to those treated with extracts and fractions of *A. chevalieri* as well as MLD of venom only. The images of the tissues in plate V and VI showed severe hepatic necrosis and micro vesicular steatosis in the liver while acute tubular necrosis was seen in the kidney of envenomated mice treated with the various plant extracts and fractions. On the contrast, there were no significant changes observed (alterations of the cell structure as compared to the normal, labelled 'a') in the architecture of the cell tissues of envenomated mice treated with EAC_1 and HXC_1. On a broad view, it can generally be opined that gross histological changes such as glomerular congestions, vacuolar degeneration of tubular cells with haemorrhagic regions were observed in kidneys of envenomated mice treated with plant extracts, while cholesterol granuloma (cleft), disturbances in circulation of protein, dystrophy in hepatocytes and degenerated hepatocytes were seen also in different regions of liver tissue of envenomated mice treated with plant extracts. Comparatively, the latter listed histological effects were not observed in envenomated mice treated with isolated compound HXC_1 and EAC_1. The evaluated LD_{50} of the n-butanol fraction (288.5mgkg^{-1}, Table 4.14) had somehow, indicated the likelihood of toxicity. The phytochemical screening earlier showed the n-butanol fraction to be rich in saponins. Saponins could be quite toxic, hence this may be responsible for the haemorrhagic and some other histological changes observed alongside the venom's lethal effect on the animal's homeostasis.

CHAPTER SIX

6.0. SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1. Summary

The Biodiversity of plant and their resources calls for biodiversity prospecting. Hence, natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Cos *et al.,* 2006).

The plant *A. chevalieri* has been reported to be bioactive from the various evaluations of its extracts but not until this work, the active constituent responsible for some of these activities have not been investigated. Isolation of pure active compound from the extracts of this plant is reported for the first time. Therefore, in this research the antivenom activity of *A. chevalieri* has been evaluated with the view of isolating bioactive compounds from the stem bark of the plant. Four (4) bioactive compounds have been isolated through series of column chromatography and characterized using spectroscopic techniques [Mass Spectrometry (MS), Nuclear Magnetic Resonance (NMR), Infra-Red (IR) and Ultra-Violet Light (UV)]. β-sitosterol was conclusively identified while other three (3) were tentatively identified as friedelin, 3-β-friedelinol, and lupeol. These compounds are reported for the first time as constituents of *A. chevalieri*. The n-butanol fraction and hexane extract of *A. chevalieri* with the exception of the ethyl acetate fraction, showed some significant antivenom activity (at $200mgkg^{-1}$) by delaying time of death of envenomated mice challenged with lethal dose (MLD) of venom, hence offering some significant protection to the animals. More so, two of the isolated compounds evaluated (friedelin and β-sitosterol) showed higher activity by exhibiting a good antivenom property, especially β-sitosterol, where there was some 60% survival rate of envenomated treated mice offered as a prophylaxis. This was so, considering

that the animals survived well above the maximum observed period of 24 hours. Consequently, gross histopathological changes in the extracted organs (liver and kidney tissues) of envenomated mice treated with two of the isolated compounds were noted to be less obvious than seen in the tissues from envenomated mice treated with extracts and fractions of *A. chevalieri*.

This study has successfully made the following new findings:

i. First time report of isolation of β-sitosterol, friedelin, friedelinol and lupeol from *A. chevalieri*
ii. Report of friedelin as a bioactive antivenom compound (at 4 and $8 mgkg^{-1}$ activity)
iii. Report on the LD_{50} of hexane (> $2900 mgkg^{-1}$) and methanol ($565.7 mgkg^{-1}$) extracts; ethyl acetate (> $5000 mgkg^{-1}$) and n-butanol ($288.5 mgkg^{-1}$) fractions of *A. chevalieri*

6.2. Conclusions

Chromatographic separation of ethyl acetate and hexane extract fractions led to the isolation of β-sitosterol, and three pentacyclic triterpenoids (Friedelin, Friedelinol and Lupeol) in *A. chevalieri* as well as the evaluation of these compounds (β-sitosterol and friedelin) for anti-venom property. The antivenom activities of β-sitosterol and friedelin against cobra venom tested *in vivo* using mice showed strong antivenom activity at a concentration of 8mg per animal with 60% survival rate, while lesser concentrations showed different mortality rates. Hence the compounds tested were found to be dose dependent against cobra venom. It has been found that the solubility of β-sitosterol in water poses a challenge, otherwise this ubiquitously highly potent bioactive molecule which has earlier been reported to be a good antivenom agent, is a candidate drug lead that can be developed further through clinical trials. The results obtained may therefore be said to have provided scientific basis for the use of *A. chevalieri* in folklore medicine in the treatment of snake bites.

6.3. Recommendations

Albizia chevalieri has a long list of folklore therapeutic practice and most of its phytoconstituents are still very much not isolated and assayed. The task of isolation of bioactive novel compounds can be very much achieved when the highly polar constituents resident in the methanol extract and n-butanol fractions are chromatograph using a preparative or semi-preparative HPLC to separate and isolate effective pure novel bioactive molecule in an isolation-bioactive-targeted-molecule research design. The biochemical reaction-mechanism of action as regard structural activity relationship (SAR) of the isolated compounds, modification to the scaffold structure for increase antivenom activity through semi or complete synthesis are all areas that can be explore as further works of this research.

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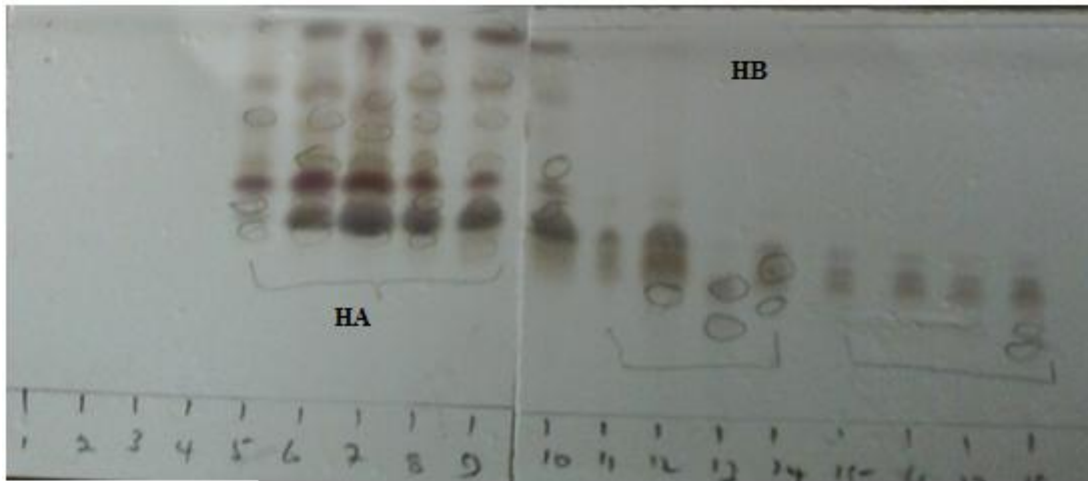
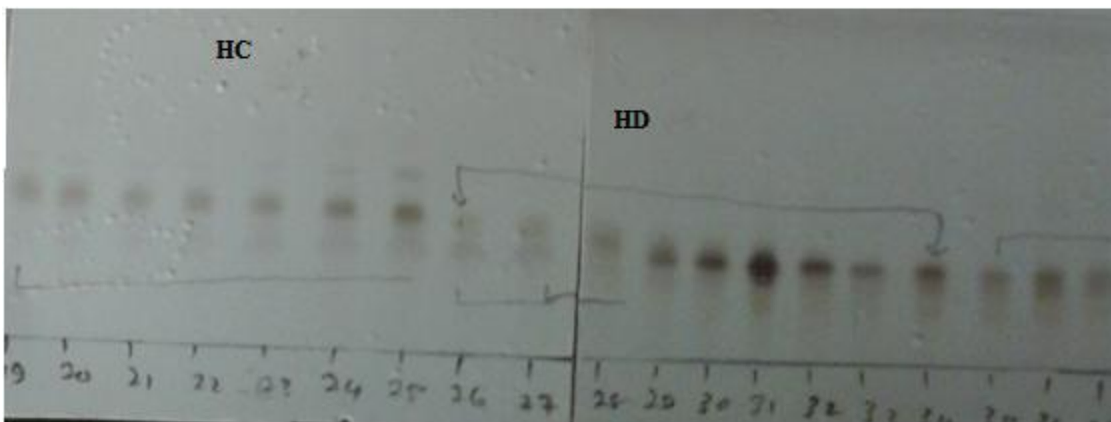
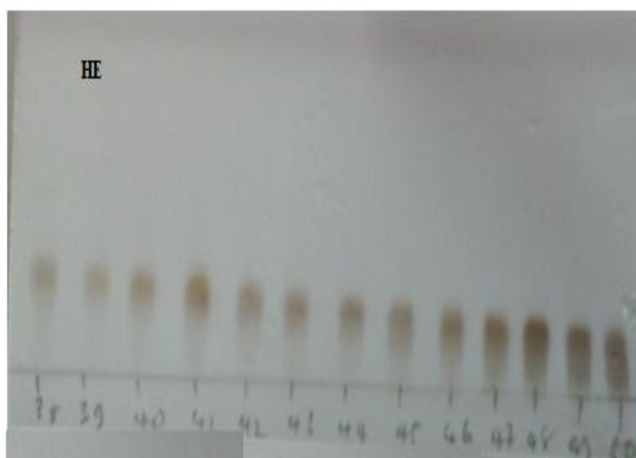
APPENDIX I

(Column Fraction TLC Profile)

Appendix I (a): The TLC profile of some fractions of *A. chevalieri* Hexane extract







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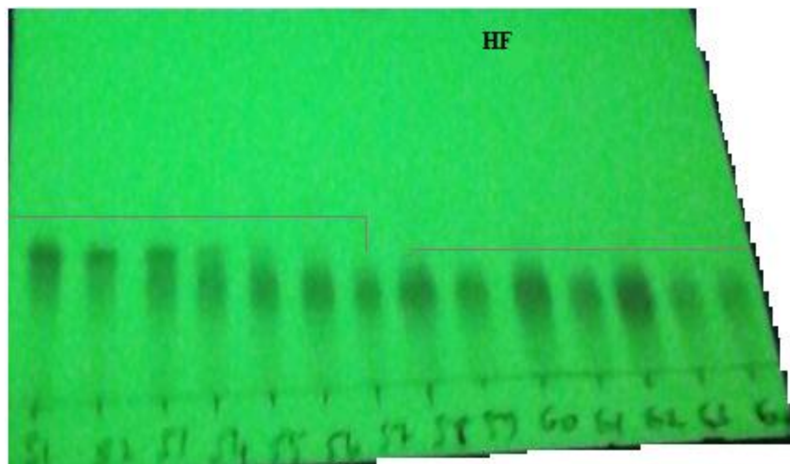
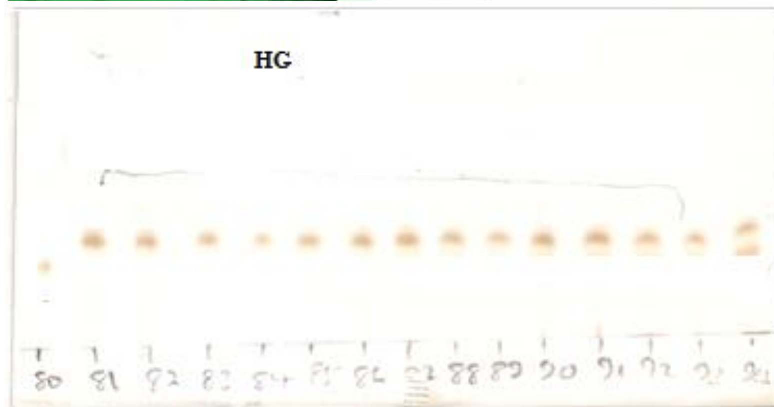
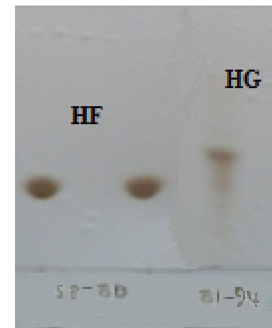
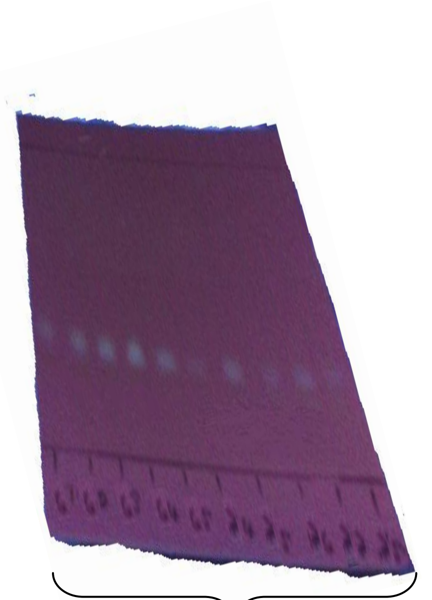
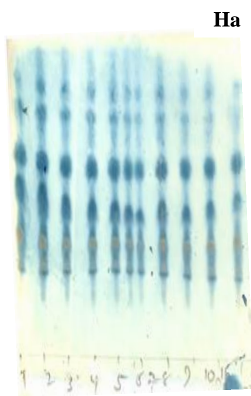


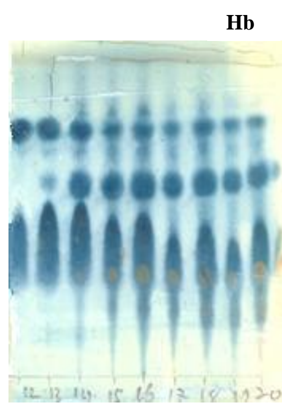


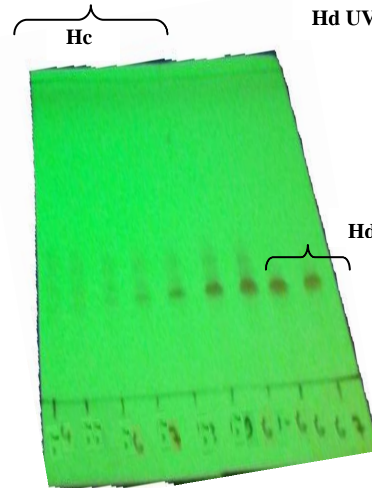




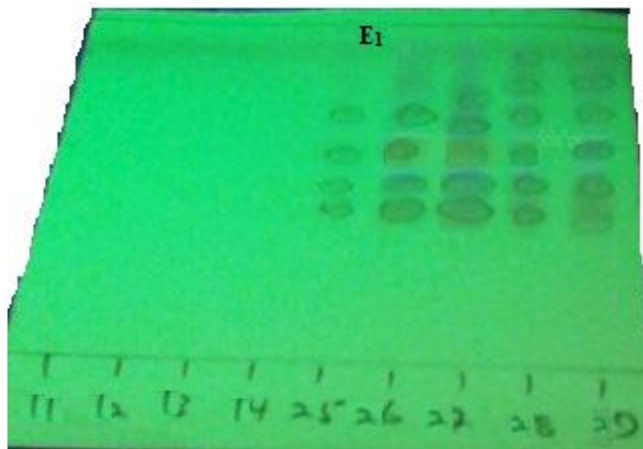
Hd UV active at 365nm

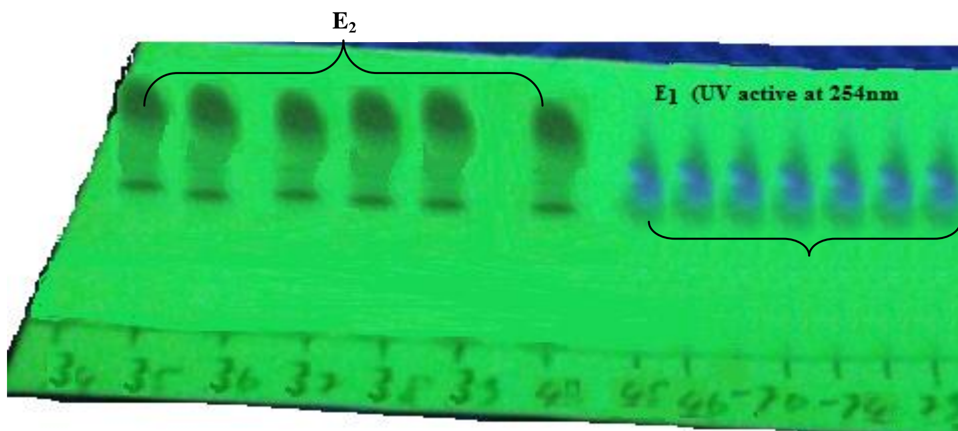


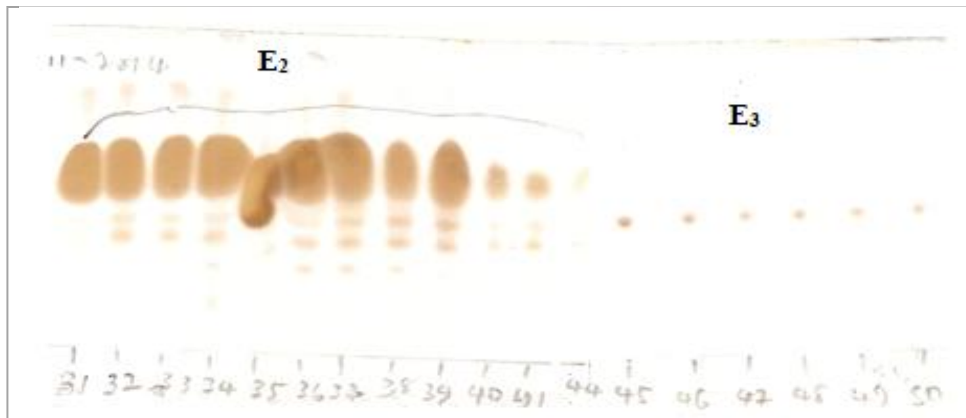




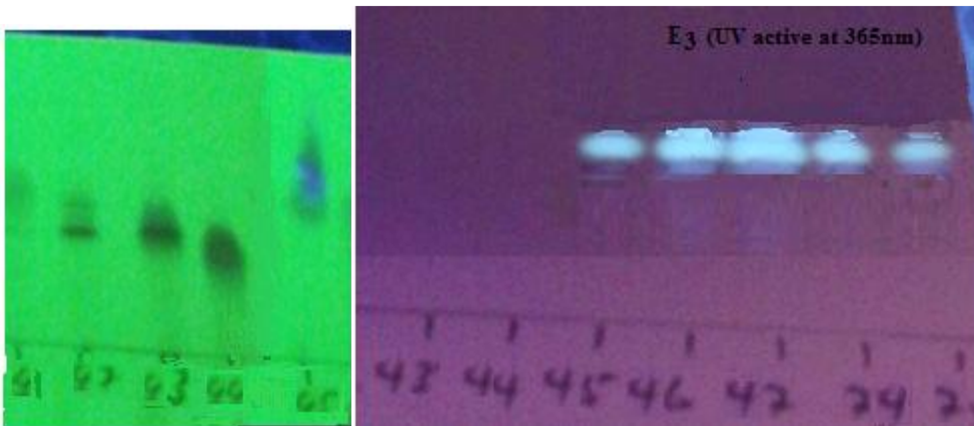
Appendix I (b): The TLC profile of some fractions of *A. chevalieri* Ethyl acetate extract







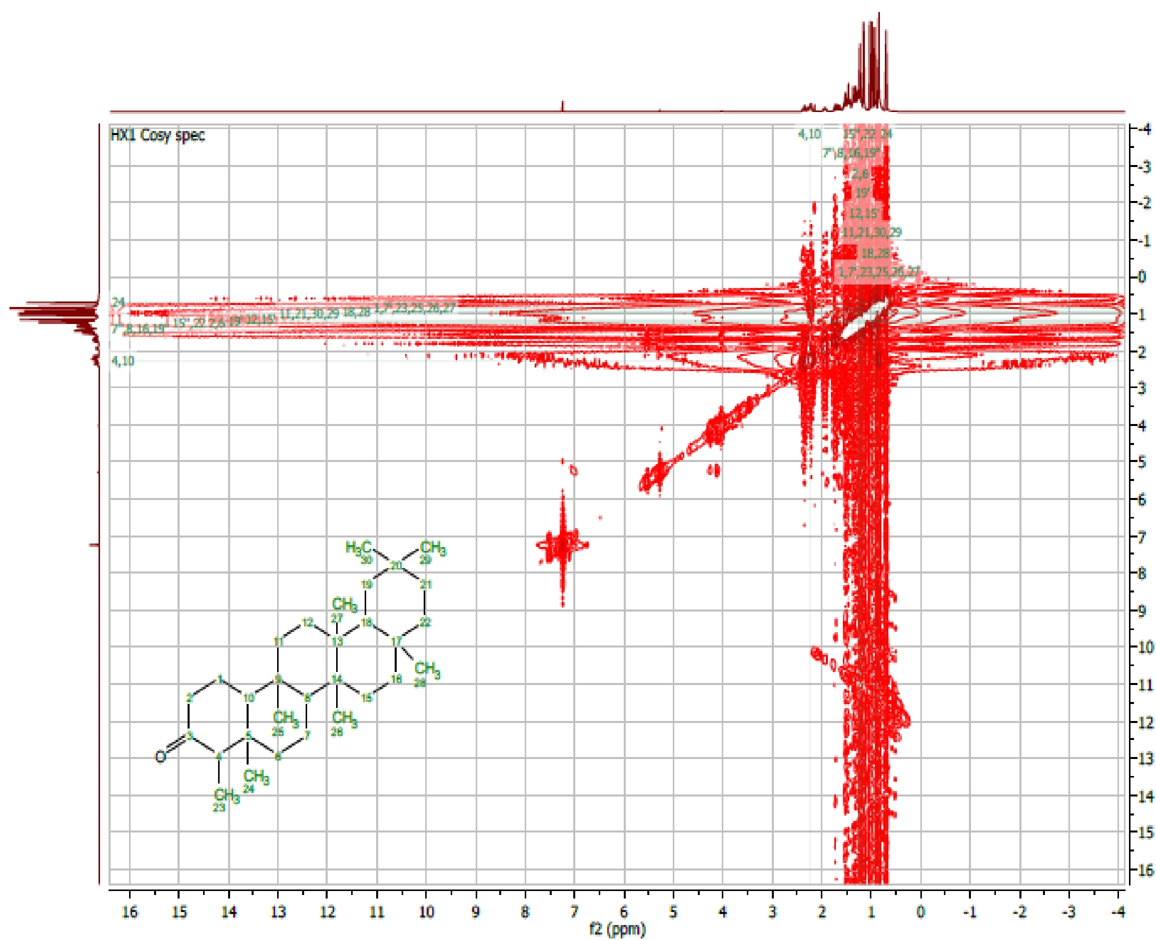
Tlc C'togram of EAc1 viewed under UV @ 365nm



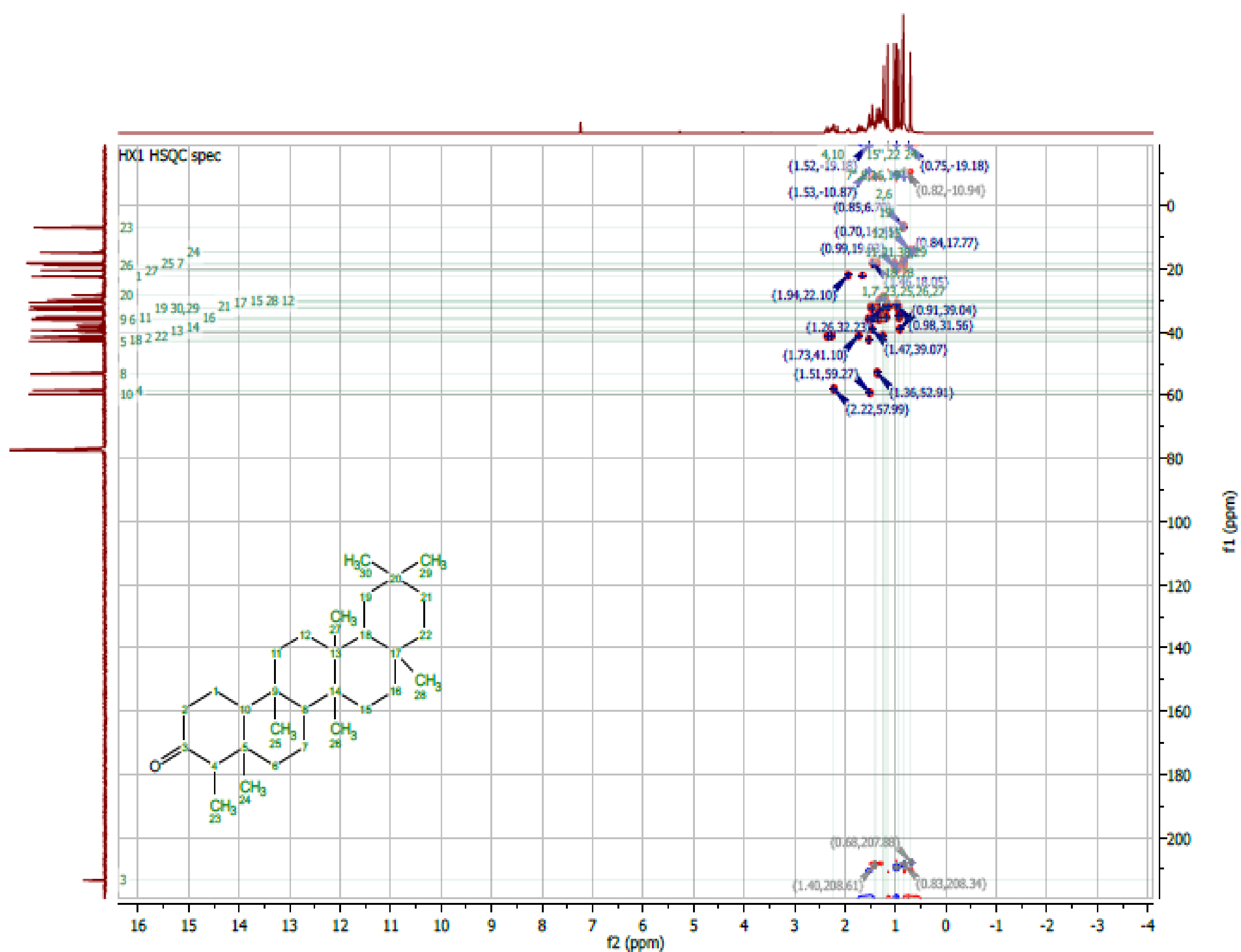
APPENDIX II

(2D NMR Spectra of Compounds HXC1 and EAC1)

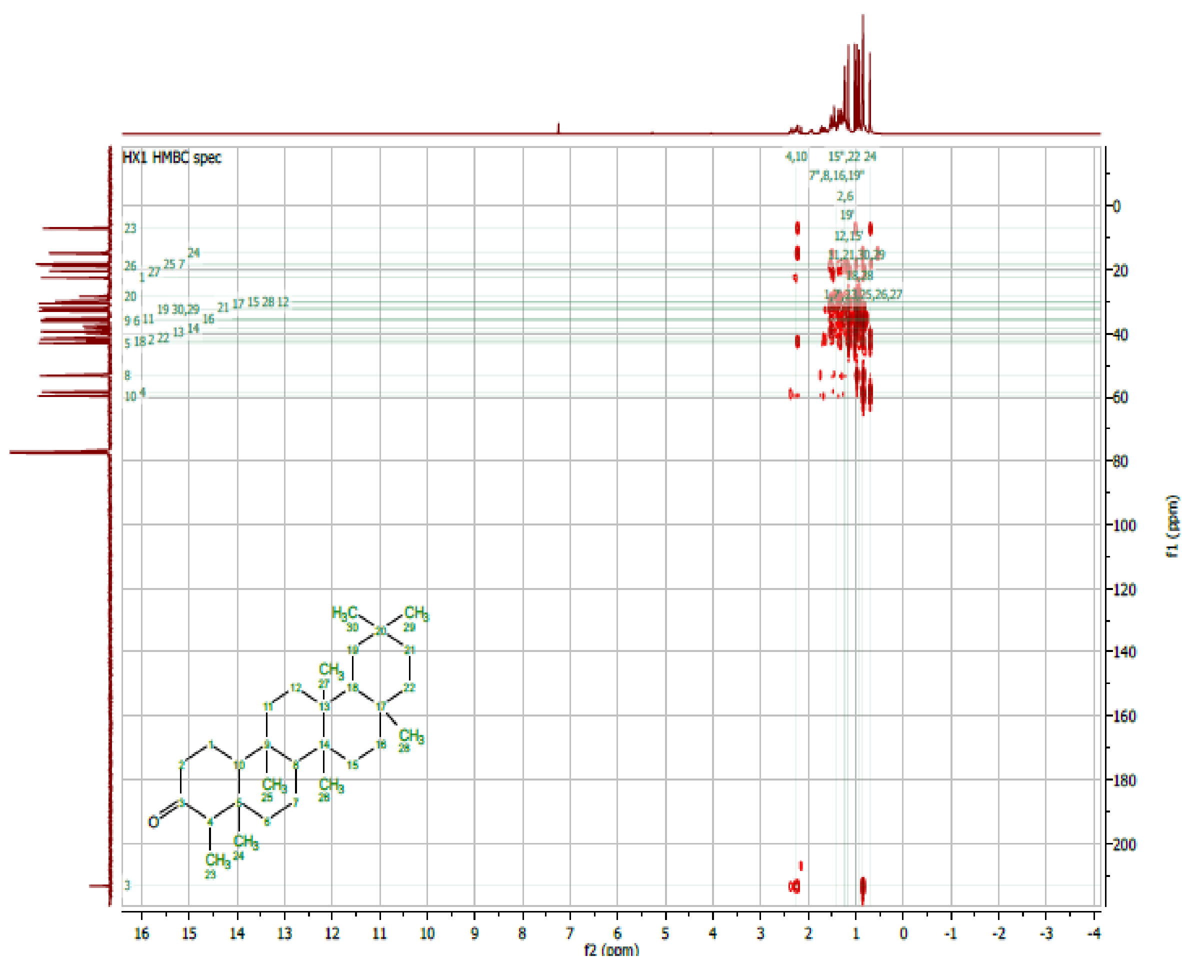
Appendix II (a): The ^{1}H-^{1}H COSY spectrum of compound HXC_1



Appendix II (b): The ^{1}H-^{13}C HSQC spectrum of compound HXC_1

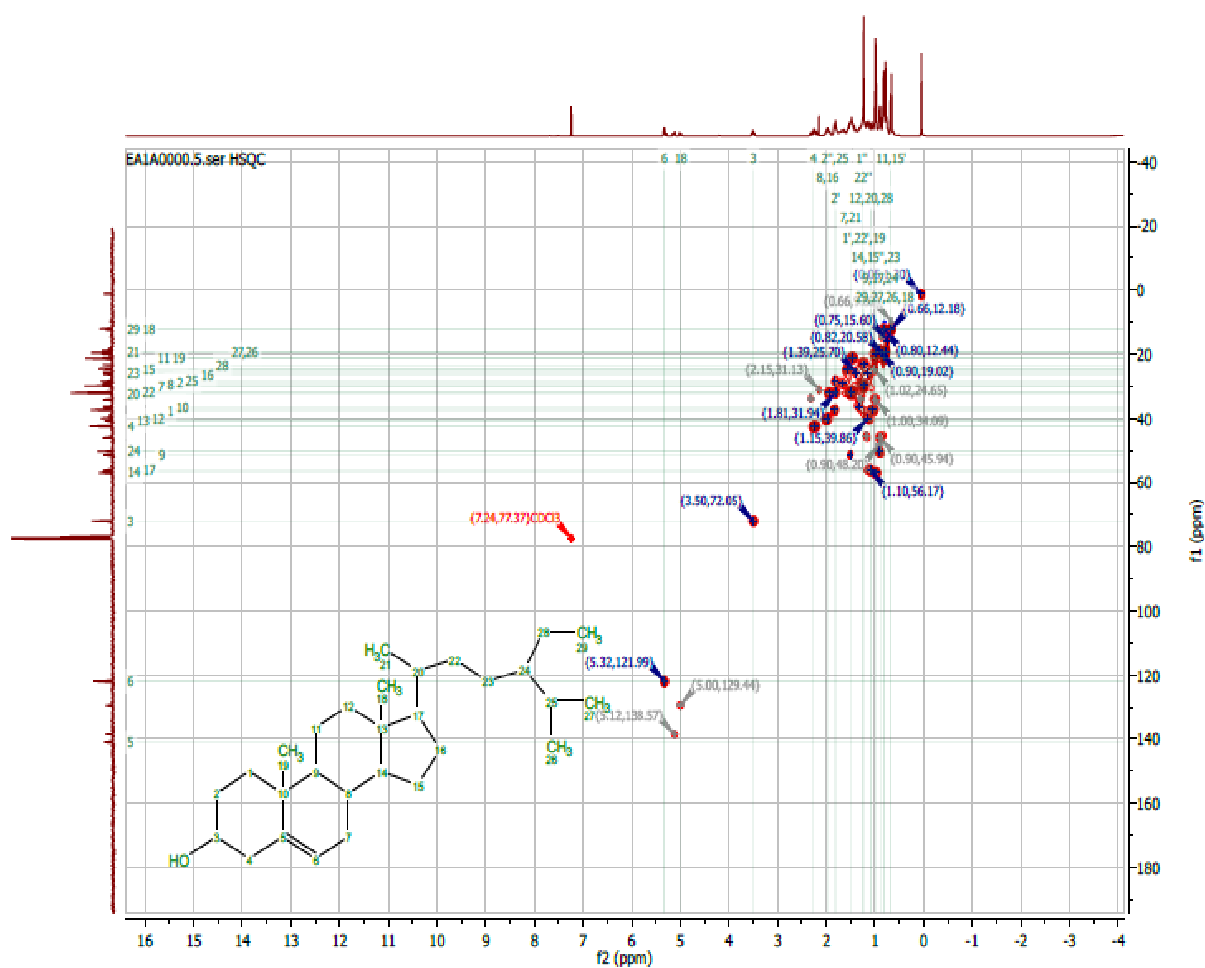


Appendix II (c): The ^{1}H-^{13}C HMBC spectrum of compound HXC_1

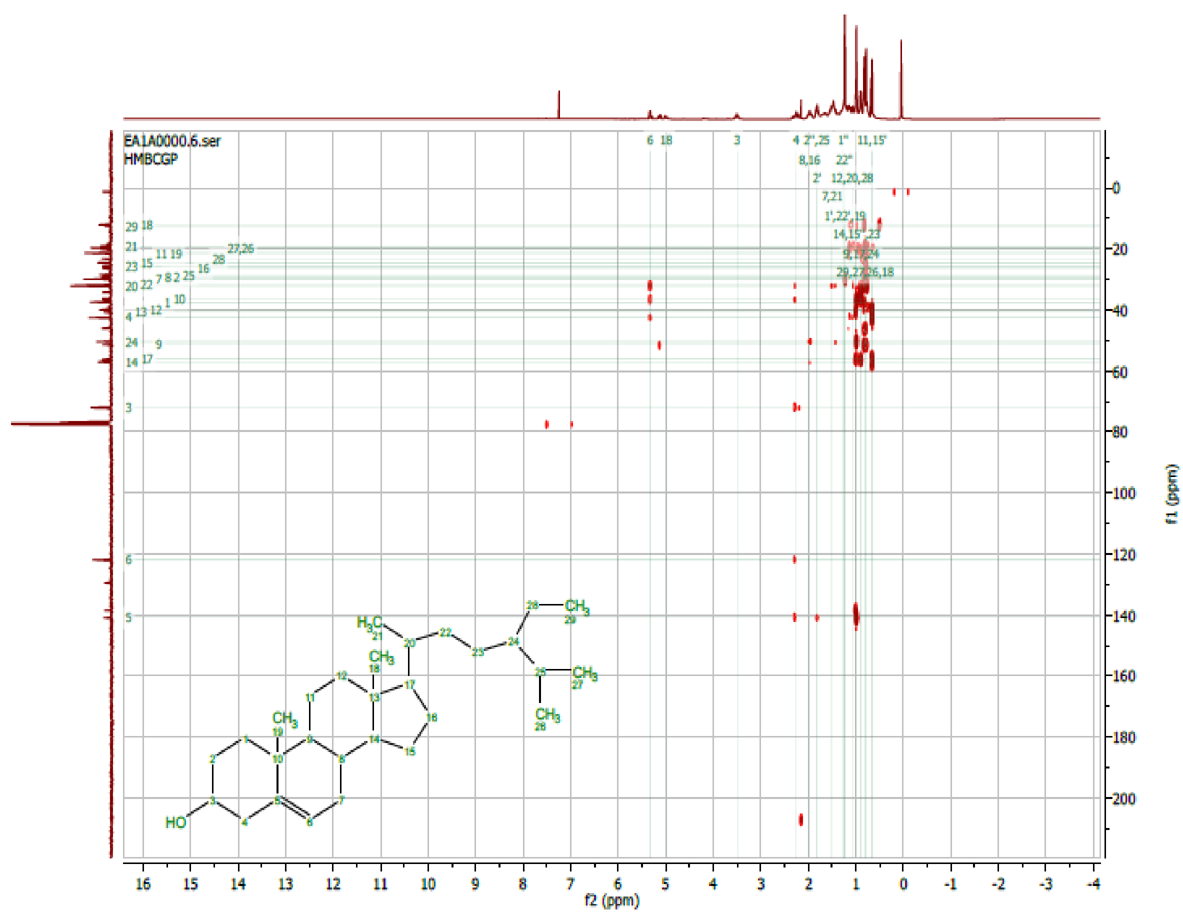


Appendix II (d): The ^1H-^1H COSY spectrum of compound EAC_1

Appendix II (e): The ^{1}H-^{13}C HSQC spectrum of compound EAC_1



Appendix II (f): The ^{1}H-^{13}C HMBC spectrum of compound EAC_1



Appendix III

[Probit Analysis Determination of MLD (LD_{99}) of *Naja nigricollis* venom]

```
PROBIT Mortality OF Total WITH Concn
  /LOG 10
  /MODEL PROBIT
  /PRINT FREQ CI

  /CRITERIA P (0.15) ITERATE (20) STEPLIMIT (.1).
```

Probit Analysis

Notes

Output Created		08-Dec-2014 16:31:05
Comments		
Input	Active Dataset	DataSet0
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	6
Missing Value Handling	Definition of Missing	User-defined missing values are treated as
	Cases Used	Statistics are based on all cases with valid data
Syntax		PROBIT Motality OF Total WITH Concn
Resources	Processor Time	00:00:00.312
	Elapsed Time	00:00:00.359

[DataSet0]

Warnings

Relative Median Potency Estimates are not displayed because there is no grouping variable in the model.

Data Information

		N of Cases
Valid		4
Rejected	Missing	0
	LOG Transform Cannot be Done	0
	Number of Responses > Number of	0
Control Group		2

	Number of Iterations	Optimal Solution
PROBIT	20	No[a]

a. Parameter estimates did not converge.

Parameter Estimates

	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concn	4.516	5.484	.823	.410	-6.232	15.263

	Intercept	-1.584	4.166	-.380	.704	-5.750	2.582

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Cell Counts and Residuals

Chi-Square Tests

		Chi-Square	df[a]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	.495	2	.781[b]

a. Statistics based on individual cases differ from statistics based on aggregated cases.

b. Since the significance level is greater than .150, no heterogeneity factor is used in the calculation of confidence limits.

	Number	Concn	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability
PROBIT	1	.699	10	9	9.420	-.420	.942
	2	.845	10	10	9.872	.128	.987
	3	.954	10	10	9.968	.032	.997
	4	1.041	10	10	9.991	.009	.999

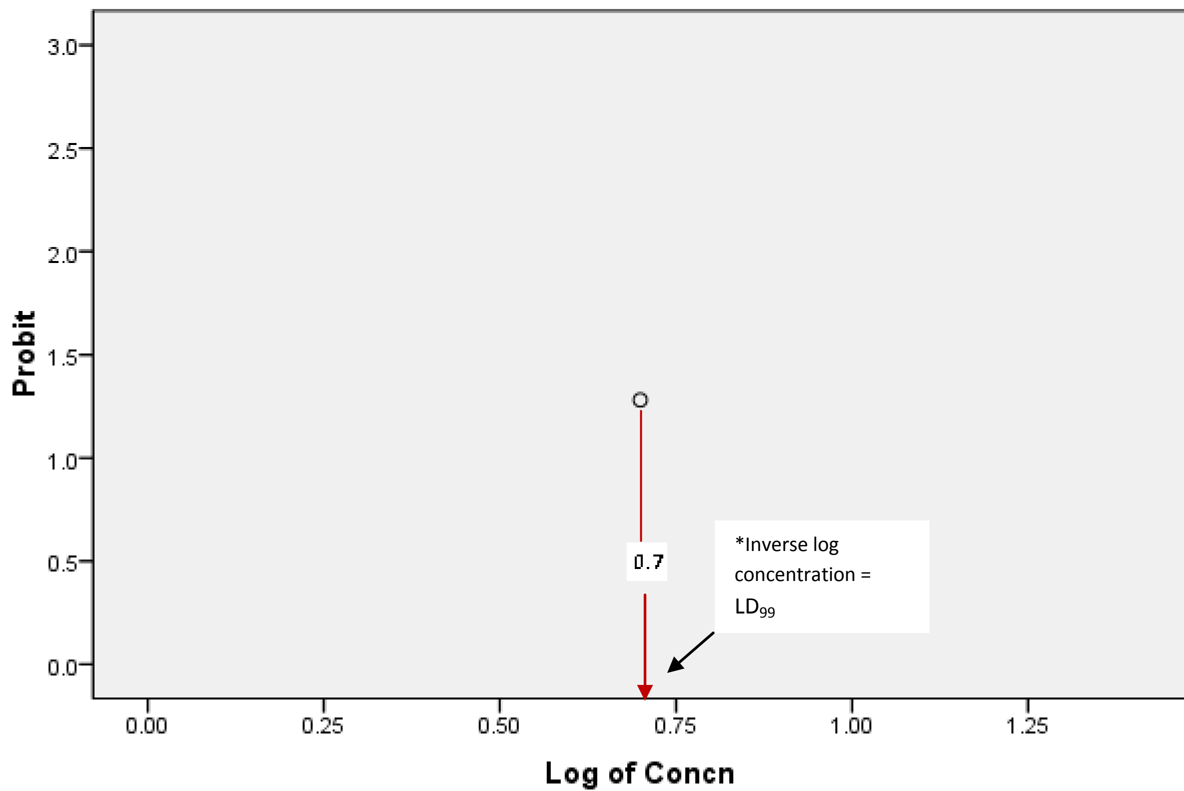
Confidence Limits

	Probability	95% Confidence Limits for Concn			95% Confidence Limits for log(Concn)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	0.01	.685	.	.	-.164	.	.
	0.02	.787	.	.	-.104	.	.
	0.03	.860	.	.	-.066	.	.
	0.04	.919	.	.	-.037	.	.
	0.05	.970	.	.	-.013	.	.
	0.06	1.015	.	.	.007	.	.
	0.07	1.057	.	.	.024	.	.
	0.08	1.096	.	.	.040	.	.
	0.09	1.132	.	.	.054	.	.
	0.1	1.167	.	.	.067	.	.
	0.15	1.322	.	.	.121	.	.
	0.2	1.460	.	.	.164	.	.
	0.25	1.590	.	.	.201	.	.
	0.3	1.717	.	.	.235	.	.
	0.35	1.843	.	.	.265	.	.
	0.4	1.971	.	.	.295	.	.
	0.45	2.104	.	.	.323	.	.
	0.5	2.243	.	.	.351	.	.
	0.55	2.391	.	.	.379	.	.
	0.6	2.552	.	.	.407	.	.
	0.65	2.730	.	.	.436	.	.
	0.7	2.931	.	.	.467	.	.
	0.75	3.164	.	.	.500	.	.
	0.8	3.445	.	.	.537	.	.
	0.85	3.805	.	.	.580	.	.
	0.9	4.312	.	.	.635	.	.
	0.91	4.444	.	.	.648	.	.

0.92	4.592	.	.	.662	.	.
0.93	4.760	.	.	.678	.	.
0.94	4.956	.	.	.695	.	.
0.95	5.189	.	.	.715	.	.
0.96	5.477	.	.	.739	.	.
0.97	5.852	.	.	.767	.	.
0.98	6.392	.	.	.806	.	.
0.99	7.345	.	.	.866	.	.

a. Logarithm base = 10.

Probit Transformed Responses



*Therefore, inverse log concentration of 0.7 = 5.0 = LD_{99} = MLD

Title of Journal Publications/ Manuscript from this Thesis

1. **Mathias, S.N**.; Abubakar, M.S. and October, N. (2016). Pentacyclic Triterpenoids from the Stem-bark of Albizia chevalieri Hams (Mimosaceae). *Int'l Journal of Pharmacy and Pharmaceutical Sciences,* Vol 8, Issue 5, 261-5.

2. **Mathias, S.N**.; Abubakar, K.; October, N.; Abubakar, M.S. and Mshelia, H.E. (2016). Antivenom Potentials of Friedelin isolated from Hexane Extract Fraction of *Albizia chevalieri* Hams (Mimosaceae*). Ife Journal of Science*, 18(2):473-482

3. **Mathias, S.N**., Abubakar, A., October, N., Abubakar, K. and Bulama, J.S. (2016). Antivenom Potentials of β-Sitosterol Isolated from the Ethyl acetate Extract Fraction of *Albizia chevalieri* Hams (Mimosaceae).

   (Accepted for publication: *Nigerian Journal of Biomedical Research, University of Maiduguri*)

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119,120,121,153-161,164

Black:

1. Preliminary pages
2. 1-48,51-62,63-65,67,
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   114,115,116,117,118,122-152,162,163,165