UNIVERSITY OF CAPE COAST

EXTRACTION, ISOLATION AND CHARACTERIZATION OF SOME LIMONOIDS AND SOME CONSTITUENTS OF THE ROOT BARK OF *TURRAEA HETEROPHYLLA* - SMITH (MELIACEAE)

AKROFI ROBERTSON

2011

UNIVERSITY OF CAPE COAST

EXTRACTION, ISOLATION AND CHARACTERIZATION OF SOME LIMONOIDS AND CONSTITUENTS OF THE ROOT BARK OF *TURRAEA HETEROPHYLLA* - SMITH (MELIACEAE)

BY

ROBERTSON AKROFI

THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY OF THE SCHOOL OF PHYSICAL SCIENCES, UNIVERSITY OF CAPE COAST IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR AWARD OF MASTER OF PHILOSOPHY DEGREE IN CHEMISTRY

JUNE, 2011

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my original work and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature: ------------------------------- Date: -------------------

Robertson Akrofi

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature: ------------------------ Date: -------------------

Professor F. S. K. Tayman

Co-Supervisor's Signature: ------------------------------- Date: -------------------

Professor Y. O. Boahen

ABSTRACT

The root bark of the plant *Turraea heterophylla* belonging to the family Meliaceae was investigated for its chemical constituents. Phytochemical screening of both the methanolic and methylene chloride extracts indicated the presence of tannins, saponnins, terpenes, steroids and about eight different limonoids.

Exhaustive extraction, chromatography and spectral analyses led to isolation of three compounds. The spectrometric analyses and the resulting data - including IR, NMR(H-H COSY and ^{1}HNMR) and the LC - ESI - MS spectra and their interpretation helped to propose the structures of the three compounds. Among the isolates, 2 compounds (**1&2**) are limonoids (tetranortriterpenoids) and the third compound **(3)** is a diterpenoid. All the compounds are known. The limonoids were elucidated and identified as rohituka-7(**1**) (ring A,B-*seco*), and obacunone **(2)** **(**A,D-*seco***)**. These tetranortriterpenoids are classified as two different carbon-frame types: (I) Prieurianin limonoid with ring A, B-*seco* (**1**), and (II) Obacunol limonoid with rings A, D-*seco*. The H-NMR shifts of the furan protons were in the range of δHs (7.95 to 6.0) and is typical of limonoids.

The plant extract had antimicrobial activity against the following bacteria: *Staphylococcus aureus, Proteus mirabilis* and *Pseudomanas aeruginosa.* The fungus *Candida albican* also experienced some growth inhibition.

ACKNOWLEDGEMENTS

I am very grateful to Prof. F.S. Tayman and Prof Yaw Opoku Boahen for their insight and assistance in research and editing. They proved to be helpful throughout my work. I owe them a lot of thank you. For the spectral interpretation and elucidation of the structure of the compound, Prof. Ernest Owusu Ansah painstakingly gave me ideas for the reporting of work and I am highly grateful. I am also indebted to Professors B.A. Dadson, D.K. Essumang, Very Rev D.K. Dodoo for their encouragement and advice. To all SRAs in the Chemistry Department, I am grateful. I am especially grateful to Dr Glenn Foulds and his group. They run the LC-MS analysis for me. I cannot forget my brother and a friend who meticulously did the ^{1}HNMR analysis for me, Mr Christian Adoko (South Africa).

I am also indebted to my wife, daughter, mother and three sisters, who prayed and motivated me throughout my research. I appreciate the backing and encouragement I received from all Church members of Apewosika SDA and J.K. Koomson. I am thankful to all the staffs of Chemistry Department, UCC for the various ways they supported me - Mr Nkrumah, Sis. Florence, Auntie Becky and Mad. Faith. Words are limited for the expression of gratitude to all who prayed and lent a hand to me. Summing up, may your effort be crowned with success and be blessed for all your sacrifices for me. I acknowledge the diverse ways you all helped me. Advice, encouragement, care, love and prayers filled your being and your heart for me. I am ever grateful for having you.

DEDICATION

This work is dedicated to my wife, Akosua O. Akrofi, Adwoa Essumanba Akrofi(daughter), mother, Mary Aba Essumanba, Sisters M. Agyiri, Veronica Akrofi, and Theresa Akrofi.

TABLE OF CONTENTS

Contents	Page
Declaration	ii
Abstract	iii
Acknowledgements	iv
Dedication	v
LIST OF TABLES	xi
LIST OF PLATES	xii
LIST OF FIGURES	xiii
LIST OF SCHEMES	xiv
CHAPTER ONE: BACKGROUND TO THE STUDY	1
Introduction	1
Meliaceae	3
The Plant *Turraea heterophylla* Smith (Meliaceae)	6
Statement Of Problem	9
Rationale For The Study	9
Objectives of Study	10
Scope of work	10
Organization Of Thesis	11
CHAPTER TWO: CHEMISTRY OF THE MELIACEAE	12
General Description (Definition)	12
Limonoids	12
Terpenes	15

Triterpenoids 17

Biosynthesis of Triterpenoids 17

Tetranortriterpenoids 23

Biosynthesis of Tetranortriterpenoids 24

Identification of Limonoids 26

Rearrangement of Tetranortriterpenoids 26

Types of Tetranortriterpenoids Found in Meliaceae 30

NMR Features of Terpenoids 32

Triterpenoids – Dammarane and Tirucallane 32

Tetranortriterpenoids – Mexicanolide 33

Other Limonoids and their NMR Features 34

Structures of the Trichilins 34

Reactions and Formation of Certain Limonoid Groups 35

Compounds Isolated from Meliaceae family (a) 38

Multidrug Resistance (MDR) Reversal Activity of Tetranortriterpenoids 47

Limonoids, Neuroprotective Compounds 49

Biopesticidal Activity 52

Pharmacological Activities of Limonoids 53

Anti-cancer Activity 53

Anti-malarial Activity 55

Anti-microbial Activity 56

Anti-HIV Activity 56

Other Miscellaneous Activity 56

Structure Activity Relationships 58

Plants from Meliaceae 60

Compounds Isolated from Meliaceae 63

LC-MS in Structural Elucidation of Limonoids and other Compounds 69

Limonoids Determined With APCI 71

CHAPTER THREE: EXPERIMENTAL 76

General Experimental Procedure 76

Plant Material 77

Extraction and Partition 77

Phytochemical Screening 78

Purification and Isolation 78

Extraction and Partition (Between 2006 and 2007) 82

Methods of Analysis 84

Bioassay (Antimicrobial activities of Plant Extract) 85

Media Preparation 85

Sterilization of Petri Dish 86

Preparation of Concentration 86

Preparation of Disc and Petri Dish For Bioactivity 86

Innoculation of Bacterial and Fungus 86

CHAPTER FOUR: RESULTS AND DISCUSSION 88

STRUCTURAL DETERMINATION OF THE ISOLATES 88

Introduction 88

Results 90

Mass Spectra/UV- Chromatogram And Their Retention Times 91

Compound 1: Structure Identification of Rohituka-7 97

Infrared Spectroscopy (Compound 2) 100

^{1}HNMR Of Compound 2 101

Bioassay 104

Results of Measurement of Zone Inhibition 104

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS 105

Conclusion 105

Recommendations 106

REFERENCES 107

APPENDICES 124

A: Antifeeding Activities of Limonoids from *Melia Azedarach* 124

B: Accurate ESI-MS spectrum of khayanone 126

C: ^{1}HNMR spectrum Of Rohituka -7 (C ^{1}HNMR (Band 1A)) 127

D: 2D HNMR Spectrum of Rohituka -7 (Band1A) ^{1}H-^{1}H COSY 128

C2: ^{1}HNMR Spectrum of Isolate 1A(Band 1A) 129

E1: 2D ^{1}HNMR of Isolate 2A(Band 2A) -^{1}H-^{1}H COSY 130

E2: ^{1}HNMR Spectrum Isolate 2A (band 2A) 131

F: LC - ESI - MS spectrum of Margocinin (GF1-1a) 132

G: Summary of ^{1}HNMR(Provided by analyst) 133

H:LC-ESI-MS spectrum of Rohituka -7(1) and Obacunone(2)(GF1-1B) 134

I: LC-ESI-MS spectra Of Isolate 1C (GF1-1C) 135

J: **LC -** ESI - MS spectra Of Isolate 1D (GF1-1D) 136

K: ^{1}HNMR spectrum Four[1] 137

L : Ionisation Sources Of Various Mass Spectrometers 138

M : ^{1}HNMR spectrum of Isolate Six(Band 6) 140

N: ^{1}HNMR spectrum of Isolate five (Band 5) 142

O: ^{1}HNMR spectrum of Isolate Four (Band 4) 144

P: An earlier HNMR spectrum of 1A[1] 146

B2: ESI-MS (positive ion mode) spectrum of 6 (khayanolide E) 148

Q: ^{1}HNMR spectrum of isolate 1a(Band 1- US) 149

R**:** ^{1}HNMR spectrum of Rohituka -7 [1b](US) 150

S: 2-D NMR Showing proton correlation of Rohituka -7 151

LIST OF TABLES

Table		Page
1	Common Classification of Terpene Groups	16
2	Refers to Limonoid Groups, from protolimonoids to Prieurianin group, are twelve (12) tables (I to X)	63
3	Shows molecular weight of limonoids determined with APCI	71
4	Phytochemical Screening of *Turrea heterophylla* extracts	78
5	Preparative Chromatography	82
6	Summary of HPLC-MS Analysis	85
7	HPLC-MS Analysis	90
8	Summary of ^{1}HNMR	93
9	Bioactivities of the extract on some microbes	104

LIST OF PLATES

Plate		Page
1	*Turraea Heterophylla* plant: leaves, seed and stem.	7
2	Entire plant: leaves, seed, stem and root	8
3	Inoculation of Bacteria and Fungus	87

LIST OF FIGURES

Figure		Page
1	Examples of limonoids from Meliaceae	8
2	Neuroprotective Limonoids	52
3	Representative EI/MS of citrus limonoids	72
4	Citrus Limonoids Analyzed with APCI/MS	73
5	Template structure identification with LC-MS	74
6	Summary of the work (2009) In flow Chart	81
7	Summary of the work for 2008	83
8	The compounds isolated, includes 1, 2, and 3	89
9	LC - ESI - MS spectrum of Margocinin	92
10	NMR Spectrum for Rohituka -7	95
11	LC - ESI - MS spectrum of Isolate 1B (Band 1B)	99
12	IR Spectrum of Isolate 1B	102

LIST OF SCHEMES

Scheme		Page
1	Origin of Terpenes	20
2	Biosynthetic Pathway Leading to the Formation of a Basic limonoid Skeleton	25
3	Biogenetic Map of Limonoids	28
4	Trichilin B	35
5	Oxidation To Form Ring D	36
6	Relationship Between the limonoid groups	39
7	Reaction of A/B to Form Carapolide E	48

CHAPTER ONE

BACKGROUND TO THE STUDY

Introduction

From the on-set of civilization dating from pre-historic times, medicine consisted mainly of application to herbs and concoction (potion). The use of herb dates from the time the first illness was seen. By definition, plant medicine and food as drug, originally, dates from antiquity. Since prehistoric time, alleviation of diseases has been one of the primary concerns of mankind. Local practitioners have used indigenous plants and herbs showing definitive pharmacological actions such as purgative, emetics, CNS stimulants and depressants for the treatment of a variety of ailments. Plants producing toxic effects were used in hunting or warfare, while plant-derived products like opium and hashish have long been used as hallucinating agents. Traditional medicines which evolved over the millennia through the acquired experience and accumulated knowledge of man for the beneficial effects of plant materials have served mankind for long in the treatment of various ailments.

The origin of modern medicine goes back to the Greek, followed by Romans. It was then taken over by the Arabs from whom, after its enrichment with Chinese and Indian medicine, it was taken over by medieval Europe. The Muslim rulers introduced it in India and incorporated with it the native Ayurvedic medicine. This mixture is now known as Unani medicine or Eastern medicine (Tibb-e-Mashrique). The earliest traces of therapeutic use of plants are recorded in the Rigveda and Ayurveda, which were compiled between 4500-1600 and 2500-

600 BC respectively. Charaka cited about fifty groups of herbs while Sushruta has explained 760 herbs in 37 sets.

The seeds of Chinese medicine, sown by Fu Hsi (2953 B.C.) flourished under the Chang dynasty. The emperor Shen Nung (2735 BC) himself compiled a Pharmacopoeia, "Pen Tsao" on medicinal plants. He reported antifebrile effects of drug "Chang Sheng" (Dichroa febrifuga), that has now proven to contain anti-malarial alkaloids.

The stimulatory and diaphoretic effects of the drug Ma Huang (Ephedra sinica), known as ephedrine alkaloids are still derived from this plant and its related species. Most of the Chinese traditional medicinal literature is found in "Ben Cao Gang Mu" and "Nei Ching". The father of medicine, Hippocrates (460 BC) laid the foundation of Greek Pharmacy. He described and named nearly 400 samples as medicines. Thus, primitive practice of treating diseases with herbs potions, referred to as traditional medicine, is the sum total of all the knowledge and practices explicable or not, used in diagnosis, prevention and elimination of physical, mental and social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing. Contemporary medicine has its origin from traditional medicine. The latter existed long before the application of science and technology to medical practice.

Like herbal medicine, the plant, *Turraea heterophylla,* has been very useful traditionally in folk-medicine for many years. For instance, this plant is used in parts of Ivory Coast for Asthma, emphysema (i.e. loss of elasticity of lungs), lumbago and headaches, the root-pulp in water being used as an enema. In Ivory

Coast, an enema from the root decoction is used for an illness caused by trypanosomes. A root extract is used by the Krobos for stomach troubles and as a tonic. The juice from the pulped bark is used as nasal or eye drop and leaf-pulp is used as liniment, the leaf-juice as eye drops. A decoction of leaf tips is used as a drink or for baths for fever by the Anyis (Ivory Coast), who also use the pulped roots, with Guinea-grains , as a liniment for fever (Kerharo & Bouquet) . In Ghana, the leaves are considered to be of considerable medicinal value and are used for bitters put in rums and drunk by men for impotency (Irvine, 1961). The above mentioned plant belongs to the Meliaceae family. Summarily, this plant is one of the most popular medicinal meliaceous plants in traditional African remedies, used as a bitter tonic, folk and popular medicine against malaria, fever, mucous diarrhea, and venereal diseases as well as an anthelmintic and a taeniacide remedy. Its extracts and chemical constituents have been the subject of extensive phytochemical and pharmacological investigations.

Turraea heterophylla belongs to the family Meliaceae. Encyclopaedia Britannica, Academic Edition, (2010) refers to the Meliaceae**,** as the mahogany family of flowering plants, of the order Sapindales, comprising 51 genera and about 575 species of trees and (rarely) shrubs, native to tropical and subtropical regions. Most members of the family have large compound leaves, with the leaflets arranged in the form of a feather, and branched flower clusters. The fruit is fleshy and coloured or a leathery capsule. The China tree (*Melia azedarach*), also called chinaberry, bead tree, and Persian lilac, is an ornamental Asian tree with round yellow fruits, often cultivated in many tropical and warm temperate areas. Trees of the genus *Swietenia* and *Entandophragma,* commonly called mahogany,

and of the genus *Cedrela* (especially the cigar-box cedar, *C. odorata*) are economically important timber trees. The neem, or nim tree, also called the margosa tree (genus *Azadirachta*), grown throughout the Old World tropics, notably in India and Southeast Asia, is a source of timber and medicinal oils and resins. Plants in the Meliaceae family are a rich source of triterpenoids and tetranortriterpenoids (known as limonoids)). This family is extremely useful to man for the high quality timbers, ease with which some species can be grown in plantations (Pennington & Styles, 1975) and bioactive compounds isolated. Pennington and Styles (1975) in their *Generic Monograph of the Meliaceae* divided the Meliaceae into four subfamilies. The Ptaeroxylaceae is a small family comprising two genera, *Ptaeroxylon,* a monotypic genus comprising only the Southern African species *Ptaeroxylon obliquum*, commonly known as the sneezewood, and *Cedrelopsis,* a genus limited to Madagascar containing several species. Previously *Ptaeroxylon* has been placed in the Sapindaceae, the Rutaceae, and most commonly, the Meliaceae family. They have wide-range uses in ethnomedicine, prompting extensive investigation. Compounds isolated from the Meliaceae family, include limonoids, mono-, di-, sesqui-, and triterpenoids, coumarins, chromones, lignans, flavonoids and other phenolics. Relatively few of the compounds and extracts from these species have been screened for biological activity, probably due to the limited screening facilities available. However, properties including cytotoxicity against tumour cell lines, insect anti-feedant and anti-malarial activity, and uterotonic activity suggest that further extensive biological screening of compounds from this family is warranted. Similar chemistry of the genera *Ptaeroxylon* and *Cedrelopsis* supports their grouping

together in the distinct family Ptaeroxylaceae. Examination of the chemistry of species from this family suggests a close relationship with the Cneoraceae family (Mulholland *et al.,* 1988). The Meliaceae family has 51 genera and it is explained in the review, that, some 22 of the total number occur in the Southern and Eastern Africa and Madagascar including representatives from all four subfamilies.

Studies have been carried out on the whole root to find the chemical constituents of the plant. However, the root bark and root wood have not been investigated separately on their own. The plant has been used locally in many treatments; stomach-ache, asthma, emphysema, impotency and lumbago in Ghana and in West Africa. The preliminary test or phytochemical screening of the methanolic extracts of the root bark and wood, revealed the presence of Tanins, Saponins, Terpenes, Steroids and Limonoids. Limonoids are known for their special medicinal and agricultural values. Limonoids have been used as agent for anti-cancer and anti-insect activities. Blood disorders, hepatitis, ulcers, diabetes, boils, malaria, cholera, measles, snakebite, rheumatism, and syphilis are among many disorders which plant preparations from the family Meliaceae, including *Azadiracta indica, Turraea heterophylla*, *Khaya senegalensis* etc have been used to treat. Typical examples of limonoids are shown in Figure 1. The health benefits of limonoids have made scientists find methods to synthesize them in laboratory (Fernandez-Mateos *et al.,* 2002). Patents have been obtained for industrial scale method for manufacturing limonoid glucosides contained in citrus fruit (Hasegawa *et al.,* 2005.) Scientists are trying to design food products, fortified with limonoids to provide prophylactic benefits against cancer and many other diseases (Charleston, 2002).

Methods have also been established to purify limonoids (Sunthanont *et al.,* 2005) and increase their yields through better extraction procedures (Miyake *et al.,* 2005). But caution has been advocated in consumption of limonoids as they may interfere with activity of other drugs (Lien *et al.,* 2004) or may even produce harmful effects if consumed in very high quantities (Gibbins *et al.,* 2005). But except for a few exceptions in most of the studies, long term consumption of limonoids have produced no adverse effects and have been found to be safe (Jacob *et al.,* 2000; Manners *et al.,* 2003). It has also been suggested that limonoids may interact with other bioactive components present in fruits and vegetables and may reduce the risk of degenerative diseases, hypertension, cataract and stroke and in particular cancers (Silalahi, 2002). Hence, the reason for this work.

Turraea Heterophylla belongs to the family Meliaceae and the Order Rutales. The family is also known for the famous bioactive chemical, the limonoids. Plants known to contai+n these compounds have been useful generally. *Turraea heterophylla* is locally known in Twi as AHUNANYANKWA (literally translated as 'when you see it, you get life'), because of its valuable medicinal qualities. The Ada's refer to it as Owunyunkwa(Thomas). Their habitat is undergrowth in closed and secondary forests in wet condition.

The Plant *Turraea heterophylla* Sm (Meliaceae)

The plant (Plate 1) is found in many places in Ghana. Colony: Abokobi, Dawa Mate Kole , Achimota, Accra Plains, Aburi Road, Cape Coast, Ashanti, Kumasi, Banso, Jimira Res. and Mampong. Also, Togo, Plateau Res., St Cl.; distribution in West Africa, begins from Sierra Leone to Nigeria. It is also found in Uganda in Central Africa. The *Turraea heterophylla* of the family Meliaceae is a

plant found in the tropics, sub-tropic and Southern Africa, Madagascar, Masc, and Asia to Austria. *Turraea heterophylla*, is an evergreen shrub up to 6ft. in height (Plate 2). Its leaves vary greatly in shape, 4x2 in. oblong or obviate, sinuate lobulate margins, with 4-5 pairs of lateral nerves. It flowers between February and March, white, sweet-scented, solitary to few, petals 1in. long, with united stamens 1in. long stigma exerted, club-shaped. It bears fruits over ½ in. long in March. The seeds are shiny black with red fleshy arils. The plant is of ornamental value because of its sweet-centred white flowers. The wood is considerably harder than Mahogany.

Plate 1: *Turraea Heterophylla* plant: leaves, seed and stem.

Plate 2: Entire plant: leaves, seed, stem and root

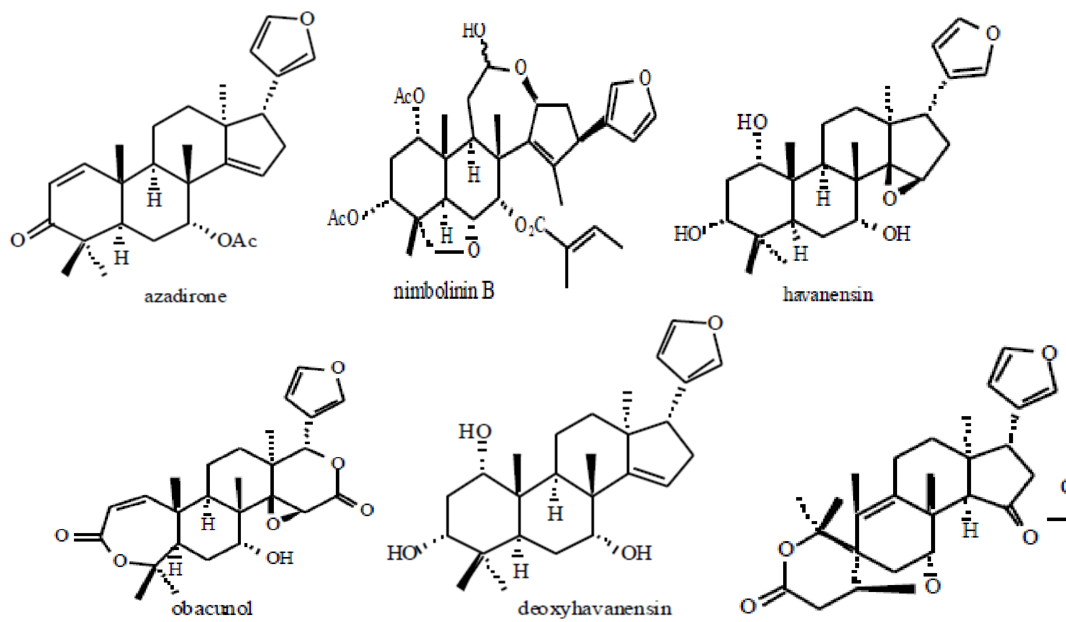


Figure 1: Examples of limonoids from meliaceae

Statement of problem

Plant diseases often reduce quality and quantity of agricultural commodities. In fact, plant pathogens are estimated to cause yield reductions in crops of almost 20% worldwide. The fungicides made by synthesis provide the primary means for controlling post-harvest fungal decay of cereals, fruits and vegetables. On the other hand, the extensive use of these synthetic fungicides causes uncontrolled residues and proliferation of resistance in the pathogen populations. Therefore, studies concerning the possible use of biologically active natural products to control decay and prolong storage life of crops. Besides agricultural pests, pathogens have developed resistant strain for many synthetic drugs.

Rationale for the study

. Compounds obtained from plants are found to be potent against the pests, fungi, malaria- parasites and human disease parasites, that develop resistant strains. Recently, plant extracts and phytochemicals with either antibacterial or antifungal properties have been investigated actively as alternatives to synthetic pesticides due to their perceived increased level of safety and minimal environmental impact. The plant, *Azadirachta indica and* many others belong to the family meliaceae as *Turraea heterophylla* and are ethnomedically very useful, treating diverse local diseases and problems assosiated with pests, human diseases -including cancers. Specifically, various parts of the plants, T. *heterophylla* are used locally for Asthma, Emphysema - loss of elasticity of lungs, Lumbago – Back ache, Headaches, Impotency- treated with bitters in Ghana and Fever bath - among the Anyis (Ivory Coast) treats stomach troubles and tonic(Krobos)

Objectives of Study

1) To isolate some major chemical constituents from *Turraea heterophylla.*
2) To identify some isolated constituents by means of spectroscopic methods; NMR, UV-chromatogram, IR, and mass spectrometry (LC-ESI-MS).
3) To screen the crude extract from the plant against some parasites (bacteria).

Scope of work

The study involved collecting plant sample, turraea heterophylla from Abrafo near the Kakum forest. the root bark of the plant is peeled off or scraped, dried and ground into powder. The powdered plant is extracted with Hexane, Dichrolomethane and methanol on the mechanical shaker. Thin layer chromatography(TLC) is done on the extracts to determine the number of component of compounds present and the solvent system respectively. Isolation of the compounds is followed using flash chromatography. Further purification is done on isolates using preparative thin layer chromatography and repeated to obtain the pure compounds. The pure isolates are analyzed in the US and South Africa respectively using liquid chromatography mass spectrometer (LCMS) and the Nuclear Magnetic Resonance Spectrometer. The LC - ESI - MS, ^{1}HNMR, ^{1}H-^{1}H correlation spectra results are interpreted and deduced into the structure of compounds Rohituka-7, Obacunone and Margocinin. The crude extract of the plant root bark is also subjected to bioactivity to find their effect on some microorganisms, *Staphlococcus aureus, Proteus mirabilis*, *Candida albican* and *Pseudomonas aeruginosa*.

Organization of thesis

This thesis is organized as follows chapter one gives the background of research, statement of problem, rational for study and research objectives. Chapter two reviews limonoids, their sources biosynthetic paths, relationship of the limonoid groups and their uses. The general experimental procedures, extraction of root bark chromatographic separation, analyses of compounds employing the mass spectrometer and the NMR analyses are in chapter three. In chapter four, the ^{1}HNMR, ^{1}H-^{1}H correlation spectra and LC-ESI-MS results are interpreted and compounds are elucidated and discussion of the whole results. The findings will be summarized in the conclusion and recommendations are made in chapter five.

CHAPTER TWO

CHEMISTRY OF THE MELIACEAE

This study focuses on the constituent of the root bark of *Turraea heterophylla* Sm., belonging to the family Meliaceae and genus, *Turraea* which contains limonoids as a major constituent.

General Description (Definition)

Plants in the Meliaceae family are a rich source of triterpenoids and tetranortriterpenoids (known as limonoids or meliacins). Previous works have established a wide range of biological activities from these compounds such as insect antifeedant, growth inhibition, bactericidal, antiviral effects and many others (Chong *et al.,* 2008). This review will look at some of the biological activities mentioned above as well as cytotoxicity tumour (cancer), classification of limonoids, formation or some reactions in this group of compounds. It will also review some spectroscopic works on limonoids and give a general overview of LC-ESI-MS which is new and very little is known about it in the country.

Extraction and isolation methods make up a bulk of recent publications, with the ultimate study of the compounds as to biological activities exhibited towards insect species. Therefore a brief overview about limonoids and studies carried out on that is useful.

Limonoids

Limonoids are an important group of metabolically altered triterpene, which are limited in their distribution. In recent years, a large number of pharmacological studies have been carried out to indicate their beneficial effects. The medicinal properties reported include anti-cancer, anti-malarial, antimicrobial,

anti-HIV, anti-viral and several others. Post-study health evaluation has established no ill effects among study subjects consuming high doses of limonin glucoside. Some of them have also shown allelopathic potential. The citrus limonoids exhibit promising health benefits (anti-cancer, cardio-protective, anti-oxidant etc.) but are the major cause of concern due to their extreme bitterness. There is a need to develop an acceptable and versatile debittering method that can substantially remove or mitigate the bitterness of fruits and juice, for example, the LARL and NARL methods of detecting bitterness (Breksa III *et al.,* 2005). *Azadirachta indica*, a related plant , *Melia azedarach* along with several other plant species belonging to the Meliaceae family are a store-house of limonoids like azadirachtin and other related compounds that have feeding deterrent, insect-repellant, anti-hormone and other insect control properties against the bulk of insect pests. Amit Roy's group, in concluding a paper on limonoids research, reported that these plant species are also useful as larvicides for destroying larvae of the *Anopheles* mosquito. And it provides an impetus to evaluate these compounds alone or in combination to identify their potential in commercial formulations that can be used as bio-pesticides in integrated pest management. However, because these are high cost biochemicals and at the same time complexity of structures precludes their synthesis, biotechnology and tissue culture techniques may be extensively investigated to enhance their production to meet the increasing demands. Regarding the biological activities of limonoids, the focus is directed towards detailed characterization, quantification, and designing a simple as well as versatile synthetic route of apparently important limonoids. Extraction methods too should be optimized; evaluation and establishment of pharmaco-

dynamic and kinetic principles, and structure activity relationships should be a key goal associated with limonoids so that they can be safely introduced in our arsenal of pharmaceuticals to safeguard the humanity from the wrath of disease and its discomfort (Roy *et al.,* 2006).

Limonoids are tetranortriterpenoids derived from euphane (H-20β) or tirucallane (H-20α) triterpenoids with a 4, 4, 8-trimethyl-17-furanylsteroidal skeleton (Siddiqui *et al.,* 1988). Over 300 limonoids have been isolated to date, and they are the most distinctive secondary metabolites of the plants in the order Rutales. Particularly, they characterize members of the family Meliaceae, where they are abundant and varied (Connolly *et al.,* 1983; Taylor *et al.,* 1983). Almost every part of the tree of this family has been used in folkloric and traditional systems of medicine (Kokwaro *et al.,* 1976; Watt *et al.,* 1962). Recent work has established a wide range of biological activities for these compounds, a variety of medicinal effects in animals and humans, and antifungal, bactericidal and antiviral activities. The biological activities of limonoids from Rutales have been reviewed (Champagne et al, 1992). In particular, the limonoids from the neem tree *Azadirachta indica* Juss and the Chinaberry tree *Melia azedarach* Linn. have attracted considerable interest because of their marked insect antifeedant properties and intriguing structural variety. The most potent insect antifeedant include azadirachtin and related highly oxidized C-seco limonoids from *Azadirachta indica*. Their antifeedant activities and structure–activity relationships have been reviewed (Rembold, 1988; Rembold, 1989; Ley, 1993).

Similarly, *Melia azedarach* is known to produce azadirachtin-type limonoids that are a potent antifeedant such as the meliacarpinins, and C-19/C-29

bridged lactols and acyl acetals such as the azedarachins, and trichilins. Nakatani's group investigated the limonoid constituents of *M. azedarach* and some related plants and evaluated the antifeedant properties of more than 30 limonoids in this work, which include all of the types of limonoids isolated from *M. azedarach* excluding glycosides. They included apo-euphol and C-seco limonoids. The structures and antifeedant activities of these compounds are detailed in the sections below.

Terpenes

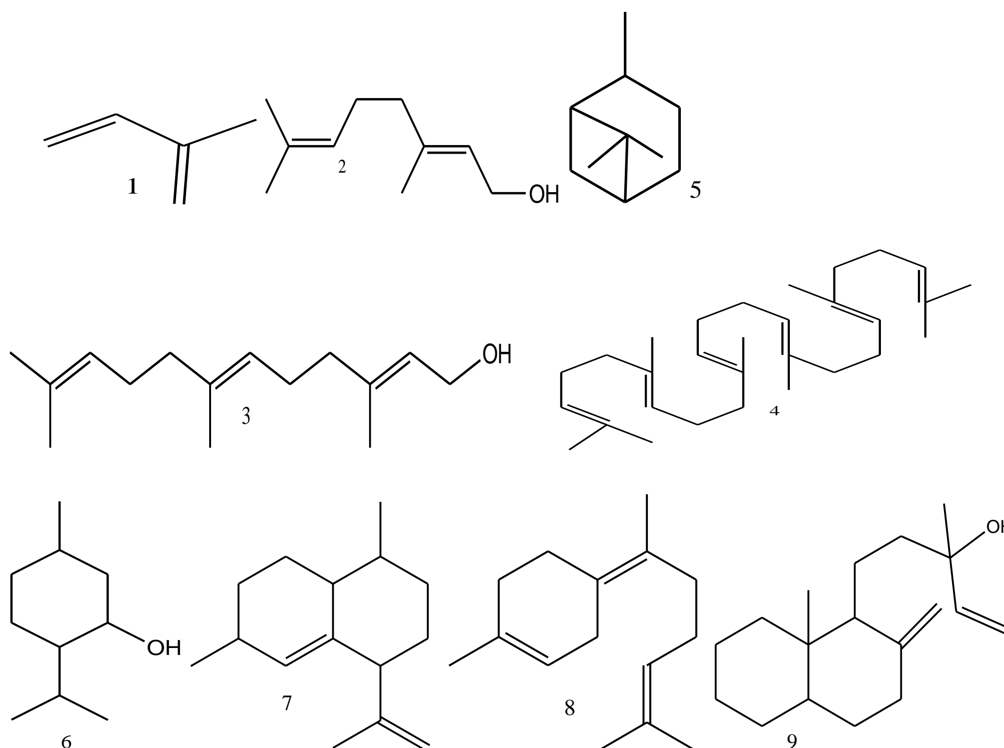
Terpenes constitute the largest known group of plant secondary metabolites. Terpenes are natural products structurally related to 2-methylbutadiene **1** or better known as isoprene. An isoprene unit is the carbon skeleton of isoprene (ignoring double bonds). In general, the term "terpenes" refers to terpenes and terpenoids. Terpenoids are modified terpenes, where methyl groups are moved or removed, or oxygen atom added. According to the isoprene rule, monoterpenes are defined as a compound having carbon skeletons with two 5-carbon isoprene units, sesquiterpenes with three isoprene units and so on. This

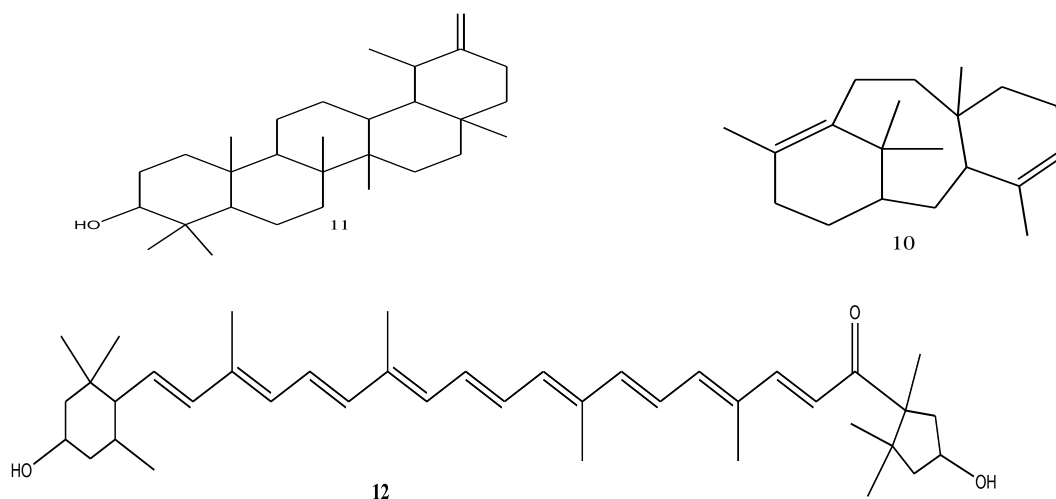
Table 1: Common Classification of Terpene Groups

Group	No. of carbon	Isoprene units	Examples
Monoterpene	10	2	Pinane **5**, menthol **6**
Sesquiterpene	15	3	Arteannuic acid **7** bisabolene **8**
Diterpene	20	4	Manool **9**, taxadiene **10**
Triterpene	30	6	Taraxasterol **11**
polyisoprenoids	> 30	> 6	Capsanthin **12**

(Source: Bruneton, 1999; Source modified: Chong Soon Lim, 2008)

classification is listed in **Table 1**. Among all possible structures of a terpene, the favoured one can be derived mechanistically by the cyclization of an aliphatic precursor such as geraniol **2**, farnesol **3** and squalene **4**. Each group of terpenes arises from the head-to-tail condensation of a variable number of isoprene units. (Bruneton, 1999 & Cseke, 2006)





Triterpenoids (Jean Bruneton, Springer, 1999)

Triterpenoid is a group of terpenes that comprises thirty carbon atoms. The triterpenoids-over 4000 compounds built upon over 40 different skeletons - form the largest group among the terpene classes, and are widely distributed in the plant kingdom, either in the free state or as esters or glycosides. Plants contain a huge variety of cyclic triterpenoids that do not occur at all in animals and fungi. These triterpenoids play an important role mediating plant-plant, plant-insect and plant-pathogen interactions. The corresponding biosynthesis is discussed in the following section.

Biosynthesis of Triterpenoids (Jean Bruneton, Springer, 1999**)**

Triterpenoids are biosynthetically derived from squalene **4**. A significant number of triterpenoids are believed to be cyclization products synthesized via the intermediate oxidosqualene **13**. Almost always hydroxylated at the 3-position (because they arise from the opening of the epoxide), triterpenoids present very high structural homogeneity. The major differences are in configuration and are linked to the conformation adopted by the squalene prior to cyclization; the cation

resulting from this cyclization can subsequently undergo a series of 1, 2 proton and methyl group shifts (Wagner-Meerwein rearrangement), which can be used to rationalize the occurrence of the different tetra- and pentacyclic skeletal characteristic of this group. The opening of the intermediate oxidosqualene **13** initiates the cyclization. This initial step is protonation-dependent. The initial conformation of the oxidosqualene 13 will determine the orientation of the biosysnthesis towards either steroids or triterpenes. If oxidosqualene 13 is suitably positioned and folded on the enzyme surface, a series of cyclization will occur. It is then followed by a sequence of concerted Wagner-Meerwein migrations of methyls and hydrides. The protonation of the epoxide group will allow opening of the ring. In typical triterpene cyclization, twelve to twenty covalent bonds are broken or formed, seven to nine chiral centres are established and four to five rings are built. There are four possibilities of biosynthesic pathways leading to the final product:

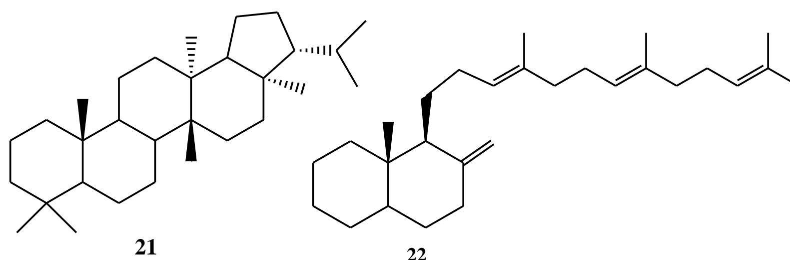
a) The oxidosqualene 13 is maintained in a chair-boat-chair-boat conformation. In this case, the cyclization leads to a protostane cation 14, which is the immediate precursor of cucurbitacines **15** and cycloartanes **16**, by a series of Wagner-Meerwein 1, 2-proton and methyl group shifts. These shifts are made possible by the trans-antiparellel arrangement of the protons and methyl groups in the 17-, 13-, 14-, and 8-positions. The migrating groups are positioned *anti* to each other, one group entering while the other leaves from the opposite side of the stereocentre. This migration inverts configurations at each appropriate centre. The reaction is terminated by the loss of proton H-9, as there is no *anti* group available to migrate to C-9. About eleven cyclization products of this group are known. Following the

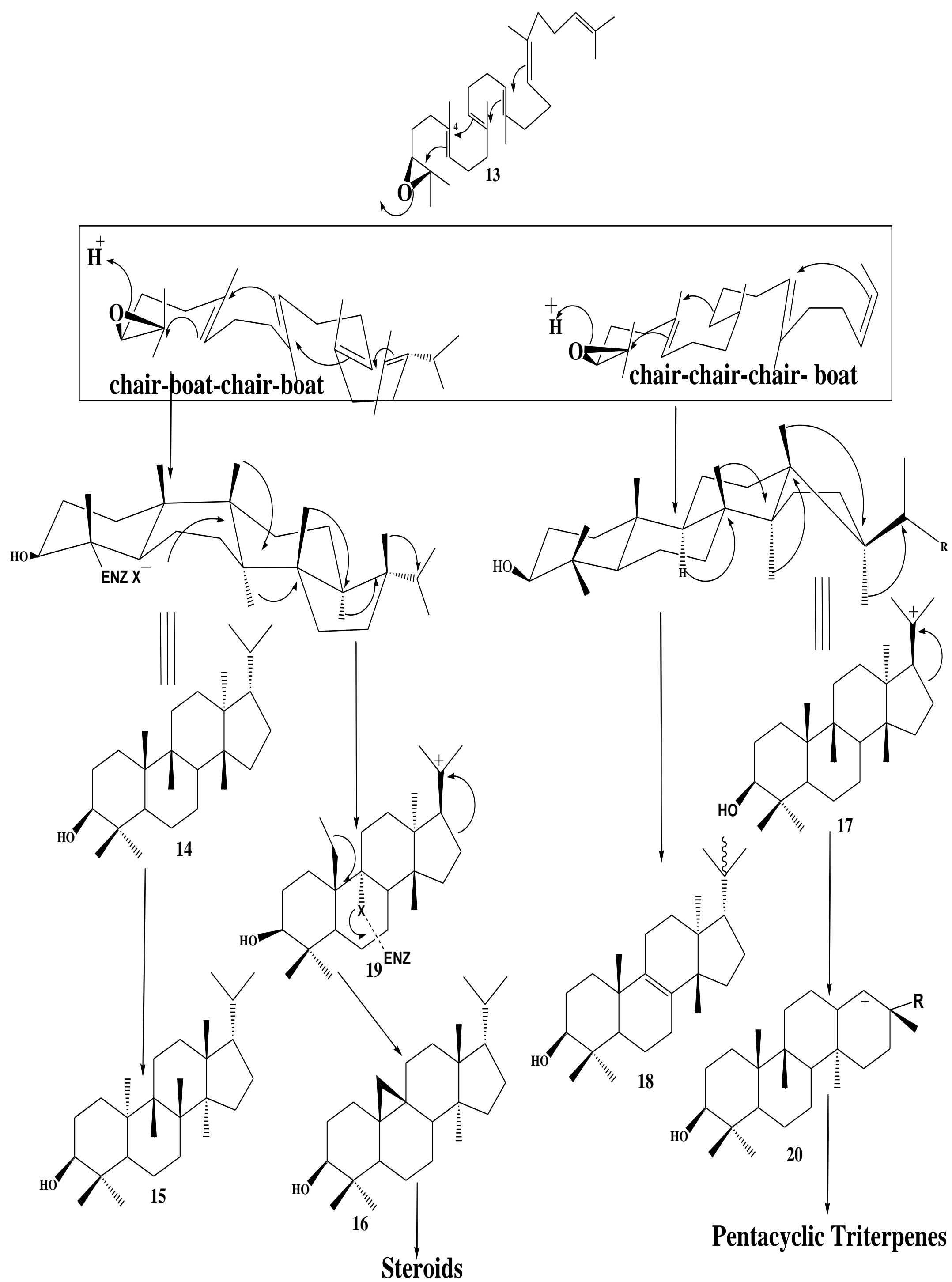
carbon skeleton rearrangement, the methyl groups at C-13 and C-14 have a configuration opposite to that of dammarane derivatives (Scheme **1**).

b) The oxidosqualene 13 is maintained in a chair-chair-chair-boat conformation. The identical carbocation mechanism as above followed, and the intermediate dammarane cation **17** was formed. The dammarane cation **17** will typically undergo further carbocation promoted cyclizations either by concerted migrations leading to tirucallol **19** and euphol **18**, the precursors of limonoids and quassinoids; - or, in most cases, by the formation of an extra ring, leading to pentacyclic triterpenes **20**, - or, in rare cases, to form tetracyclic compounds with a six-membered D ring (Taraxasterol 11).

c) A special case is of triterpenes devoid of a hydroxyl group in the 3-position. They are generally derived from the direct cyclization of squalene, e.g. fernanes **21** (the conformation of the precursor is of the chair-chair-chair-boat type).

d) In some cases, the cyclization is only partial (polypodatetraenes **22**), or on the contrary it is complete, and completely concerted or even initiated from both ends of the precursor. Some unusual structures have also been described, especially in the animal kingdom (siphonales from the Spongie).

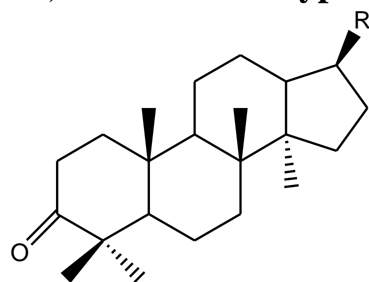


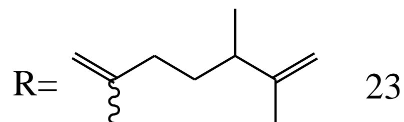


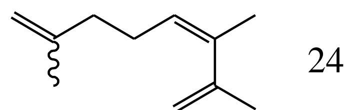
Scheme 1: Origin of Terpenes (source: Chong Soon Lim, 2008)

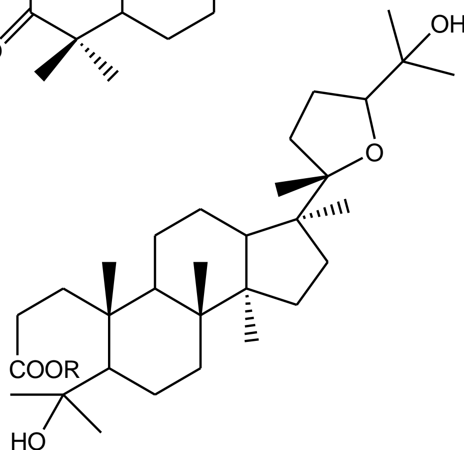
Types of Triterpenoids In Meliaceae

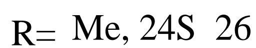
i) **Dammarane Type**

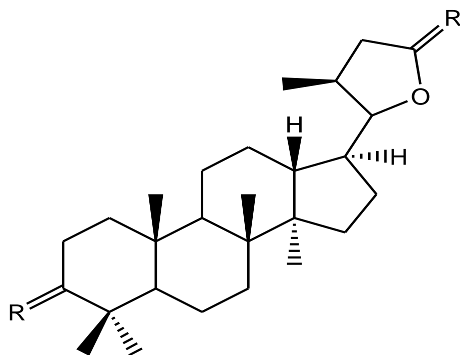


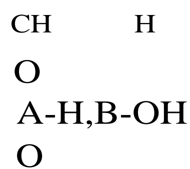


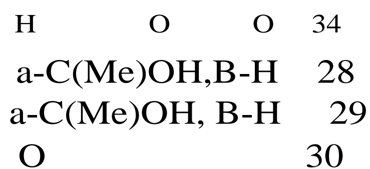




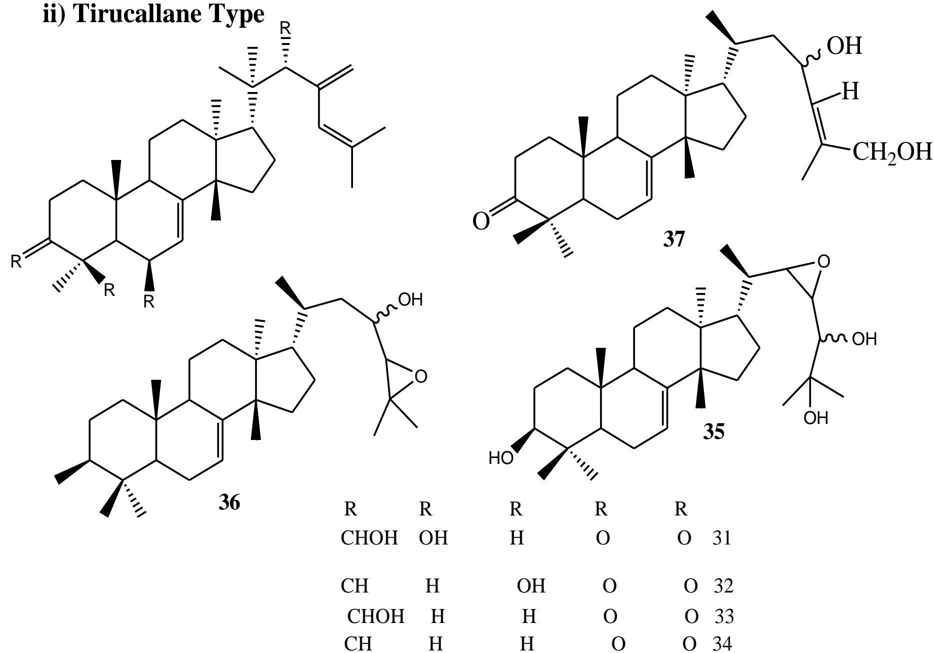




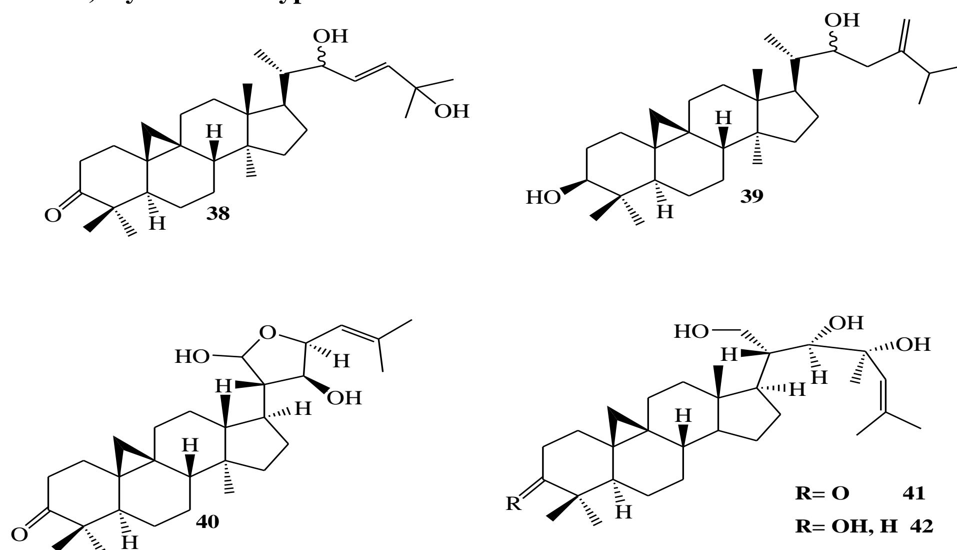




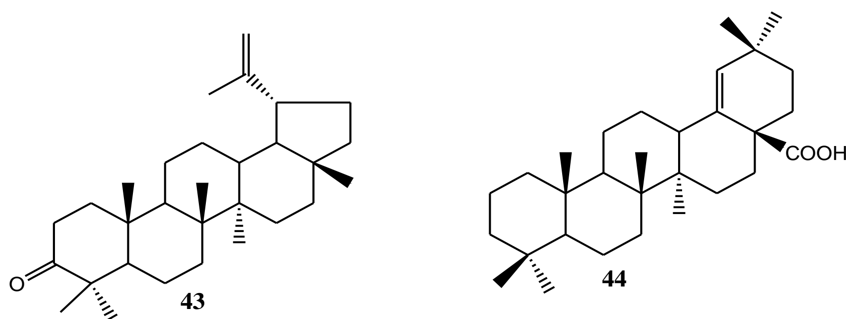
ii) Tirucallane Type



iii) Cycloartane Type



Pentacyclic Triterpene

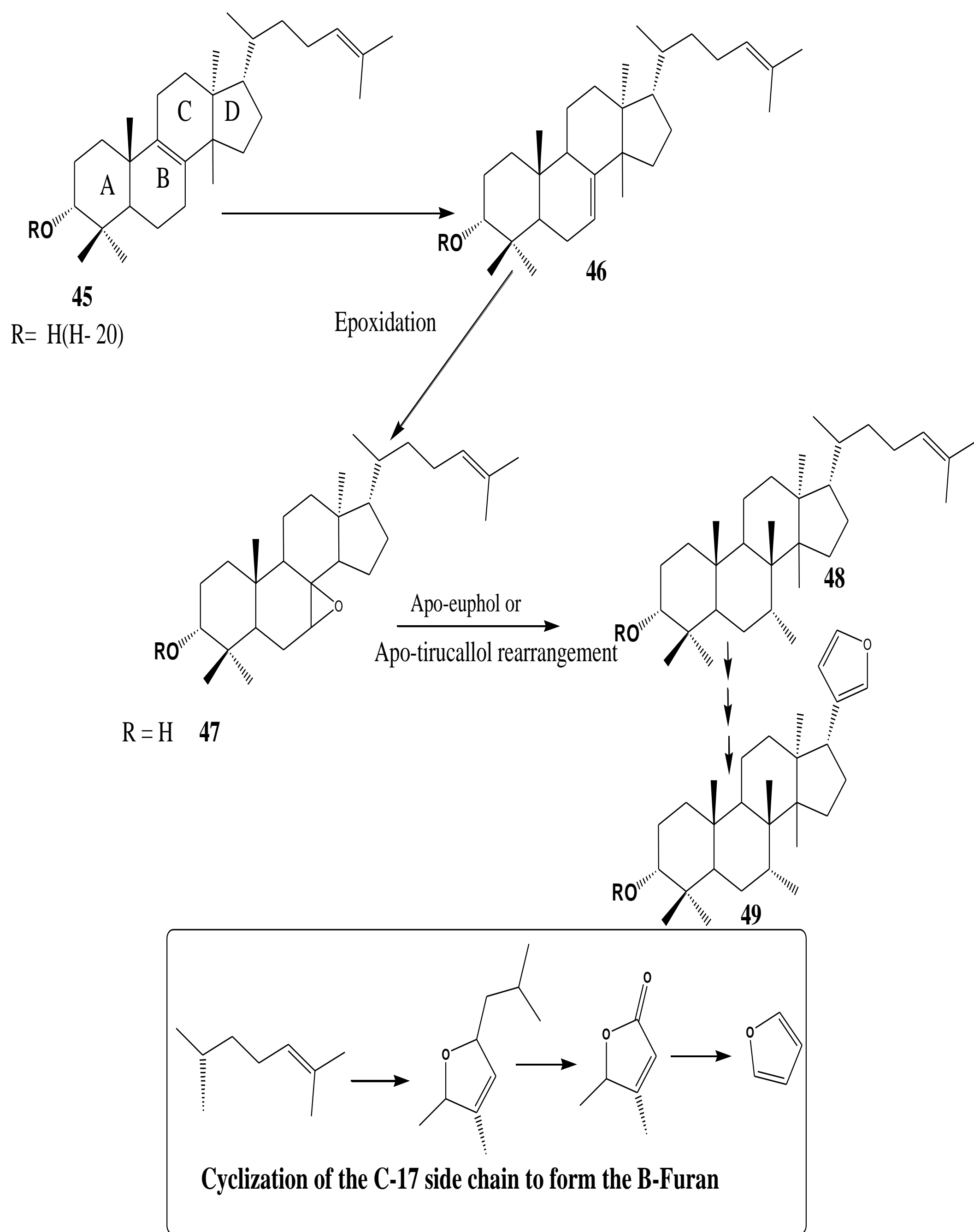


Tetranortriterpenoids

Tetranortriterpenoids are degraded triterpenoids, in which four terminal carbons from the side-chain are removed. Despite the term 'degraded', this type of compound is structurally more advanced than triterpenoids. It is capable of achieving profound modifications of the initial tetracyclic skeleton through oxidation, lactonization, ring opening and closure, side chain elimination, functionalizations of the angular methyl groups, and many others. Tetranortriterpenoids are derived from the metabolism of a 4,4, 8-trimethyl-17-furanylsteroid skeleton **48**. To date, over 300 tetranortriterpenoids have been isolated. Tetranortriterpenoids - also known as limonoids - are confined to plants in the order Rutales; Rutaceae, Meliaceae, Cneoraceae, and Simaroubaceae. In particular, they characterize members of the family Meliaceae, where their skeletons are structurally diverse and abundant. Up to mid 1982, 51 (out of about 550) species from 20 (out of 51) genera had been shown to contain such compounds. A more limited range of structures is found in the family Cneoraceae. This class of compound has attracted considerable study because of its cytotoxic, antimalarial, and amoebicidal properties.(Dewick,2002; Taylor, 1984).

Biosynthesis of Tetranortriterpenoids

Tetranortriterpenoids are thought to arise from Δ7–tirucallol [H-20, C-20(R)] **45** or Δ7–euphol [H-20, C-20(S)]. The stereochemistry of the precursor is unknown but tirucallol **46** is generally accepted as the precursor. According to the generally accepted scheme **2,** the Δ7 –bond is epoxidized to a 7–epoxide, which is then opened, inducing a Wagner-Meerwein shift of Me-14 to C-8 position, formation of the 7-OH, and introduction of an unsaturation at C-14/15 **48**. The Scheme 2 accounts for both the ubiquitous presence of oxygen at C-7 and the correct stereochemistry of the C-30 methyl group. Subsequently the side chain is cyclized with the loss of four carbons to form the 17 β-furan ring **49**. Thus, the latter step is accomplished after the formation of the 4,4,8-trimethyl-steroid skeleton, which is indicated by the occurrence of several protolimonoids and 4,4,8-trimethylsteroid compounds with an intact C-8 side chain. (Champagne *et al.,* 1992)



Scheme 2: Biosynthetic Pathway Leading to the Formation of a Basic Limonoid Skeleton

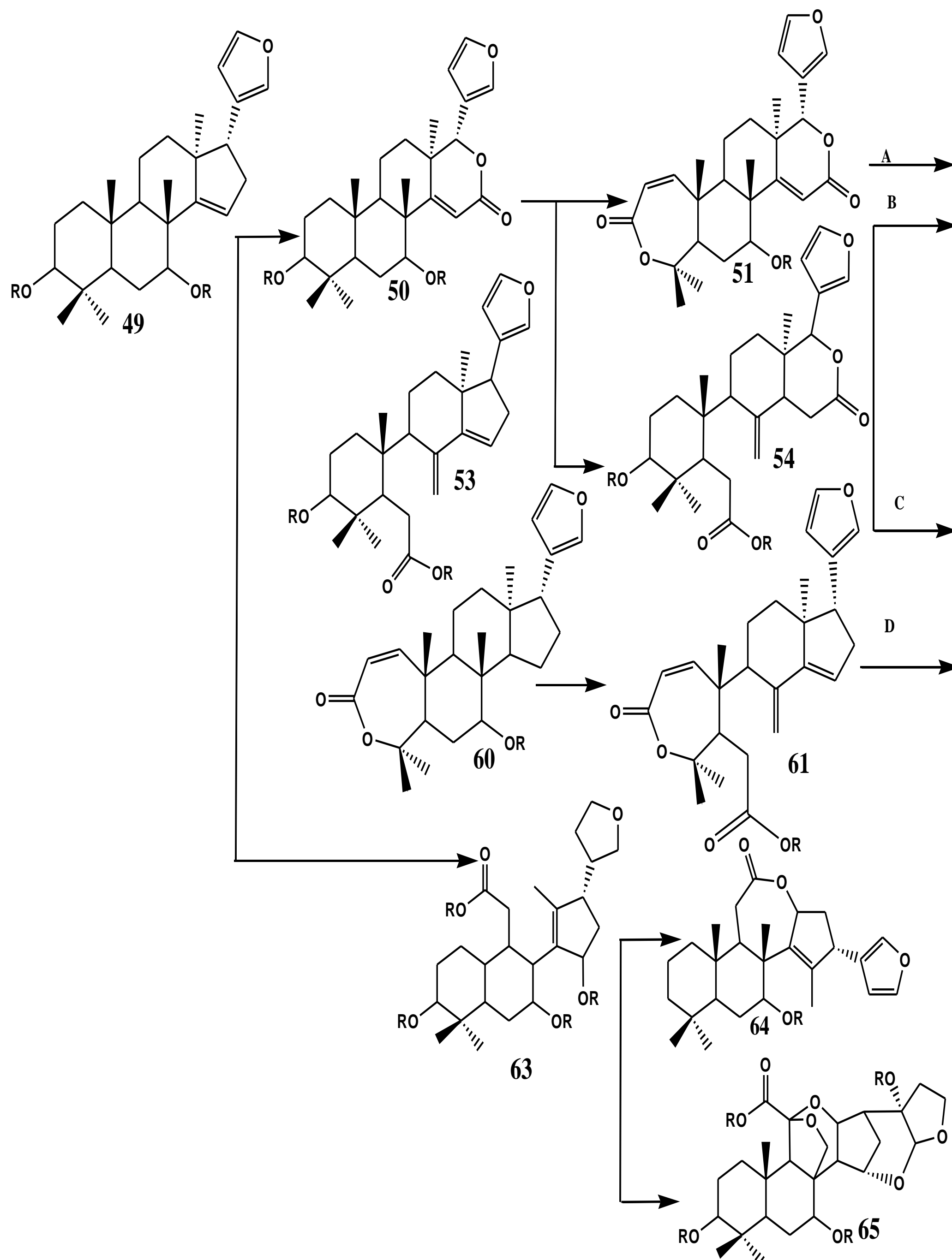
Identification of Limonoids

The Erhlich's, Lieberman-Buchard and Salkowski tests identify limonoids. In the Erhlich's test, 2.0g of p-amino benzaldehyde dissolved in 20ml of ethanol forms the solution. The reagent is sprayed on a developed TLC plate of distinct separations (chromatogram) of the limonoids. The sprayed chromatogram is placed in a tank with an inlet tube connecting from a source tank containing NH_4Cl salt and H_2SO_4 which generates HCl gas. This exposure resulted in colours such as yellow, orange, blue and purple which indicate the presence of limonoids. In the Salkowski test, a terpenoid in chloroform, treated with concentrated sulphuric acid results in a deep brown colouration in the chloroform layer but with the Lieberman-Buchard test , the terpenoid inchloroform treated with the same amount of acetic anhydride and concentrated sulphuric acid result in black colouration.

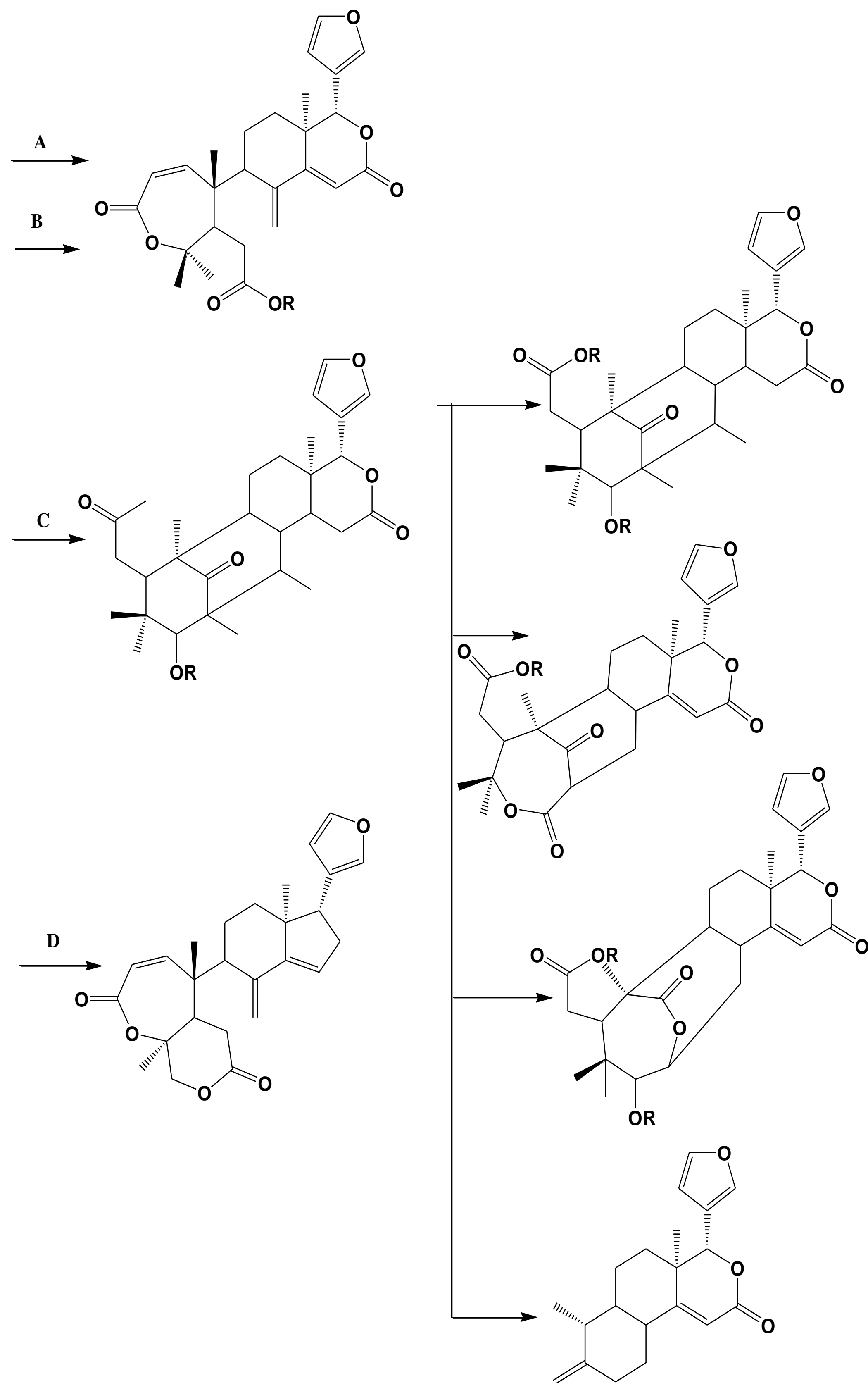
Rearrangement of Tetranortriterpenoids

Limonoids are usually categorized according to the changes they undergo in one or more of their four-ring structures. The rings are designated as A, B, C or D. There are about 10 types of skeletons, based on how these specific rings are formed or chemical groups that are incorporated into or onto them. Scheme 3 below illustrates how the types of limonoids are derived. Although the skeletal types of tetranortriterpenoids are structurally quite diverse, where a variety of oxidations and skeletal rearrangements may occur, they are derived from a common precursor; 4,4,8-trimethyl-17-furanylsteroid type skeleton **48**. Commonly, the ring D is oxidized to a lactone (D-*seco* limonoids) **49**. The ring A-*seco* **60** may be oxidized by a similar mechanism. A,D-*seco* limonoids **51** are

found in all four families of the Rutales. In the subfamily Swietenioideae of the Meliaceae, D-seco limonoids **50** are further oxidized to B, D-*seco* structures **54, 55, 56**, which may then be extensively rearranged to yield complex structures. In contrast, members of the subfamily Melioideae produce limonoids through a variety of pathways, leading to B- **53**, A- **61**, A,B- **55, 56**, and C-*seco* **63, 64, 65** compounds. Chemically, the limonoids are varied but the prototypical structure consists of four cyclohexane rings and a furan ring.



Scheme 3: Biogenetic Map of LimonoidsFeaturing All Structuring Types in Meliaceae



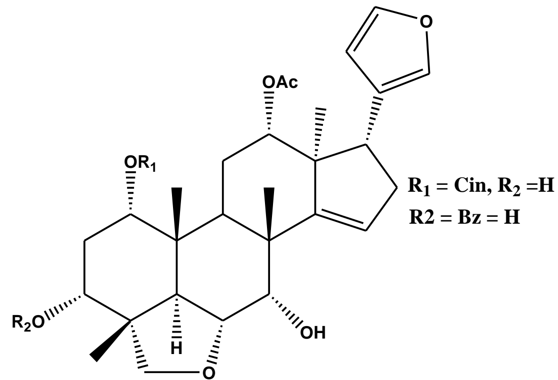
Biogenetic Map continues (Source: Chong Soon Lim, 2008)

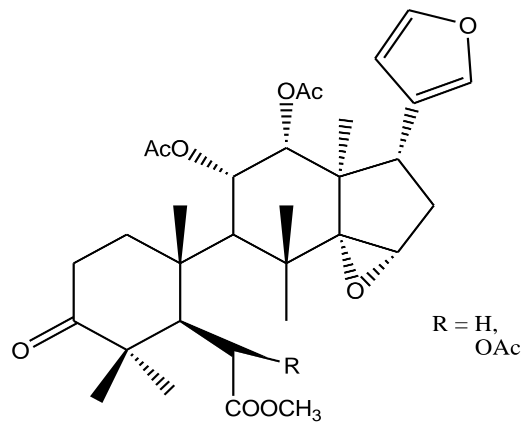
Types of Tetranortriterpenoids Found in Meliaceae

There are five types of tetranortriterpenoids:

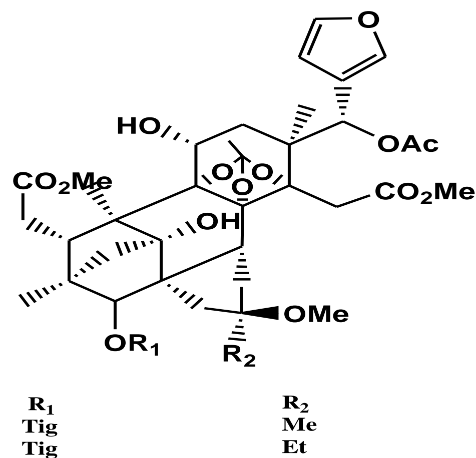
Protolimonoid

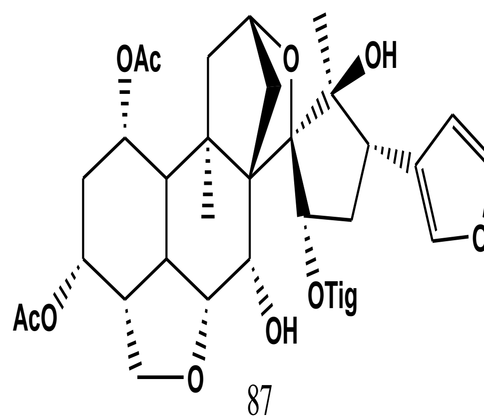
ii) Apo- Euphol

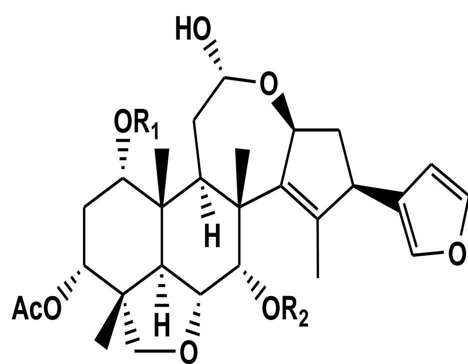




iii) B,D-seco

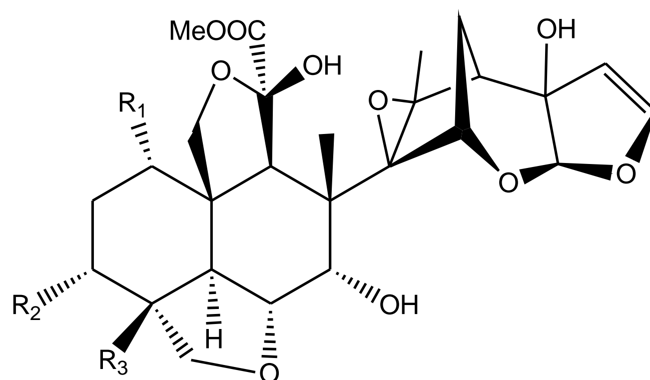






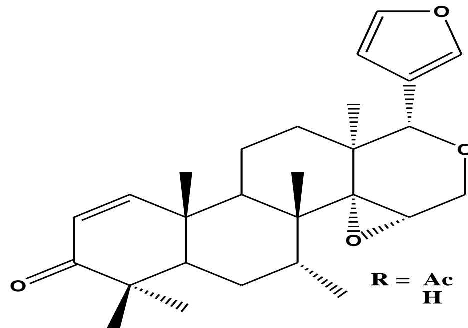
R_1 = H, R_2 = Bz
R_1 = Ac, R_2 = Tig
R_1 = H, R_2 = Tig

iv) C-seco



R_1	R_2	R_3
OCin	OCin	CH_3
OCin	$OCOC(CH_3)=CH_2$	CH_3
OCin	OAc	CH_3
OCin	OFer	CH_3
OTig	OAc	CO_2CH_3

v) D-seco



NMR FEATURES OF TERPENOIDS

The isolated compounds from this plant were characterized mainly based on NMR. Below are some important NMR features of the skeletal types of the isolated compounds; tirucallane, dammarane, and mexicanolide.

Triterpenoids – Dammarane and Tirucallane

The basic skeletal structures of dammarane **93** and tirucallane **94** are quite similar, having fused tetracyclic ring and the same number of methyl groups. However, there are two main differences. Firstly, it is the position of the methyl group. Dammarane has a methyl group at C-8, while tirucallane has it at position C-13. Secondly, there is a double bond at C-7 position in tirucallane, whereas dammarane does not have. The general features of 1H and 13C NMR of both skeletons are listed below:

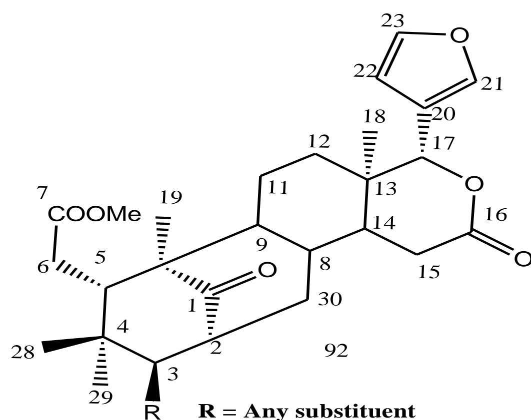
i) Total count of exactly thirty carbon atoms.

ii) Seven or eight methyl groups (basic skeleton and its derivatives) observable in 1H NMR spectrum in the range δH 0.5 – 2.5. 35

iii) Carbon at position 3 always oxygenated – in the form of carbonyl (~ δC 210) or hydroxyl (~ δC 65 - 85).

iv) An unsaturation (double bond) at carbon C7-C8 position for tirucallane skeleton. (Luo *et al.,* 2000, 54, 801.)

Tetranortriterpenoids – Mexicanolide



Tetranortriterpenoids are degraded triterpenoids in which four terminal carbons from the side chain are removed. Despite the term 'degraded', this type of compound is structurally more advanced. It is very different from its parent, triterpenoids, owing to its remarkable ability to undergo various kinds of reactions; oxidation, lactonization, ring opening and closure, side chain elimination, and many others. The general features of ^{1}H and ^{13}C NMR of basic mexicanolide skeleton, one type of tetranortriterpenoids, are listed below:

i) Universal presence of a β-furan or its derivatives – which is located at the C-17 position. Three signals are detectable in ^{1}H NMR spectrum, where two of them can be observed at δ_H 7.0 – 7.6, and the other signal at ~ δ_H 6.5.

ii) Ubiquitous presence of oxygen - which is established from biosynthetic pathway - C-7 position.(Champagne, *et al.,* 1992)

iii) A δ-lactone substructure – which forms ring-D in the basic skeleton. (Silva, 1984)

iv) Four tertiary methyl groups observable in the ^{1}H NMR spectrum

v) A propionate ester function attached as a side chain at C-5. (Silva, 1984)

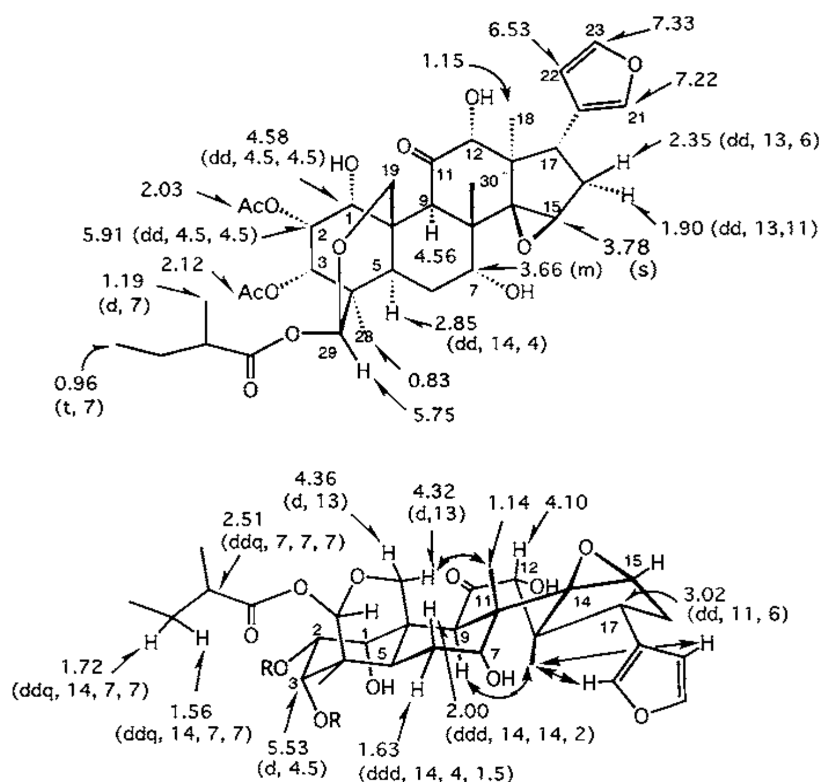
vi) Presence of a six-member ketone carbonyl carbon at ~ δ 212 (Daniewski *et al.,* 1993)

OTHER LIMONOIDS AND THEIR NMR FEATURES

Structures of the Trichilins

The series of limonoids called the trichilins was first isolated from the root bark of the East African medicinal plant *Trichilia roka* (Meliaceae) (M. Nakatani *et al.,* 1981). The structures of trichilin B (**17**) and its 12-epimer (trichilin A) were elucidated by extensive 1H and 13C nuclear magnetic resonance imaging (NMR) studies and through chemical correlation. Some pertinent points related to these structural studies are listed as follows: 1) Irradiation of the 13- and 8-Me peaks induced 30% and 12% Nuclear Overhauser Effect (NOE) enhancements of the 9-H and 19-H signals respectively, (Scheme 4). 2) The assignment of the 12-OH stereochemistry as in trichilin A and in trichilin B was deduced from the finding that in their 12-bromobenzoates, the aromatic protons of the benzoate and furan rings were at higher field in trichilin B. Thus, the shifts of the *p*-bromobenzoate protons were (for trichilins A/B) *o*-H 7.99/7.65, *m*-H 7.51/7.59, and those of the furan were 21-H 7.20/7.02, 22-H 6.36/5.98, and 23-H 37/7.10. The higher-field chemical shifts of these aromatic protons in trichilin B can be accounted for by the mutual shielding induced by the ring currents of the two aromatic rings, which are located on the same side of the molecule. More directly, however, the 12-OH configurations were independently derived from a new additivity relation found in

the Cotton effects of multiple coupled chromophores in the CD spectra. (Stonard *et al.,* 1983).



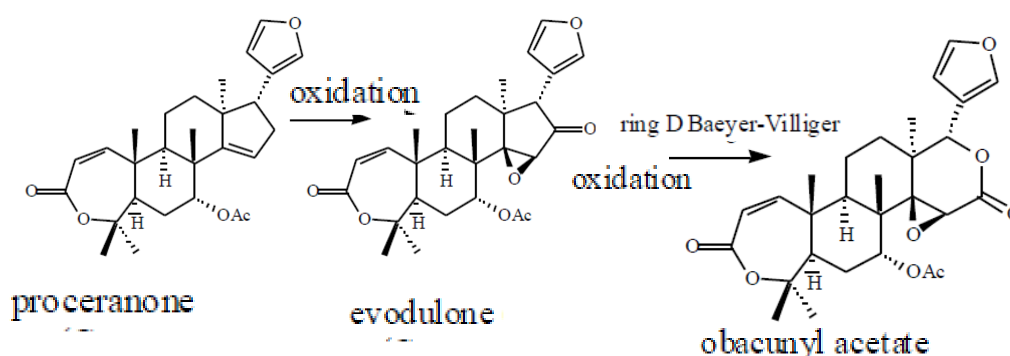
Scheme 4 Trichilin B(Source: Munehiro Nakatani 1998)

REACTIONS AND FORMATION OF CERTAIN LIMONOID GROUPS

(Note: oxidation of ring D takes place independently of modifications of rings A and B, as compounds have been isolated at different stages of ring A/B modification with different ring D structures) (scheme **5**)

Carapa has yielded limonoids of the evodulone, obacunol, mexicanolide and phragmalin groups, and the carapolides, an interesting group of compounds which appear to arise from the evodulone group by oxidation of ring D to a lactone, contraction of ring A by formation of a C1,C5-bond, followed by cleavage of the C1,C10-bond to give a spirolactone. A literature search of compounds from

other *Carapa* species (Kimbu, et al 1984; Ayafor, *et al.,* 1994), allows one to suggest a possible biosynthetic pathway for the formation of these compounds based on the occurrence of possible intermediates. Oxidation of ring D appears to occur independently of ring A modifications, for example, carapa procera spirolactone A has an intact ring D, but contraction of ring A and cleavage of the C1, C10- bond has occurred to form the spirolactone. This compound co-exists with obacunol acetate in which the ring A lactone has not undergone modification, but ring D has been oxidised to the lactone. The proposed pathway is supported by the isolation of intermediates either from *Carapa procera* or the related West African species *C. grandiflora* (Kimbu et al 1984; Ayafor *et al.,* 1994) .



Scheme 5 Oxidation to form Ring D (Dulcie *et al.,* 2000)

A suggested scheme for the formation of the carapolides from an evodulone-type compound is given in Scheme 5. (source: Mulholland *et al.,* 2000).

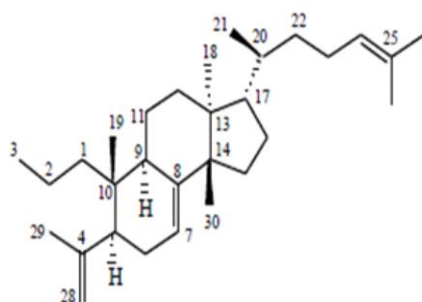
It has been mentioned from the beginning of the review that limonoids from Meliaceae generally have four main groups aside the protolimonoids, in their review on the chemistry of the family in relation to the limonoid groups). In his review, Taylor (1984) divided the compounds from the Meliaceae into the protolimonoids (triterpenoid precursors of the limonoids; the term is often used to

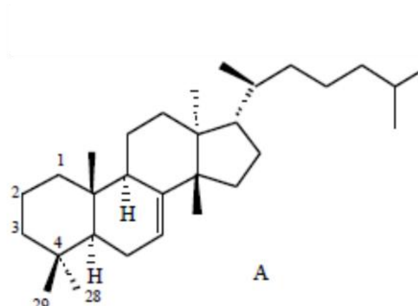
include compounds that may not be direct precursors of the limonoids, but have oxygenated side chains), and nine classes of limonoids based on which the four triterpenoid skeleton rings had been oxidatively opened. Two further subgroups, the trijugins (group IVb) and entilins (group IVe) and the carapolide group (group XI) have subsequently been added (Table 1), and the criterion for grouping compounds into the phragmalin group has been modified. The relationship between the groups is shown in scheme 6. (Mulholland *et al.,* 2000). The type of limonoids produced has been used for taxonomic purposes. An example of this is the monospecific South African genus *Nymania,* which had previously been placed in several other families, especially in the Sapindaceae because its external appearance was so unlike that of most Meliaceae. Close examination of morphology, wood anatomy and pollen indicated it was closely related to *Turraea* in the subfamily Melioideae (Pennington & Styles, 1975). Chemical work on *Nymania capensis* and *Turraeai obtusifolia* has confirmed this close relationship (MacLachlan,*et al.,*1982). Subfamilies 1 and 4 may be readily distinguished on the basis of their chemistry. The Swietenioideae produce complex limonoids of the mexicanolide and phragmalin classes where ring D has been oxidised and ring B opened and recyclised, but the Melioideae typically produce prieurianin or evodulone limonoids with rings A and B or ring A only oxidised. In both cases simpler, less oxidised limonoids and protolimonoids often occur together with the highly oxidised limonoids. Little work has been done on the Madagascan subfamilies 2 and 3, the Capuronianthoideae and Quivisianthoideae, but the single publication on these sub-families indicates the Capuronianthoideae is closely related to the Swietenioideae and suggests a relationship between

Quivisianthoideae and *Ekebergia* of the Melioideae (Mulholland & Taylor,1988). However, the recent isolation of only mexicanolide group limonoids from *Quivisianthe papinae,* contradicts this and shows that the Quivisianthoideae are also more closely related to the Swietenioideae.

Compounds Isolated from Meliaceae family(a)

Protolimonoid Group



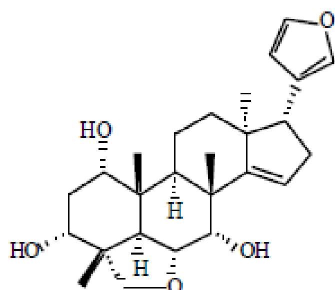


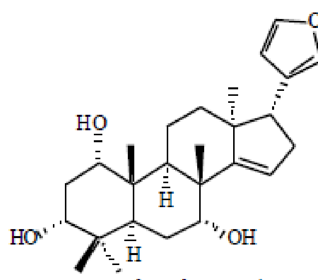
3,4-secotirucalla-4(28)-7,24-triene

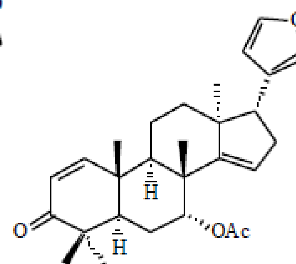
delevoyin A

Limonoid Group

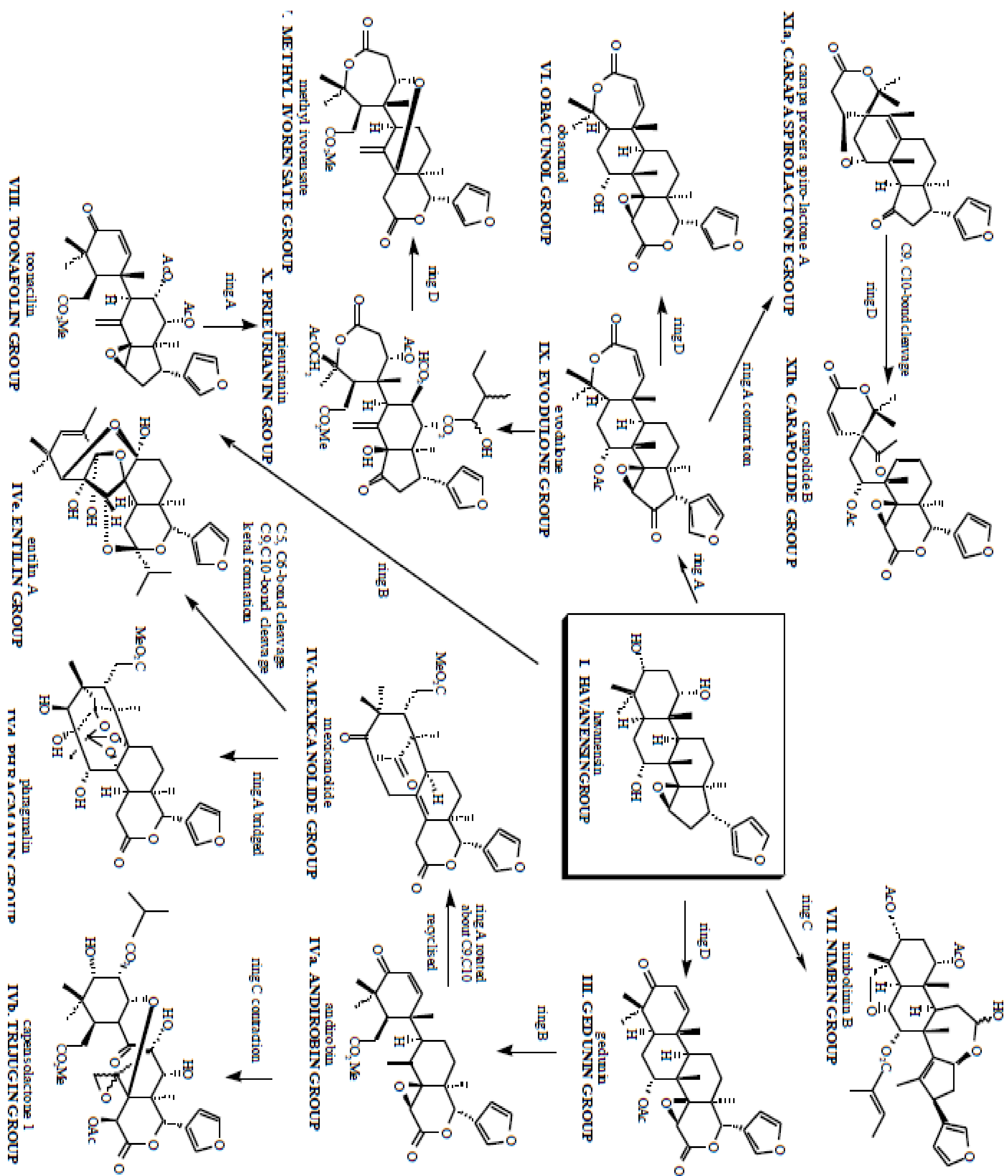
Havanensin Group





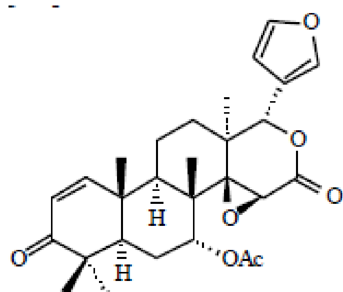


deoxyhavanensin azadirone vilasinin

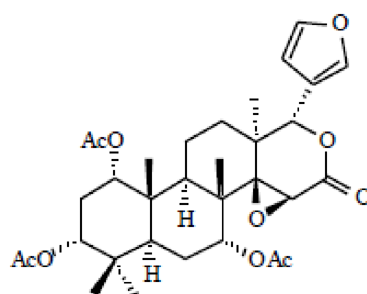


Scheme 6 : The Relationship between the limonoid Groups

Gedunin Group

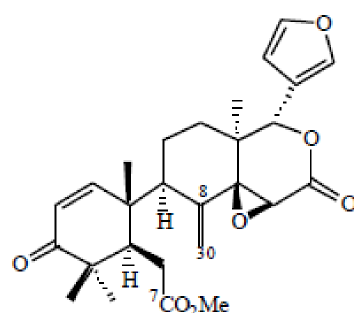


Gedunin

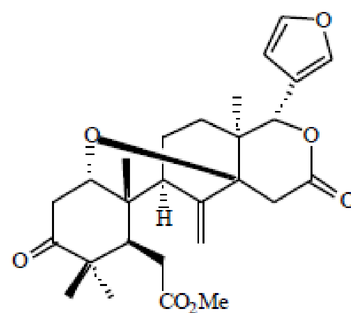


Khivorin

Andirobin Group

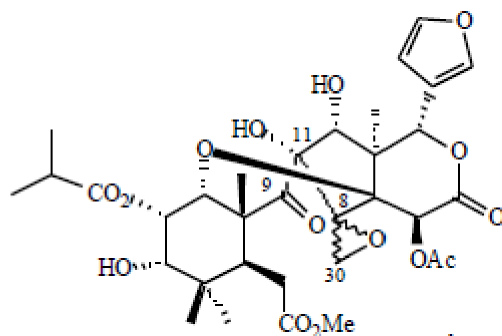


andirobin

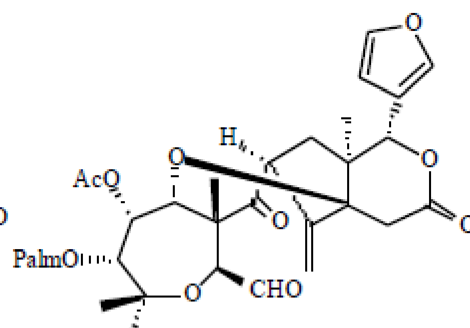


methyl angolensate

Trijugin Group

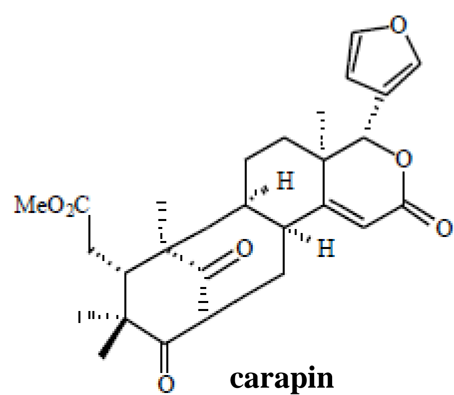


capensolactone 1

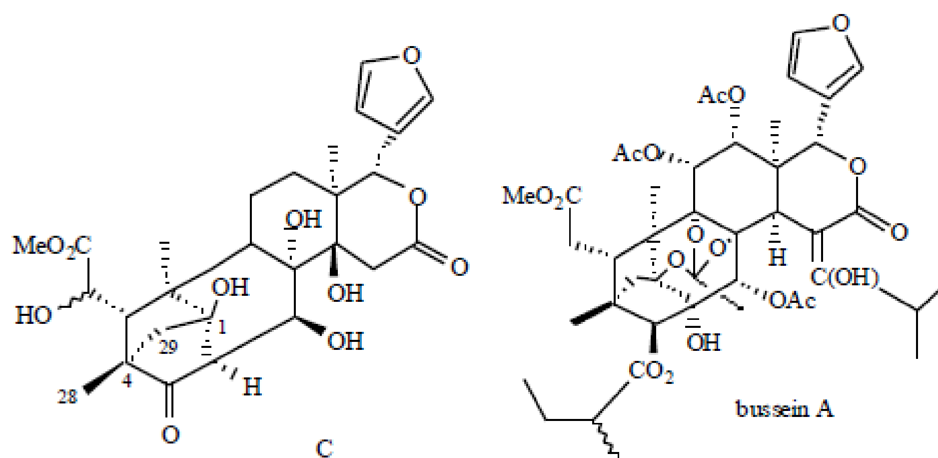


voamatin C

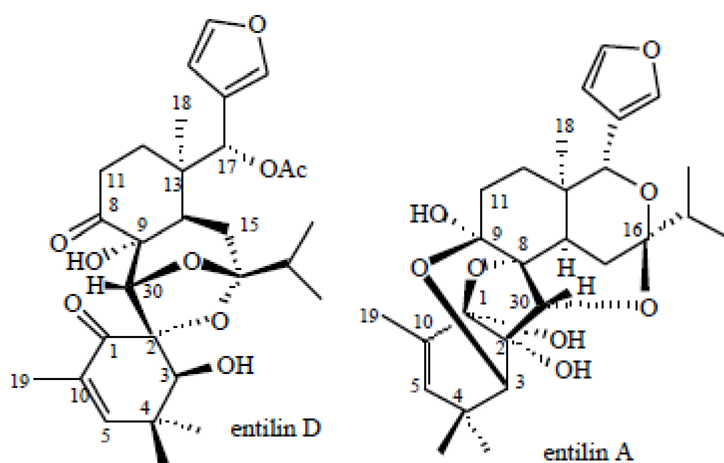
Mexicanolide Group



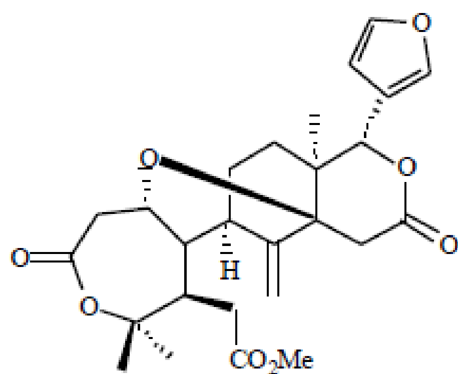
IVD. Phragmalin Group



IVE. Entilin Group

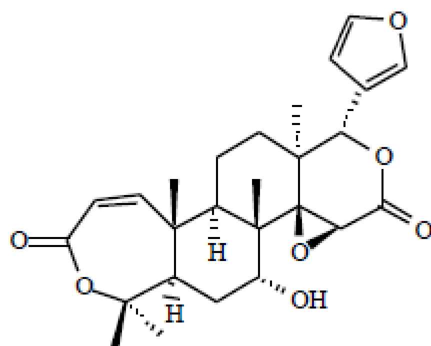


V. Methyl Ivorensate Group



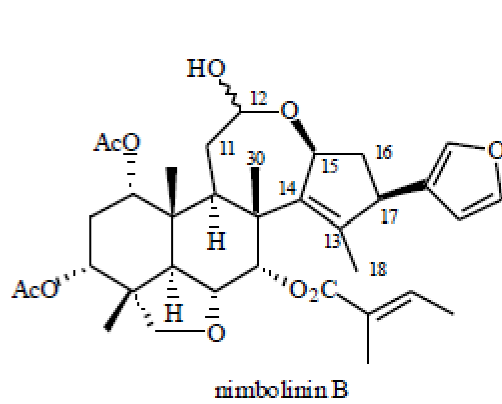
methyl ivorensate

VI. Obacunol Group

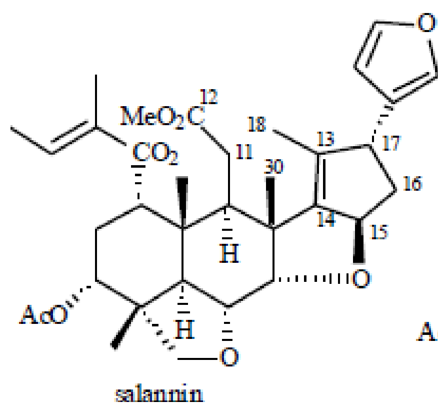


obacunol

VII. Nimbin Group

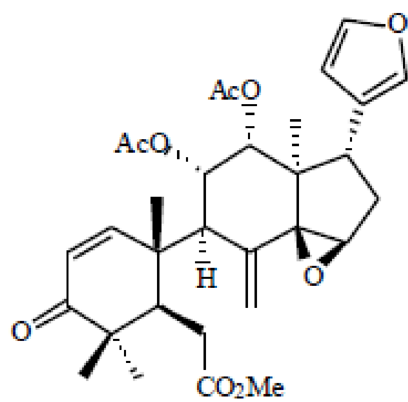


nimbolinin B



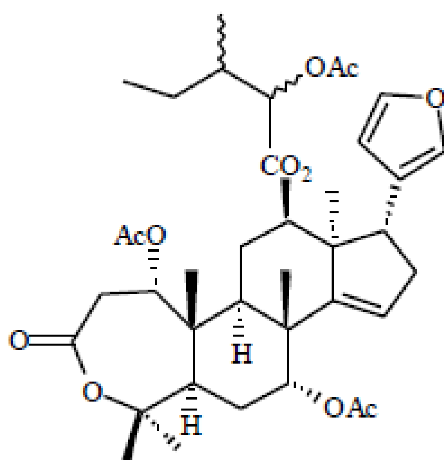
salannin

VIII. Toonafolin Group

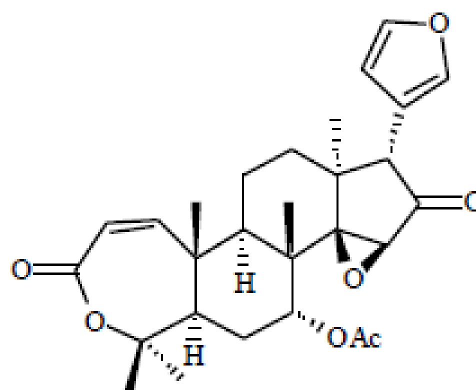


toonacilin

IX. Evodulone Group

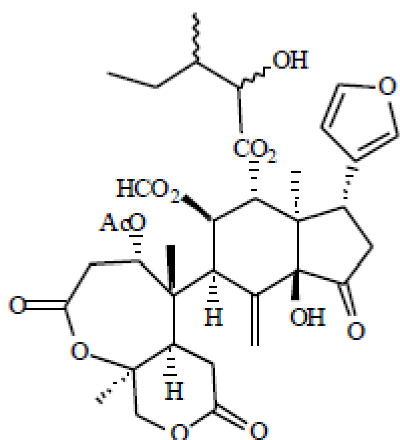


dregeana-3

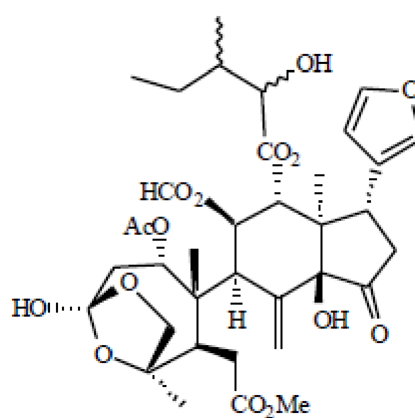


evodulone

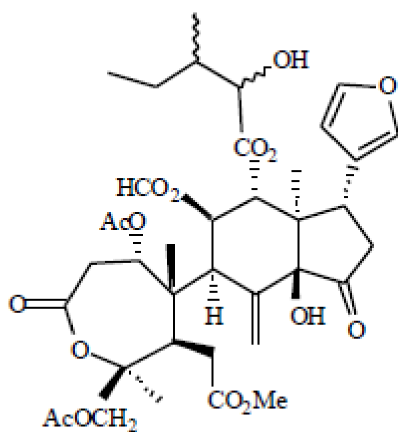
X. Prieurianin Group



trichilia substance Tr-B

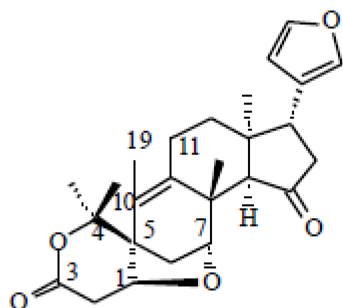


nymania 1



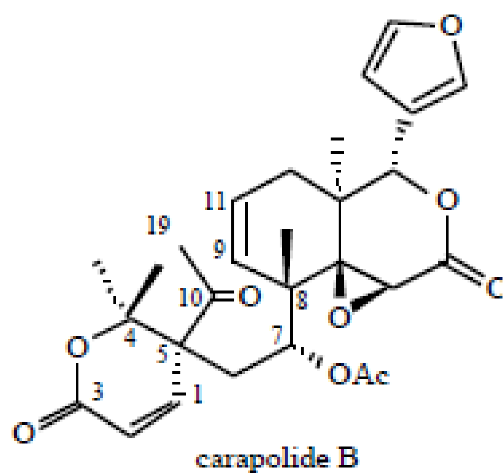
prieurianin

XIA. Carapa Spirolactone Group



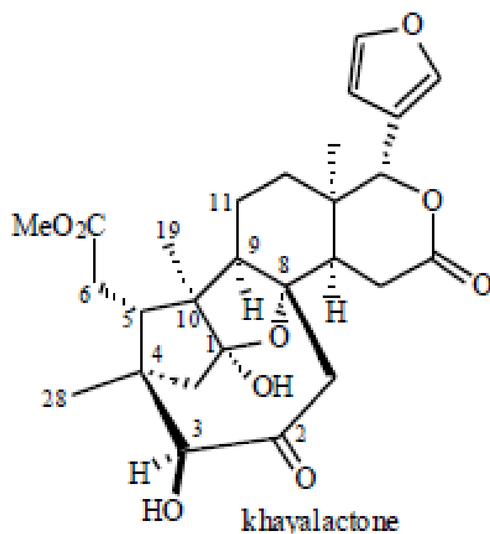
carapa procera spiro-lactone A

XIB. Carapolide Group



carapolide B

Miscellaneous

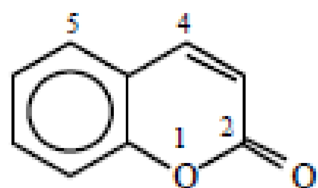


khayalactone

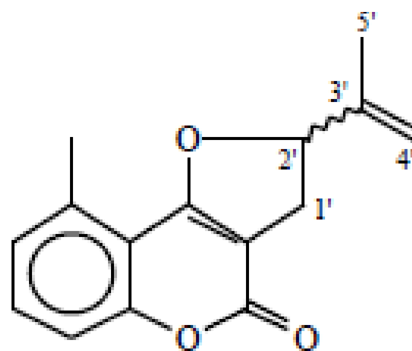
Several compounds are listed in tables (Mulholland *et al.,* 2000) from these groups and their sources where the compounds were isolated.

Other Compounds

I. Coumarins

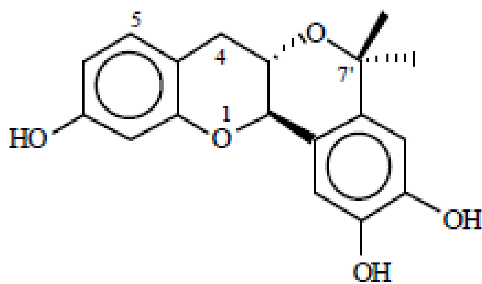


coumarin



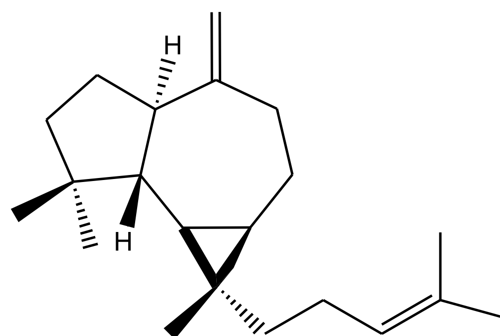
pterophyllin 1

Flavonoids



pubeschin

Diterpenoids



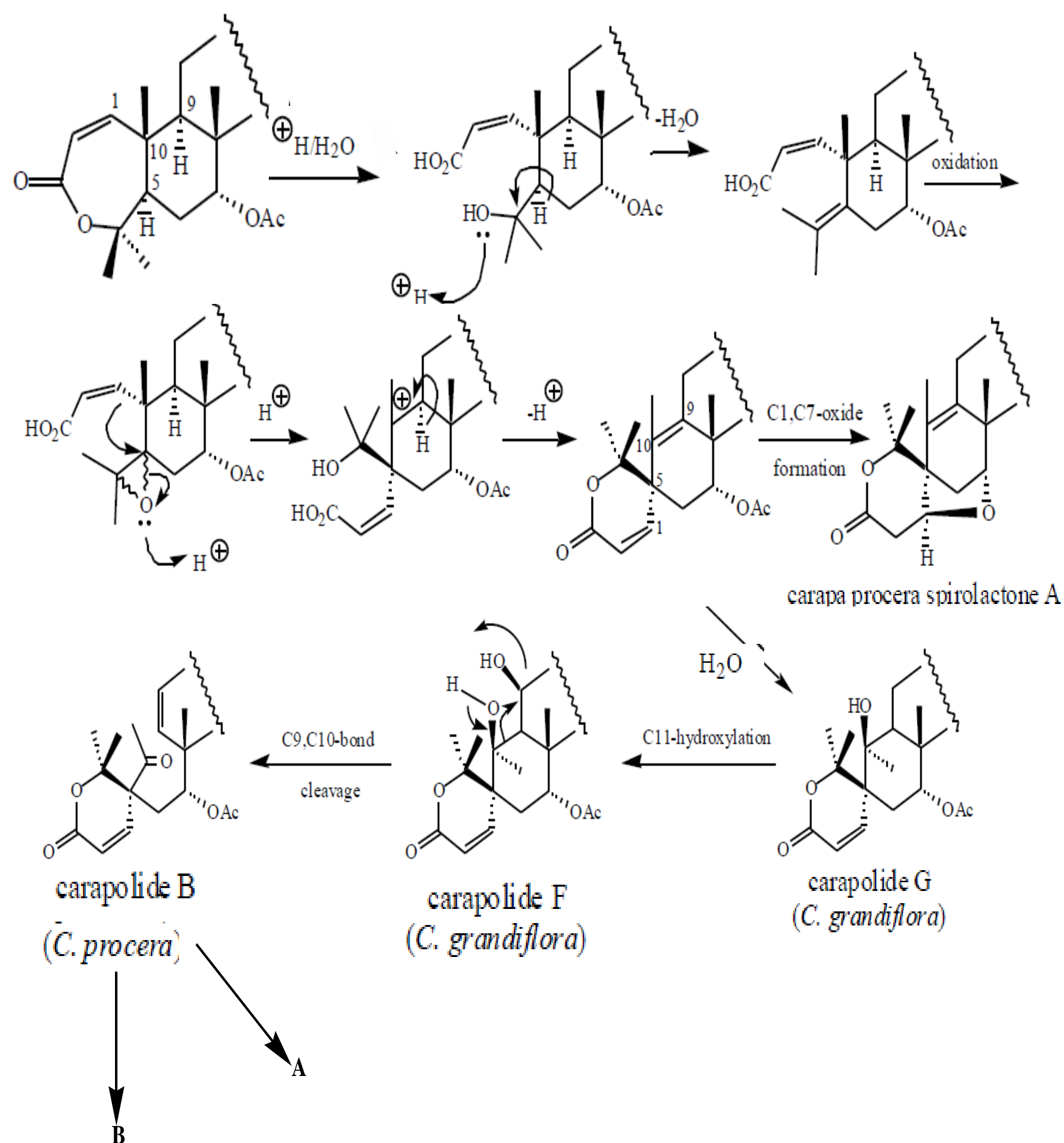
CneorubinX

These are but a few isolates or compounds from the meliaceae. In **Table 2,** a few more of the compounds from Meliaceae family are as well listed.

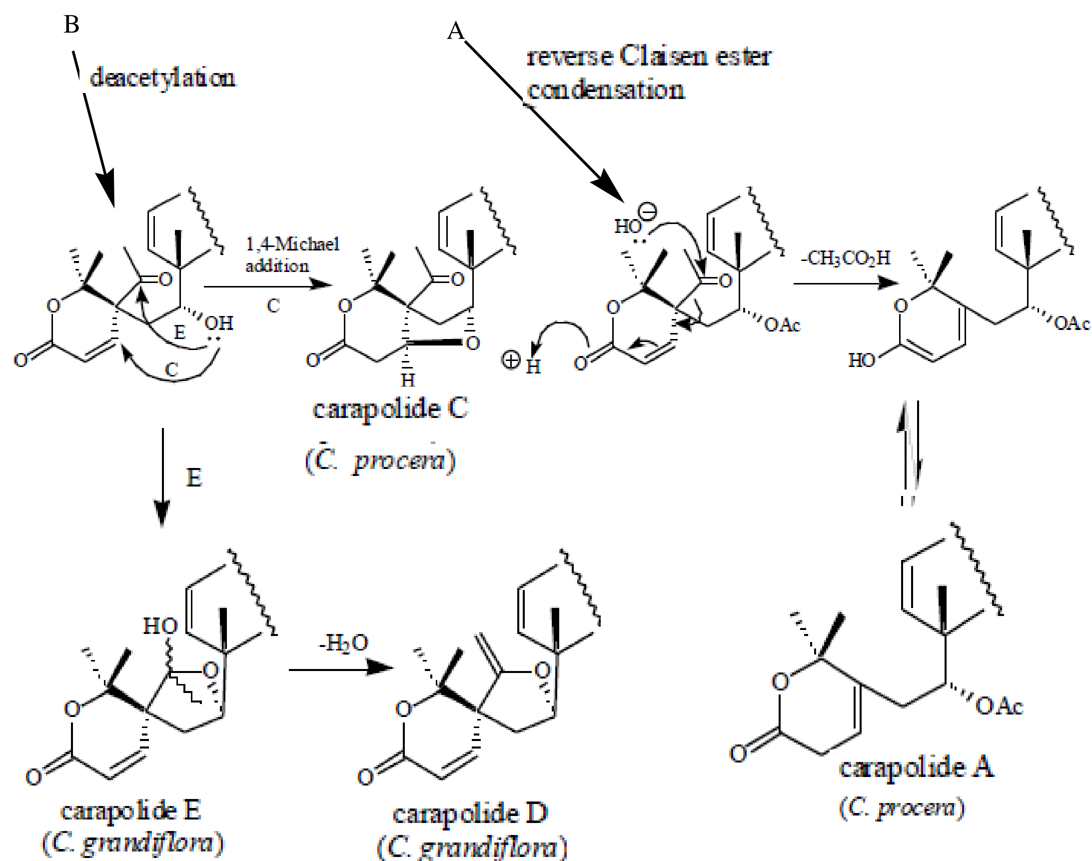
Multidrug Resistance (MDR) Reversal Activity of Tetranortriterpenoids

In traditional Chinese medicine, Phellodendri Cortex (The stem bark of *Phellodendron amurense* Rupr., Rutaceae) has been used to treat dysentery, jaundice, yellow thick foul leukorrhagia, swelling pain in the knees and feet, urinary tract infections, and infections on the body surfaces (Yan *et al.,* 1999). Isoquinoline alkaloids, phenolic compounds, butenolides and limonoids from the bark of this plant have been reported (Kondo *et al.,* 1985; Wada *et al.,* 1990; Miyaki *et al.,* 1992; Kishi *et al.,* 1992; Ida *et al.,* 1994). Indolopyridoquinazoline alkaloids, furoquinoline alkaloids and isoquinoline alkaloids were also extracted from the callus tissues of bark of this plant (Ikuta *et al.,* 1995, 1998a, 1998b). As part of an ongoing search for multidrug resistance (MDR) reversal compounds from Korean medicinal plants, the study examined Phellodendri Cortex because the MeOH extract was found to show P-gp mediated MDR reversal activity in human cancer cells. The chromatographic separation of the chloroform fraction of the MeOH extract from the bark of P. amurense led to the isolation of three limonoids and five alkaloids. Their structures were characterized as obacunone (1), limonin (2), 12α-hydroxylimonin (3), γ-fagarine, oxyberberine, canthin-6-one (6), 4-methoxy-N-methyl-2-quinolone and oxypalmatine based on the physicochemical and spectroscopic data. The compounds were tested for their in vitro cytotoxicity against five tumour cell lines using the SRB method. The marginal or non-cytotoxic compounds (1, 2, and 3) were tested for their MDR reversal activity. . Compound 1 showed significant P-gp MDR inhibition activity in MES-SA/DX5

and HCT15 cells with an ED50 value of 0.028 µg/mL and 0.0011 µg/mL, respectively. Compound 5 showed significant cytotoxicity against the five tumour cell lines with ED50 values ranging from 0.30 to 3.0 µg/mL. (Yong Deuk Min et. al., 2006) Limonoids also have neuroprotecting, therapeutic effects



Scheme 7 Ring A/B Modification:



Scheme 7: Reaction of A/B rings to form Carapolide E (Continuation)

Limonoids, Neuroprotective Compounds (from Root Bark of *Dictamnus dasycarpus*)

Glutamate is known to be associated with central excitatory neurotransmission as occurs in neuronal survival, synaptogenesis, neuronal plasticity, learning, and memory processes in the brain. However, high concentration of glutamate causes neuronal cell death within the central nervous system and may be involved in neuropsychiatric and neuropathological disorders such as Alzhemer's disease, Parkinson's disease, ischemic stroke, and spinal cord trauma. Thus, neuroprotection against glutamate-induced neurotoxicity has been a therapeutic strategy to treat neurodegenerative disease. In the course of searching

for neuroprotective compounds from natural sources using primary cultures of rat cortical cells injured by glutamate as an *in* V*itro* assay system, it was found that a methanolic extract of the root bark of *Dictamnus dasycarpus* Turcz. (Rutaceae) showed significant neuroprotective activity. *D. dasycarpus* is widely distributed in Asia, and root bark of this plant has been used for treatment of various ailments such as skin inflammation, eczema, rubella, scabies, acute rheumatoid arthritis, jaundice, cold, and headache in Korean traditional medicine. Known constituents of *D. dasycarpus* root bark include limonoids, furoquinoline alkaloids, flavonoids, coumarin sesquiterpenes, sesquiterpene glycosides, and phenolic glycosides. To date, however, there has been no report related to neuroprotective constituents of this plant. Thus, an attempt has been made to isolate compounds having neuroprotective activity from a methanolic extract of *D. dasycarpus* root bark using a bioactivity-guided fractionation technique. As a result, four new degraded limonoids (1–4), five known degraded limonoids (5–9), and three limonoid derivatives (10–12) were obtained. In the present study, we report the isolation and structural elucidation of compounds 1–4 and the neuroprotective activities of compounds 1–12. *Dictamnus dasycarpus* root bark afforded four new degraded limonoids, 9R-hydroxyfraxinellone-9-*O*-_-D-glucoside (1), dictamnusine (2), dictamdiol A (3), and dictamdiol B (4), together with eight known compounds, dictamdiol (5), fraxinellone (6), fraxinellonone (7), 9_-hydroxyfraxinellone (8), calodendrolide (9), obacunone (10), limonin (11), and rutaevin (12). The source of the work is not modified, hence the numbering. Compounds 2, 3, 6, 9, 10 and 11 showed significant neuroprotective activity against glutamate-induced

neurotoxicity in primary cultures of rat cortical cells at a concentration of 0.1 μM. (Jeong *et al.,* 2008).

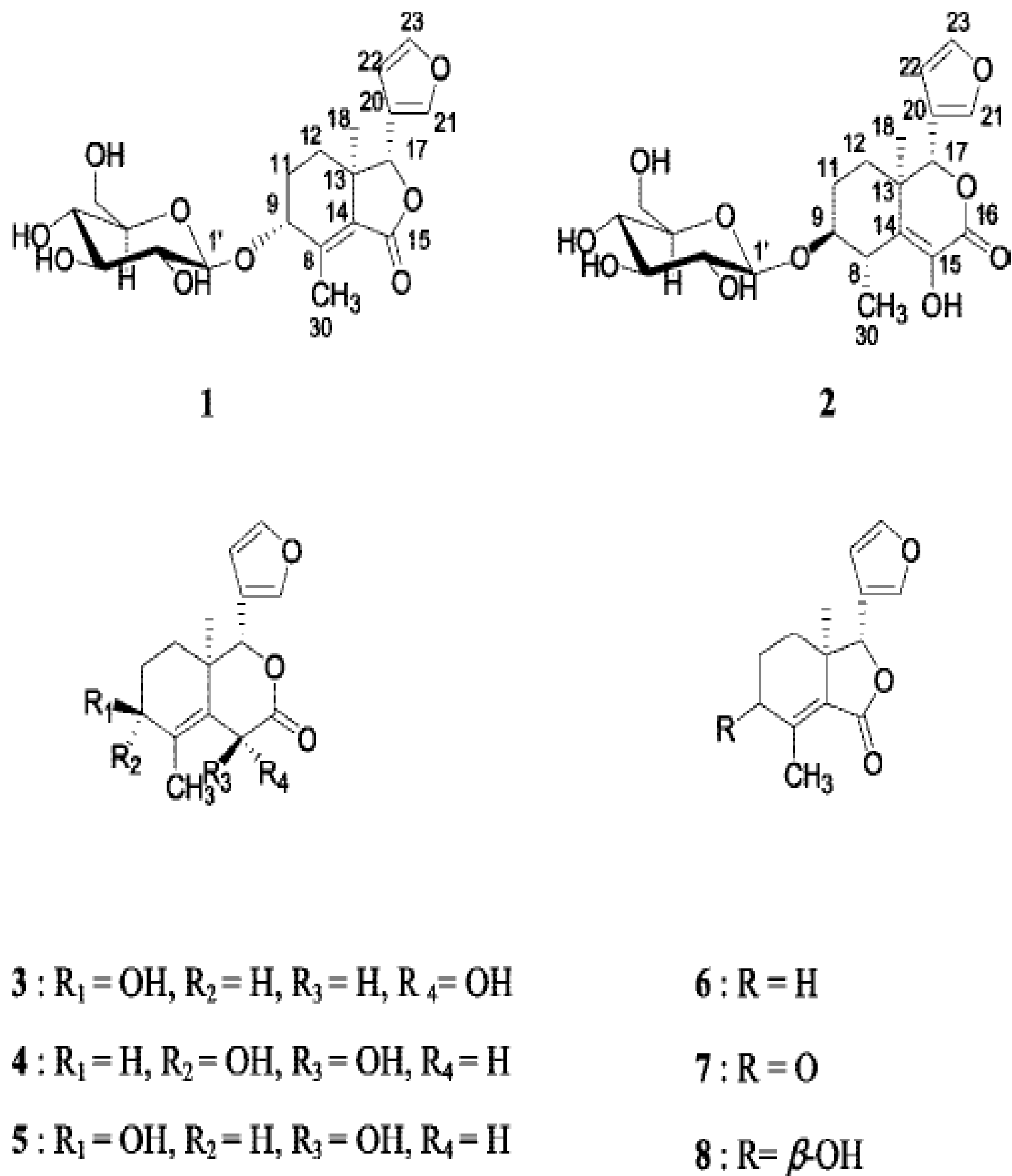


Figure 2: Neuroprotective Limonoids (Source: Jeong *et al.,* 2008)

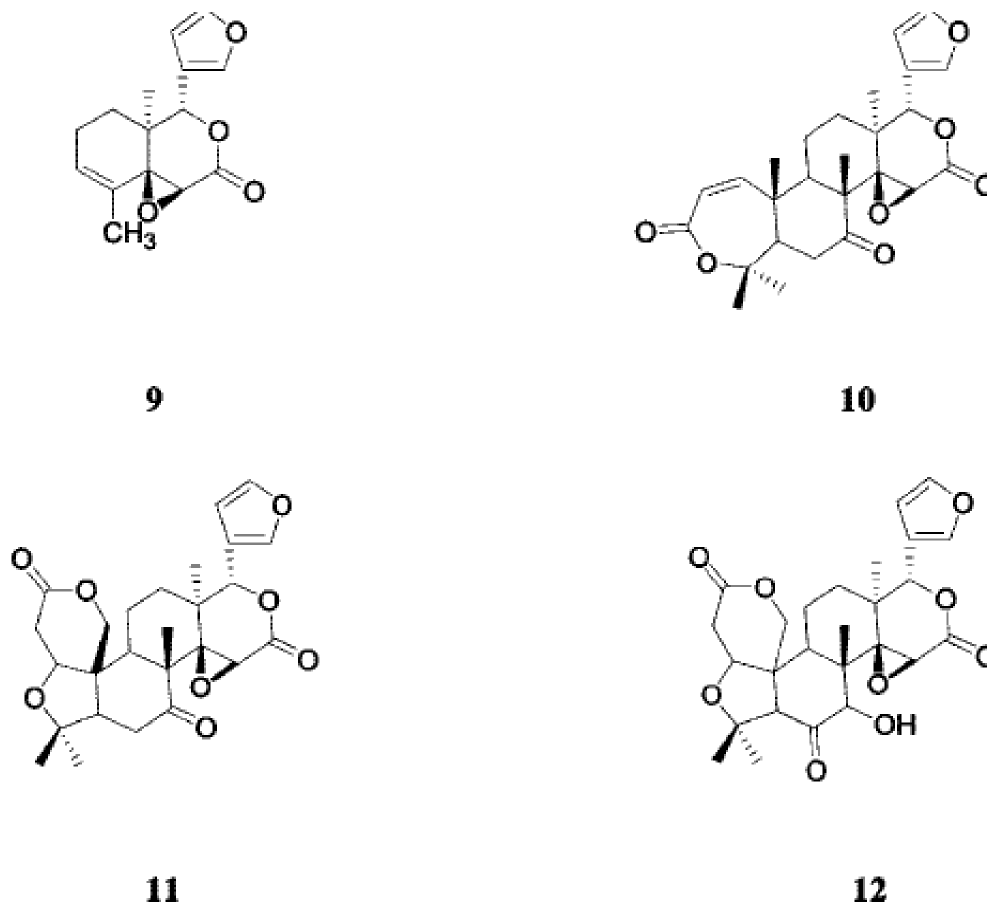


Figure 2: Neuroprotective Limonoids (Source: Jeong *et al.,* 2008)

Biopesticidal activity

Phenolic compounds act as natural pesticides, providing plants with resistance to pathogens, parasites, and predators (Ames *et al.,* 1990; Stoewsand, 1995; Salad, 1998). Limonoids are such compounds and being tetranotriterpenoid in nature, are included in the category of phenols. These compounds occur in abundance in the plants belonging to family Meliaceae. Abdelgaleil and Nakatani, 2003; Nakatani *et al.,* 1981, 1984, 2000; Nakatani, 1999 and Saad *et al.,* 2003 isolated several types of compounds (limonoids) as insect antifeedent from the members of Meliaceae family. In a leading step to their research, Nakatani et al.

(2004) further isolated 6 new phragmalin limonoids (tabulalin and tabulalides (A - E)) from the root bark of *C. tabularis* using droplet countercurrent chromatography (DCCC) and reversed phase HPLC. Nakatani et al. further analyzed the antifeedant activity of the isolated compounds in 2004s using conventional leaf disc method given by Wada and Munakata (1968) against the third instar larvae of *Spodoptera littoralis* (Boisd.). Furthermore, the antifeedant activity was also studied by Abdelgaleil and Aswad (2005) against third instar larvae of cotton leaf worm, Spodoptera littoralis (Boisd.) by using leaf disc choice bioassay given by Kubo and Nakanishi (1977). In their analysis, they reported that tabulalin and tabulalide D were strongly active at 500 with 50 ppm concentration corresponding to concentration of ca.1 _g/leaf/cm^2 . The antifeedent activity was found to be comparable to many other limonoids from the meliaceae plants (Abdelgaleil *et al.,* 2000; Huang *et al.,* 1995; Mootoo *et al.,* 1996). The other compound under investigation showed weak activity at 1000 ppm while tabulalide C was not active at the same concentration. In Appendix A, is a table showing the antifeeding activity of limonoids from *Melia azedaracht*. (Munerohiro Nakatani *et al.,* 1999)

Pharmacological Activities Of Limonoids

Anti-cancer Activity

Many experimental evidences have revealed that limonoids present in citrus fruits and their juice and some plants in the family Meliaceae, have cancer chemopreventive property. Limonoids have been shown to inhibit the growth of estrogen receptor-negative and -positive human breast cancer cells in culture. They have also been found to target and stop neuroblastoma cells (Jacob *et al.,*

2000; Poulose *et al.,* 2005; Miller *et al.,* 2004; Tian, 2001). Hesperidin, other flavonoids, limonin 17-beta-D-glucopyranoside, and other limonoid glucosides are potential chemopreventive agents in orange juice that could account for the decreased colon tumour-genesis associated with feeding orange juice (Miyagi *et al.,* 2000). Significant cytotoxic activity has also been exhibited by limonoids isolated from *Melia azedarach*, 18) *Melia toosendan* (Tada *et al.,* 1999) and azadirachtin A. (Akudugu *et al.,* 2001). The citrus limonoids obacunone, limonin, nomilin and their glucosides and some aglycones inhibit chemically induced carcinogenesis and a series of human cancer cell lines, with remarkable cytotoxicity against lung, colon, oral and skin cancer in animal test system and human breast cancer cells (Nakagawa *et al.,* 2001; Manners, 2003; Silalahi, 2002; Berhow *et al.,* 2000; Tanaka *et al.,* 2001.; Tanaka *et al.,* 2000.; Schmandke *et al.,* 2005.) Obacunone was found to enhance the cytotoxicity of vincristine against L1210 cells by approximately 10- fold. Further, it was found that the cytotoxicity of other microtubule inhibitors such as vinblastine and taxol in drugsensitive KB-3-1 cells as well as in multidrug-resistant KB-V1 cells was enhanced greatly in the presence of obacunone (Jung *et al.,* 2000). Pure limonin glucoside and limonin, its water insoluble relative lacking glucose, have been found to possess significant anti-tumour properties in animal tests and with human cells (Manners *et al.,* 2003, 2000). All these studies have reported the lack of toxicity of the limonoids in mammals and also have presented their modifying effect on the development of aberrant crypt foci as well as the ability of these compounds to induce specific carcinogen-metabolizing enzymes, glutathione Stransferace and quinine reductase in the liver and mucosa of the small intestine to detoxify chemical carcinogenesis.

Studies show that the activity of phase II enzyme glutathione-Stransferase in the liver of the rats, fed diets containing limonin and nomilin, increased significantly in dose dependent manner. While simultaneously, the limonoids nomilin and limonin were found to have no significant effect on the phase I enzyme Cytochrome P450. A dose dependent increase in small intestinal GST activity was also observed in nomilin fed animals, where as some citrus limonoids were able to inhibit the development of 7,12-dimethylbenz[*a*]anthracene-induced oral tumours. The data from these studies have suggested that certain rings in the limonoid nucleus may be critical to antineoplastic activity. The results with deoxylimonin were significant, *p*_0.05.2). Nutritional research on health benefits of chemicals present in plant foods advocate that citrus limonoids possess substantial anticancer activity and they are free of any toxic effects in animal models (Jacob *et al.,* 2000).

Guthrie *et al.,* (2001) were awarded a patent, recently, for proposing composition and methods for treatment of neoplastic diseases with limonoids in combination with flavonoids and tocotrienols.

Anti-malarial Activity

Gedunin, nimbin, nimbolide and many more limonoids isolated from *Azadirachta indica*, *Cedrela odorata*, *Guarea mltiflora* and *Khaya grandifoliola* have been identified for their *in-vitro* antimalarial activity on *P. falciperum* (Kayser *et al.,* 2003; Saxena *et al.,* 2003). Gedunin was found to be most effective, against *Plasmodium falciperum*, out of several limonoids isolated from *Khaya grandifoliola* and it exhibited additive effect in combination with Chloroquine (Bickii *et al.,* 2000). Novel antimalarial limonoids were isolated

following a veterinary and self-medicative behavioural survey of wild chimpanzees in Uganda, from leaves of *Trichilia rubescens* (Krief *et al.,* 2004).

Anti-microbial Activity

Germano *et al*. (2005) have recently reported the presence of limonoids in *Trichilia emetica*, which can be considered responsible for activity against many clinically, isolated bacterial strains. Limonoids obtained from some *Khaya* species showed good antibacterial and antifungal activity (Abdelgaleil *et al.,* 2005). In another study, limonoids from several plants belonging to Meliaceae as well as Rutaceae families were reported to have significant antifungal activity (Abdelgaleil *et al.,* 2005; Govindachari *et al.,* 2000). In these studies, the importance of structural features on activity was also illustrated.

Anti-HIV Activity

Limonin and nomilin have shown to inhibit the replication of HIV-1 in a number of cellular systems (Battinelli *et al.,* 2003). A novel limonoid isolated from *Clausena excavate* has shown HIV-1 inhibitory activity (Sunthitikawinsakul *et al.,* 2003).

Other Miscellaneous Activities

Limonoid 1-cinnamoyl- 3,11-dihydroxymeliacarpin, isolated from *Melia azedarach* showed IC50 value of 6m ml and 20m ml for vesicular stomatitis and herpes simplex (HSV-1) viruses respectively (Alche *et al.,* 2003). Limonoids in *Trichilia emetica* were considered to be responsible for hepatoprotective activity on CCl4 induced damage in rat hepatocytes. Radical scavenging and anti-oxidant activity were demonstrated by some limonoids, which are supposed to play a role in their anti-proliferative activity (Yu *et al.,* 2005). A reduction of low-density

cholesterol in rabbits was observed after substituting orange juice and grapefruit juice for water. Further tests suggest that the limonoid contributes to cholesterol-lowering action of citrus juices (Manners *et al.,* 2000). Raphael and Kuttan (2003) have reported immuno-modulatory activity of nomilin.

Limonin is supposed to be specifically directed towards protection of lungs for clearing congestive mucus (Rohr *et al.,* 2002). Zimmerman (2005) has cited reports of cardioprotective effect of limonoids from mandarin oranges. In an *in-vitro* study, limonoids isolated from *Swietenia humilis* have exhibited a concentration dependant and non-reversible spasmogenic and uterotonic activity (Perusquia *et al.,* 1998). *In-vitro* anti-sickling activity of a rearranged limonoid isolated from *Khaya senegalensis* has been reported by Fall and co-workers in 1999. They found the limonoid to have much higher activity at every concentration and incubation conditions, in comparison to the standard drug. Schmandke and Rehbrücke (2006) have reported that some limonoids decrease cholesterol release in cultured human liver cells. Biswas *et al.* (2002) in their review have reported a number of other pharmacological activities of limonoids derived from neem tree, like- anti-inflammatory, anti-arthritic, antipyretic, hypoglycemic, anti-gastric ulcer, spermicidal and diuretic. Cedrelanolide, the most abundant limonoid from *Cedrela salvadorensis* has shown to interfere with monocot pre-emergence properties and also inhibits their photophosphorylation, H_ uptake and non-cyclic electron flow which results in inhibition of germination, seed respiration and seedling dry weights of some plant species (Cespedes *et al.,* 2001).

Structure activity relationships

Madyastha and Venkatakrishnan (2000) have described the studies carried out on the structure–activity relationships amongst limonoids, showing that limonoids with an intact apoeuphol skeleton, a 14, 15 b epoxide, and a reactive site such as either a 19—28 lactol bridge or a cyclohexanone 'A' ring are biologically very active, and the absence of these structural features results in reduced activity. *C*-seco limonoids with an enone system in ring 'A' are potent cytotoxic and anti-malarial agents. In some of these (e.g. nimbolide 5,28-deoxonimbolide and gedunin) a ,b -unsaturated ketone in ring 'A' has been proposed as a common feature that is primarily responsible for their biological activity. They further say that the *C*-seco limonoids are two to three times more active than other limonoids and they are highly active against herbivorous insects. Data from the studies conducted by Miller *et al*. (2004) have suggested that certain rings in the limonoid nucleus may be critical to anti-neoplastic activity. Changes in the A ring of the limonoid nucleus can lead to a loss of anti-cancer activity, whereas changes in the D ring can be tolerated without any apparent loss of biological activity. Studies carried out on azadirachtin and some of its derivatives as insect feeding deterrents revealed that neither hydrogenation of Δ^{22} double bonds nor deacetylation caused any change in effect but blocking of hydroxyl group affected the feeding inhibitory activity. While acetylation of azadirachtin caused a decrease in the activity to 75%, etherification with a bulky trimethylsilyl group eliminated it altogether. Thus, the stereochemical environment around hemiacetal region seemed to be critical for its activity (Devakumar *et al.,* 1996). Screening of eight derivatives of azadirachtin for their insect growth-inhibitory effect on first instar larvae of tobacco bud worm

(*Heliothis virescens*) gave a similar conclusion that the free hydroxyls are essential for maximum activity. On comparing the relative efficacy of isomeric azadirachtins and their derivatives for growth inhibition of larvae of the Mexican beetle (*Epilachna varivestis*), it was found that LC50 values (ppm) were 1.66, 1.30, 12.97, 1.57, 2.80, 1.15, and 7.69 for azadirachtins A—G respectively. Interestingly, 3-detigloylazadirachtin B was the most active with LC50 value of 0.08 ppm and the hydrogenated derivatives were more active than the parent molecules. On structural modifications and screening the new products for insect feeding deterrent action, the following conclusions were derived: even a simple analogue retaining the hydroxydihydrofuran portion of the molecule was 50—60% as active as azadirachtin. Compounds showing gross structural rearrangements of this portion were less active. Considering the structural homology with salannin, the uniquely high level of activity of azadirachtin apparently stems from the hydroxydihydrofuran portion of the molecule (Devakumar *et al.,* 1996; Anonymous, 2005).

In structure–activity studies of limonin, it has been determined that the furan ring and epoxide groups in the citrus limonoid structure are critical for the antifeedant activity of the limonoids against Colorado potato beetle larvae (Danielson, 2005). Ruberto *et al*. (2000) evaluated the antifeedant activity of citrus-derived limonoids limonin, nomilin, and obacunone and their semi synthetic derivatives against a commercially important pest, *Spodoptera frugiperda*. These conversions focused on functional groups considered important for the biological activity, namely the C-7 carbonyl and the furan ring. In particular, reduction at C-7 afforded the related alcohols, and from these their acetates, oximes, and

methoximes were prepared. Hydrogenation of the furan ring was also performed on limonin and obacunone and on comparison with previously reported data it showed that insect species vary in their behavioural responses to these structural modifications. Highly significant antifeedant activity (*p*_0.01) for two natural (limonin and obacunone) and three semi synthetic limonoids (Umonol, Umonin-7-oxime, and Limonin-7-oxime acetate) was observed against *S. frugiperda.*

Extraction and isolation methods make up a bulk of recent publications, with eventual study of the compounds as to biological activities exhibited towards insect species (Chong, 2008). In this section, the Meliaceae as a family, the general chemical characteristics, reactivity of some major constituents, bioactivities, and chemical constituents found in the family Meliaceae are discussed.

Plants from meliaceae

Medicinal usage of the species in this family is enormous. *Khaya senegaleneis* is one of the most popular medicinal meliaceous plants used in traditional African remedies. The decoction of its stem bark is commonly used as a bitter tonic in folk and popular medicines for malaria, fever, mucous diarrhoea, and venereal diseases as well as for an anthelmintic and a taeniacide remedy (Iwu, 1993; Olayinka *et al.,* 1992). The stem bark extracts and the chemical constituent profile have been subject of extensive phytochemical and pharmacological investigations since the 1960s (Adesida *et al.,* 1971; Mulholland *et al.,* 2000; Narender *et al.,* 2007). These plant extracts have been reported to exhibit anti-inflammatory effects (Lompo *et al.,* 1998) as well as anti-bacterial (Koné *et al.,* 2004), anthelmintic (Ademola *et al.,* 2004), anti-tumour, anti-oxidant (Androulakis

et al., 2006), and anti-plasmodial activities (El-Tahir *et al.,* 1998). Two dimeric flavonoids as well as 2,6-dihydroxy-p-benzoquinone, b-sistosterol, and 3-O-glucose-b-sistosterol, catechin, tannins, saponins, coumarins, and polysaccharides (Kayser & Abreu, 2001) with immunostimulating activity have been isolated from the extracts of its stem bark. Approximately 45 limonoids (Adesida *et al.,* 1971; Olmo *et al.,* 1996; Olmo *et al.,* 1997, Govindachari & Kumari 1998; Khalid *et al.,* 1998; Govindachari *et al.,* 1999; Mulholland *et al.,* 2000; Abdelgaleil *et al.,* 2003; Tchimene *et al.,* 2006) isolated from the different parts (leaf, seed, and stem bark) of this plant constitute the bitter principles of the plant extracts. Furthermore, some limonoids exhibited feeding deterrent and growth inhibitory properties (El-Aswad *et al.,* 2003) and anti-feeding (Abdelgaleil & Nakatani, 2003), anti-fungal (Govindachari *et al.,* 1998; Abdelgaleil *et al.,* 2004) and anti-sickling activities (Fall *et al.,* 1999).

The bark and leaves of *C. tabularis* in Ayurveda are also said to have great medicinal properties as antipyretic and antidiarrheal activities (Kirtikar and Basu, 1981). The twig and bark extract of this plant are reported to have antifeedant activity against *Pieris rapae* (cabbage white butterfly) and third instar larvae of Spodoptera littoralis (Boisd.) respectively due to which it might be used as natural insecticide (Kalinganire & Pinyopusarek, 2000; Nakatani *et al.,* 2004; Abdelgaleil & Aswad, 2005). Of the two species considered, not all is exhausted on their medicinal value/uses and other economic importance. Turraea Heterophylla is virtually similar to all the species as mentioned above. Locally, it has been used to treat lumbago, emphysema, impotency and stomach-ache. South African Meliaceae species are used in a variety of ways in traditional medicine, including

the treatment of stomach complaints, backache, fever, kidney complaints, rheumatism, dropsy and heart disease, and by diviners to put themselves into a trance prior to performing divining dances (Hutchings *et al.,* 1996; Mulholland *et al.*, 2000).

Limonoids have been found to have a range of biological activities, including insect anti-feedant and growth regulating properties, anti-bacterial, anti-fungal and anti-viral activities (Champagne *et al.,* 1992), anti-protozoal (Khalid *et al.,* 1998) and anti-sickling properties (Fall *et al.,* 1999). Several species of Meliaceae have been used in traditional medicine to treat cancer (Hartwell, 1970) and several limonoids have been found to be active in the inhibition of a murine P388 lympho-cytic leukemia cell line (Pettit *et al.,* 1983). Gedunin (**91**) has been found to exhibit anti-malarial activity (Khalid S.A.*et al.,* 1**989**) and methyl angolensate to have anti-ulcer properties. Limonoids from *Trichilia* have been shown to have potent cell adhesion inhibitory properties and limonoids from *Swietenia* have been shown to have platelet aggregation inhibiting properties. Citrus limonoids, including obacunol, which has been isolated from several Meliaceae species, have been found to be potent inducers of glutathione *S*-transferase activity in mice. Glutathione *S*-transferase may reduce the carcinogenic activity of chemical carcinogens by facilitating their rapid excretion The last major review of the chemistry of the Meliaceae appeared in 1983 (Taylor, 1984), but much chemical work has been done on the family, especially on species from the geographical region under review, since this time.

Compounds Isolated from Meliaceae

From novel to known ones, many compounds have been isolated from Meliaceae family. As enumerated earlier, they include limonoids, mono-, di-, sesqui-, and triterpenoids, coumarins, chromones, lignans, flavonoids and other phenolics.

I. Protolimonoids Group

Table 2: Refers to Limonoid Groups, from protolimonoids to Prieurianin group are twelve (12) tables (I to X)

Name	Structure	Source
melianone	-	*Turraea obtusifolia* *Entandrophragma caudatum*
turraeanthin	3β-acetoxy	*Turraeanthus africanus* *Turraea obtusifolia* *Entandrophragma caudatum*
melianodiol	24β,25-dihydroxy	*Turraea obtusifolia*
prieurone	11-acetoxy, Δ^{24}	*Trichilia prieuriana*
29-hydroxyprieurone	11-acetoxy-29-hydroxy, Δ^{24}	*Trichilia prieuriana*
glabretal	-	-
-	3α-acetyl-7α-deacetyl	*Turraea obtusifolia*
holstinone B	-	*Turraea holstii*
meliavolkenin	1α-acetoxy-7α-acetyl-3α-benzoyloxy-21,24α-dihydroxy, 1,2-dihydro	*Melia volkensii*
holstinone A	25-methoxy	*Turraea holstii*
holstinone C	25-chloro*	*Turraea holstii*

II. Havanensin group

Name	Structure	Source
deoxyhavanensin	-	-
-	1α-acetyl-11β-hydroxy-12α-(2'-methylbutanoyloxy), 28-oic acid, methyl ester	*Turraea floribunda*
-	11β-acetoxy-1α-acetyl-12α-(2'-methylbutanoyloxy), 28-oic acid, methyl ester	*Turraea floribunda*
-	11β-acetoxy-1α,7α-diacetyl-12α-(2'-methylbutanoyloxy), 28-oic acid, methyl ester	*Turraea floribunda*
-	11β-acetoxy-1α-acetyl-7-keto-12α-(2'-methylbutanoyloxy), 28-oic acid, methyl ester	*Turraea floribunda*
-	3α,7α-diacetyl	*Khaya anthotheca*
azadirone	-	*Turraea robusta* *Entandrophragma delevoyi* *Khaya anthotheca*
-	1,2-dihydro	*Turraea robusta*
mzikonone	12α-acetoxy-7α-deacetyl, 1,2-dihydro	*Turraea robusta*

III. Gedunin Group

Name	Structure	Source
gedunin	-	*Xylocarpus granatum* *Xylocarpus moluccensis* *Entandrophragma angolense* *Entandrophragma delevoyi*
-	7-keto	*Xylocarpus moluccensis* *Carapa procera* *Khaya senegalensis* *Pseudocedrela kotschyii*
-	7α-deacetyl	*Pseudocedrela kotschyii* *Khaya grandifoliola*
-	11β-acetoxy	*Entandrophragma delevoyi*
khivorin	-	*Khaya anthotheca* *Khaya senegalensis* *Khaya nyasica* *Khaya madagascariensis* *Khaya grandifoliola*

IVa. Andirobin group

Name	Structure	Source
andirobin	-	*Carapa procera*
-	Δ^{14}	*Khaya grandifoliola*
astrotrichilin a* astrotrichilin b*	12α,14α-diacetoxy-3α-hydroxy-2α-cinnamoyloxy-3α-nicotinoyloxy, 1,2-dihydro, Δ^{14} 12α,14α-diacetoxy-3α-hydroxy-3α-cinnamoyloxy-2α-nicotinoyloxy, 1,2-dihydro, Δ^{14}	*Astrotrichilia asterotricha*
methyl angolensate	-	*Xylocarpus moluccensis* *Carapa procera* *Entandrophragma angolense* *Entandrophragma utile* *Khaya senegalensis* *Khaya grandifoliola*
	6-hydroxy	*Khaya senegalensis* *Khaya grandifoliola*
	6-acetoxy	*Khaya senegalensis* *Khaya grandifoliola*

IVb. Trijugin group

Name	Structure	Source
capensolactone 1	-	*Ekebergia capensis*
capensolactone 2a* capensolactone 2b*	12α-acetyl-2α-(2'-methylbutanoyloxy)-3α-nicotinoyl 12α-acetyl-3α-(2'-methylbutanoyloxy)-2α-nicotinoyl	*Ekebergia capensis*
capensolactone 3a* capensolactone 3b*	12α-acetyl-2α-(2'-methylbutanoyloxy)-3α-nicotinoyl, $\Delta^{8,30}$ 12-acetyl-3α-(2'-methylbutanoyloxy)-2α-nicotinoyl, $\Delta^{8,30}$	*Ekebergia capensis*
E.P.4	2α-acetoxy-12α-acetyl-3α-angeloyl, $\Delta^{8,30}$	*Ekebergia pterophylla*
E.P.5	2α-acetoxy-3α,12α-diacetyl-11-dehydroxy	*Ekebergia pterophylla*
voamatin A	-	*Astrotrichilia voamatata*
voamatin B	9β-hydroxy	*Astrotrichilia voamatata*
voamatin C	-	*Astrotrichilia voamatata*
voamatin D	3α-cinnamoyl	*Astrotrichilia voamatata*

IVc. Mexicanolide group†

mexicanolide	-	*Carapa procera* *Xylocarpus moluccensis* *Xylocarpus granatum* *Khaya senegalensis* *Khaya madagascariensis* *Khaya grandifoliola*
-	6α-hydroxy	*Khaya senegalensis*
fissinolide	3β-acetoxy	*Khaya grandifoliola* *Khaya madagascariensis* *Khaya nyasica* *Khaya senegalensis*

IVd. Phragmalin group

Name	Structure	Source
phragmalin	-	-
-	3β,30α-diacetyl	*Xylocarpus moluccensis*
xyloccensin E	2α,3β,30α-triacetyl	*Xylocarpus moluccensis*
pseudrelone A_1	15β-acetoxy-30α-acetyl-3β-(2'-methylpropanoyl)	*Pseudocedrela kotschyii*
pseudrelone A_2	30α-acetyl-3β-(2'-methylpropanoyl)-15β-(2'-methylpropanoyloxy)	*Pseudocedrela kotschyii* *Neobeguea mahafalensis*
-	3β-nicotinoyl-30α-(2'-methylpropanoyl)	*Entandrophragma caudatum*
-	12α-acetoxy-3β-nicotinoyl-30α-(2'-methylpropanoyl)	*Entandrophragma caudatum*
-	30α-nicotinoyl-3β-(2'-methylpropanoyl)	*Entandrophragma caudatum*
-	3β,30α-di(2'-methylpropanoyl)	*Entandrophragma caudatum*
-	3β-(2'-methylpropanoyl)-30α-propanoyl	*Entandrophragma caudatum*

VI. Obacunol Group

Name	Structure	Source
obacunol	-	*Lovoa trichiliodes*
-	7α-acetyl	*Carapa procera*
dihydronomilin acetate	1α-acetoxy-7α-acetyl, 1,2-dihydro	*Xylocarpus granatum*†

VII. Nimbin Group

Name	Structure	Source
nimbolinin B	-	*Turraea robusta* *Melia volkensii*
-	12-O-methyl	*Turraea holstii*
volkensin	1α-tigloyoxy-7α-detigloyl	*Melia volkensii*
salannin	-	*Melia volkensii*
	1α-(2-methylpropanoyloxy)	*Melia volkensii*
	1α-(2'-methylbutanoyloxy)	*Melia volkensii*
ohchinin acetate	1α-cinnamoyloxy	*Melia volkensii*
volkensinin	-	*Melia volkensii*

VIII Toonafolin Group

Name	Structure	Source
toonacilin	-	-
-	11β-acetoxy	*Turraea holstii*
turraflorin A	11β-acetoxy; Δ^{14}	*Turraea floribunda*
turraflorin B	11β-acetoxy-12α-deacetyl, Δ^{14}	*Turraea floribunda*
turraflorin C	1α,2α,11β-triacetoxy, 1,2-dihydro, Δ^{14}	*Turraea floribunda*

IX. Evodulone Group

Name	Structure	Source
evodulone	-	*Carapa procera*
proceranone	Δ^{14}	*Carapa procera*
delevoyin B	$1\alpha,6\alpha$-acetoxy, 1,2-dihydro, Δ^{14}	*Entandrophragma delevoyi*
dregeana-3	-	*Trichilia dregeana*
dregeana-4	29-hydroxy-7α-(2'-hydroxy-3'-methylbutanoyloxy)	*Trichilia dregeana* *Trichilia emetica*
dregeana-5	1-deacetoxy-29-hydroxy-7α-(2'-hydroxy-3'-methylbutanoyloxy), Δ^{1}	*Trichilia dregeana*

X. Prieurianin group

Name	Structure	Source
prieurianin	-	*Turraea obtusifolia* *Nymania capensis* *Trichilia prieuriana*
mombasone	1-deacetoxy-2'-keto, Δ^{1}	*Turraea mombasana*
mombasol	1-deacetoxy, Δ^{1}	*Turraea mombasana*
nymania 3	1,29-dideacetoxy-$11\beta,12\alpha$-diacetoxy, $14\beta,15\beta$-epoxy, Δ^{1}	*Nymania capensis*
nymania 4	$11\beta,12\alpha$-diacetoxy, $14\beta,15\beta$-epoxy	*Nymania capensis*
trichilia substance Tr-A	29-deacetyl-15β-acetoxy, ethyl ester	*Trichilia emetica*
trichilia substance Tr-C	29-deacetyl-15β-acetoxy	*Trichilia emetica*
dregeana 2	29-deacetyl-$11\beta,12\alpha$-diacetoxy	*Trichilia dregeana*
nymania 1		*Turraea obtusifolia* *Nymania capensis* *Trichilia emetica*

Over the past decade, improvements of LC-MS systems have made great progress. The combination of classic Liquid Chromatography (LC) with Mass Spectrometry (MS) has led to a remarkable increase of analytical applications. Today LC-MS is an important technique for identification and quantification of metabolites in pharmaceutical laboratories as well as for pharmacokinetics and

protein, peptide and oligonucleotide analysis. With advancements in ionization methods and instrumentation, liquid chromatography /mass spectrometry (LC/MS) has become a powerful technology for the characterization of small molecules and proteins (Guodong *et al.,* 2007).

The development of electrospray ionization (ESI) (Fenn *et al.,* 1989), matrix-assisted laser desorption/ionization (MALDI) (Karas *et al.,* 1987 and Hillenkamp *et al.,* 1991) or soft ionization, (Tanaka *et al.,* 1988) and ambient MS (Cooks *et al.,* 2006) 11 has further augmented the role of MS in the studies of the small molecules and proteins (Guodong *et al.,* 2007).

LC-MS has currently become the rapid and accurate way of determining the molecular weight and identifying compounds. It has been used in analyzing drugs and substances in forensic laboratory (Andrew *et al.,* 2005). A rapid and sensitive LC-ESI-MS method for quantification of limonoids, for instance, quantification of limonoate A-ring lactones (LARL) and nomilinoate A-ring lactones (NARL) in a wide variety of citrus juices was described. This method provides a valuable tool for citrus growers and juice producers to evaluate the susceptibility of fruit or juice to delayed bitterness. The LC-MS of accurate mass and data base searching has been used to determine/identify unknown empirical formulas to chemical structures, for example, unknown pesticides on tomato skins (Andrew *et al.,* 2005).

LC-MS in Structural Elucidation of Limonoids and Other compounds

Several chemists/researchers in their work have used the reverse phase HPLC in isolation of limonoids and determined their molecular weights in decades now. These include Nakatani *et al*. (2004) in their isolation of 6 new phragmalin

limonoids (tabulalin and tabulalides (A - E)) from the root bark of *C. tabularis* using droplet countercurrent chromatography (DCCC) and reversed phase HPLC/MS and Manners *et al.,* 2004 which isolated a number of limonoids and compounds with the same tool (isolated Limonin, obacunone, nomilin, Deacetylnomilin, Methyl isoobacunoate, Ichangensin, Methyl deacetylnomilinate, Methyl isoobacunoate diosphenol and more using LC-APCI-MS). The APCI, atmospheric pressure chemical ionization is one of many soft ionization sources in LC-MS (Fenn *et al.,* 1989). Manners' group isolated 17 compounds and used EI/MS for the fragmentation pattern but in all cases used the APCI (Manners *et al.,* 2004). Schoch et al. (2001) also used the LC-ESI-MS.

ESI is an extremely gentle ionization method, accompanied by very little fragmentation of the formed molecular ions. Consequently, weak bonds are often preserved and analysis of intact post-translationally modified peptides/proteins and noncovalently bound complexes, such as protein–ligand complexes, can be successfully performed with ESI-MS (Li *et al.,* 1991; Karas *et al.,* 2000; Hofstadler *et al.,* 2006). Even though fragments are seldom produced in ESI, the ions generated are especially favorable for collision induced dissociation (CID), because the high charge state of the molecular ions increases the energy available for the collision event. (Smith *et al.,* 1990). Analyte signal suppression caused by charge competition between electrolytes and, for example, other analytes, is a major problem in ESI and may in practice prevent thorough analysis of complex mixtures if chromatographic prefractionation is not applied. These charge competition phenomena as well as the analyte signal's strong dependence on

experimental conditions, such as pH, solvent composition, and salt concentration, make it risky to draw quantitative conclusions from ESI-MS data.

Limonoids Determined With APCI

Zhang *et al.,* in 2007 isolated 11 limonoids from *Khaya senegalensis* using ESI-MS, LC-ESI-MS and other spectroscopic methods. The limonoids, (1-8) were elucidated as 3α, 7α-dideacetylkhivorin (**1**), 1α, 3α, 7α-trideacetylkhivorin (**2**), khayanone (**3**), 1-*O*-acetylkhayanolide B (**4**), khayanolide B (**5**), khayanolide E (**6**), 1-*O*-deacetylkhayanolide E (**7**), and 6-dehydroxylkhayanolide E (**8**). Each of them was elucidated with the accurate ESI-MS (LC-ESI-MS). Compound **3** as indicated in the Appendix is one of the 8 limonoids whose molecular weight was determined with the accurate LC- ESI-MS. The high molecular ion that is recorded on the spectrum is due to incomplete dissolvation in the ion source of the mass mass spectrometer. In their work, mostly limonoids, are observed in the spectra. One of the compounds isolated is khayanone. Typical spectrum of khayanone from the work is the Compound **3** in the appendix **B**.

Table 3 shows molecular weight of limonoids determined with APCI.

Compounds	**APCI(*m/z*)**
Limonin .	[M+H] +: 471.3.
Nomilin	*m/z*[M+H] +: 515.3.
Deacetylnomilin	[M+H] +: 473.4.
Obacunone	[M+H]+: 455.4.
Ichangensin.	[M+H]+: 445.4.
Methylisoobacunoate diosphenol	[M+H]+: 505.3.
7α-Limonol	*m/z*[(M+H)+72]+:545.4.
Cyclocalamin	[M+H] +: 503.2

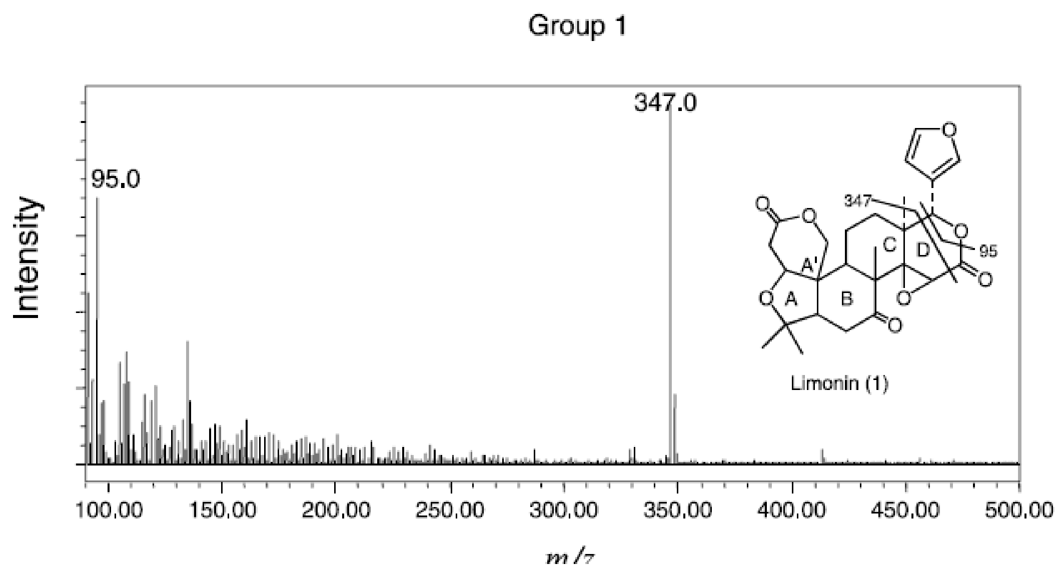


Figure 3a. Representative EI/MS of citrus limonoids categorised into groups

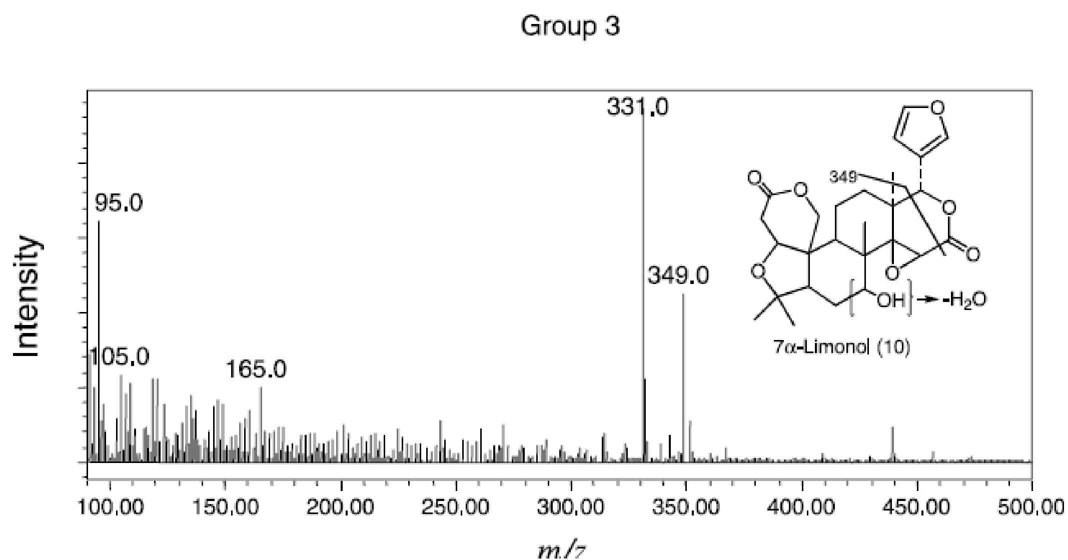


Figure 3b. Representative EI/MS of citrus limonoids categorised into groups

From Manners *et al.*'s work, it is observed that the accuracy of EI/MS is limited while APCI/MS a source presents *m/z* at 471 and APCI/MS *m/z* [(M+H) + 72]+: 545.4., the EI-MS *m/z* 347 and 349 for limonin and 7α Limonol respectively . In this work Manners *et al.,* modified their previously described normal-phase

HPLC-UV method (Manners & Hasegawa, 1999) for application as an HPLC-MS method which could utilize APCI or EI detection in order to identify neutral limonoids in citrus seed extracts. The modified method was found effectively to resolve 13 of 17 neutral limonoid standards on a spherical silica gel column with cyclohexane:tetrahydrofuran gradient elution (Figure 3). In this work , the LC-MS is used coupled with the ESI source and UV- chromatogram. Limonoids which were chromatographically resolved by the normal-phase method were detected as TIC chromatograms. In the HPLC-APCI/MS method, the mass spectrometer was operated in the positive ion mode and, with the exception of 7α-limonol , 7α-limonyl acetate, deoxylimonin and deoxylimonol, each standard limonoid provided a protonated molecular ion [M+H]+. The 17 compounds isolated are modified below:

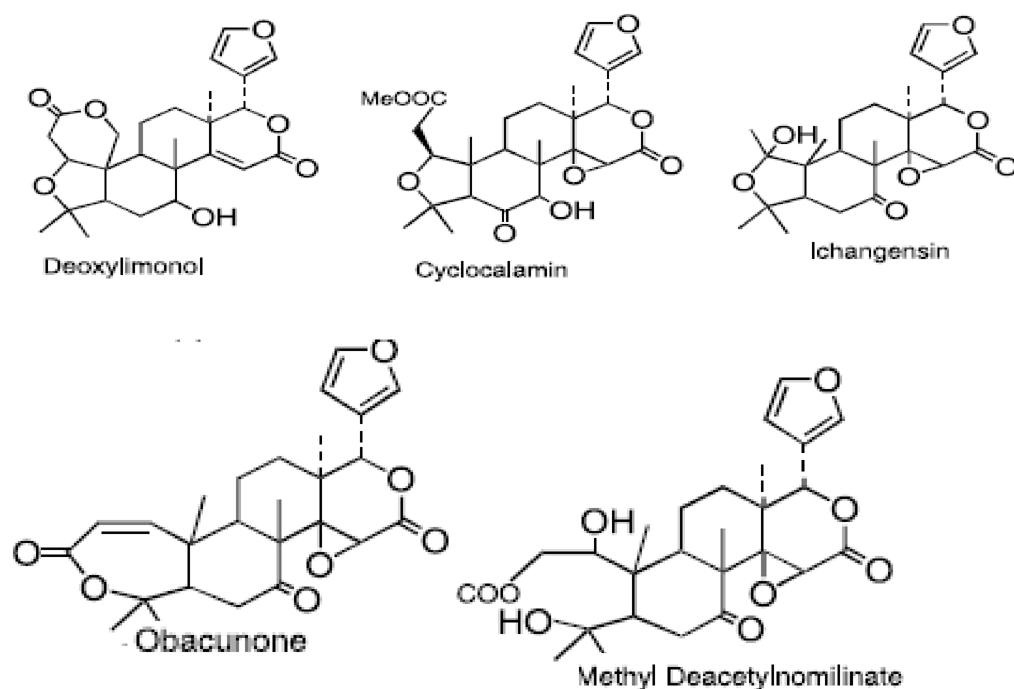


Figure 4: Citrus Limonoids Analyzed with APCI/MS (Source Modified: Manners *et al.,* 2004)

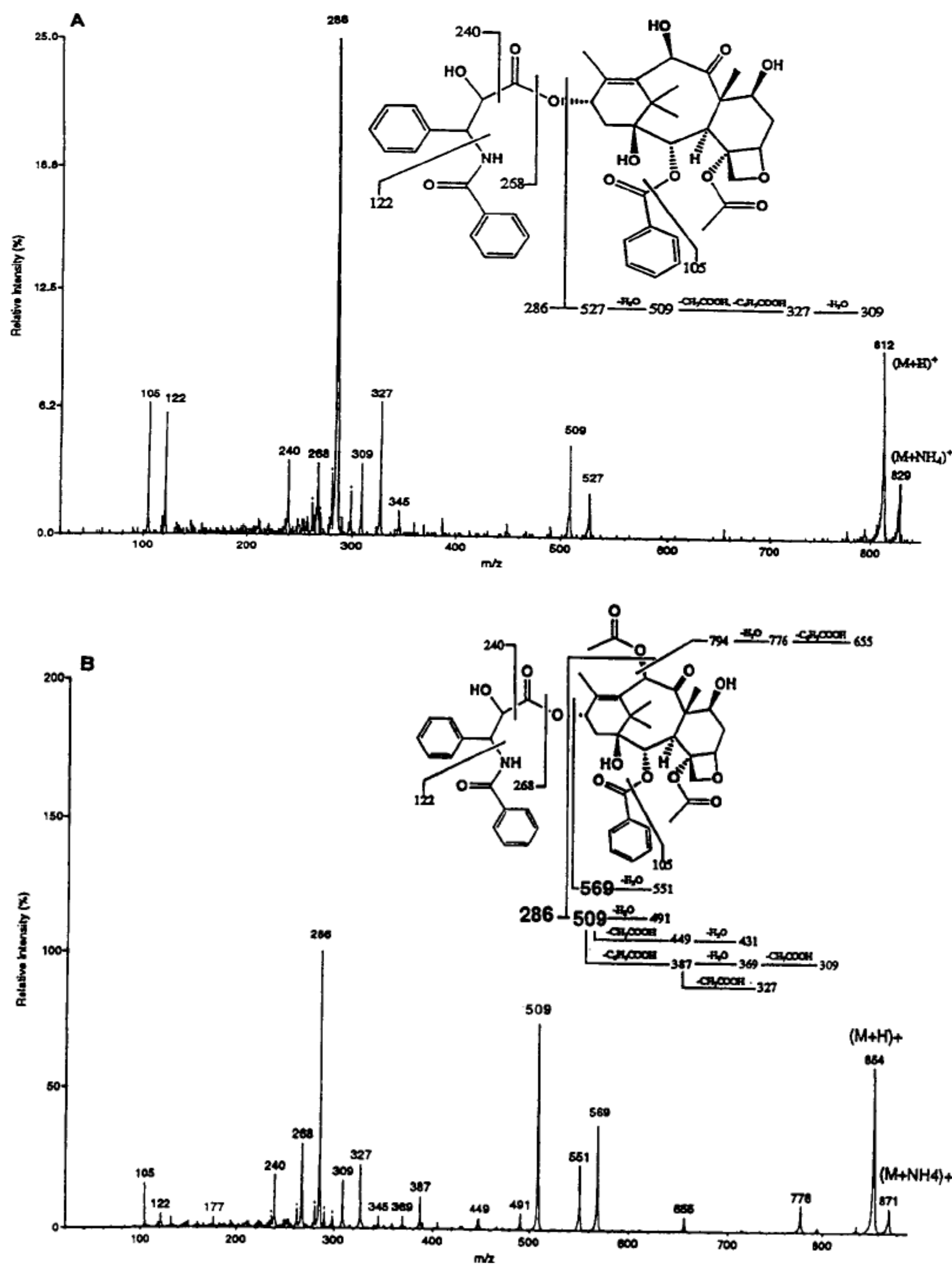


Figure 5. Template structure identification of a base-induced degradant of paclitaxel. (A) Product-ion spectrum of the ion at *m/z* 829 (H.NH4) . of a base-induced degradant of paxlitaxel. (B) Product-ion spectrum of the *m/z* 871 (H.NH4) ion of paclitaxel used as a template. The product ions and neutral losses that correspond to specific substructures are indicated. Diagnostic product ions at *m/z* 286, 268, and 240 indicate the presence of the paclitaxel sidechain. The *m/z* 509 ion in the degradant is indicative of the deacetylated paclitaxel core ring structure. The presence of the *m/z* 527 ion instead of the *m/z* 569 ion of paclitaxel indicates the loss of an acetyl substructure from the core ring structure. This result is consistent with the MW difference of 42 Da of the degradant, which is indicative of an acetyl substructure (Volk *et al.,* 1997).

LC –MS interface with ESI or APCI softly ionizes a huge compound as in figure 5 and by means of collision induced dissociation result in just a few fragments as shown above (Volk *et al.,* 1997).

CHAPTER THREE

EXPERIMENTAL

General experimental procedures

All chemicals used for extraction and separation were distilled and some few bottles analytical grade and were used directly without further purification.

The Infrared (IR) spectra were recorded on KBr discs using Shimadzu FT-IR 820/A spectrometer. Appendix T are UV-spectra for 1A; 4th, 5th,1b, and 2A

Nuclear Magnetic Resonance (NMR) analyses were performed on a Varian VNMRS 400MHz NMR spectrometer (Sample dissolved in deuterated chloroform) in the US.(Appendix .R ^{1}HNMR 1a(Band 1) and Appendix S 1b(A))

Second analyses were done on Bruker 400 NMR spectrometer- South Africa. Appendices ..C, D, C2, E, K, L, N, O, P, and Q

Third analyses were performed NMR spectrometer in McMaster University in Canada. Appendix T.

Mass Spectrometer(MS)

LC-MS analyses were performed Using an Agilent 1200 series HPLC system with Electrospray Mass spectrometer in Positive ion Mode with a reverse phase (RP) C-18 analytical column (ZORBAX).

Second analysis MS was done with Electrospray Mass spectrometer in both Positive and Negative ion Modes in McMaster University, Canada.

Chromatography: For Colomn, silica gel, Merck Kieselgel 230-400 mesh used. For Thin Layer Chromatography, however, silica gel 60HF was used with thickness of 0.25mm. Wihout sparing, precoated TLC cards (0.25mmsilica gel with flouriscent indicator; polygram(R) SILG-/UV_{250}) were also used at the

various stages to check purity,. With the TLC the Spots were detected under UV-light. Erhlich reagent (p-aminobenzaldehyde dissolved in 50ml ethanol) was sprayed on the developed chromatogram and followed by exposure to HCl gas evolved (generated) from a reaction between NH_4Cl and H_2SO_4. Purple to Blue coloured distinct separations indicated the presence of limonoids

Plant Material

The stem bark of *Turraea heterophylla* was collected from Abrafo in the Kakum Forest near Cape Coast, Central Region, Ghana (West Africa), in May 2009 under the supervision of Prof. Tayman, and was identified by Mr. Agyakwa in the Botany Department, University of Cape Coast, Ghana.

Extraction and partition (2009)

Air dried root bark (544 g) of *Turraea heterophylla* was ground using a mechanic machine and sieved through a 100 mesh sifter. Then 200 g of the plant powder was extracted using 2.5 L each of the solvents Hexane, Dichloromethane (CH_2Cl_2), and Methanol (MeOH) for 72 h on a Mechanical Shaker, with a total of 3 batches needed to process the 544 g of bark. The pooled Hexane, Dichloromethane (DCM), and MeOH extracts were in that order filtered through glass wool, and concentrated with the rotary evaporator in vacuum to remove the solvents. The 5g extract was obtained for hexane, 42g for DCM and MeOH, 30g. The 2 extracts, DCM and MeOH were bioassayed using bacteria. For anti-tumor and antioxidant tests, the isolates waited for their turn of the test but because of several thousand waiting, it could not be done. The same extraction method (procedure) was followed for the root wood alone and TLC checks were done for

all the extracts to determine their solvent systems. The work has been summarized in the flow chart (**Figure 6**).

Phytochemical Screening

The crude extract of the plant was subjected to phytochemical screening to find the group of compounds present (Table 4.)

Purification and Isolation

The concentrated CH_2Cl_2 extract was subjected to flash chromatography over silica gel (particle size 32-63 μM), and the elution was conducted in an increasing polarity of solvent system using *n*-hexane, 10% ethylacetate in hexane, 20% ethylacetate in hexane, 30% ethylacetate in hexane, 40% ethylacetate in hexane, 50% ethylacetate in hexane and 60% ethylacetate in hexane respectively.

Table 4 Phytochemical Screening of *T. Heterophylla* extracts

Test	Observation	Inference
ALKALOIDS (1) 2ml MeOH extract + 2ml $CHCl_3$ +1.0g of Fe(III) and heating with 10ml of HCl(2M) in a boiling tube	Deep brown Coloration	Alkaloids absent
(2)Few drops ofH_2SO_4 + 2ml MeOH extract	Deep black solution observed	Alkaloids absent
(3)2ml Dragendorf'reagent + 1ml methanol	Deep black solution No red or orange coloration	00.Alkaloids absent
TANNINS 1ml MeOH extract + 3ml of 80% MeOHin $FeCl_3$ solution	Solution turned green black	Tanins may be present
SAPONINS 1ml MeOH extract + 8ml distilled water shaken vigorously in a test tube and allowed to stand vertically.	Formation of honey comb froth about 3.5 cm above surface of the solution. Froth persisted for 2hours	Saponins may present
LIEBERMAN – BUCHARD 2ml MeOH extract + 2ml of chloroform + 3ml of acetic anhydride. Swirled and a drop of concentrated H_2SO_4 added	Appearance of a reddish pink to purple colouration	Saponin, may triterpenoids present
SALKOWISKI 2ml Conc. H_2O_4 allowed to run down the inside of a test tube containing plant sample (0.50g)	Deep brown coloration i.e. A reddish brown ring developrd at the interface	Unsaturated Terpenoids and / or steroids present

Continuation Table 4

Test	Observation	Inference
CARDIAC GLYCOSIDES		
5ml extract evaporated to dryness. Residue is dissolved in 10ml distilled water plus 3ml strong lead acetate solution, plus $CHCl_3$ layer. 2ml of the $CHCl_3$ extract plus 4ml of 5% Ferric Sulphate: glacial acetic acid 1:99 volumes plus 2 drops of conc. H_2SO_4.	Blue colour produced in the acetic acid layer	Cardiac glycosides probably present
REDUCING SUGARS		
2ml methanol extract plus Fehling's solution A + b heated to boiling.	Brick red precipitate	Reducing sugars present
LIMONOID		
Methanol extract spotted on TLC plate was developed in ethyl acetate/Pet Ether 1:1 solvent mixture. The plate was sprayed with Ehrlich's reagent (prepared by dissolving 4g p-N, N-dimethylaminobenzaldehyde in 100ml ethanol to make 4% solution), from ammonium chloride and conc. Sulphuric acid.	Over ten spots with colours ranging between yellow and orange were observed.	The spots were indicative of the number of limonoids present.

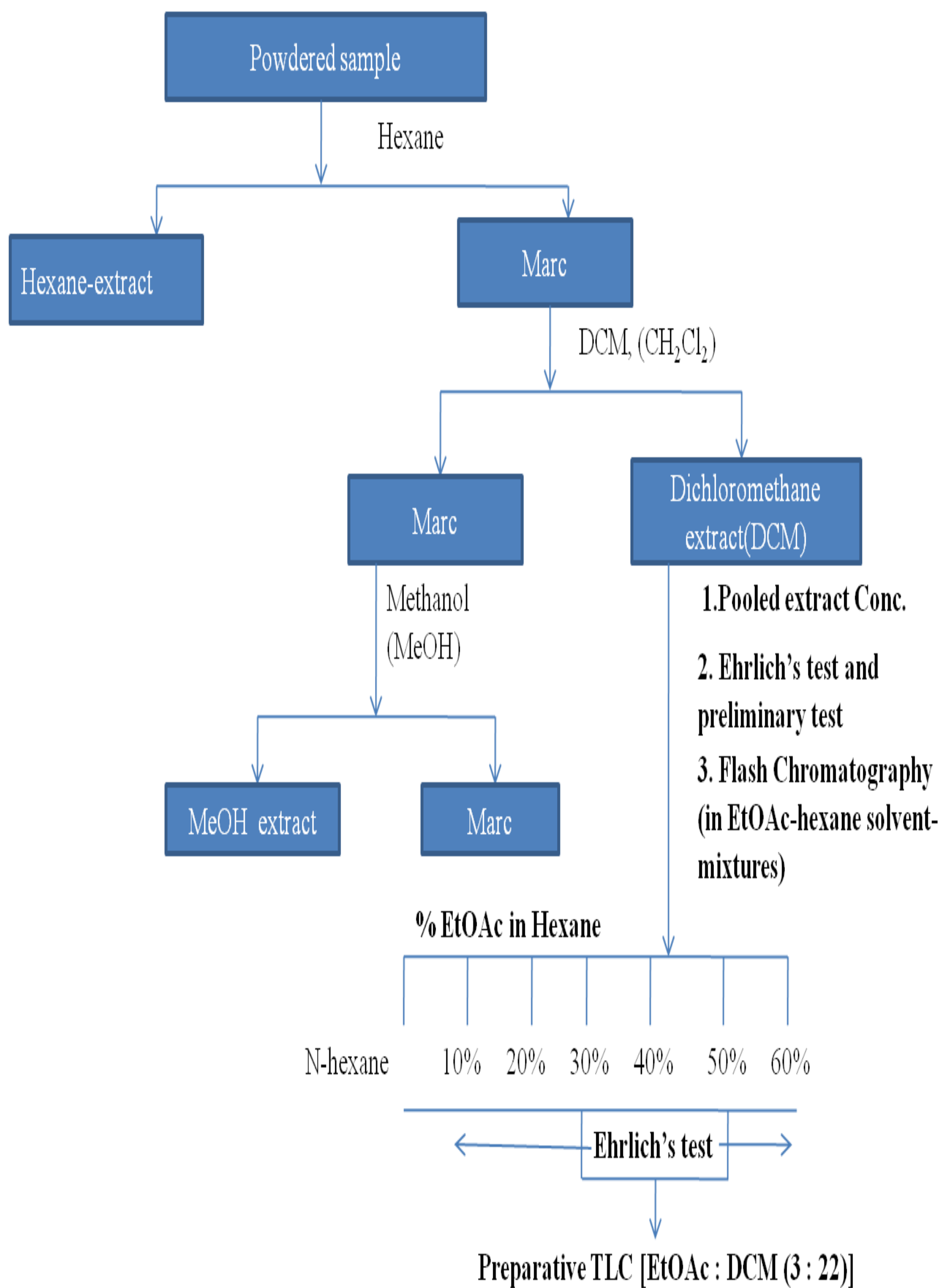


Figure 6: Summary of the work (2009)

. **Table 5: Preparative Chromatography**

Solvent Systems	Components(No. of separations)		Comment
	40% fraction	30% fraction	
DCM : EtOAc (22:3)	6	6	Were both very distinct

Extraction and partition (Between 2006 and 2007 and was analysed in 2008)

Air dried root bark (400g) of *Turraea heterophylla* was ground using a mechanical machine (Miller). Then 200 g of the plant powder was extracted using 2.5 L each of the solvents [Dichloromethane (CH_2Cl_2) - Methanol (MeOH)] in the ratio 1:1 for 72 h on a Mechanical Shaker. The marc was subjected to 100% MeOH extraction on the shaker for 48hours and the two extracts were put together. The pooled Dichloromethane (DCM), and MeOH extracts were decanted and concentrated with the rotary evaporator in vacuum to remove the solvents. The 70g extracts obtained were suspended in 300 ml of 80% MeOH and then partitioned with 400 ml n-hexane. The hexane extract, 19g was run slowly on the column with 5% (fractions 1-20), 10%(fractions 21-40), 20%(fractions 41-60) and then 30% ethylacetate in hexane (fractions 61-80) 500ml each. The 72nd and 73rd after concentration had formed some crystals but was mixed with some oily component of the fraction and was so difficult to purify and still obtain a sizeable amount for analysis. The summary of the work is in the flow chart (**Figure 7**).

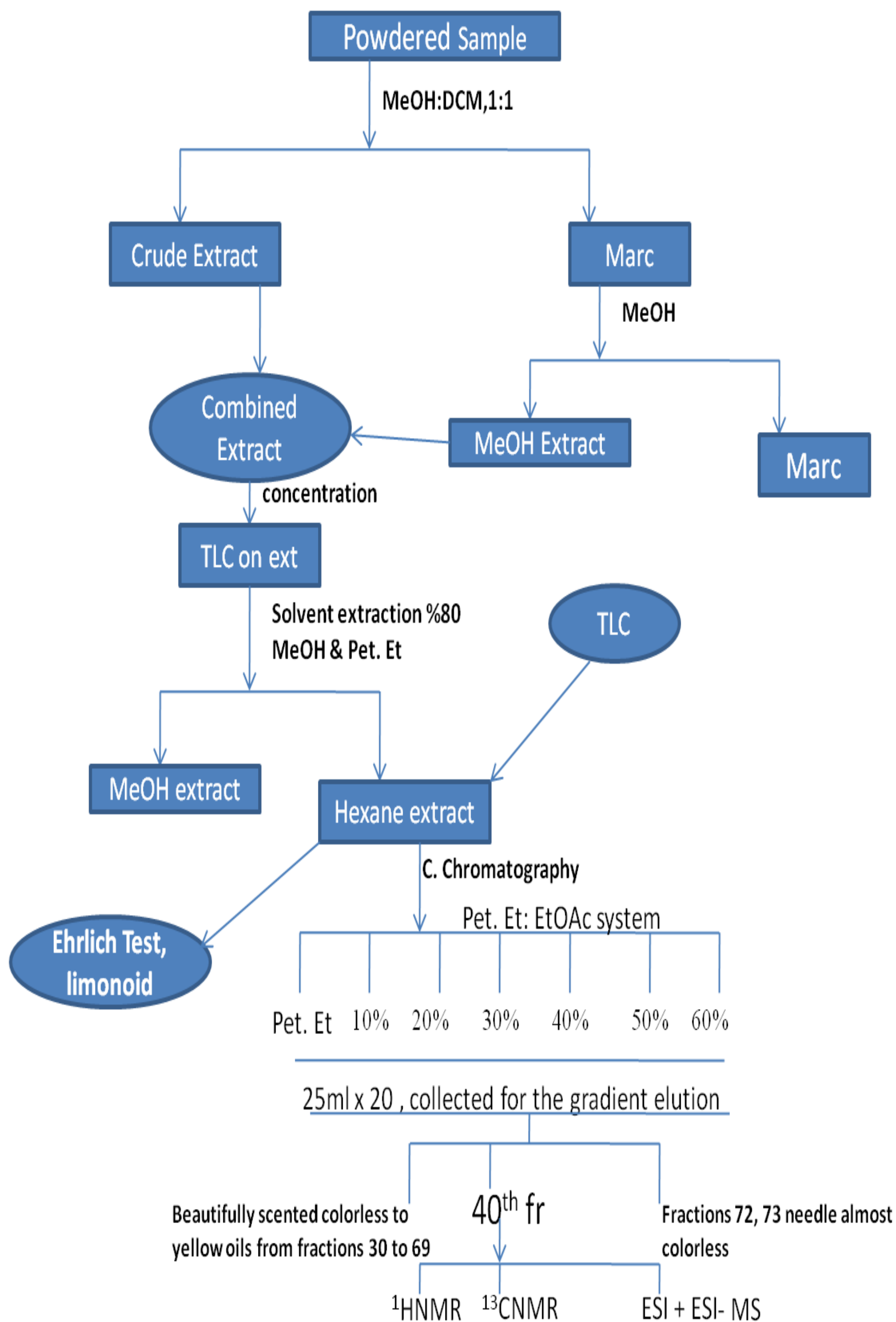


Figure 7: Summary of the work for 2008

Methods of Analysis

Each sample was analyzed by ^{1}H NMR and HPLC-ESMS.

^{1}H NMR analysis was performed on a Varian VNMRS 400MHz NMR spectrometer. Samples were dissolved in deuterated chloroform ($CDCl_3$) for analysis unless otherwise noted. Deuterated chloroform is observed in the ^{1}H nmr spectra as a singlet peak at 7.26 ppm, due to presence of trace amounts of $CHCl_3$.

HPLC-ESMS analysis was performed using an Agilent 1200 series HPLC system with positive ion mode electrospray mass spectrometer (ESMS) with a reverse phase (RP) C18 analytical 5µm column (ZORBAX). Purification by reverse phase C-18 chromatography usually gives the reverse elution of polar/non-polar compounds as compared to silica chromatography. HPLC elution was using a 5% to 95% gradient of an acetonitrile/water solvent system containing 0.01% of trifluoroacetic acid (TFA) to ensure a reproducible elution and positive ionization in the ESMS. In ESMS, analytes were usually observed as ions with a *m/z* value (mass to charge ratio) corresponding to ionic species such as MH^+ (protonated parent ion), MNa^+ (sodium adduct), M_2H^+ (protonated dimer), M_2Na^+ (sodium adduct dimer), or a protonated fragment (i.e., the molecule was fragmented in the mass spectrometer to produce a fragment that has a smaller *m/z* value than the parent MH^+.

Table 6: Summary of HPLC-MS Analysis

HPLC retention time (min)	Fraction 1a Major ion species MH+	Fraction 1b Major ion species MH+	Fraction 1c Major ion species	Fraction 1d Major ion species
0.6-1.0		673.2, 609.2, 555.2		
0.9-1.8	331.2 , 301.1			335.2, etc
10.2-10.5			943.7, 509.3	
10.8-11.1		455.3 (MH+)		495.3, 705.5, etc
11.1-11.3				
12.2-12.8			665.5 (MH+), 413.3	413.3, etc
12.6-13.3	391.2 (MH+)			

Bioassay (Antimicrobial Activity of Plant Extract)

Media Preparation

An amount of 10.0g of commercially prepared nutrient Agar was suspended in 50ml of distilled water and shaken vigorously to mix. The bottle was then plastered with 2cm autoclave tape (this showed a black striped colour on the tape, indicating the completion of the sterilization). The mixture in the bottle was then autoclaved for 15minutes at 121°C in a current of steam. The prepared media was allowed to stand for 15 minutes, then poured in the Petri dish and covered until it dried for the inoculating of bacteria samples. This was then packed into a fridge for storage.

Sterilization of Petri Dish

Petri dishes were washed thoroughly and dried in the oven at 50°C. It was then sterilized in the oven for one hour at 160°C. After the sterilization it was allowed to cool; thus to contain the prepared media.

Preparation of Concentration

A quantity of 5mg of the extract of *Turraea heterophylla* was weighed and dissolved in 1ml of 10% Dimethylsulphoxide, (10ml of DMSO topped up to 100ml) in an ependorf tube. The concentrations were decreased in serial dilution succession by pipetting 0.5ml of the stock (1st ependort tube) into a 2nd ependorf tube, topped up with 10% DMSO to 1ml graduation mark and then the procedure was repeated for three other ependorf tubes with the sixth containing 5mg of the extract dissolved in 1ml methanol for comparison.

Preparation of Disc and Petri Dish for Bioactivity

A very small disc of diameter 5mm was made from filter paper and sterilized in an oven at 30°C and dipped into the various concentrations from tube 1-6. It was allowed to soak the solution and was then removed to air dry. This was then ready for the administering of the antimicrobial activity experiment.

Inoculation of Bacteria and Fungus

Identified (bacteria and fungi) microbes were picked into peptone water and shaken gently for inoculation. After 10mintues, the peptone water (containing the microbes) was used to flood the prepared media in the Petri dish and completely poured onto another Petri dish until all the 5 Petri dishes were flooded with the peptone water (containing microbes) and the rest eventually poured away. The prepared disc was then placed in the respective concentration divisions

numbered 1-5 divisions. The 6th division disc, after using forceps and sterilizing it, was picked and placed on the surface. Antibiotic disc, cefuroxime sodium (CXM 30) was used as a control to all the microorganisms except for one which was a fungal microbe. This was then left for 24 hours in an incubator at room temperature after which an observation and the zone of inhibition was measured and recorded.

Inoculated Petri-dish

Six divisions of the inoculated plate with the concentrated disc

Plate 3: Inoculation of Bacteria and Fungus

CHAPTER FOUR

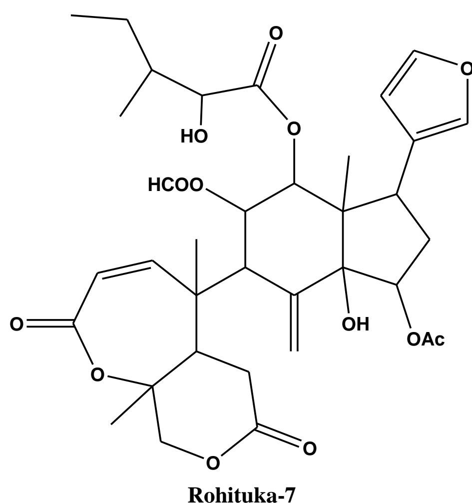
Results and Discussion

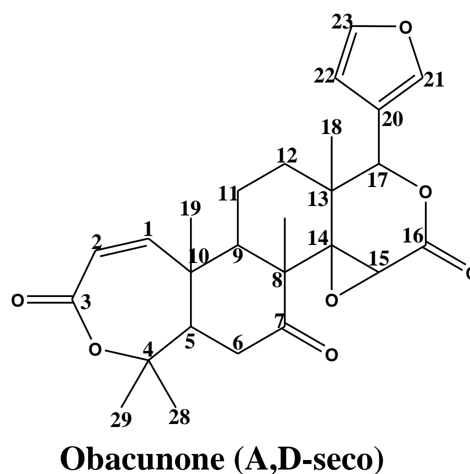
STRUCTURAL DETERMINATION OF THE ISOLATES

Introduction

The structures of the 3 isolated natural products (Figure 8) were characterized through various spectroscopic methods including IR, MS (LC-ESI-MS), ^{1}HNMR. Among the isolates, 2 compounds (**1&2**) belong to limonoids (tetranortriterpenoid) group and **3** compound is a diterpenoid. All the compounds are known ones. The limonoids were elucidated as rohituka-7(**1**) with ring A,B-*seco*(1), and obacunone limonoid with AD-seco- (**2**) and (**3**), diterpenoid. These tetranortriterpenoids are classified as three different carbon-frame types: (I) Prieurianin limonoid with ring

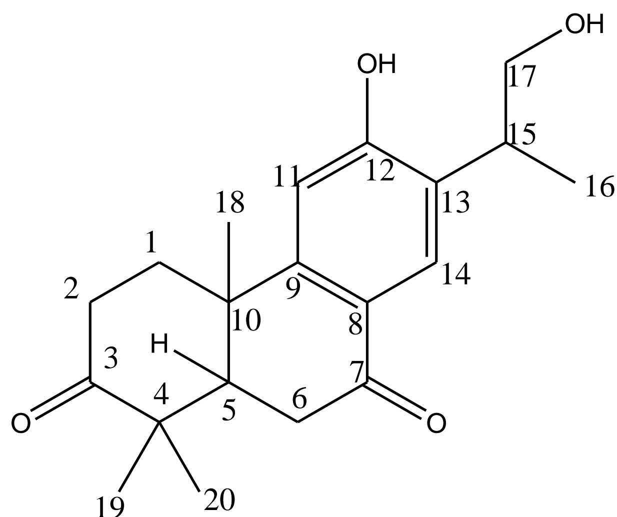
A, B-*seco* (**1**), and (2) Obacunol limonoid with rings A, D-*seco*.





Compound 1 ($C_{35}H_{44}O_{13}$)

Compound 2 ($C_{26}H_{30}O_{7}$)



Margocinin **3** ($C_{20}H_{26}O_4$)

Figure 8: The compounds isolated, includes 1, 2, and 3

Four samples (isolates) analysed with LC-MS instrument are GF1-1a, GF1-1b, GF1-1c and GF1-1d. They are analysed using electrospray mass spectrometer (ESMS) with a reverse phase (RP) C-18 analytical 5µm column (ZORBAX) very good for the analysis of non-polar (Taylor and Niessen, 2007) and medium polar small molecules. The ESMS results showed TIC (total ion counts) and the UV-chromatograms in the spetra. In ESMS, analytes were usually observed as ions with a *m/z* value (mass to charge ratio) corresponding to ionic species such as MH^+ (protonated parent ion), MNa^+ (sodium adduct), M_2H^+ (protonated dimer), M_2Na^+ (sodium adduct dimer), or a protonated fragment (i.e., the molecule was fragmented in the mass spectrometer to produce fragment(s) that has a smaller *m/z* value than the parent MH^+.

Results

Table 7: Results of HPLC-MS Analysis

HPLC retention time (min)	Fraction 1a Major ion species MH+	Fraction 1b Major ion species MH+	Fraction 1c Major ion species	Fraction 1d Major ion species
0.6-1.0		673.2, 609.2, 555.2		
0.9-1.8	331.2 , 301.1			335.2, etc
10.2-10.5			943.7, 509.3	
10.8-11.1		455.3 (MH+)		495.3, 705.5, etc
11.1-11.3				
12.2-12.8			665.5 (MH+), 413.3	413.3, etc
12.6-13.3	391.2 (MH+)			

Such fragmentation provided information as to the structure of the molecule being analysed. The dimmer species are clusters that contain 2 molecules due to e.g., incomplete desolvation in the ion source of the mass spectrometer. Unlike our traditional ionization sources example, Electron ionization, Chemical ionization (appendix M, they include most of the modern ones and the literature review on ESI and LC-MS), these points are considered:

1. The complex pattern of multiple-charged ions makes interpretation of ESI spectra from complex mixtures difficult and in practice computers are used

to transform the charge state envelopes to single peaks at the respective molecular mass (or zero charge state).

2. ESI is an extremely gentle ionization method, accompanied by very little fragmentation of the formed molecular ions. Consequently, weak bonds are often preserved and analysis of intact post-translationally modified peptides/proteins and noncovalently bonded complexes, such as protein–ligand complexes, can be successfully performed with ESI-MS (Li *et al.,* 1991; Karas *et al.,* 2000; Hofstadler *et al.,* 2006).
3. Even though fragments are seldom produced in ESI, the ions generated are especially favorable for collision induced dissociation (CID), because the high charge state of the molecular ions increases the energy available for the collision event. (Smith *et al.,* 1990).

The LC-ESI-MS results in this work have similar characteristics, and most of the compounds identified had either i) just the molecular ion or

ii) few fragments between 2-3 and

iii) analyst make comparison from their LCMS data bank coupled with other literature compounds.

Mass Spectra/UV-Chromatogram and their retention times

The ESI-MS result, GF1-1a of the TIC-UV-chromatogram showed two compounds with molecular ions of ESIMS *m/z* at 331.2 $[M+H]^+$ and 391.2 [M+H] respectively

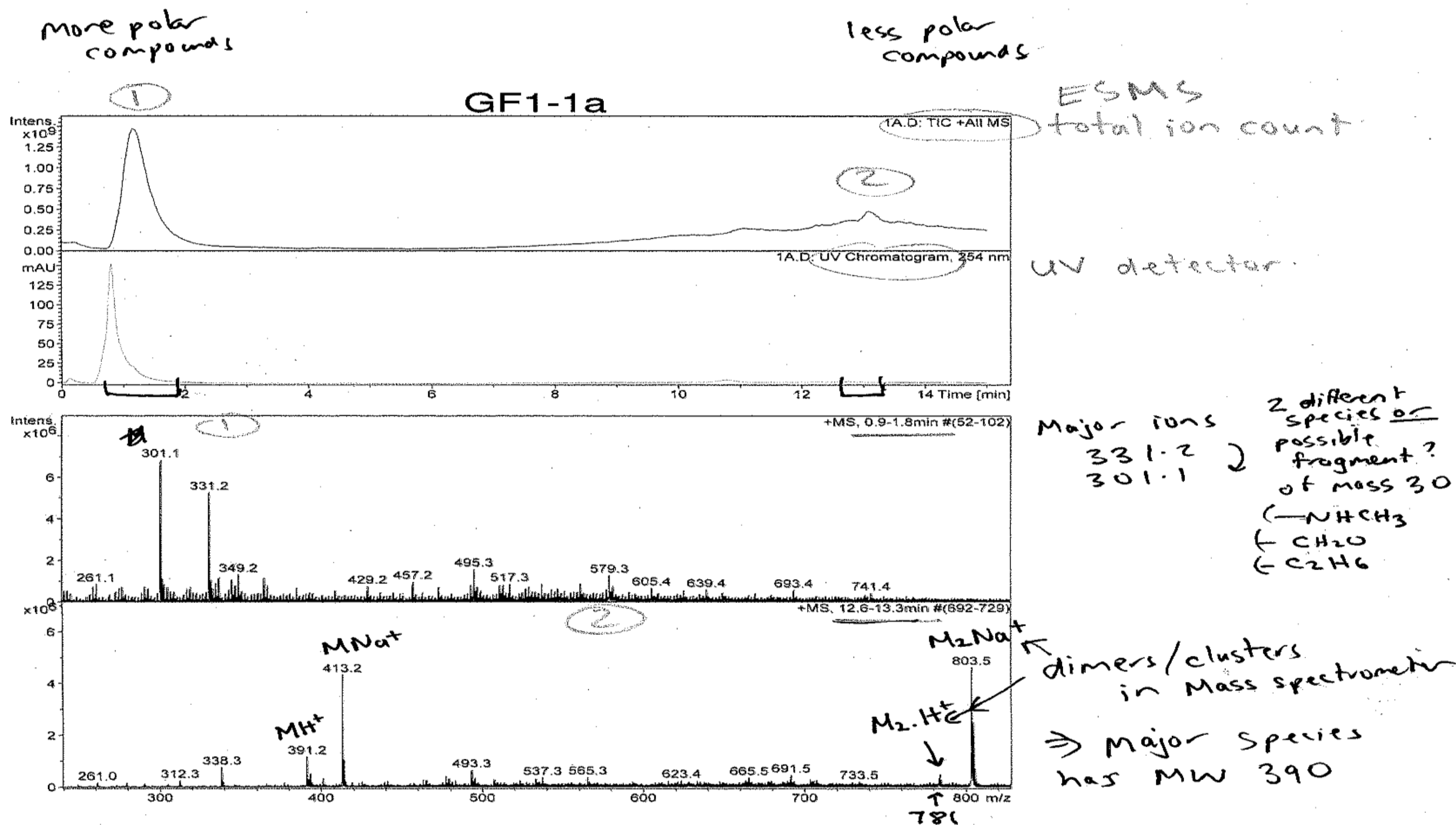


Figure 9: LC - ESI - MS spectrum of Margocinin

They are indicated as Ms **1** and Ms **2** accordingly.

The Ms2 in GF1-1a indicates *m/z* values of 391.2, 413.2, 781 and 803.5. The *m/z* 391.2 is $[M+H]^+$ and 413.2 is the sodium adduct of the compound $[M+H]^+$ with *m/z* $[M+Na]^+$.The MS value (*m/z*) 781 is $M_2.H^+$ and 803.5, sodium adduct in the form, $M_2.Na^+$. They are dimmers or a cluster of the compound in the mass spectrometer. The compound, 390 was identified by comparison with other spectroscopic data from the mass data bank, had characteristics of a protolimonoid but have not yet enough data for its structural elucidation. Hence, could be a new compound but a spectroscopic data is scanty to help in the full identification of the structure.

Table 8: Summary of ^{1}HNMR

Fraction 1a Selected Characteristic Peaks (ppm)
0.88 s, 1.0 s, 1.25 s, 1.6 s
2.04 m, 2.3 m, 2.62 s
3.42 d
3.68 d
4.1 dd
4.35 q
4.87s, 4.98 s
5.35 m
6.83, 7.87

The compound with *m/z* 331 [$M+H^+$] by the same mechanism of identification was found to be a diterpenoid. The HNMR data, though scanty to explain all the structural characteristics, the characteristic chemical shift for tricyclic diterpenoids with the C-11 and C-14 shifts recorded down field at 7.87 and 6.83 respectively, are typical of aromatic ring deshielding and additional deshielding due to the OH at C-12 and the carbonyl C-7(Figure 8).

The four methyl-groups have chemical shift (0.88, 1.6, 1.1, 2.5). There are two gem-dimethyl groups at C-19 and C-20. The rest of the chemical shift includes δ_H (2.04, 2.3, 3.42, 3.68, 4.1, 4.35, 4.87, 4.98 and 5.35) as shown in table 8.

The accurate molecular weight determined by the LC-ESI-MS and its comparison with the data in the mass bank of LCMS spectra showed consistency with those isolated from the *Azadiracta indica* (Ara, 1990; Hanson,1991). Now, the point listed above comes in here, that ESI molecular ions seldom form fragments because of their soft ionization. (Li *et al.,* 1991; Karas *et al.,* 2000; Hofstadler *et al.,* 2006).

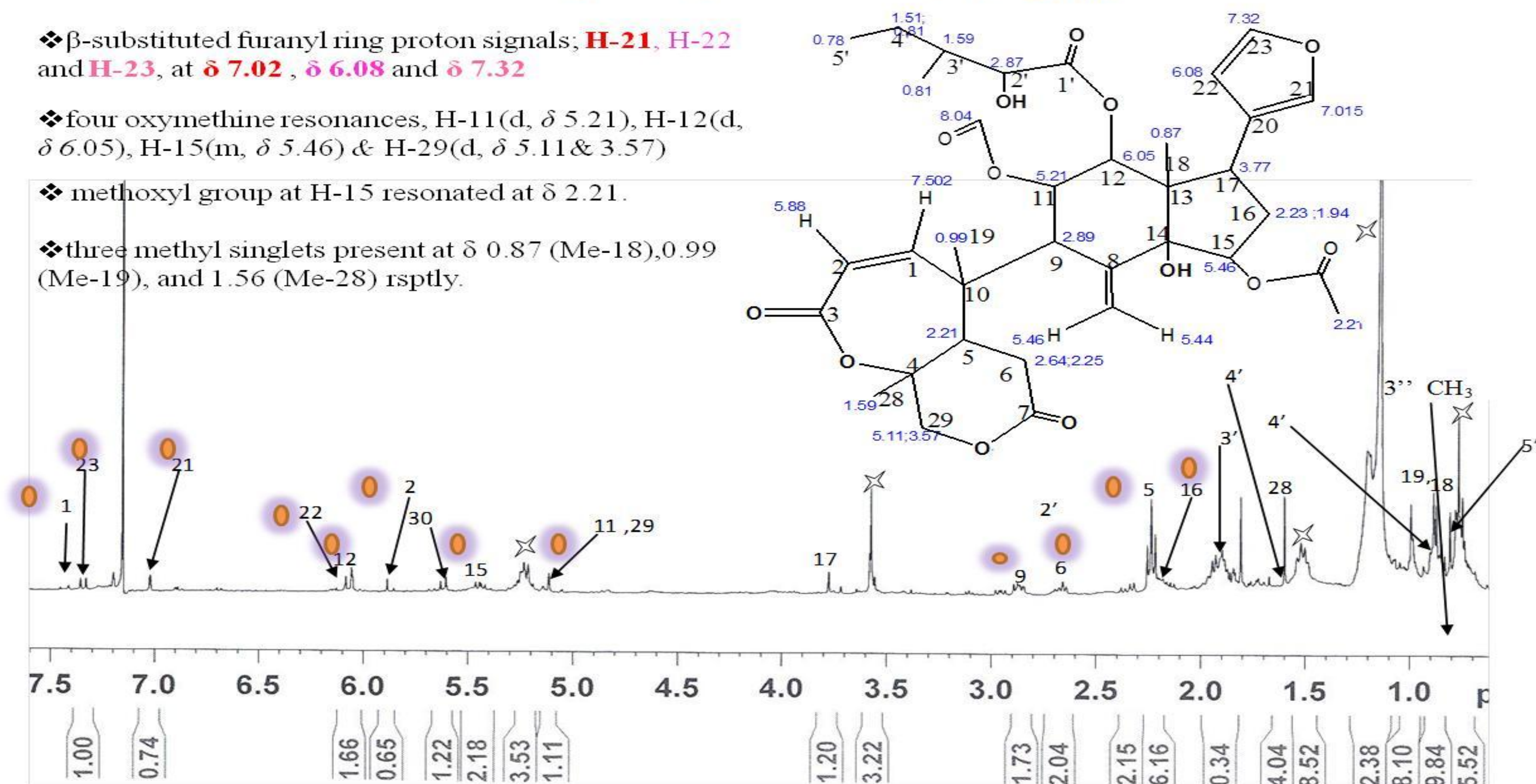


Figure 10: ^{1}H NMR Spectrum for **Rohituka -7**

The spectroscopic data available in comparison with the molecular weight of the compound is consistent with Margocinin. The sample isolate, GF1-1b equally had two mass spectra. The TIC-UV-chromatogram indicated two compounds; all of them pronounced with their accurate ESI-MS molecular ions for spectra (1) and (2) being *m/z*: 673.2 $[M+H]^+$ and *m/z* $[M+H]^+$ = 455.3 and *m/z* $[M+Na]^+$ = 477.3. Compound 1 is proposed as rohituka-7.

The extremely gentle ionization resulted in very few fragments, the MH^+ at *m/z* 673.2 with two other fragments- *m/z* 609 = $[M+H – CH_4O_3]$ and *m/z* 555.2 = $[M+H- C_3H_2O]$. The analyst compared the data with the information on rohituka-7 from the mass data bank and it consistently agreed with the literature or Huaping *et al's* publication in 2007. The data available on it, the H NMR coupled with the accurate ES1-MS data helped propose, *m/z* 672.2 as rohituka-7. (Appendix D, C_2 and Q) and (GF1-1b) in appendix H.

Compound 2 is identified as obacunone. The accurate ES1-MS results gave *m/z* $[M+H]^+$ =455.2 and its sodium adduct at *m/z* $[M + Na]^+$ = 477.2 agrees with the molecular weight 454.3. The undissociated dimeric sodium adduct at the ESI source at the time of analysis is indicated on the spectrum at *m/z* 931.6 $[2M + Na]^+$ which agrees with the molecular weight 454.3g/mol. This leaves no doubt on the fact that the proposed structure in comparison with Gary *et al.,* 2004 agrees with Obacunone. This major species has molecular weight of 454.3g/mol, similar to the APCI (*m/z*) results by Gary *et al.,* in 2004. The information at hand, what was confirmed from literature and analyst proposed that the compound is obacunone. The retention time for the elution of obacunone was between 10.8-11.3 minutes in the reverse phase C-18 analytical 5um (zorbax) column, resolved on a

mobile phase of the 5-95% acetonitrile in water with 0.01% of triflouroacetic acid (TFA). Shibu M.P. *et al.,* 2005 isolated obacunone at a very high retention time, resolved on 10-50% acetonitrile in water with 0.003% phosphoric acid using a reverse phase C-18 column at 1 ml/min. flow rate. The flow rates are different and in the case of this work the total time is 13.2 min. The non-polar solvent might have been more to reduce the retention time (Snyder *et al.,* 1974). These were other results, obtained from South Africa (SA) 2009 and Canada 2008. The spectra from Canada Mc Master University was also analysed with ES1-MS spectrometer in both positive and negative ion mode. The work (experiment) is described in chapter 3. The ES1-MS mass spectra on both the normal and expanded form indicated the molecular ion at *m/z*; $[M+H^+]$ = 552.4. The work involved data on ^{1}H NMR, ^{13}C H NMR and LC-ES1-MS. The isotopic peaks were 554 and 553. This aided in the determination of the molecular ion peak. They are shown in the appendices. The negative ion mode gave highest peak as *m/z* 485 in the spectrum. The ^{1}HNMR analysis from SA gave nice expanded form of spectra 6th, 5th, fourth, and 1A. The rest were the two gCOSY spectra.

Compound 1

Structure identification of Rohituka-7 ($C_{35}H_{44}O_{13}$)

Compound **1** (Fig10) was found to have the molecular formula of $\mathbf{C_{35}H_{44}O_{13}}$ (molecular weight 672.2) as determined through ESI-MS (*m/z* 673.2 $[M+H]^+$, 609.2 [M-63] equivalent to $[M-CH_3O_3]$ and NMR experiment. There was not enough NMR data; however, chemical shift at δ_H-21, δ_H-22 and δ_H-23 were observed 7.32, 6.082, and 7.35 indicates the presence of the furan protons on the same sample analysed in South Africa as observed in Fig 11a. The characteristic

chemical shift of the quaternary methyl groups typical of tetranortriterpenoids are also observed at δ_H (0.99, 1.2, 1.59, 5.21) corresponding to δ_H-18, δ_H-19, δ_H-28, and δ_H-29 in the spectrum. In fact, the NMR data is not sufficient to account for all the protons in the structure of compound **1,** however as mentioned earlier the furan ring is in good agreement with those reported on tetranortriterpenoids as well as the accurate ESI-MS explained below and reported in literature (Jolad S.D. *et al.,* 1981) with no specific reference but in general, as in limonoids chemistry (Abdelgaleil *et al.,* 2001). A typical mass spectrum from a qualitative analysis run is shown in (GF1-1b) of **Appendix H.**

The Huaping Zhang *et al.,* 2007 and Jolad S.D. *et al.,* 1981 respectively reported the following EI-MS *m/z* (rel.int.) 672 [M]C (10), 626 (11), 481 (7), 452 (13), 434 (17), 418 (10), 312 (10), 226 (40), 209 (44), 195 (24), 177 (27), 135 (26), 121 (45), 95 (47), 76 (67), 57 (100). Based on these data, it is concluded that peak 673.2 $[M+H]^+$ is the molecular ion peak. (2) *Aphanamixis polystachya* (synonyms: *Amoora rohituka* Weight and Arn.; *Aphanamixis rohituka* (Roxb.) Pierre) (Huaping Zhang *et al.,* 2007), *Trichilia hispida* (Mulholland A. Mulholland *et al.,* 2000 and *Turraea heterophylla* are all in the same family Meliaceae and the same limonoid group prieurianin and usually most compounds isolated in a species is also isolated in others. For instance rohituka-7 isolated from *Amoora rohituka* and *Trichilia hispida.* On these bases, it is conclusive that the molecular ion peak showed in the spectrum GF1-1B at HPLC retention time of 0.6-1.0(min.) may be Rohituka-7. From previous data obtained on rohituka-7 with EI/MS *m/z* 672.2 [M+], where Jolad SD *et al* in 1981 could not completely assign the ^{1}HNMR and ^{13}CNMR, Huaping *et al* did that in 2007. This work with the help of the LC-ESI-

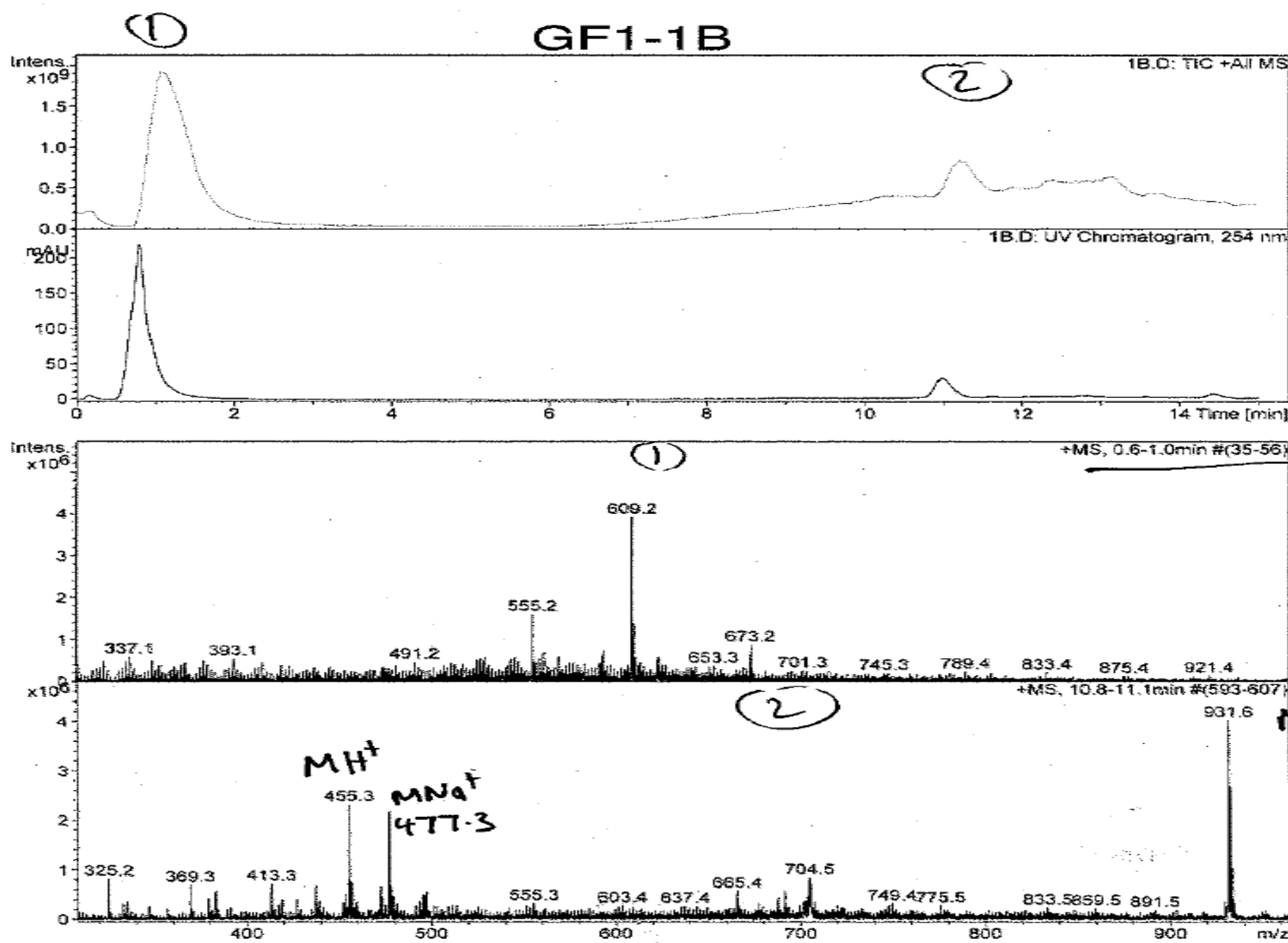


Figure 11: LC - ESI - MS spectrum of Isolate 1B (Band 1B)

MS is able to determine the molecular ion peak of the rohituka-7 from LC-MS spectrum as *m/z* 673.2[M+H] $^{+}$ = [M-H] =672.2, [M+H-CH_4O_3] and [M+H – C_3H_2O].

Infrared Spectroscopy (Compound 2)

The infrared spectroscopy of the fraction B (Band A in the PTLC plate), appendix II indicates hydroxyl group (3439 cm^{-1}) and carbonyl group (1645 cm^{-1}), β-substituted furan ring moiety (1519 cm^{-1}), a carbon singly bonded to oxygen as in epoxy (1099 cm^{-1}), overtones between (2000 cm^{-1}) and (1645 cm^{-1}) indicates aromaticity and confirms the presence of furan ring.

^{1}HNMR Of Compound 2

The^1 HNMR of compound (2) , was done in, $CDCl_3$, at appendix III is contained chemical shifts typical of tetranortriterpenoids including (δ_H 1.0, 1.2, 1.4, 1.6, and 2.0) five quaternary methyl groups H-28 and H-29, gem-dimethyl substituents, characteristic feature of Evodulone, Havenensine, Gedenin, and Obacunone groups of limonoids. The rest of the methyl groups are angular substituted (H-18, H-19, and H-30). The chemical shifts (δ) at H-21, H-22, and H-23 are (δ_H 6.85, 7.45, and 7.96), aromatic protons (showing the furan ring). The furan ring has a heteroatom, the oxygen, responsible for the de-shielding of H-21, H-22, and H-23 protons. The other chemical shift on the spectrum includes δ_H-1 and δ_H-2 at 5.35 and the others which are slightly de-shielded due to the oxygen atoms in the ester close to the H-1 and the H-2. There were not sufficient data from the ^{1}HNMR spectrum to help explain all the δ_H-1…δ_H-30, however in comparison with the spectrum obtained from an isolate by Asamoah in 2003 on the root of the same plant (*Turraea heterophylla*), small data obtained are in

agreement. Coupled with the HPLC-ESI-MS data, that showed an accurate molecular weight of 455.2 $[M+H]^+$, ESI-MS. This agrees with the APCI/MS mass spectrum obtained in Gary *et al*., 2004.

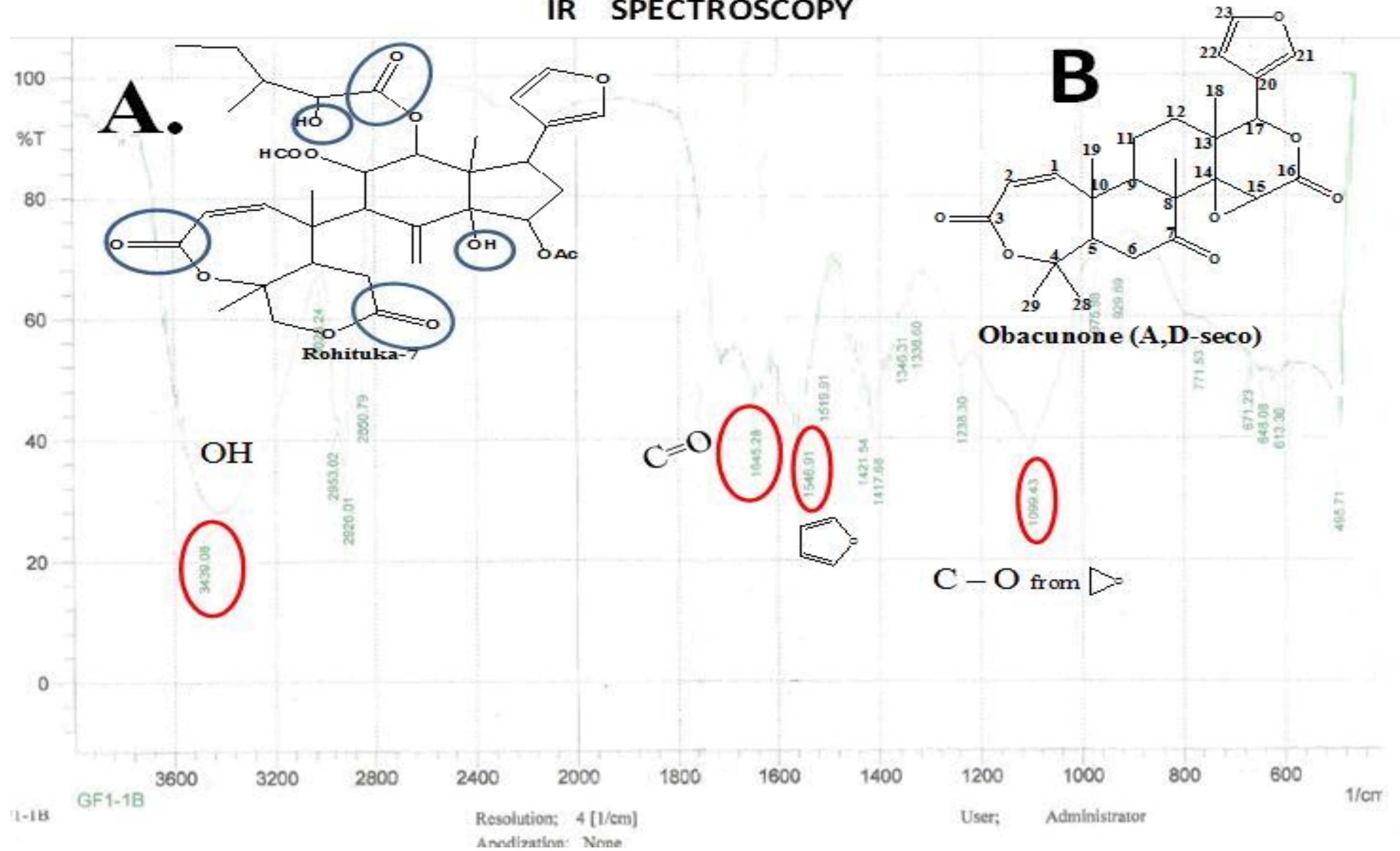


Figure 12: IR Spectrum of Isolate 1B

Bioassay

Results of Measurement of Zone of Inhibition

The zone of growth inhibition sizes were interpreted as follows:

Susceptibility: ≥16; Intermediate : 11-15; Resistant: ≥10

Results (Table 9) indicate the potential of *Turraea Heterophylla* extract to control microbial activities. The concentrations for the serial dilution are between 5 and 0.3153 mg/ml and the zone of inhibition for *Staphylococcus aureus*

was 15 under division 1 but in the division 2,3,4,5 no significant inhibition were observed except with division 6 where extract was dissolved in methanol(making up 5mg/ml) and the inhibition observed was 10mm. Inhibition for the control was observed at 30mm. The rest oserved with the other organisms did not do that well. The reason is that the concentration were that low. Yet some amount of inhibition was observed but not as wide as the control. In general, it can be seen that *T. Heterophylla* extract had some growth inhibition. The plant extract had both antimicrobial activity against all the bacteria: *Staphylococcus aureus, Proteus mirabilis* and *Pseudomanas aeruginosa. Candida albican* also experienced some growth inhibition. They are recorded in the table (Table 9). However it inactive was against *Salmonella typhi,* which showed complete resistance to the concentrations prepared (this was not recorded).

They all showed some activity against the microbes, however, most of the concentrations were too low to inhibit their growth of microbes. The records indicated ≥10mm zone of growth inhibition with a few of the microbes recording between 11-15mm zone of growth inhibition. Only one concentration was made of methanol (5mg/ml) and this recorded 18mm zone of growth inhibition against

Table 9: Bioactivities of the extract on some microbes

Micro-organism	Serial dilution with DMSO						
	Staphylococcus aureus					MeOH solvent	
Division	1	2	3	4	5	6	Control
Concentration mg/ml	5	2.5	1.25	0.625	0.3153	5	0.03
Zone of inhibition/mm	15	0	0	0	0	10	30
Micro-organism	***Proteus mirabilis***						
Division	1	2	3	4	5	6	Control
Concentration mg/ml	5	2.5	1.25	0.625	0.3153	5	0.03
Zone of inhibition/mm	0	10	10	8	8	0	0
Micro-organism	***Candida albican***						
Division	1	2	3	4	5	6	No control
Concentration mg/ml	5	2.5	1.25	0.625	0.3153	5	
Zone of inhibition/mm	8	7	6.5	0	0	8	
Micro-organism	***Pseudomonas aeruginosa***						
Division	1	2	3	4	5	6	Control
Concentration mg/ml	5	2.5	1.25	0.625	0.3153	5	0.03
Zone of inhibition/mm	12	7	0	8	8	18	17

candida albican organism. Cefuroxime sodium (CXM 30) was used as a control for entire experiment, except for fungal (microorganism) microbe.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

Conclusion

Limonoids, as reviewed, their medicinal value and uses in other fields are enormous. The health benefits such as Obacunone, found to enhance the cytotoxicity of vincristine against L1210 cells by approximately 10-fold(Amit *et al.,* 2006), being neuroprotective agents, antineoplastic agent, anti-oxidant, multidrug resistant reversal agent, anti-malaria agent and others as mentioned in this work, report their worth. They have countless other uses unexplored and aside their use as biopesticides. *Turraea heterophylla*(Meliaceae) Smith, has been determined as a rich source of limonoids. It has been used in the treatment of lumbago and impotency locally. The LC-MS, IR and the ^{1}HNMR analysis of *Turraea heterophylla* extracts and isolates revealed that limonoids are major constituent of this plant. The extraction isolation and characterization in this work resulted in the identification of three compounds. Two of which are limonoids, rohituka- 7, obacunone and the third one margocinin.

The NMR chemical shifts δHs (7.95 to 6.0), indicating aromaticity of the furan rings present, can be found in the spectra at the appendices may tell us, how many may not be known which should be investigated.

The bioassay of the extract also showed the potential of this plant extract, indication that limonoids, the major constituent of the plant, play a major role in our health.

Recommendations

More should be done on the dichloromethane (DCM) extract of the root of *T heterophylla* . Limonoids as countless as their uses and potential in bioactivities is not been investigated much. More screening and bioassays will help a lot of inverstigation. Instrument for analysis must be made available and more collaboration outside should be established to aid research work. Research groups must be formed or encouraged in the Dept to source funds for research works.

May be the most potent limonoids for anti-HIV, anti- Cancer agent, Hepatitis B is (are) in *Turraea heterophylla*, more work on this plant is required.

REFERENCES

Abdelgaleil, S. A. M., Hashinaga, F. & Nakatani, M. (2005) Antifungal activity of limonoids from Khaya Ivorensis. *Pest management science*. **61**, 186—190.

Abdelgaleil, S. A. M. & El-Aswad, A. F. (2005). Antifeedant and growth inhibitory effects of tetranortriterpenoids isolated from three Meliaceous species on the cotton leafworm, spodoptera littoralis (Boisd.). *J. App. Sci. Res*. **1**, 234-241.

Abdelgaleil, S. A. M. & Nakatani, M. (2003). Antifeeding activity of limonoids from Khaya senegalensis (Meliaceae*). J. App. Entomol*. **127**, 236–239.

Akudugu, J., Gade, G. & Bohm, L. (2001). Cytotoxicity of Axadirachtin A in human glioblastoma cell lines. *Journal of Life Science.* **68**, 1153-1160.

Alche, L. E., Ferek, G. A., Meo, M., Coto, C. E. & Maier, M. S. (2003). Clinica Botanical Medicine 2nd Ed - Revised & amp ; Expanded. Z. Nat. forsch. C,. published. Retrieved from http://webtutor.tamu.edu/ students/vikram/ Project /limonoid.html

Ara, I., Siddiqui, B. S., Faizi, S. & Siddiqui, S. (1990). Structurally Novel Diterpenoid Constituents from the Stem Bark of Azadirachta indica. Phytochemistry. **28,** 1177.

Ara, I., Siddiqui, B. S. Faisi, & Siddiqui S. (1990). Two new Terpenoids fromroot bark of Azadirachta indica. *J. Nat. Prod.* 1989, **52,** 1209 – 1213.

. Ara, I., Siddiqui, B. S., Faizi, S. & Siddiqui, S. (1989). Structurally Novel Diterpenoid Constituents from the Stem Bark of Azadirachta indica. *Fitoterapia*. **60,** 519.

Arpino, P. (1990). Combined liquid chromatography mass spectrometry II: Techniques and mechanisms of thermospray. *Mass Spectrom. Rev*. **9,** 631–669.

Asamoah, P. (2003). *Phytochemical constituents of the roots of Turraea Heterophylla, (Meliaceae) Smith.* (Master of Philosophy(Chemistry) Hard Copy), University of Cape Coast, Unpublished work.

Ayafor, J. F., Kimbu, S. F., Ngadjui, B. T., Akam, T. M., Dongo, E., Sondengam, B.L., Connolly, D. & Rycroft, D. S. (1994). Antitrichomonal and antioxidant activities of Dorstenia barteri and Dorstenia convexa. *Tetrahedron*. ***50*****,** 9343.

Battinelli, L., Mengoni, F., Lichtner, M., Mazzanti, G., Saija, A., Mastroianni, C. M. & Vullo, V. (2003). Screening of adhatoda vasica nees as a putative hiv-protease Inhibitor. *Planta Med.* **69**, 910—913.

Beckmann, H., Su., L. K. & Kadesch, T. (1990). Modulation of the IgH enhancer's cell type. *Genes Dev*. **4,** 167.

Berhow, M. A., Hasegawa, S. & Manners, G. D. (2000). *Citrus limonoids: Functional chemicals in agriculture and foods*. Washington, D.C.: American Chemical Society.

Bickii, J., Njifutie, N., Ayafor, F. J., Basco, L. K. & Ringwald, P. (2000). In vitro antimalarial activity of limonoids from Khaya – AAPS. *J. Ethnopharmacology.* **69**, 27—33.

Biswas, K., Chattopadhyay, I., Banerjee, R. K. & Bandopadhyay, U. (2002). Biological activities and medicinal properties of neem (Azadirachta indica). *Curr. Sci.* **11**, 1336—1345.

Carrascal, M., Schneider, K., Calaf, R. E., van Leeuwen, S., Canosa, D., Gelpi, E. & Abian, J. (1998). Quantitative electrospray LC-MS and LC-MS/MS in biomedicine. *J. Pharm. Biomed. Anal*. **17,** 1129–1138.

Carroll, D. I., Dzidic, I., Stillwell, R. N., Haegele, K. D. & Horning, E. C. (1975). Atmospheric pressure ionization mass spectrometry: Corona discharge ion source for use in a liquid chromatograph-mass spectrometer-computer analytical system. *Anal. Chem*. **47,** 2369–2373.

Cespedes, C. L., Calderon, J. S., Salazar, J. R., Lotina-Hennsen, B. & Segura, R. (2001). The Sesquiterpenes β-Caryophyllene and Caryophyllene Oxide Isolated from Senecio salignus Act as Phytogrowth and Photosynthesis Inhibitors. *J. Chem. Ecol.* **27**, 137—149.

Champagne, D. E., Koul, O., Isman, M. B., Scudder, G. G. & Towers, G. H. N. (1992). *Phytochemistry*. **31,** 377.

Charleston, D. (2002). Research Council‑ Plant Protection. *Plant Protection News*. **60**, 7—9.

Connolly, J. D. (1983). *Chemistry and chemical taxonomy of the Rutales*. Aterman, P. G. & Grunden, M. F. (Eds.). New York: Academic Press, p. 175.

Cooks, R. G, Ouyang, Z., Takats, Z. & Wiseman, J. M. (2006). Ambient mass spectrometry. *Science*. **311,** 1566.

Danielson, E. J. (2005). Retrieved April 10th, 2005, from http://www.colostate.edu/Depts/Entomology/courses/en570/papers_1996/danielson.html.

Daniewski, W. M., Gumulka, M., Danikiewicz, W., Gluzinski, P., Krajewski, J., Pankowska, E., Bloszyk, E., Ulla, J., Norin, T. & Szafranski, F. (1993). Chemical constituents of *chisocheton Erythrocarpus* hiern *Phytochemistry*. **33,** 1534.

Devakumar, C. & Sukh, D. (1996). *Neem research and development society of pesticide science*. In Randhawa, N. S. and Parmar, B. S. (Eds.). Chemistry. pp. 63—99. New Delhi, India.

Dewick, P. M. (2002). *Medicinal natural products: A biosynthetic approach*. John Wiley & Sons.

Dulcie, A., Mulholland, B. P. & Coombes, P. H. (2000). The chemistry of the Meliaceae and Ptaeroxylaceae of Southern and Eastern Africa and Madagascar. *Current Organic Chemistry*. **4,** 1011-1054.

Ekong, D. E. U., Fakunle, C. O., Fasina, A. K. & Okogun, J. I. (1969). Structure of Azadirachtin Functional Groups. *Journal of Chem. Soc. Chem. Commun.*, 1166.

Fall, A. B., Vanhaelen-Fastre, R., Vanhaelen, M., Hartwell, J. L. & Lloydia, P. (1999). *Planta Medica*. **65,** 209.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. & Whitehouse, C. M. (1989). Electrospray ionization for mass spectrometry of large biomolecules. *Science*. **246,** 64.

Fernandez-Mateos, A., Martin de la Nava, E. M. & Gonzalez, R. R. (2002). Synthesis. *J. Synthetic Organic Chem*. **12,** 1728—1734.

Germano, M. P., D'Angelo, V., Sanogo, R., Catania S., Alma R., De Pasquale, R. & Bisignano, G. (2005). Hepatoprotective and antibacterial effects of

extracts from Trichilia emetica Vahl. (Meliaceae). *J. Ethnopharmacology*. **96,** 227—232.

Gibbins, R. P., Miller, E. G., Taylor, S. E., Mcintosh, J. E., & Patil, B. S. (2005) Limonoids: Overview of Significant Bioactive Triterpenes. Retrieved July 27th, 2005. from http://iadr.confex.com /iadr/2004 Hawaii/techprogram/abstract_38997.html

Govindachari, T. R., Suresh, G., Gopalakrishnan, G., Masilamani, S. & Banumathi, B. (2000). Antifungal activity of some tetranortriterpenoids *Fitoterapia*. **71**, 317—320.

Guodong, C., Pramanik, B. N., Yan-Hui, L. & Mirza, U. A. (2007). Applications of LC/MS in structure identifications of small molecules *J. Mass Spectrom*. **42,** 279–287.

Guthrie N., Elzbieta M. V., Carroll K. & Kitchener, C. K. (Inventors) (2001). Compositions and methods of treatment of neoplastic diseases and hypercholesterolemia with citrus limonoids and flavonoids and tocotrienols; Patent Number: US6251400;Publication date: 2001-06-26.

Hanold, K. A., Fischer, S. M., Cormia, P. H., Miller, C. E. & Syage, J. A. (2004). Atmospheric pressure photoionization. I. general properties for LC/MS. *Anal. Chem*. **76,** 2842–2851.

Hasegawa, S. & Miyake, M. (2005). Retrieved April 10th, 2005. from http://ars.usda.gov/research/ patents/patents.htm?serialnum_08595607

.Hillenkamp, F., Karas, M., Beavis, R. C. & Chait, B. T. (1991). Matrix-assisted laser desorption/ ionization mass spectrometry of biopolymers. *Anal. Chem.* **63,** 1193A.

Hofstadler, S. A. & Sannes-Lowery, K. A. (2006). Applications of ESI-MS in drug discovery: Interrogation of noncovalent complexes. *Nat. Rev. Drug Discov*. **5,** 585–595.

Huaping, Z., Xi, W., Feng, C., XiaO, M. A., & Michael, J. W. (2007). *Anticancer ativity of limonoids from Khaya Senegalensis.* Phytotherapy Research Wiley interscience Published by Wiley and Sons Ltd.

Hutchings, A., Scott, A. H., Lewis, G. & Cunningham, A. (1996). *Zulu medicinal plants: An inventory*. Pietermaritzburg: University of Natal Press.

Ida, Y., Satoh, Y., Ohtsuka, M., Nagasao, M. & Shoji, J. (1994). Phenolic constituents of Phellodendron amurense bark. *Phytochemistry*. **35,** 209-215.

Ikuta, A. & Nakamura, T. (1995). Canthin-6-one from the roots of Phellodendron amurense. *Planta Med*. **61,** 581-582.

Ikuta, A., Nakamura, T. & Urabe, H. (1998a). Indolopyridoquinazoline, furoquinoline and canthinone type alkaloid from Phellodendron amurense callus tissues. *Phytochemistry*. **48,** 285-291.

Ikuta, A., Urabe, H. & Nakamura, T. (1998b). A new indolopyridoquinazoline-type alkaloid from Phellodendron amurense callus tissues. *J. Nat. Prod*. **61,** 1012-1014.

Iribarne, J. V. & Thomson, B. A. (1976). On the evaporation of small ions from charged droplets. *J. Chem. Phys*. **64,** 2287–2294.

Jacob, R., Hasegawa, S. & Manners, G. (2000). The potential of citrus limonoids as anticancer agents. *Perishables handling*. **102**, 6—8.

Jeong, S. Y., Sang, H. S. & Young, C. K. (2008). Inhibitory effects of Cnidium officinale Makino and Tabanus fulvus Meigan on the high glucose-induced proliferation of glomerular mesangial cells. *J. Nat. Prod.* **71,** 208–211.

Jolad, S. D., Hoffmann, J. J., Schram, K. H., Cole, J. R., Tempesta, M. S. & Bates, R. B. (1981). Monthly Variations in Biological Activity of Asimina triloba *Journal Org. Chem.* **46,** 641.

Jung, H. J., Sok, D. E., Kim, Y. H., Min, B. S., Lee, J. P. & Bae, K. H. (2000). Structure Elucidation of Malleastrone C. *Planta Med.* **66,** 74-76.

Kalinganire, A. and Pinyopusarek, K. (2000). Chukrasia: Biology, cultivation and utilisation. *ACIAR Technical Reports*. **49,** 1-35.

Karas, M. & Hillenkamp, F. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal. Chem*. 602299–2301.

Karas, M., Bachmann, D., Bahr, U. & Hellenkamp, F. (1987). Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Processes*. **78**, 53.

Karas, M., Bahr, U. & Dulcks, T. (2000). Nano-electrospray ionization mass spectrometry: Addressing analytical problems beyond routine. *Fresenius. J. Anal. Chem*. **366,** 669–676.

Kayser, O., Kiderlen, A. F. & Croft, S. L. (2003). Natural products as antiparasitic drugs. *Parasitol Res.* **90**, S55-S62.

Khalid, S. A., Duddeck, H. & Gonzales-Sierra, M. (1989). Characterization of bioactive phytochemicals from the stem bark of African mahogany Khaya senegalensis (Meliaceae). *J. Nat. Prod.* **52,** 922.

Khalid, S .A., Friedrichsen, G. M., Kharazami, A., Theander, T. G., Olsen, C. E. & Christensen, S. B. (1998). Limonoids from Khaya senegalensis. *Phytochemistry*. **49,** 1769.

Khalijah, A., Chong, S. L., Khalit, M., Hiroshi, M., Yusuke, H., Koichi, Takeyad, O. T., Hamid, A. & Hadia, A. (2007). Vasorelaxant effect of isoquinoline derivatives from two species of Popowia perakensis and Phaeanthus crassipetalus on rat aortic artery. *Bioorganic and medicinal* chemistry. **15,** 5997–6002.

Kimbu, S. F., Ayafor, J. F., Sondengam, B. L., Tsamo, E., Connolly, J. D. & Rycroft, D. S. (1984). Meliaceous Limonoids: Chemistry and Biological Activities *Tetrahedron Letters*. **25,** 1617.

Kirtikar, K. R. & Basu, B. D. (1981). *Indian medicinal plants* (2nd ed.). New Delhi, India: Periodical Expert Book Agency.

Kishi, K., Yoshikawa, K. & Arihara, S. (1992). Limonoids and protolimonoids from the fruits of Phellodendron amurense. *Phytochemistry*, **31,** 1335-1338.

Koike, K. & Ohmoto, T. (1985). Carbon-13 nuclear magnetic resonance study of canthin-6-one alkaloids. *Chem. Pharm. Bull*. **33,** 5239-5244.

Kokwaro, J. O. (1976). *Medicinal plants of East Africa: East African Literature Nairobi,* Kenya: Bureau.

Kondo, Y., Suzuki, H. & Nozoe, S. (1985). Two γ-Hydroxybutenolides from the bark of Phellodendron amurense and photo oxidation of limonoids. *Yakugaku Zasshi*. **105,** 742-746.

Krief, S., Martin, M. T., Grellier, P., Kasenene, J. & Sevenet, T. (2004). *Antimicrob. Agents Chemother.* **48,** 3196-3199.

Ley, S. V., Denholm, A. A. & Wood, A. (1993). The chemistry of azadirachtin *Nat. Prod. Rep*. **109.**

Li, Y.-T. Hsieh, Y.-L. Henion, J. Senko, M. W. McLafferty, F. & Ganem, B. (1993). Mass spectrometric studies on noncovalent dimers of leucine zipper peptides. *J. Am. Chem. Soc*. **115,** 8409–8415.

Lien, E. J., Wang, T. & Lien, L. L. (2004). Retrieved September 21th, 2004, from http://jwc1.mc.ntu.edu.tw/cpj/Material/54-2/542077abs.htm

MacLachlan, L. K.; Taylor, D. A. H (1982). Limonoids from nymania capensis *Phytochemistry* **21,** 1701– 1703.

Madyastha, M. K. & Venkatakrishnan, K. (2000). Structural flexibility in the biocatalyst-mediated functionalization of ring 'A' in salannin, a tetranortriterpene from *Azadirachta indica. J. Chem. Soc. (*Perkin Trans.) **1,** 3055—3062.

Manners, G. D., Jacob, R. A., Breksa, A. P., Hasegawa, S. & Schoch, T. K. (2003). Bioavailability of Citrus Limonoids in Humans. *J. Agric. Food Chem.* **51,** 4156-4161.

McDonnell, L. A. & Heeren, R. M. A. (2007). Imaging mass spectrometry. *Mass Spectrom. Rev*. **26,** 606–643.

Miller, E. G., Porter, J. L., Binnie, W. H., Guo, I. Y. & Hasegawa, S. (2004). Citrus Limonoids Induce Apoptosis in Human Neuroblastoma Cells and Have Radical Scavenging Activity. *J. Agric. Food Chem.*, **52,** 4908—4912.

Mitra, C. R., Garg, H. S. & Pandy, G. N. (1970). Biological activity of Neem *Tetrahedron Lett*. **5,** 2761.

Miyagi, Y., Om, A. S., Chee, K. M. & Bennink, M. R. (2000). Inhibition of azoxymethane-induced colon cancer by orange juice. *Nutr. Cancer.* **36**, 224—229.

Miyake, M. & Ifuku, Y. (2005). Retrieved 2005, April 10th from http://www. ffcr.or.jp/zaidan/FFCRHOME.nsf/.

Miyaki, M., Inaba, N., Ayano, S., Ozaki, Y., Maeda, H. & Ifuku, Y. (1992). Limonoids in Phellodendron amurense (Kihada). *Yakugaku Zasshi*. **112,** 343-347.

Mora, J. F., Van Berkel, G. J., Enke, C. G., Cole, R. B., Martinez-Sanchez, M. & Fenn, J. B. (2000). Electrochemical processes in electrospray ionization mass spectrometry. *J. Mass Spectrom*. **35,** 939–952.

Mulholland, D.A. & Taylor, D.A.H., (1988). Limonoid extractives from the genera Capuronianthus, Neobeguea and Quivisianthe. *Phytochemistry* **27(6),** 1741–1743

Nakagawa, H., Duan, H. & Takaishi, Y. (2001). Limonoids from Citrus sudachi, Chemical & Pharmaceutical Bulletin. *Chem. Pharm. Bull.* **49**, 649—651.

Nakatani, M., Zhou, J.-B. Tadera, K. & Naoki, H. (1998). Antifeedant and Insecticide Properties of a Limonoid from Melia azedarach (Meliaceae) with Potential Use for Pest Management. *Chem. Lett*. 1279.

Nakatani, M., James, J. C. & Nakanishi, K. (1981). Study of the metabolites of Phyllosticta maydis: Isolation and partial identification *J. Am. Chem. Soc*. **103,** 1228

Pennington, T.D. & Styles, B.T. (1975). A generic monograph of the Meliaceae. Blumea. **22** 419-540.

Perusquia, M., Hernandez, R., Jimenez, M. A., Pereda-Miranda, R. & Mata, R. (1998). Limonoids: Overview of Significant Bioactive Triterpenes Distributed in Plants Kingdom. *Phytother Res.* **11,** 354-357.

Pettit, G. R., Barton, D. H. R., Herald, C. L., Polonsky, J., Schmidt, J. M. & Connolly, J. D. (1983). Evaluation of limonoids against the murine P388 lymphocytic leukemia cell line. *J. Nat. Prod.* **46,** 379.

Poulose, S. M., Harris E. D. & Patil, B. S. (2005). Citrus limonoids induce apoptosis in human neuroblastoma cells and have radical scavenging activity. *Journal of Nutrition,* **135,** 870-877.

Price, P. (1991) Standard definitions of terms relating to MS, *J. Am. Soc. Mass Spectrom.* **2,** 336.

Rajbir, K. & Saroj, A. (2009). Chemical constituents and biological activities of Chukrasia tabularis A. Juss. - A review. *Journal of Medicinal Plants Research*. **3,** 196-216.

Raymond, C. & John, K. (1999). Snyder: The biology-chemistry interface- insect antifeeding limonoids from the chinaberry tree melia azedarach linn. and related compounds. Marcel Dekker, Inc. 2-38.

Rembold, H. (1988). Focus on phytochemical pesticides. In Jacobson, M. (Eds.), *The Neem Tree* (pp. 47). Boca Raton, Fla: CRC Press,

Rembold, H. (1989) Isomeric Azadirachtins and their mode of action. 1988 *Focus on Phytochemical Pesticides. Volume 1. The Neem Tree,* Jacobson, M., ed. Boca Raton, Florida, USA, CRC Press Inc.

Revel'skii, I. A., Yashin, Y. S. Voznesenskii, V. N. Kurochkin, V. K. & Kostyanovskii , R. G. (1986). Mass spectrometry with Photoionization of n-alkanes, alcohols, ketones, esters, and amines at atmospheric pressure. *Russ. Chem. Bull*. **35,** 1806–1810.

Robb, D. R., Covey, T. R. & Bruins, A. P. (2000). Atmospheric pressure photoionization: An ionization method for liquid chromatography-mass spectrometry. *Anal. Chem*., **72,** 3653–3659.

Rohr, A. C., Wilkins, C. K., Clausen, P. A., Hammer, M., Nielsen, G. D., Walkoff P. & Spengler, J. D. (2002). Limonoids: Overview of Significant Bioactive Triterpenes Distributed in Plants Kingdom. *Inhalation Toxicol.* **14**, 663-684.

Ruberto, G., Renda, A., Tringali, C., Napoli, E. M. & Simmonds, M. S. (2002). Red Mexican Grapefruit: A Novel Source for Bioactive Limonoids and their Antioxidant Activity. *J. Agric. Food Chem.* **50,** 6766-6774.

Saxena, S., Pant, N., Jain, D. C. & Bhakuni, R. S. (2003). Novel antimalarial agent (Cinnamic 3β-hydroxyolean-12-en-28-carboxylicanhydride): Synthesis, characterization and in vivo studies. *Curr. Sci.* **85,** 1314-1329.

Schmandke, H. R., B. (2005). Retrieved September 25 th, 2005, from http://www.ernaehrungs-Umschau.de/archiv/summaries /?page 2& id_1372

Shiea, J., Huang, M.-Z., Hsu, H. J., Lee, C.-Y., Yuan, C.-H., Beech, I. & Sunner, J. (2005). Electrospray-assisted laser desorption/ionization mass spectrometry for direct ambient analysis of solids. *Rapid Commun. Mass Spectrom*. **19,** 3701–3704.

Shibu, M. P. (2005). Isolation And Effects Of Citrus Limonoids On Cytochrome P450 Inhibition, Apoptotic Induction And Cytotoxicity On Human Cancer Cells

Siddiqui, S., Siddiqui, B. S., Faizi, S. & Mahmood, T. (1988). Tetracyclic Triterpenoids and their Derivatives from Azadirachta indica *J. Nat. Prod*. **51**, 30.

Silalahi, J. (2002). Anticancer and health protective properties of citrus fruit components Asia Pac. *J. Clin. Nutr.* **11**, 79-84.

Silva, M. F. D. G. F., Gottlieb, O. R. and Dreyer, D. L. (1984). Chemical constituents of *chisocheton erythrocarpus* hiern *Biochemical Systematic and Ecology*. **12**, 299.

Silverstein, R. M. Bassler, G. C. & Morrill, T. C. (1981). *Spectrometric identification of organic compounds*. (4th Ed.). New York: John Wiley & Sons.

Smith, R. D., Loo, J. A., Edmonds, C. G., Barinaga, C. J. & Udseth, H. R. (1990). New developments in biochemical mass spectrometry: Electrospray ionization. *Anal. Chem*. **62,** 882–899.

Snyder, L. R., & Kirkland J. J. (1974), *Introduction to Modern Liquid Chromatography, (*1st Ed.). Wiley-Interscience, New York, (Japanese translation, 1976).

Sparkman O.D., (2000) *Mass Spec Desk Reference*. Pittsburgh, PA, Global View Publishing.

Stonard, R. J., Trainor, D. A., Nakatani, M. & Nakanishi, K. (1983). Biology-Chemistry Interface: A Tribute to Koji Nakanishi *J. Am. Chem. Soc*. **105,** 130.

Sunthanont, K., Bennink, M. R. & Uebersax, M. A. (2005). Retrieved from 25th July 2005 http://ift.confex.com/ift/2002/techprogram/paper_14285.htm;

Sunthitikawinsakul, A., Kongkathip, N., Kongkathip, B., Phonnakhu, S., Daly J. W., Spande, T. F., Nimit, Y., Napaswat, C., Kasisit, J. & Yoosook, C. (2003). Phytother. *Res.*, **17,** 1101-1103.

Tada, K., Takido, M. & Kitanaka, S., (1999). Limonoids from fruit of Melia toosendan and their cytotoxic activity *Phytochemistry*. **51**, 787-791.

Takats, Z. J. M., Wiseman, B., Gologan, R. & Cooks. G. (2004). Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science*. **306,** 471–473.

Tanaka, K, Waki, H, Ido, Y., Akita, S., Yoshida, Y. & Yoshida, T. (1988). Protein and polymer analyses up to *m/z* 100,000 by laser ionization

time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **2,** 151.

Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y. & Yoshida, T. (1988). Protein and polymer analyses up to *m/z* 100,000 by laser ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom*. **2,** 151–153.

Tanaka, T., Kohno, H., Tsukio, Y., Honjo, S., Tanino, M., Miyake, M. & Wada, K. (2000). Citrus limonoids obacunone and limonin inhibit azoxymethane-induced colon carcinogenesis in rats *Biofactors.* **13**, 213-218.

Tanaka, T., Maeda, M., Kohno, H., Murakami, M., Kagami, S., Miyake, M. & Wada K. (2001). Inhibition of azomethan-induced colon carcinogenesis in male F344 rats by the citrus limonoids obacunone and limonin. *Carcinogenesis*, **22,** 193-198.

Taylor, D. A. H. (1981). A monograph of neotropical Meliaceae (with accounts of the subfamily Swietenioideae by BT Styles and the chemotaxonomy). *Flora Neotropica*. 120- 150

Taylor, D. A. H. (1983). The occurrence of limonoids in the Meliaceae. In Pennington, T. D. & Styles, B. T. (Eds.), *Chemotaxonomy* (p. 450). New York: Academic Press.

Taylor, D. A. H. (1983). Biogenesis, distribution and systematic significance of limonoids in the Meliaceae, Cneoraceae and allied taxa. In Waterman, P. G. & Grunden, M. F. (Eds.), *Chemistry and chemical taxonomy of the Rutales*. New York: Academic Press.

Tian, Q., Miller, E. G., Ahmad, H., Tang, L. & Patil B. S. (2001). Differential inhibition of human cancer cell proliferation by citrus limonoids. *Nutr. Cancer*, **40**, 180-184.

Tito, M. A., Tars, K., Valegard, K., Hajdu, J. & Robinson, C. V. (2000). Electrospray time-of-flight mass spectrometry of the intact MS2 virus capsid. *J. Am. Chem. Soc*. **122,** 3550–3551.

Todd J.F.J., (1995) *Recommendations for nomenclature and symbolism for mass spectroscopy*, Int. J. Mass Spectrom. Ion. Processes, **142,** 209.

Wada, K., Yagi, M., Matsumura, A., Sasaki, K., Sakata, M. & Haga, M. (1990). Isolation of limonin and obacunone from Phellodendri Cortex shorten sleeping time induced in mice by α-chloralose-urethane. *Chem. Pharm. Bull*. 38, 2332-2334.

Watt, J. M. & Breyer-Brandwick, M. G. (1962). *The medicinal and poisonous plants of Southern and Eastern Africa*, London: E. & S. Livingstone.

Wiseman, J. M., Ifa, D. R., Song, Q. & Cooks, R. G. (2006). *Tissue imaging at atmospheric pressure using desorption electrospray ionization (DESI) mass Spectrometry.* Angewandte Chemie International Edition. **45,** 7188–7192.

Yan, X., Zhou, J., & Xie, G. (1999). *Traditional Chinese medicines molecular structures, natural sources and applications*. England: Ashgate Publishing Limited.

Yong, D., Min, H., Cheol, K., Min, C. Y., Kyu, H. L., Sang, U. C. & Kang, R. L. (2006). Isolation of limonoids and alkaloids from Phellodendron amurense and their multidrug resistance (MDR) reversal activity.

Yu, J., Wang, L., Walzem, R. L., Miller, E. G., Pike, L. M. & Patil, B. S. (2005). Antioxidant activity of citrus limonoids, flavonoids, and coumarins. *J. Agric. Food Chem.* **53,** 2009-2014.

Zimmerman, M. C. N. (2005). Retrieved September 23rd, 2005, from http://exchange.healthwell.com/nutritionsciencenews/nsn_backs/Jul_00/phytochemicals.cfm

APPENDIX A

Antifeeding Activities of Limonoids from *Melia Azedarach* (Source: Munerohiro *et al*., 1999)

Table 1 Antifeeding Activity of Limonoids from *Melia azedarach*

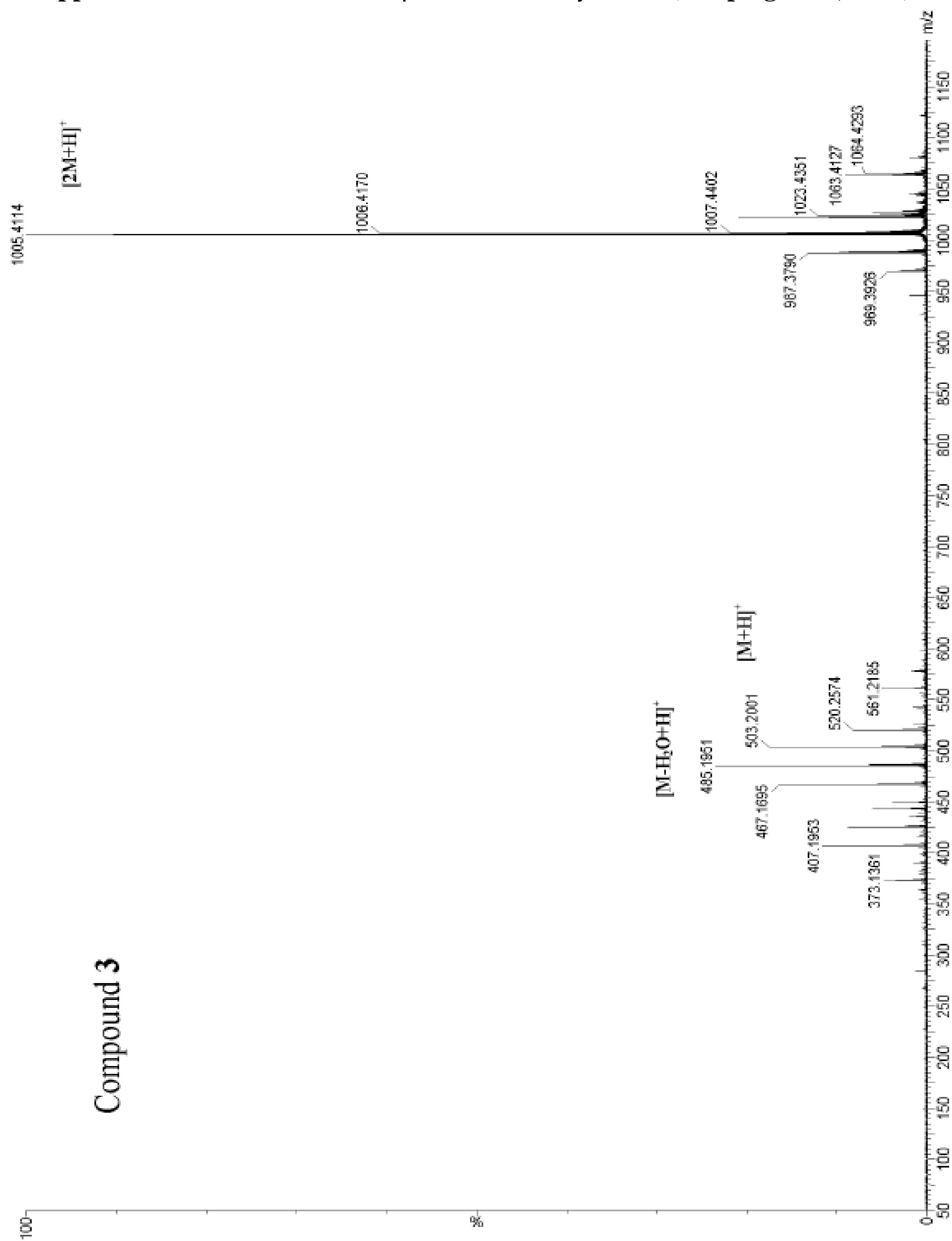
Limonoid	Test insect	Effective concentration (ppm)	References	
			Isolation	Activity[a]
Group 1: Intact apo-euphol limonoids				
Azadirone (**1**)	*Spodoptera eridania*	Inactive	27	12
Meldenin (**2**)			28,29	
6-Acetoxy-7α-hydroxy-3-oxo-14β,15β-epoxymeliac-1,5-diene (**3**)			29	NA
6-Acetoxy-3β-hydroxy-7-oxo-14β,15β-epoxymeliac-1,5-diene 3-O-β-D-glucopyranoside (**4**)			29	NA
6-Acetoxy-3β-hydroxy-7-oxo-14β,15β-epoxymeliac-1,5-diene 3-O-β-D-xylopyranoside (**5**)			30	NA
6-Acetoxy-3β,11α-dihydroxy-7-oxo-14β,15β-epoxymeliac-1,5-diene 3-O-α-L-rhamnopyranoside (**6**)			31	NA
6,11α-Diacetoxy-3β-hydroxy-7-oxo-14β,15β-epoxymeliac-1,5-diene 3-O-β-D-glucopyranoside (**7**)			32	NA
Sendanal (**8**)			33	NA
Nimbolin A (**9**)	*S. eridania*	1000	11	12
C-19/C-29 bridged acyl acetals				
Amoorastatin (**10**)	*S. eridania*	150	34,35	12
Toosendanin (**11**)	*O. furnacalis*	200	36,37	35
	S. eridania	300		20
Azedarachin A (**12**)	*S. eridania*	200	38	37
12-O-Acetylazedarachin A (**13**)	*S. eridania*	400	38	37
Azedarachin C (**14**)	*S. eridania*	400	39	37
Azedarachin B (**15**)	*S. eridania*	200	12	12
12-O-Acetylazedarachin B (**16**)	*S. eridania*	400	40	37
Trichilin B (**17**)	*S. eridania*	200	22,40	40
12-O-Acetyltrichilin B (**18**)	*S. eridania*	400	40	20
1,12-Di-O-acetyltrichilin B (**19**)	*S. eridania*	400	40	40
Trichilin D (**20**)	*S. eridania*	400	22,40	40
Trichilin H (**21**)	*S. eridania*	400	40	40
Meliatoxin A_2 (**22**)	*S. litura*	300	40,41	42
	S. eridania	400		40
Aphanastatin (**23**)	*S. eridania*	200	24	20
Amoorastatone (**24**)	*S. eridania*	400	34,35	12
12-Hydroxyamoorastatone (**25**)	*S. eridania*	300	35	12
iso-Chuanliansu (**26**)	*S. eridania*	400	37	12
Meliatoxin B_1 (**27**)	*S. eridania*	500	41	12
Meliatoxin B_2 (**28**)	*S. eridania*	500	41	12
Group 2: D-seco limonoids				
Gedunin (**29**)	*O. nubilalis*	500	11	43
7α-Acetoxy-3β-hydroxy-14β,15β-epoxygedunan-1-ene 3-O-β-D-glucopyranoside (**30**)			44	NA
Group 3: C-seco limonoids				
Ohchinal (**31**)	*S. frugiperda*	Inactive	45	46
Nimbolin B (**32**)	*S. eridania*	1000	11	12
Nimbolinin B (**33**)	*S. eridania*	1000	47	48
1-Deacetylnimbolinin B (**34**)			47	NA
Spirosendan (**35**)			12	NA
Ohchinolide A (**36**)			47,49	NA
Ohchinolide B (**37**)	*S. eridania*	700	47,49	12
Ohchinolal (**38**)	*S. eridania*	1000	50	12
Salannin (**39**)	*S. eridania*	1000	51	48
	S. littoralis	100		52
Deacetylsalannin (**40**)	*S. eridania*	1000	51	12
	E. varivestis	30		53
Ohchinin (**41**)			50	NA

Table 1 Continued

Limonoid	Test insect	Effective concentration (ppm)	References	
			Isolation	Activity[a]
Ohchinin acetate (**42**)	*S. eridania*	1000	45	12
Nimbolidin A (**43**)	*S. eridania*	500	47	20
Nimbolidin B (**44**)	*S. eridania*	500	47	20
Highly oxidized compounds				
1-Cinnamoylmelianolone (**45**)			54	NA
1-Cinnamoyl-3-acetyl-11-methoxymeliacarpinin (**46**, Meliacarpinin A)	*S. eridania*	50	25	25
3-Deacetylmeliacapinin A (**47**)			55	NA
Meliacarpinin B (**48**)	*S. eridania*	50	48	48
Meliacarpinin C (**49**)	*S. eridania*	50	48	48
Meliacarpinin E (**50**)	*S. eridania*	50	12	12
20-O-Acetylmeliacarpinin C (**51**)			55	NA
Meliacarpinin D (**52**)	*S. eridania*	50	48	48
20-O-Acetylmeliacarpinin D (**53**)			55	NA
1-Deoxy-3-methacrylyl-11-methoxymeliacarpinin (**54**)			55	NA
Azadirachtin (**55**, Azadirachtin A)	*E. varivestis*	14	56,57	53
Other trichilins from different species and reaction products				
1: Natural products				
Trichilin A (**56**) (C12-epimer of trichilin B)	*S. eridania*	300	22	22
7-O-Acetyltrichilin A (**57**)	*S. eridania*	400	59	12
Trichilin C (**58**) (15-Keto isomer of trichilin A)	*S. eridania*	500	22	12
12α-Epimer of trichilin C (**59**)	*S. eridania*	400	12	12
Trichilin E (**60**) (C12-Epimer of aphanastatin)	*S. eridania*	300	22	12
Trichilin F (**61**) (1-O-Acetyl-3-deacetyltrichilin A)	*S. eridania*	300	60	60
Trichilin G (**62**) (1-O-Acetyl-2,3-deacetyltrichilin A)	*S. eridania*	300	60	60
2: Acetylated compounds				
12-O-Acetyltrichilin A (**63**)	*S. eridania*	400	58	58
7,12-Di-O-acetyltrichilin A (**64**)	*S. eridania*	500	58	58
1,7,12-Tri-O-acetyl-2-deacetyltrichilin A (**65**)	*S. eridania*	500	61	61
1,7,12-Tri-O-acetyl-3-deacetyltrichilin A (**66**)	*S. eridania*	500	61	61
7,12-Di-O-acetyltrichilin B (**67**)	*S. eridania*	500	58	58
1-O-Acetyl-3-deacetyltrichilin B (**68**)	*S. eridania*	200	61	61
12-O-Acetyltrichilin C (**69**)	*S. eridania*	>500	12	12
3: Oxidized compounds				
7-Oxotrichilin A (**70**)	*S. eridania*	Inactive	22	58
7-Oxotrichilin B (**71**)	*S. eridania*	Inactive	22	58
12-Oxotrichilin B (**72**)	*S. eridania*	1000	22	12
1,12-Dioxotrichilin B (**73**)	*S. eridania*	Inactive	22	58
7,12-Dioxotrichilin B (**74**)	*S. eridania*	Inactive	22	58

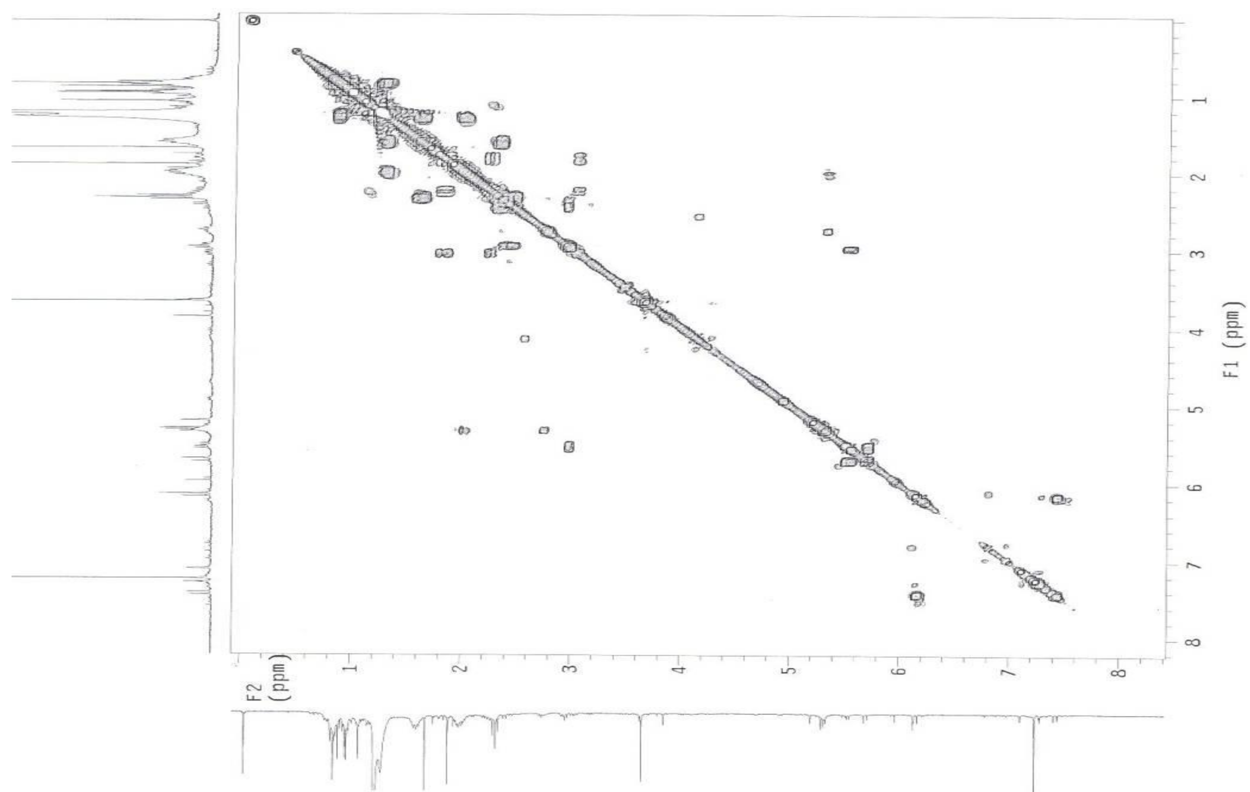
[a] NA = not available.

Appendix B: Accurate ESI-MS spectrum of khayanone (**Huaping et al., 2007**)

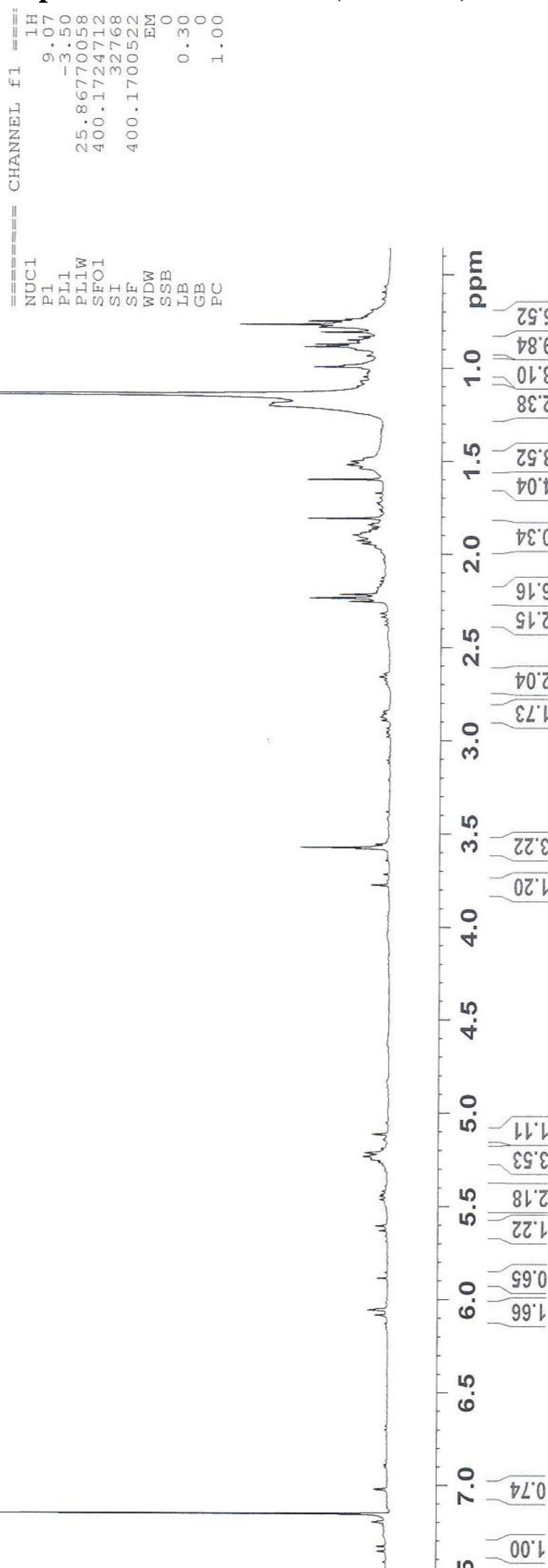


^{1}HNMR spectrum Of Rohituka -7 (C ^{1}HNMR (Band 1A))

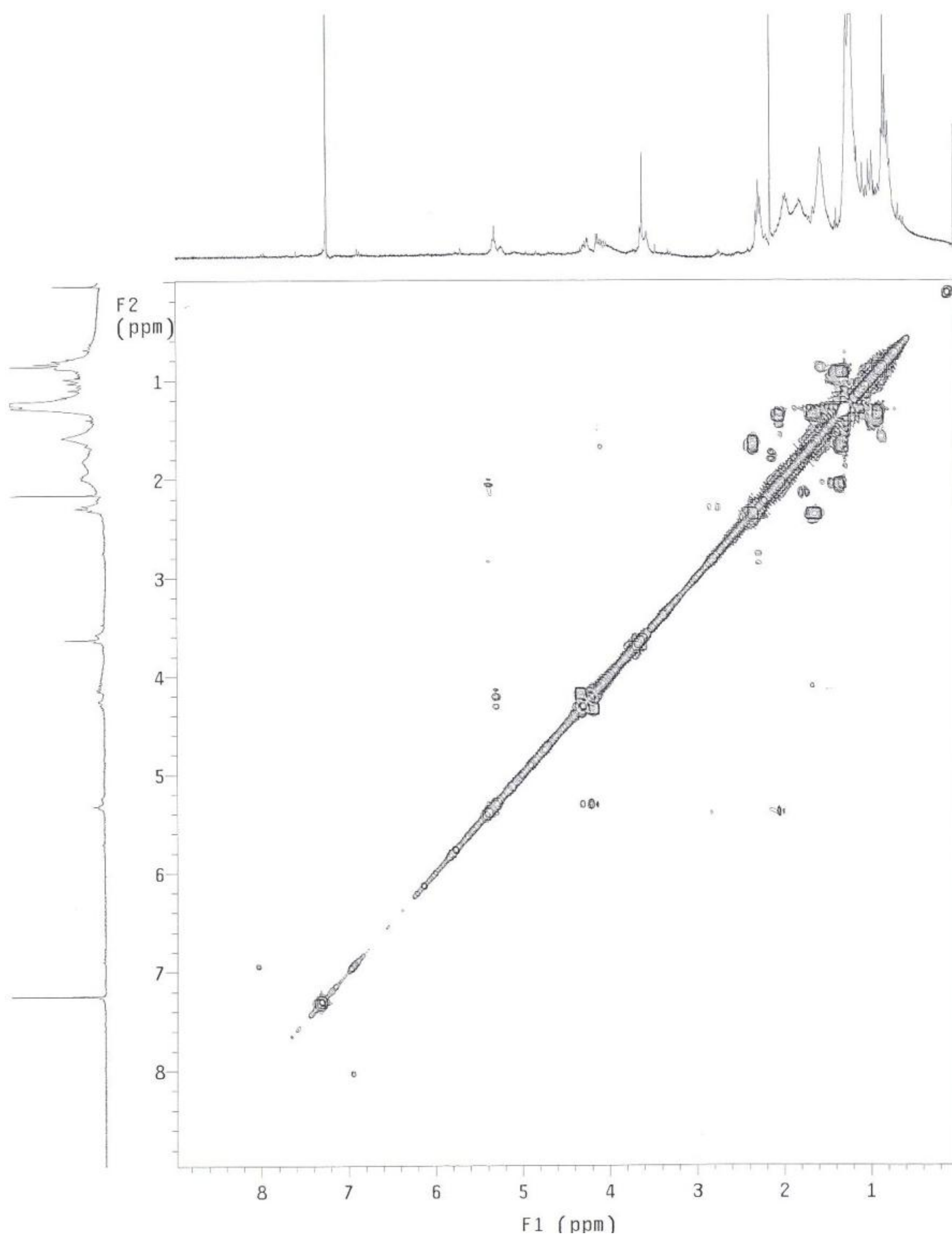
Appendix D: 2D HNMR spectrum of Rohituka -7 (Band1A) ^{1}H-^{1}H COSY



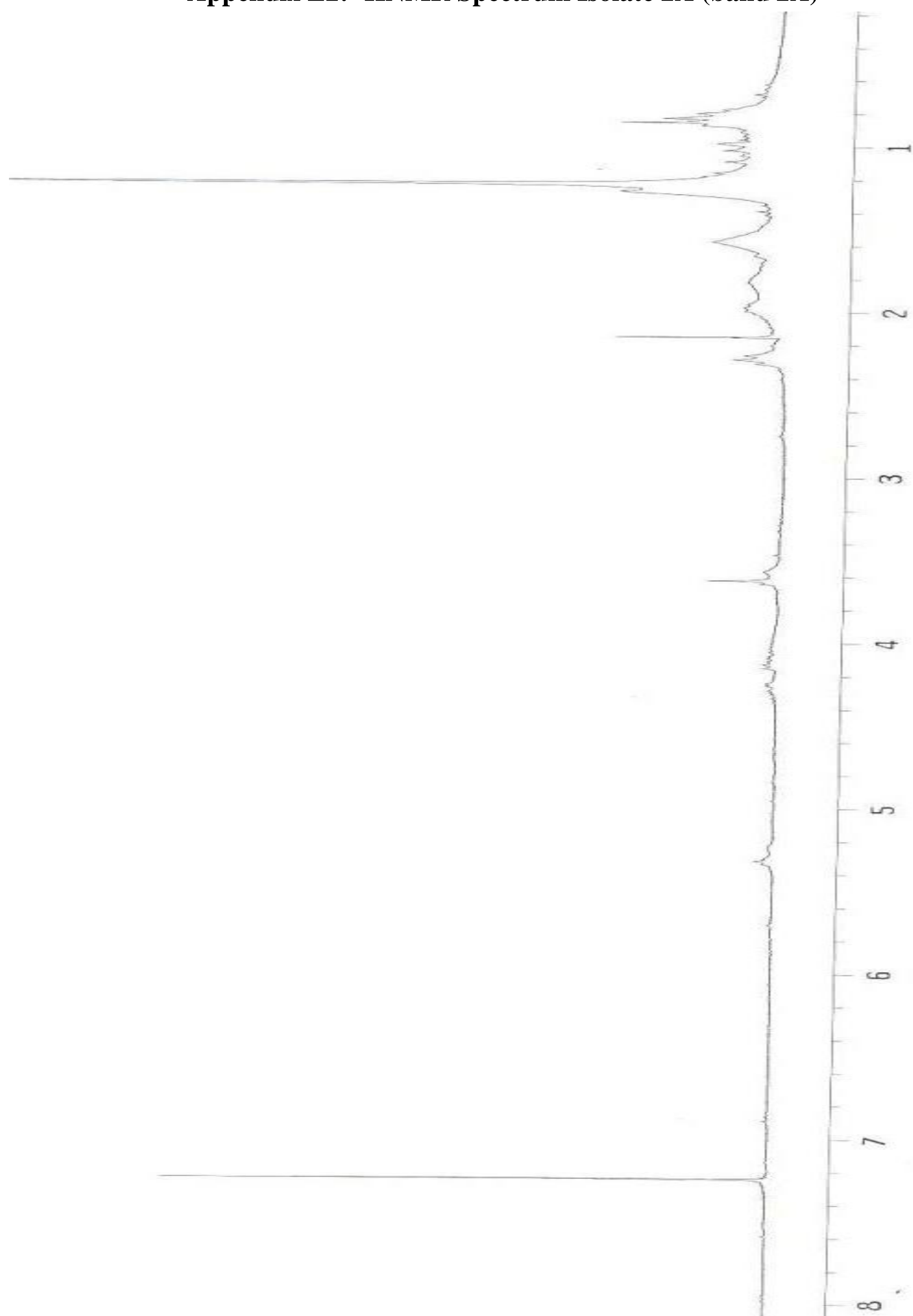
Appendix C2 **^{1}HNMR Spectrum of Isolate 1A(Band 1A)**



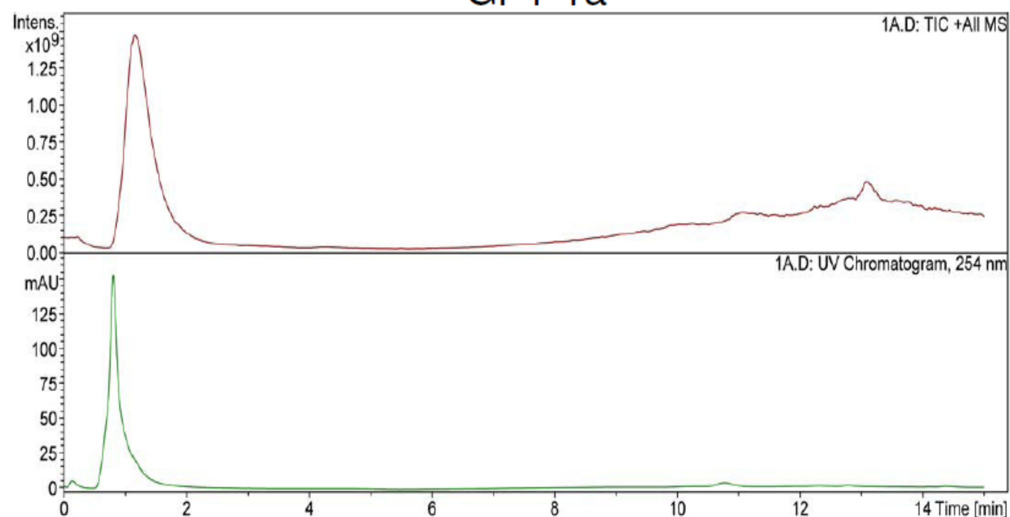
Appendix E: 2D ^{1}HNMR of Isolate 2A(Band 2A)

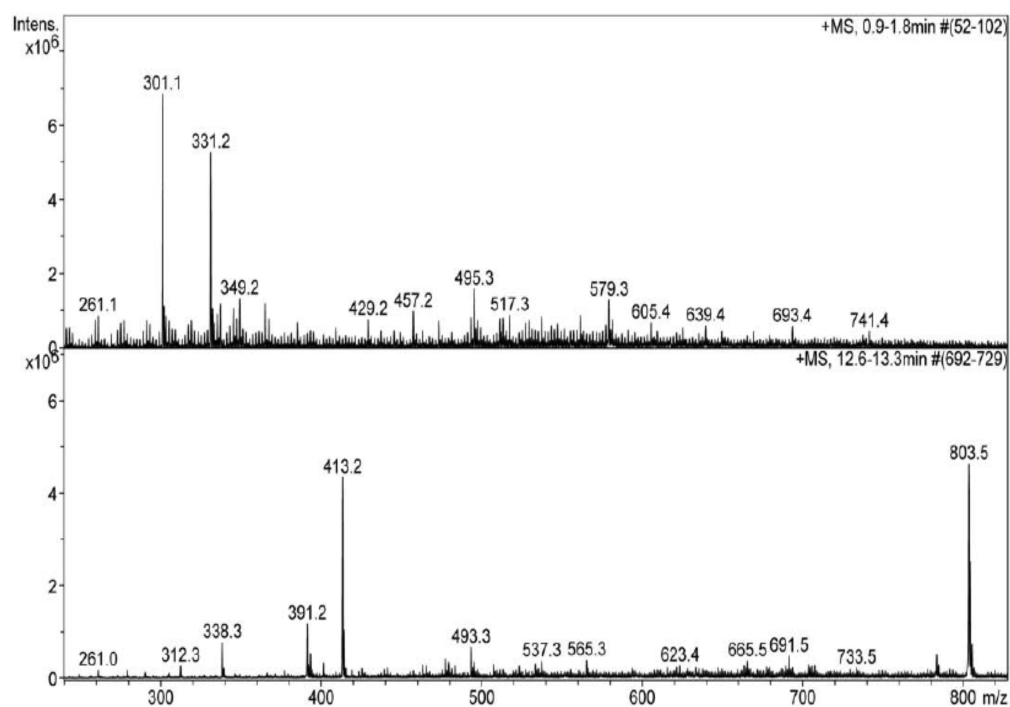


Appendix E2: ^{1}HNMR Spectrum Isolate 2A (band 2A)



Appendix F : LC - ESI - MS spectrum of Margocinin

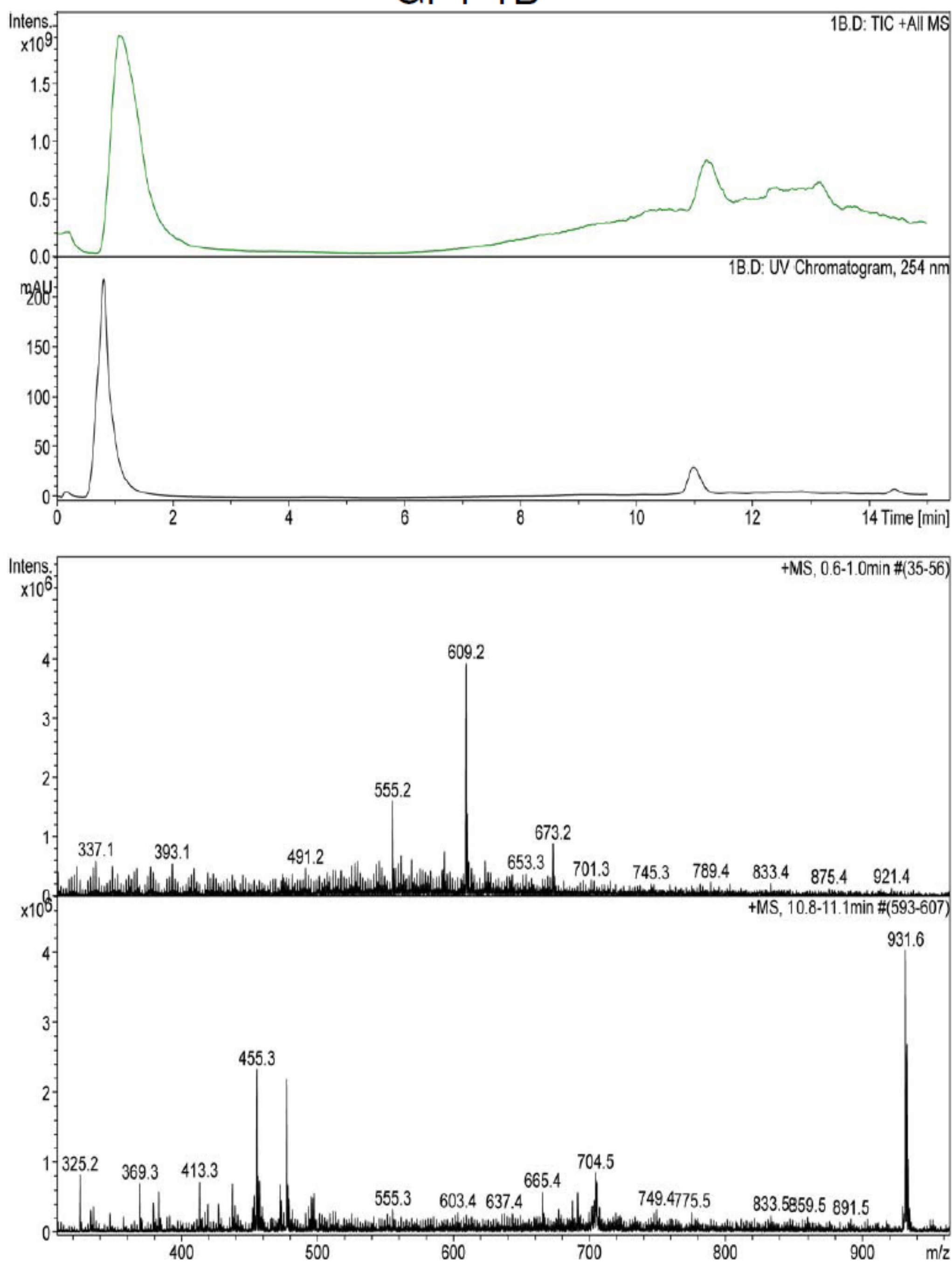




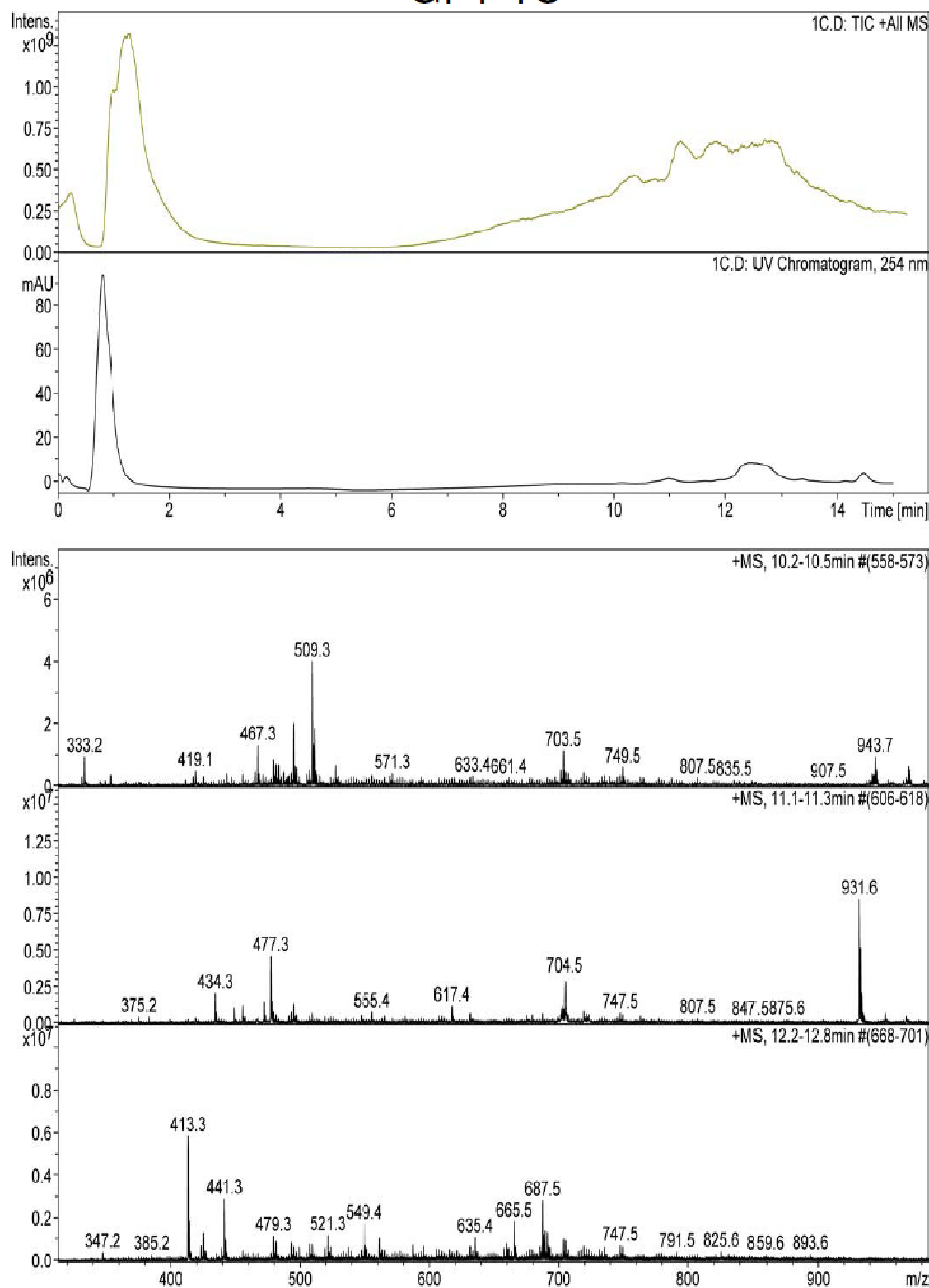
Appendix G Summary of ^{1}HNMR(Provided by analyst)

Fraction 1a Peaks (ppm)	Fraction 1b peaks(ppm)
0.88 s, 1.0 s, 1.25 s, 1.6 s	0.88 m, 1.0 m, 1.25 s, 1.6 m
2.04 m, 2.3 m, 2.62 s	2.04 m, 2.20 m
3.42 d	
3.68 d	3.68 s
4.1 dd	4.15 dd
4.35 q	4.28 dd
4.87s, 4.98 s	
5.35 m	5.35 m
6.9 d, 8.0 d	6.82 ,7.45s , 7.87

Appendix H : LC - ESI - MS spectrum of Rohituka -7(1) and Obacunone(2)

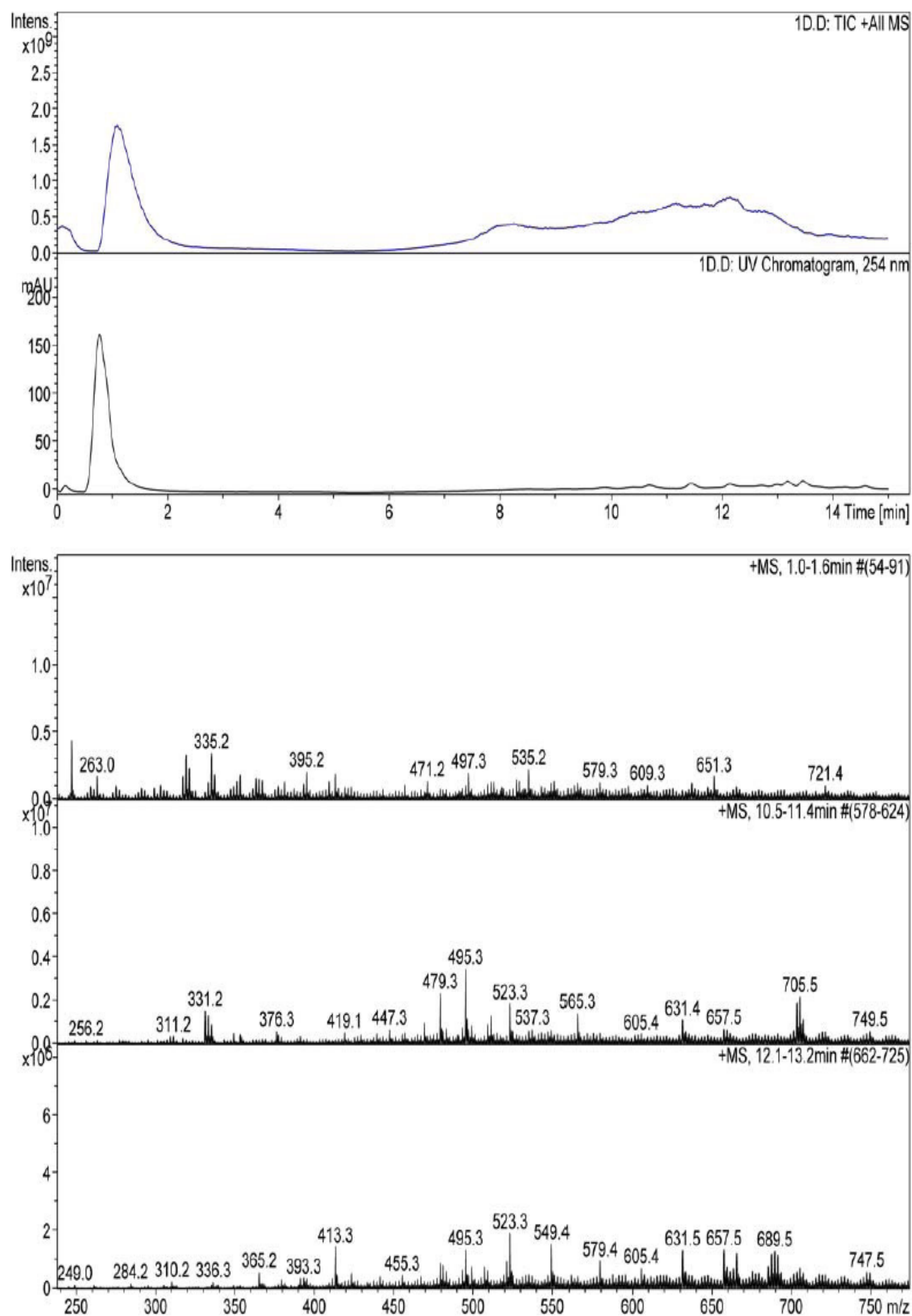


Appendix I : LC - ESI - MS spectra Of Isolate 1C



Appendix J: LC - ESI - MS spectra Of Isolate 1D

GF1-1D



Appendix K: ^{1}HNMR spectrum Four[1]

FOURTH

NAME	May11-2010-CAFOURT
EXPNO	10
PROCNO	1
Date_	20100511
Time	12.51
INSTRUM	spect
PROBHD	5 mm PABBO BB-
PULPROG	zg30
TD	65536
SOLVENT	CDCl3
NS	16
DS	2
SWH	8223.685 Hz
FIDRES	0.125483 Hz
AQ	3.9846387 sec
RG	144
DW	60.800 use
DE	6.50 use
TE	291.6 K
D1	1.00000000 sec
TD0	1
======== CHANNEL f1 =======	
NUC1	1H
P1	9.07 use
PL1	-3.50 dB
PL1W	25.86770058 W
SFO1	400.1724712 MHz
SI	32768
SF	400.1700153 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

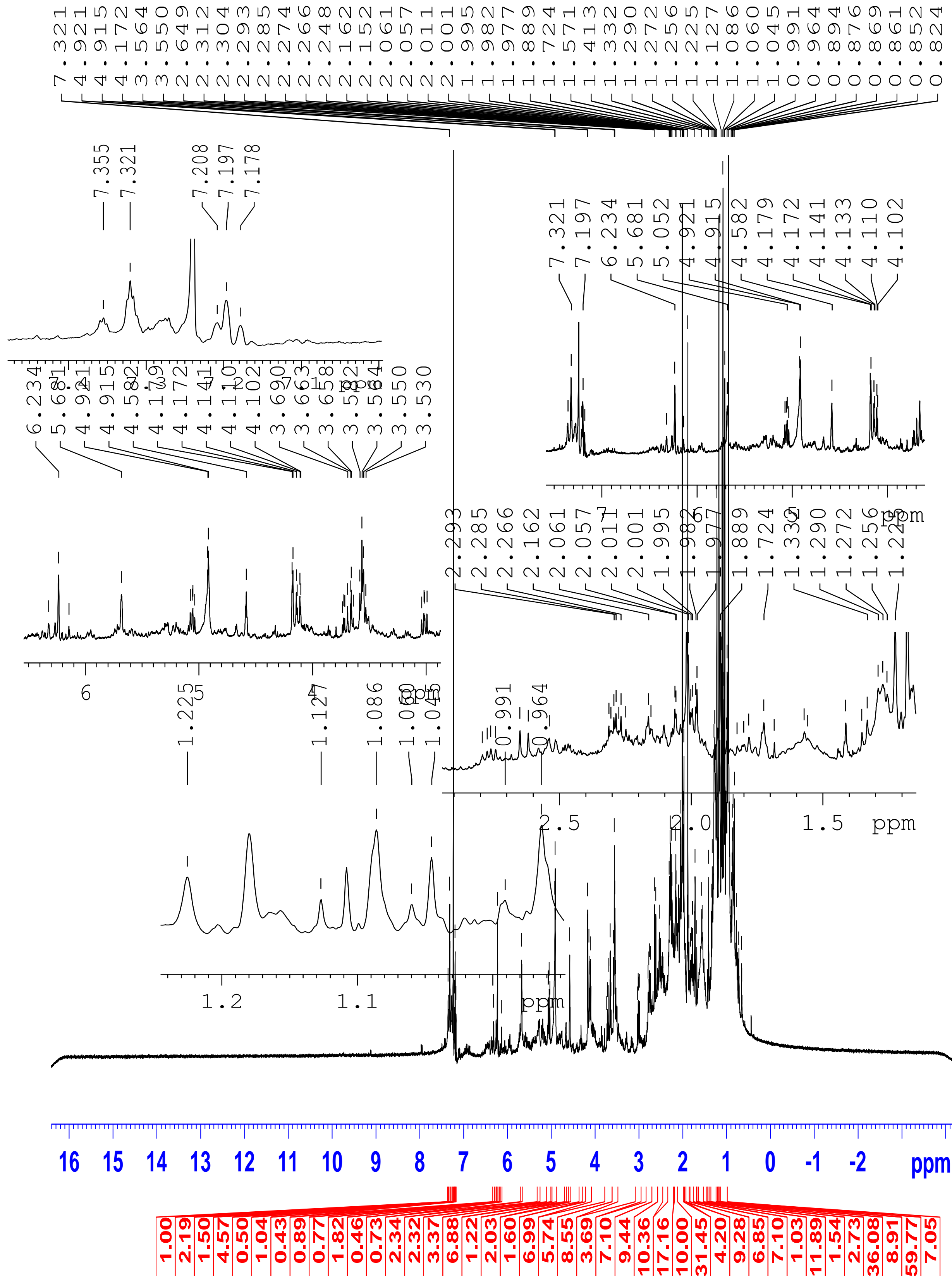
Appendix L : Ionisation Sources Of Various Mass Spectrometers

Method	Acronym	Category	Ion type	Applications[a]
Gas discharge	–	Discharge	Atomic ions	First ionization mechanism to be used in MS
Thermal ionization	TI	Ionization by heating	Atomic ions	Isotope ratio, Trace analysis; Solid samples
Spark source	SS	Discharge	Atomic ions	Trace analysis in solid samples
Glow discharge	GD	Plasma source	Atomic ions	Trace analysis
Inductively coupled plasma	ICP	Plasma source	Atomic ions	Isotope ratio; Trace analysis
Electron ionization	EI	Electron induced ionization	Volatile molecular ions	Smaller molecules; GC-MS; Extensive libraries
Chemical ionization	CI	Electron induced ionization	Volatile molecular ions	GC-MS
Atmospheric pressure chemical ionization	APCI	Electron induced ionization	Nonvolatile molecular ions	Smaller molecules; LC-MS
Photoionization	PI	Photoionization	Volatile molecular ions	Smaller molecules; GC-MS
Multiphoton ionization	MPI	Photoionization	Atomic and molecular ions	Resonance-enhanced MPI is highly selective; Trace analysis
Atmospheric pressure photoionization	APPI	Photoionization	Nonvolatile molecular ions	LC-MS; Nonpolar compounds
Field ionization	FI	Ionization by strong electric field	Volatile molecular ions	Molecular compounds
Field desorption	FD	Desorption/ionization by strong electric field	Nonvolatile molecular ions	First soft method; Large molecules
Thermospray ionization	TSI	Spray	Nonvolatile molecular ions	LC-MS
Electrospray ionization	ESI	Spray	Nonvolatile molecular ions	Soft method, LC-MS; Large molecules

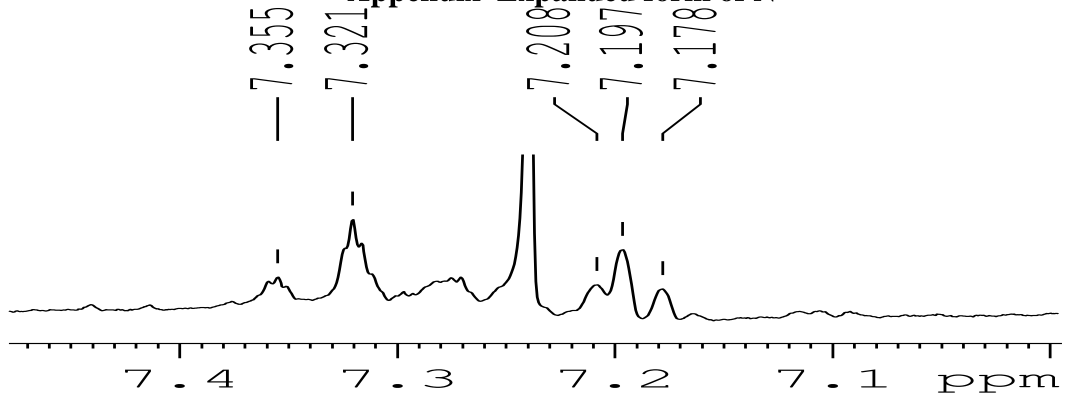
(Continued)

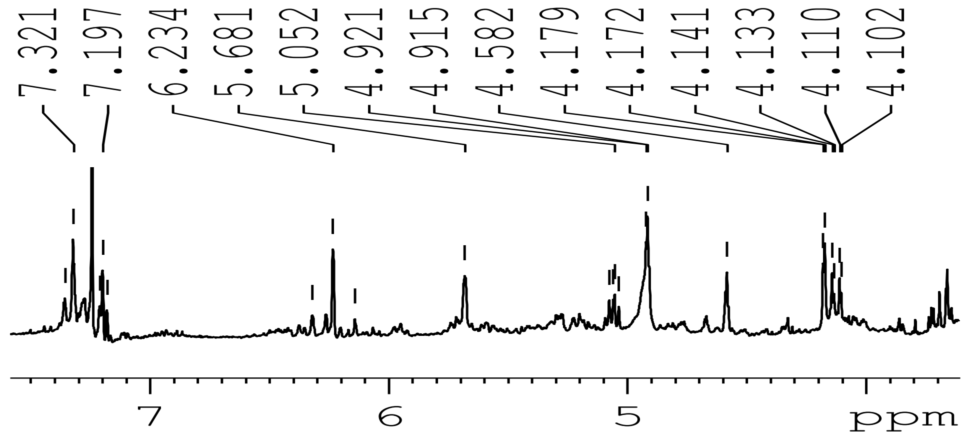
Method	Acronym	Category	Ion type	Applications[a]
Desorption electrospray ionization	DESI	Spray	Nonvolatile molecular ions	Direct, preparation-free analysis of samples
Direct analysis in real time	DART	Discharge	Nonvolatile molecular ions	Direct, preparation-free analysis of samples
Secondary ion (mass spectrometry)	SIMS	Particle induced desorption/ ionization	Nonvolatile molecular ions	Semiconductors; Surface analysis; Imaging
Fast atom bombardment	FAB	Particle induced desorption/ ionization	Nonvolatile molecular ions	Soft method; Large molecules
Plasma desorption	PD	Particle induced desorption/ ionization	Nonvolatile molecular ions	Soft method; Large molecules
Laser desorption/ ionization	LDI	Photon induced desorption/ ionization	Nonvolatile atomic and molecular ions	Isotope ratio; Trace analysis
Matrix-assisted laser desorption/ ionization	MALDI	Photon induced desorption/ ionization	Nonvolatile molecular ions	Soft method; Large molecules
Atmospheric pressure matrix-assisted laser desorption/ ionization	AP-MALDI	Photon induced desorption/ ionization	Nonvolatile molecular ions	Soft method; Large molecules

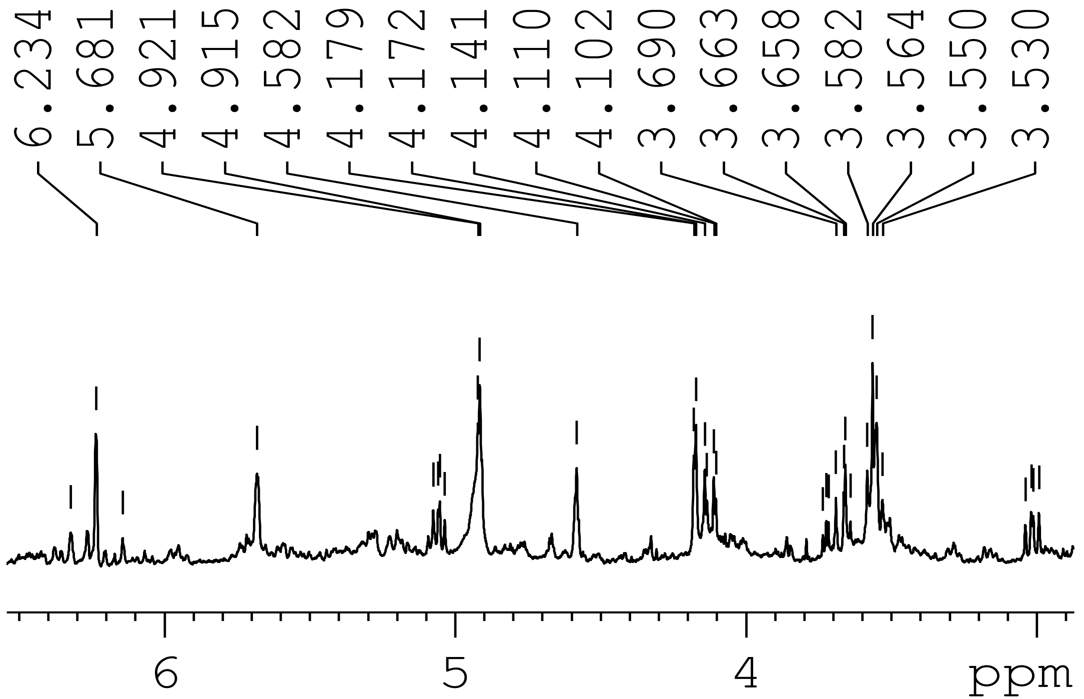
Appendix M: 1HNMR spectrum of Isolate Six(Band 6)



Appendix Expanded form of N

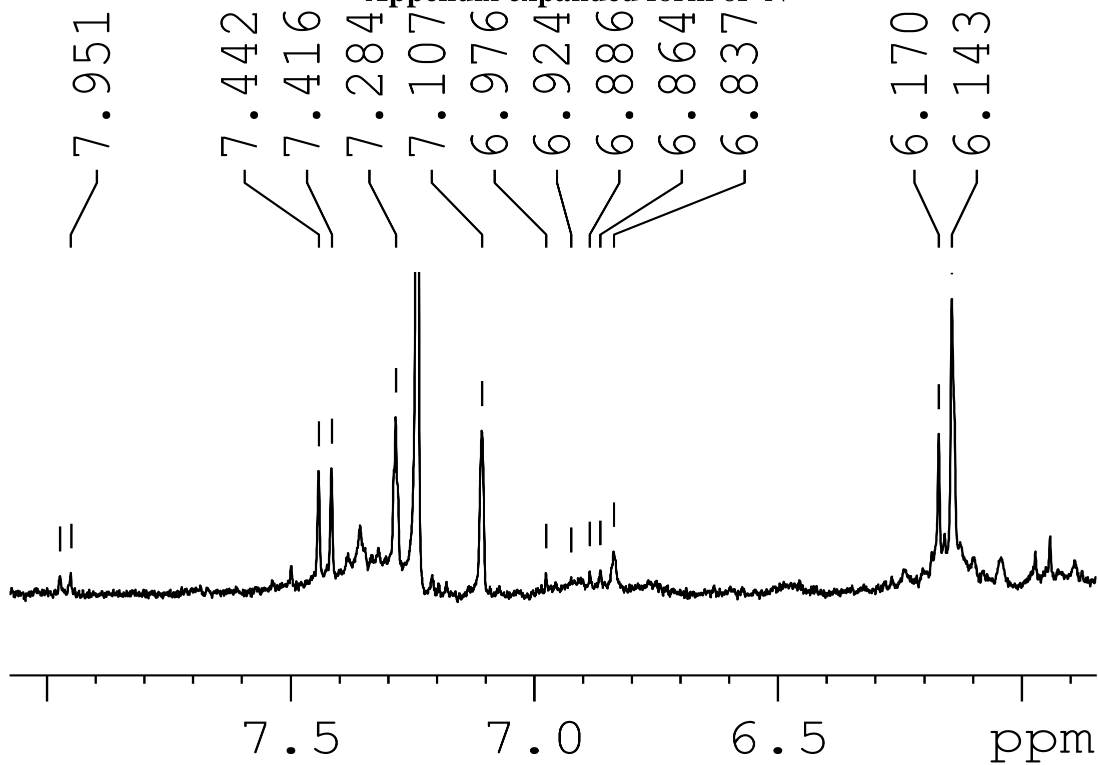


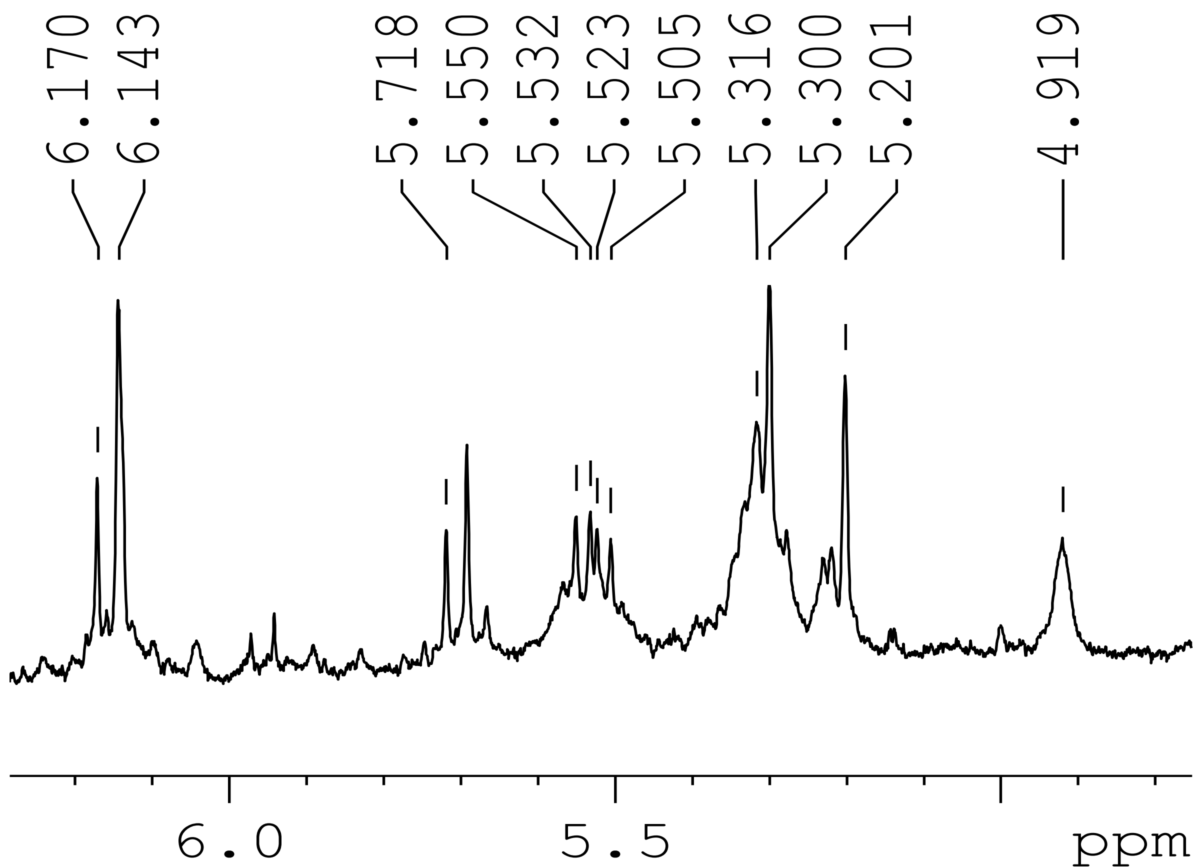




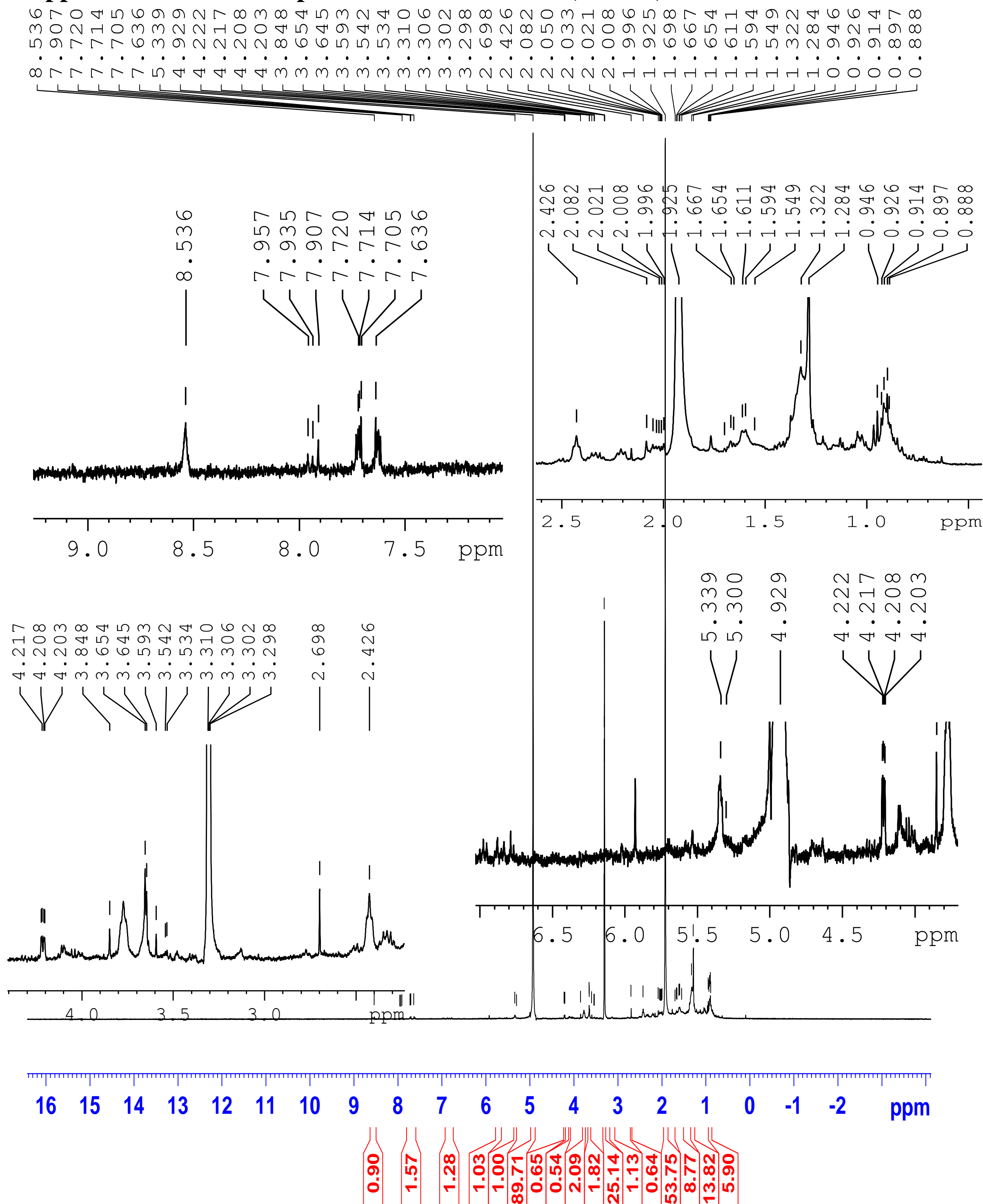
Appendix N: ^{1}HNMR spectrum of Isolate five (Band 5)

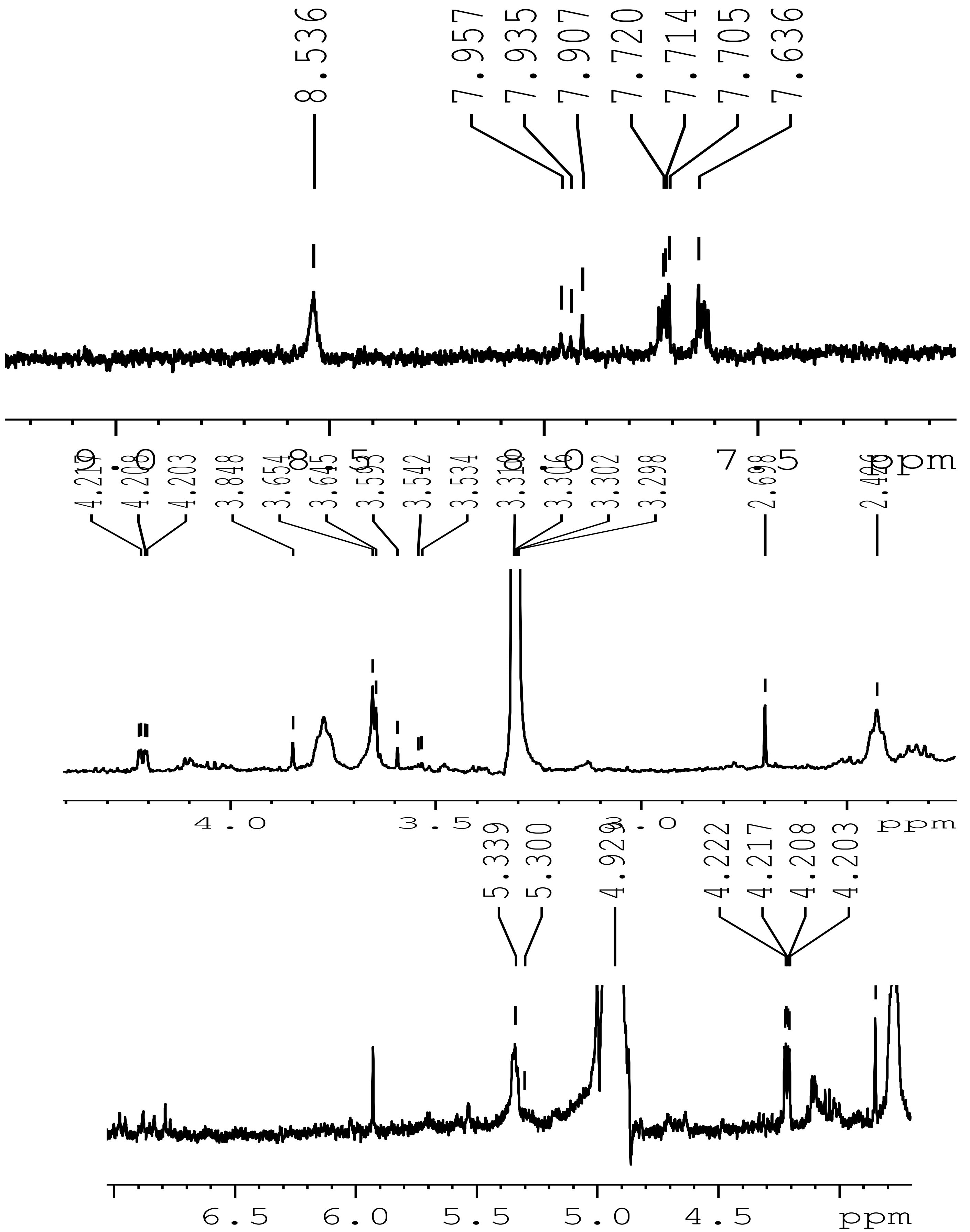
Appendix expanded form of N



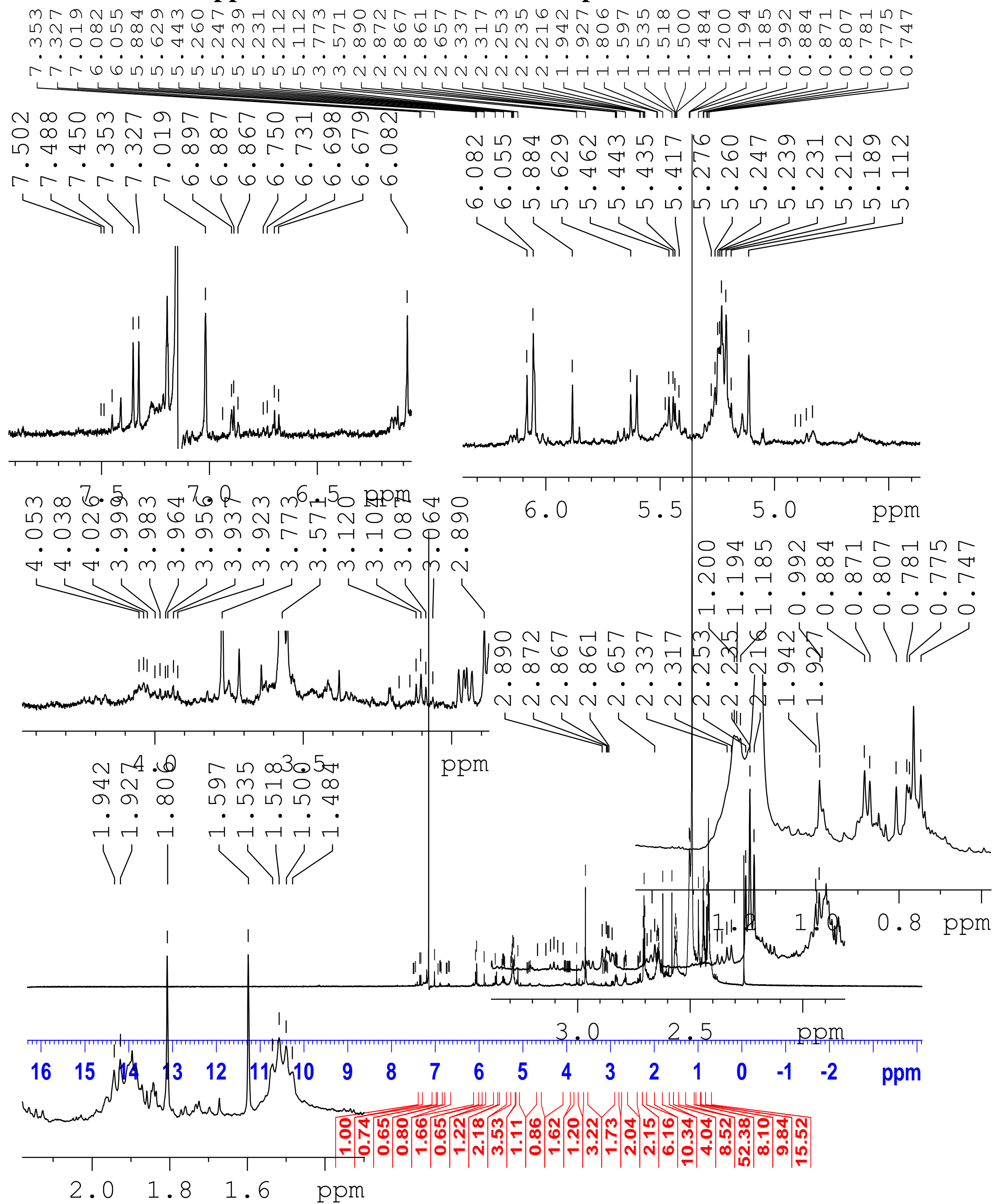


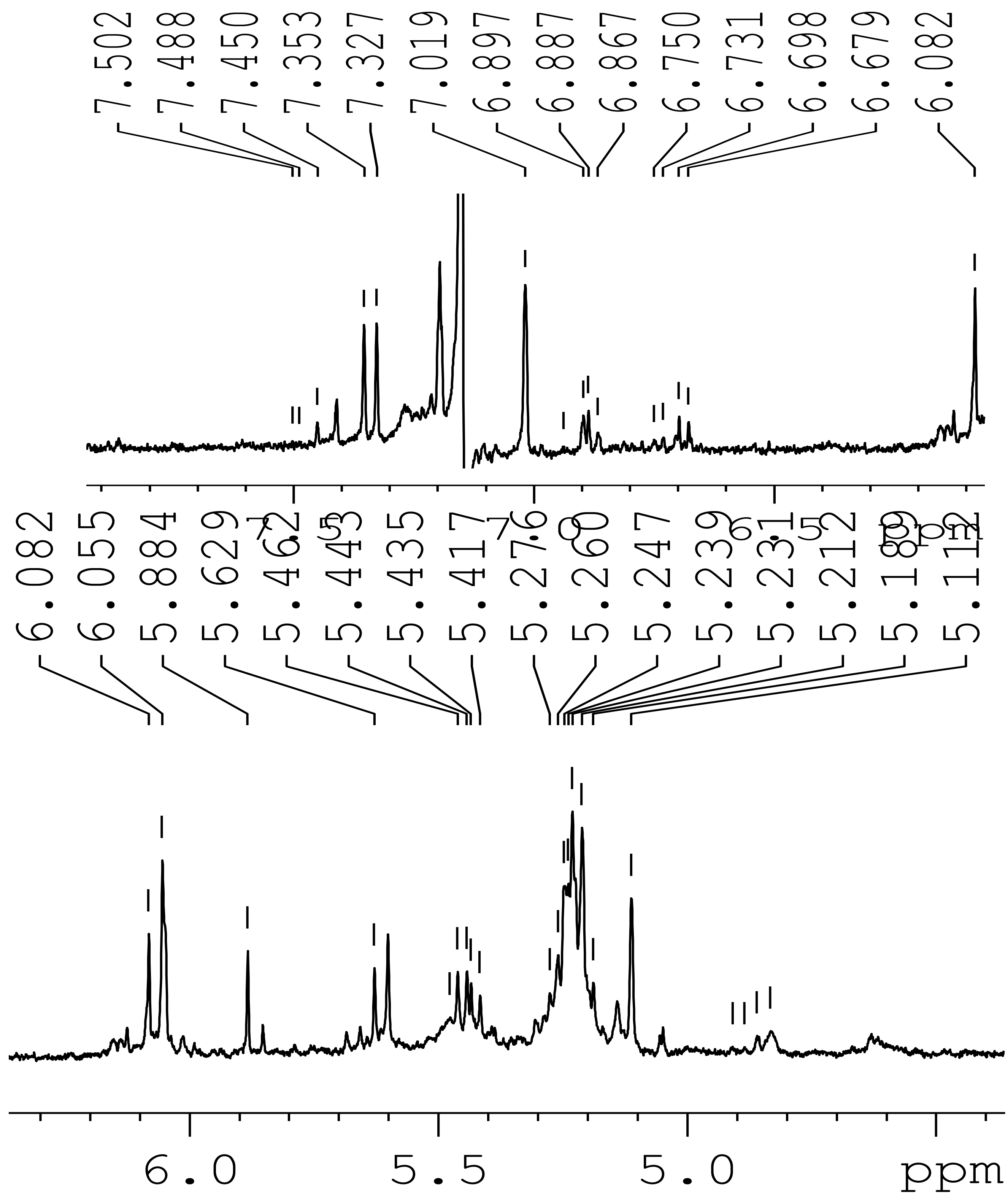
Appendix O: 1H NMR spectrum of Isolate Four (Band 4)



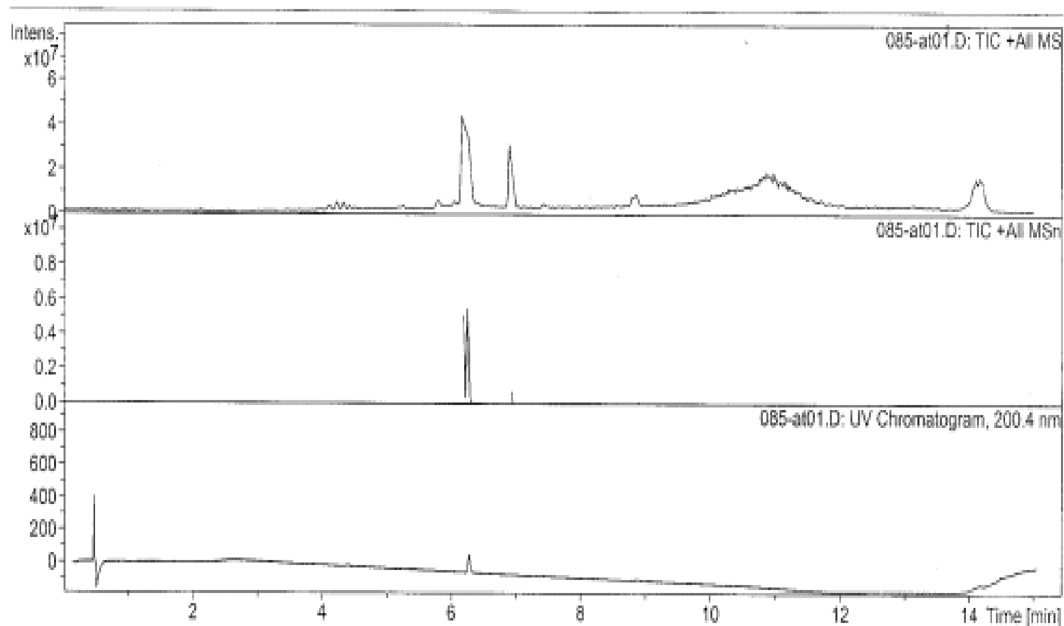

8.536
7.957
7.935
7.907
7.720
7.714
7.705
7.636
9.0
8.5
8.0
7.5
ppm
4.203
3.848
3.654
3.542
3.534
3.302
3.298
4.0
3.5
3.0
ppm
5.339
5.300
4.222
4.217
4.208
4.203
6.5
6.0
5.5
5.0
4.5
ppm

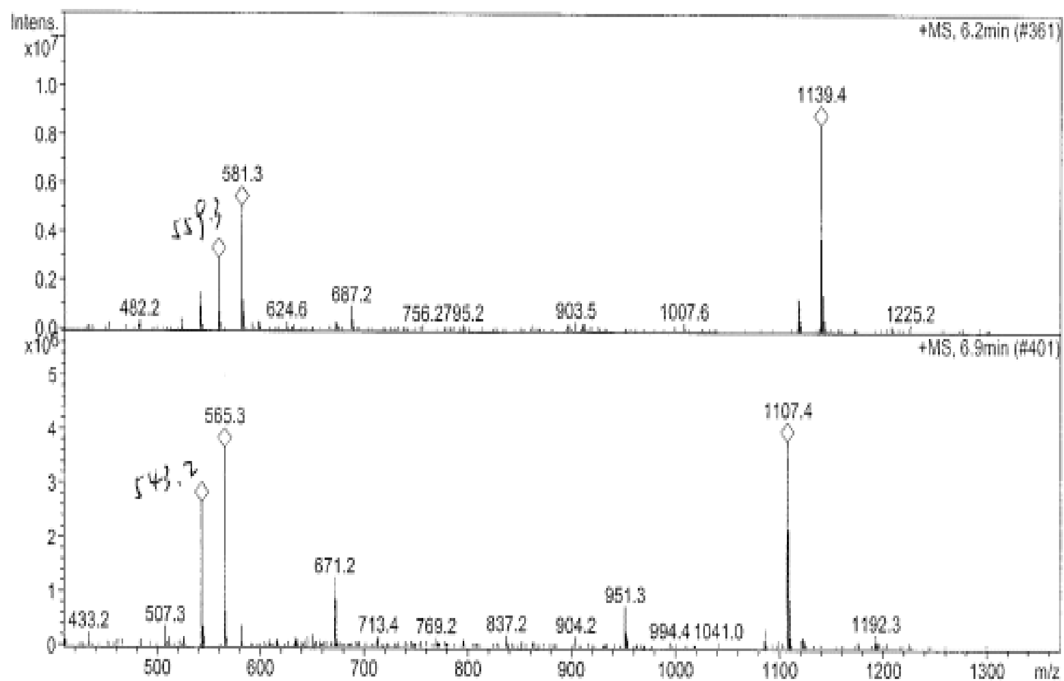
Appendix P: An earlier [1] HNMR spectrum of 1A[1]




7.502
7.488
7.450
7.353
7.327
7.019
6.897
6.887
6.867
6.750
6.731
6.698
6.679
6.082
7.5
7.0
6.5
ppm
6.082
6.055
5.884
5.629
5.462
5.443
5.435
5.417
5.276
5.260
5.247
5.239
5.231
5.212
5.189
5.112
6.0
5.5
5.0
ppm

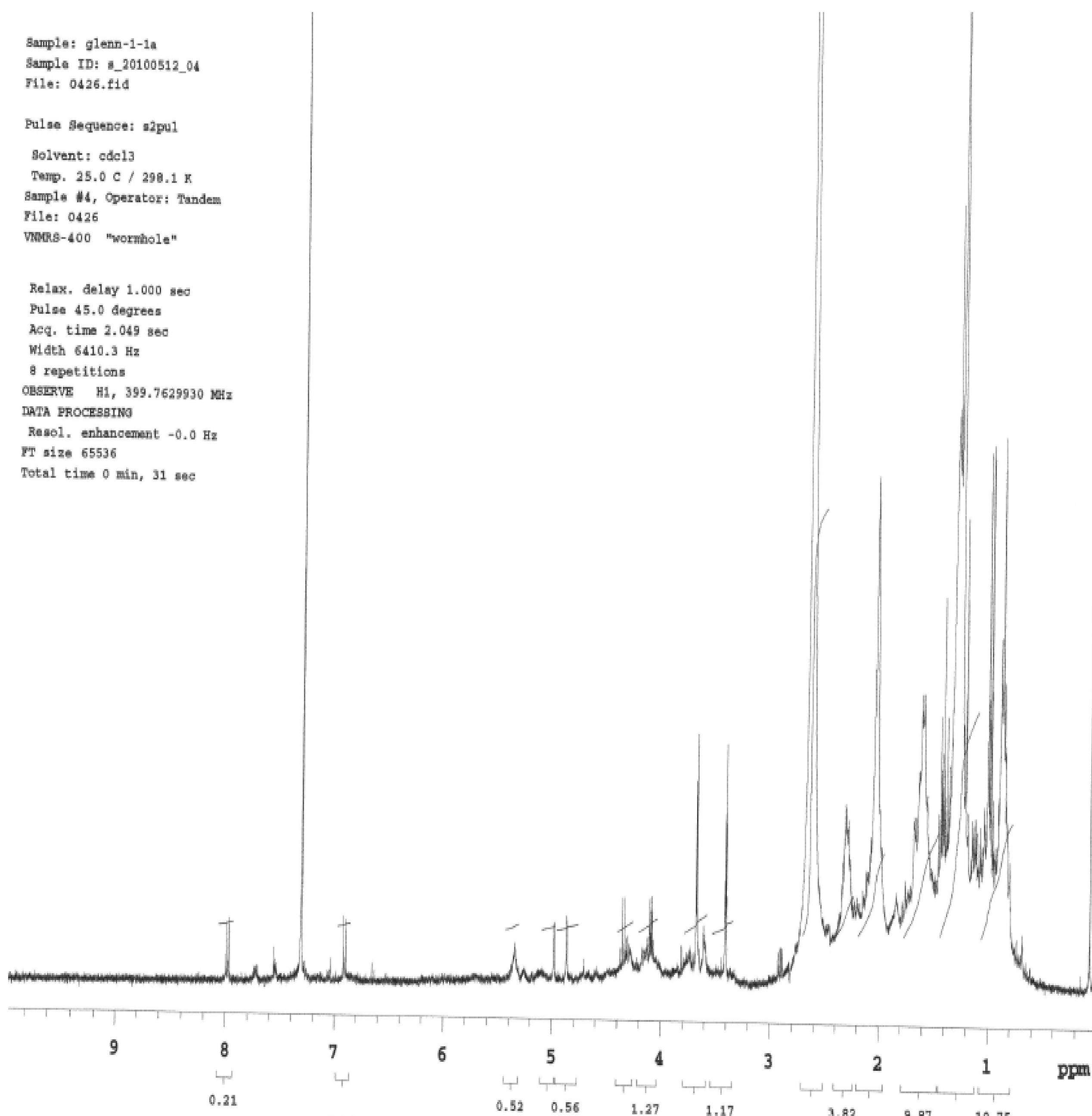
Appendix B2: ESI-MS (positive ion mode) spectrum of 6 (khayanolide E)
(source: Zhang et al, 2007)



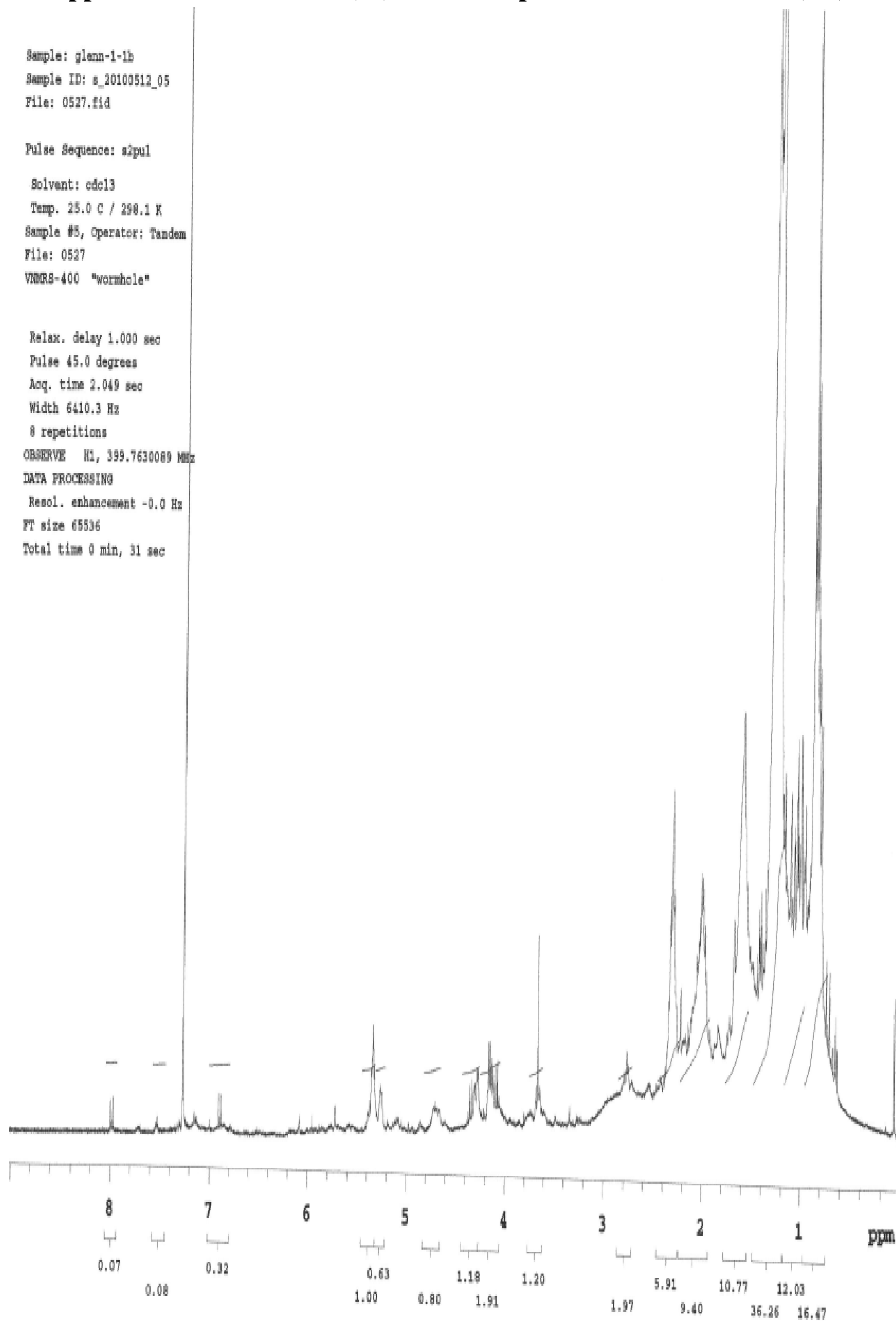


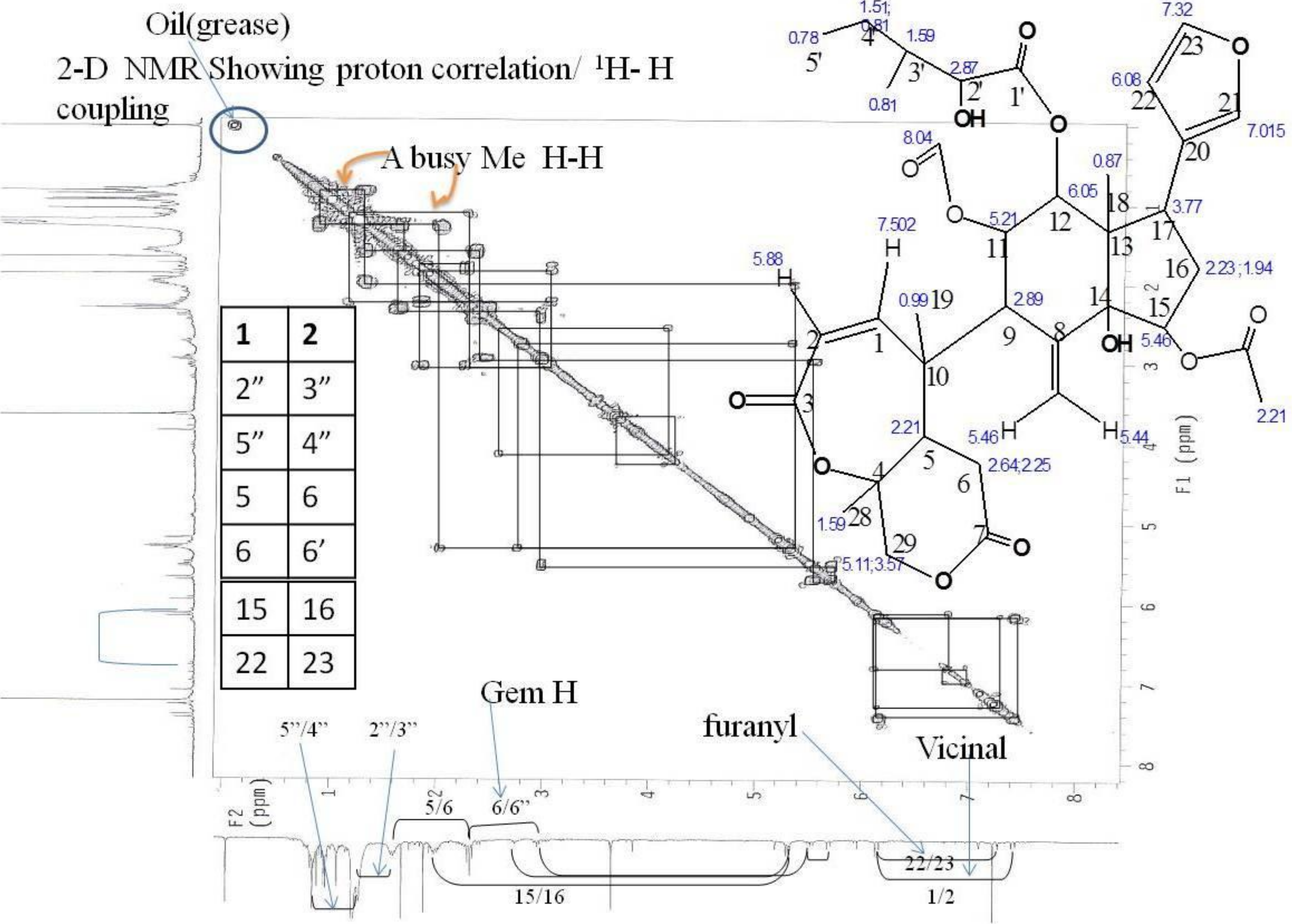
Bruker Daltonics DataAnalysis 3.0 printed: 02/29/08 08:41:54 Page 1 of 1

Appendix Q ^{1}HNMR spectrum of isolate 1a(Band 1- US)



Appendix S ¹HNMR **1b (A): ¹HNMR spectrum of Rohituka -7 (US)**





1	2
2''	3''
5''	4''
5	6
6	6'
15	16
22	23