

**PHYTOCHEMICAL, ANTIMICROBIAL, PROXIMATE AND ELEMENTAL
STUDIES OF THE LEAVES OF *TETRACARPIDIUM CONOPHORUM* (MULL.
ARG.) HUTCH AND DALZIEL**

BY

OLUBUNMI OLUKEMI AKINWANDE

**DEPARTMENT OF CHEMISTRY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA**

JUNE, 2015

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BY

**Olubunmi Olukemi AKINWANDE
Bsc.Chem, Msc.Chem, PGDE (A.B.U. Zaria)
Ph.D./SCIEN /11558 / 07-08**

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**DEPARTMENT OF CHEMISTRY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA**

JUNE, 2015

DECLARATION

I declare that the work in this dissertation entitled ‘Phytochemical, Antimicrobial, Proximate and Elemental Studies of the Leaves of *Tetracarpidium conophorum* (Mull. Arg.) Hutch and Dalziel’ was carried out by me in the Department of Chemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

Akinwande Olukemi Olubummi

Signature

Date

CERTIFICATION

This dissertation entitled ‘Phytochemical, Antimicrobial, Proximate and Elemental Studies of the Leaves of *Tetracarpidium conophorum* (Mull. Arg.) Hutch and Dalziel’ by Akinwande Olukemi Olubummi meets the regulations governing the award of the degree of Doctor of Philosophy Organic Chemistry of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

Prof. J.O. Amupitan
Chairman supervisory committee

Signature

Date

Prof. A.O. Oyewale
Member supervisory committee

Signature

Date

Dr. (Mrs.) R.G. Ayo
Member supervisory committee

Signature

Date

Prof. V.O. Ajibola
Head of Department

Signature

Date

Prof. H.Z. Hassan
Dean, School of Postgraduate Studies,
A.B.U.Zaria

Signature

Date

DEDICATION

This work is dedicated to the Lord Jesus Christ, The Lover and Bishop of my soul.

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The Ancient of days, the Almighty God, My Rock, Fortress and High Tower to Him alone be all the glory. AMEN.

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ABSTRACT

The extracts of the pulverized leaves of *Tetracarpidium conophorum* (Family – *Euphorbiaceae*) were investigated by carrying out phytochemical, antimicrobial, proximate and elemental studies using standard laboratory procedures. Natural products were extracted from pulverized leaves using a Soxhlet extractor, applying four (4) solvents of different polarities namely - petroleum ether (60-80°C), ethyl acetate, chloroform and finally methanol of analar grade successively. The chemical assays using phytochemical tests showed the presence of proteins, tannins, saponins, glycosides, alkaloids and flavonoids in the crude extracts. The bioassays using agar well diffusion technique were carried out on gram – negative and gram - positive bacteria, fungi and mould. These included *Methicillin Resistant Staphylococcus Aureus* (MRSA), *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Corynebacterium diphtheria*, *Corynebacterium ulcerans*, *Bacillus subtilis*, *Campylobacter fetus*, *Listeria monocytogenes*, *Shigella dysenteriae*, *Vibro cholerae*, *Candida albicans*, *Candida krusei*, *Helicobacter pylori*, *Aspergillus niger*, *Aspergillus fumigatus*, *Streptococcus pneumoniae* and *Klebsiella pneumoniae*. The crude extracts and the isolated compounds inhibited the growth of MRSA, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Salmonella typhi*, *Candida albicans*, *Candida krusei*, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Listeria monocytogenes*, *Shigella dysenteriae*, *Campylobacter fetus*, *Klebsiella pneumoniae* and *Helicobacter pylori* (zone of inhibition ranged from 14 – 29 mm). Control was carried out using standard drugs that are currently used to treat some illnesses caused by the above micro-organisms. These include fulcin, fluconazole, tetracycline, sparfloxacin and ciprofloxacin. The isolated compounds inhibited the growth of the same micro –organisms (zone of inhibition ranged from 20 – 35 mm). All the four extracts exhibited anti – microbial activities. Proximate analysis of the leaves showed crude protein (17.08 %), crude fiber (15.53 %), crude lipids (1.89 %), ash (13.38 %), nitrogen free - extract (51.52 %) and dry matter (91.86 %). Elemental analysis revealed the presence of fourteen elements:- magnesium, aluminum, chlorine, calcium, strontium, manganese, sodium, potassium, bromine, lanthanum, cobalt, zinc and barium. Essential elements present and their quantities are:- Zn (24 mg), Ca (33460 mg) Mg (4756 mg) and Mn (262 mg). The purification of petroleum ether crude extract by chromatographic techniques (by TLC, column and prep-TLC) yielded two pure compounds (TC 1 and TC 2). Structural elucidation of TC 1 and TC 2 were established based on spectral data; IR, 1D NMR and 2D NMR, MS and cited literatures. TC 1 was established to be 3 β , 22E- Stigmasta-5,22-dien-3-ol (C₂₉H₄₈O, 412.7g/mol – Stigmasterol) and TC 2 was established to be 3 β , hydroxyolean -12-en-28-oic acid (C₃₀H₄₈O₃, 456.7 g/mol - Oleanolic acid). Stigmasterol and oleanolic acid are triterpenoids. They inhibited the growth of *Shigella dysenteriae* (causative agent of dysentery) significantly. Triterpenoids are known to exhibit anti-inflammatory, anti-cancer and anti-diabetic properties. This is the first time they are being reported present in leaves of *Tetracarpidium conophorum*.

TABLE OF CONTENTS

	Page
Cover Page.....	i
Title Page	Error! Bookmark not defined.
Declaration.....	iii
Certification	iiv
Dedication.....	v
Acknowledgements.....	vi
Abstract.....	vii
Table of Contents.....	iix
List of Figures.....	ixvi
List of Tables	xvii
List of Plates.....	xix
Abbreviation and Symbols.....	xx
Glossary.....	xxi
CHAPTER ONE : INTRODUCTION	
1.1 Importance of Medical Plants	1
1.2 Aim and Objectives.....	5
1.3 Justification of the Research	6
CHAPTER TWO : LITERATURE REVIEW	
2.1 General Botanical Characteristics of the Family <i>Euphorbiaceae</i>	7

2.1.1 Botanical Characteristics of <i>Juglans regia</i> L.....	7
2.1.2 Botanical Characteristics of <i>Coula edulis</i> (Family: <i>Olacaceae</i>).....	10
2.2 Botanical Characteristics of <i>Tetracarpidium conophorum</i>	12
2.3 Chemical Constituents of <i>Tetracarpidium conophorum</i>	18
2.3.1 Aspirin (Acetylsalicylic Acid)	18
2.3.2 Phytosterols.....	19
2.3.3 Terpenes and Terpenoids	21
2.3.4 Phytoestrogens (Plant Estrogens)	25
2.3.5 Lectins.....	27
2.3.6 Saponins.....	29
2.3.7 Glycosides.....	29
2.2.8 Alkaloids.....	31
2.3.9 Flavonoids.....	34
2.3.10 Other Compounds found in <i>Tetracarpidium conophorum</i>	36
2.4 Medicinal and Economic Uses of the ThreeWalnuts.....	37
2.5 Microorganisms.....	44
2.5.1 As Causative agents of diseases and sickness.....	44
2.5.2 Culture media for microorganisms.....	45
2.6 Proximate Analysis	44
2.7 Elemental Analysis	46

CHAPTER THREE: MATERIALS AND METHODS

3.1 List of materials	48
3.1.1 Reagents.....	48
3.1.2 Chromatographic Analyses	48
3.1.3 Microbiological media	48
3.2 Instrumentations.....	48
3.2.1 Fourier Transform – Infra Red (FT-IR).....	48
3.2.2 Mass Spectroscopy (MS).....	49
3.2.3 Nuclear Magnetic Resonance (NMR).....	49
3.2.4 Melting point.....	49
3.3 Test organisms for antimicrobial screening	49
3.4 Sample Collection and Preparation	50
3.5 Extraction of Plant Material.....	50
3.6 Preliminary Phytochemical Screening.....	51
3.6.1 Tests for Carbohydrates	51
3.6.1.1 Molisch’s Test.....	51
3.6.1.2 Seliwanoff’s Test (for ketones).....	52
3.6.1.3 Barfoed’s Test (General Test for Monosacharrides)	52
3.6.1.4 Test for Pentoses	52
3.6.1.5 Fehling's Test.....	52
3.6.1.6 Test for Starch.....	53

3.6.1.7 Test for Agar	53
3.6.1.8 Tests for Tragacanth	53
3.6.2 Test for Glycosides	53
3.6.2.1 By Dilute Mineral Acid	53
3.6.2.2 Borntrager's Test (Test for Anthracene Derivatives)	54
3.6.2.3 Test for Steroidal Aglycone	54
3.6.2.4 Tests for Saponin Glycosides.....	54
3.6.2.5 Keller-Kiliani Test (For Cardiac Glycosides).....	55
3.6.2.6 Kedde Regent Test.....	56
3.6.2.7 Baljet Reagent Test.....	56
3.6.3 Test for Alkaloids	56
3.6.3.1 Using Mayer's Reagent.....	56
3.6.3.2 Using Dragendorff's Reagent	57
3.6.3.3 Using Wagner's Reagent	57
3.6.4 Test for Flavonoids	57
3.6.4.1 Shinoda test.....	57
3.6.4.2 Sulphuric Acid Test	58
3.6.4.3 Ferric Chloride Test.....	58
3.6.4.4 Test For Plant Phenols.....	58
3.6.5 Test for Sterols.....	58
3.6.5.1 Liebermann- Buchard Test	58

3.6.5.2 Salkowski Reaction.....	59
3.6.6 Test for Proteins	59
3.6.6.1 Millon’s Reagent (Freshly Prepared Mercuric Nitrate Solution)	59
3.6.6.2 Reaction with solution of Picric acid and Tannin Acid	59
3.6.6.3 Xanthoprotein Test.....	60
3.6.6.4 Biuret Test.....	60
3.6.7 Test for Saponins	60
3.6.7.1 Frothing Test.....	60
3.7 Antimicrobial Sensitivity Studies	61
3.7.1 Media Preparation.....	63
3.7.2 Detection of Antimicrobial Activity	63
3.7.3 Determination of Minimum Inhibitory Concentration (MIC).....	64
3.7.4 Determination of Minimum Bactericidal/Fungicidal Concentration (MBF/ MFC).....	65
3.8 Chromatographic Techniques Employed in Purification of Crude Extract.....	65
3.8.1Thin Layer Chromatography	65
3.8.2 Column Chromatography	66
3.8.3 Additional Purification of Crude Petroleum ether Extract	67
3.9 Proximate Analysis of the Leaves of <i>Tetracarpidium conophorum</i>	69
3.9.1 Determination of Crude Protein.....	69
3.9.2 Determination of Crude Lipid.....	70
3.9.3 Determination of Nitrogen-Free Extract	70
3.9.4 Determination of Ash.....	70

3.9.5	Determination of Crude Fiber	71
3.9.6	Determination of Dry Matter	71
3.10	Determination of Elemental Analysis.....	71

CHAPTER FOUR : RESULTS

4.1	Extraction of the Leaves	73
4.2	Proximate Analysis	75
4.3	Elemental Analysis by INAA	77
4.4	Phytochemical Screening of the Crude Extracts.....	79
4.5	Antimicrobial Screening of the Crude Extracts.....	81
4.6	Determination of Melting Points of TC1 and TC2	91
4.7	The IR Spectral of TC1 and TC2.....	92

CHAPTER FIVE : DISCUSSIONS

5.1	Extraction.....	117
5.2	Elemental Analysis of the leaves of <i>T.conophorum</i>	117
5.3	Proximate Analysis of the leaves of <i>T.conophorum</i>	118
5.4	Biological Functions of the Elements in the leaves of <i>T. conophorum</i>	119
5.5	Preliminary Phytochemical Screening of the Crude Extracts	120
5.6	Antimicrobial Screening of the Crude Extracts	122
5.7.1	The FT-IR spectrum of TC 1	126
5.7.2	The FT-IR spectrum of TC 2	126
5.8	NMR Spectral of TC 1	127
5.8.1	The ¹ H NMR spectrum of TC 1	127

5.8.2 The ^{13}C NMR spectrum of TC1.....	127
5.8.3 The COSY Spectrum of TC 1.....	128
5.8.4 The DEPT spectral of TC1.....	128
5.8.5 HSQC Spectrum of TC 1.....	128
5.8.6 HMBC Spectrum of TC 1.....	129
5.8.7 Mass Spectroscopy (MS) of TC 1.....	129
5.9.The NMR Spectral of TC 2.....	129
5.9.1 ^1H NMR Spectrum of TC 2 (Oleanolic acid).....	129
5.9.2 ^{13}C NMR Spectrum of TC 2.....	130
5.9.3 COSY Spectrum of TC 2.....	131
5.9.4 DEPT Spectral of TC 2	131
5.9.5 HMBC of TC 2.....	131
5.9.6 HSQC of TC 2.....	132
CHAPTER SIX:	
SUMMARY, CONCLUSION AND RECOMMENDATION	
6.1 Summary.....	133
6.2 Conclusion	135
6.3 Recommendations.....	135
REFERENCES.....	136

LIST OF FIGURES

Figure	Page
4.1: FT-IR spectrum of TC 1	98
4.2: ^1H NMR of TC 1.....	99
4.3: ^{13}C NMR of TC 1.....	100
4.4: COSY of TC 1	101
4.5: DEPT of TC 1	102
4.6: HMBC of TC 1	103
4.7: HSQC of TC 1	104
4.8: NOESY of TC 1.....	105
4.9: MS spectra of TC 1	106
4.10 FT-IR spectrum of TC 2.....	108
4.11 ^1H spectrum of TC1.....	109
4.12: ^{13}C NMR of TC 2.....	110
4.13: COSY of TC 2	111
4.14: DEPT of TC 2	112
4.15: HMBC of TC 2	113
4.16: HSQC of TC 2	114
4.17: NOESY of TC 2.....	115

LIST OF TABLES

Table	Page
2.1 Taxonomy of <i>Tetracarpidium conophorum</i>	13
2.2 Local names of <i>Tetracarpidium conophorum</i>	16
2.3 Nomenclature for phytosterols	20
2.4 Classification of terpenes.....	23
2.5 Main classes of monomeric alkaloids	32
2.6 Essential Fatty Acids in walnuts.....	36
2.7 Uses of <i>Juglans regia</i>	38
2.8 Uses of <i>Coula edulis</i>	40
2.9 Other Uses of <i>Tetracarpidium conophorum</i>	43
4.1 Extraction and percentage recovery of <i>Tetracarpidium conophorum</i> leaves.....	74
4.2 Proximate analysis of leaves of <i>Tetracarpidium conophorum</i>	76
4.3 Elemental Analysis of <i>Tetracarpidium conophorum</i> leaves	78
4.4 Preliminary phytochemical screening of the crude extracts of the leaves of <i>Tetracarpidium conophorum</i>	80
4.5 Antimicrobial screening of leaves of <i>T.conophorum</i> and control	82
4.6 Zones of Inhibition of the extracts (mm) against the test microorganisms and control	83
4.7 Minimum Inhibitory Concentration (MIC) and MBC/MFC (mg/cm ³).....	84

4.8	The Antimicrobial screening of fractions of petroleum ether extract – TCPE I, TCPE II. TCPE III and the control.	85
4.9	Zone of Inhibition of petroleum ether extract – TCPE I, TCPE II and TCPE III	86
4.10	MIC and MBC/MFC of fractions of petroleum ether extract – TCPE I, TCPE II and TCPE III (mg/cm³)	87
4.11	The antimicrobial screening of isolated pure compounds, TC1, TC2 and the control	88
4.12	Zones of Inhibition of TC 1, TC 2 and the control (mm)	89
4.13	Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal or Fungicidal Concentration (MBC/MFC)	90
4.14	Infra-red spectrum of TC 1.....	93
4.15	Comparing the ¹³ C NMR and ¹H NMR spectral data of TC 1 with that obtained from the cited literatures.....	94
4.16	Mass Spectrum readings of TC1.....	95
4.17	Infra-red spectrum of TC 2.....	96
4.18	Comparing the ¹³ C NMR and ¹H NMR spectral data of TC 2 with that obtained from the cited literatures.....	97

LIST OF PLATES

Plate I :	<i>Juglans regia</i> L. (Persian Walnut) tree	8
Plate II:	Leaves of <i>Juglans regia</i> L.	9
Plate III :	Fruits of <i>Juglans regia</i> L.....	9
Plate IV:	<i>Coula edulis</i> (African Walnut) tree.....	11
Plate V:	Leaves of <i>Coula edulis</i>	11
Plate VI:	Fruits of <i>Coula edulis</i>	12
Plate VII:	<i>Tetracarpidium conophorum</i> (African Walnut)	14
Plate VIII :	Leaves of <i>Tetracarpidium conophorum</i>	14
Plate IX :	Pods of <i>Tetracarpidium conophorum</i> containing the walnuts	15
Plate X:	Walnuts / seeds of <i>Tetracarpidium conophorum</i>	15

ABBREVIATIONS AND SYMBOLS

T. conophorum *Tetracarpidium conophorum*

TC 1	Isolated pure compound 1
TC 2	Isolated pure compound 2
TCPE I	Petroleum ether fraction I
TCPE II	Petroleum ether fraction II
TCPE III	Petroleum ether fraction III
MIC	Minimum Inhibitory Concentration
MBC/MFC	Minimum Bactericidal / Fungicidal Concentration
CC	Column chromatography
TLC	Thin Layer Chromatography
PTLC	Preparative Thin Layer Chromatography
FT-IR	Fourier Transform – Infra Red
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement by Polarization Transfer
COSY	Proton Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
HMBC	Heteronuclear Multiple Bond Correlation
NOESY	Nuclear Overhauser Effect Spectroscopy
TMS	Trimethylsilane
CDCl ₃	Deuteriochloroform
DNA	Deoxyribonucleic acid
RDA	Recommended Dietary Allowance

GLOSSARY

- Antibiotics:** Are medications used to treat and in some cases prevent bacteria infections.
- Antiseptic:** Is an antimicrobial substance that prevents the growth of disease causing microorganisms.
- Amphipathic:** When a compound has a polar end and a non-polar end.
- Arthritis:** Is a common condition that causes joint-pain, inflammation and stiffness.
- Astringent:** Is a substance that causes the contraction of body tissues, typically used to protect the skin and to reduce bleeding from minor abrasions.
- Atherosclerosis:** Is the hardening and narrowing of the arteries.
- Bacteremia (bacteraemia):** Is the presence of visible bacteria in the circulating blood.
- Cancer:** Is a class of diseases characterized by out-of-control cell growth and invading other tissues.
- Candidiasis:** Is an infection caused by a yeast-like fungus called *Candida albicans*. It can infect the mouth, vagina, skin, stomach and urinary tract. Symptoms include white patches on the tongue, genital itching and burning.
- Chemotherapeutic agents:** Can be defined as antimicrobial agents of synthetic origin useful in the treatment of microbial or viral disease.
- Cognitive skills:** Are any mental skills that are used in the process of acquiring knowledge, such as reasoning, perception and intuition.
- Diabetes:** Is a group of diseases characterized by high blood glucose levels over a prolonged period as a result of metabolic diseases.

Diabetic vascular disease: Refers to the development of blockages in the arteries throughout the body because of diabetes.

Diarrhea: Is an uncomfortable condition with passage of loose, watery stools that occur more frequently than usual.

Diphtheria: Is a highly contagious and potentially life threatening bacterial disease.

Drug: Is a chemical administered in an attempt to prevent, treat or diagnose disease.

Dysentery: Is an inflammation of the intestine causing diarrhea with blood. Other symptoms can include: painful stomach cramps, nausea or vomiting and a high temperature (fever) of 38 °C or above in children above five.

Endothelial dysfunction: Is a condition in which the endothelium (inner lining) of blood vessels does not function normally.

Hemorrhoids: are also called piles, are swollen and inflamed veins in the anal canal and lower rectum.

Hypercholesteromic: Is the presence of high levels of cholesterol in the blood.

Hypertension: Is also referred to as high blood pressure, is a condition in which the arteries have persistently elevated blood pressure.

Meningitis: Is a disease caused by the inflammation of the protective membranes covering the brain and spinal cord known as the meninges.

Nephropathy; Means kidney disease or damage caused by uncontrolled high blood sugar.

Pathogen: Anything or an infectious agent that causes disease or illness to its host.

Pelvic Inflammatory Disease (PID): Is an infection and inflammation of the uterus, ovaries and other female reproductive organs. It causes scarring in these organs.

Peptic (or gastric) ulcers: Are holes or breaks in the protective lining of the duodenum or the stomach. It occurs as a burning, gnawing abdominal pain.

Pneumonia: Is an inflammatory condition of the lung affecting primarily the microscopic air sacs known as alveoli.

Thrombophlebitis: Is an inflammation of a vein, usually in the legs, that becomes swollen due to a blood clot.

Typhoid fever: Also known as enteric fever is a potentially fatal multisystem illness. Symptoms include fever, malaise, diffuse, abdominal pain and constipation.

Retinopathy: Is due to the persistent or acute damage to the retina of the eye as seen in diabetes or hypertension.

Scalded Skin Syndrome: Is characterized by red blistering skin that looks like a burn or scald

Septic abortion: Is a form of miscarriage that is associated with a serious uterine infection. It is characterized by severe abdominal pain and heavy bleeding.

CHAPTER ONE

INTRODUCTION

1.1 Importance of medicinal plants

In the developing countries in the world, large number of people die daily of preventable or curable diseases because of lack of simple healthcare. These developing countries have certain features in common, extreme poverty, lack of education, poor or polluted water supplies, etc. (Sofowora 2008). Another special characteristic of the developing world is the nomadic life-style of some of its people. As a result of these constant movements it is difficult for conventional health services to reach these people. It is against the fore-going back-ground of the socio-economic status of developing countries, the magnitude of their health problems and the few resources available that any advantages of traditional medicine can be and should be viewed (Sofowora 2008).

Medicinal plants have been identified and used throughout human history. Plants have the ability to synthesis a wide variety of chemical compounds that are used to perform important biological functions, and to defend against attacks from predators such as insects, fungi and herbivorous mammals. At least 12,000 such compounds have been isolated so far, a number estimated to be less than 10 % of the total (Tapsell *et al.*, 2006). Traditional medicine is more accessible to most of the population in the third world. In fact, it is reported that 60-85% of the population in every country of the developing world has to rely on traditional or indigenous forms of medicine. For example, 59 % of Swazis (Bantu people of Swaziland, South Africa) use traditional healers. This is mainly because of shortage of hospitals and health centers, as well as the medical and paramedical staff needed to manage orthodox

healthcare delivery systems (Sofowora 2008). The World Health Organization (WHO) estimates that 80% of the populations of some Asian and African countries presently use primary herbal care (WHO 2008).

In 1999, the World Health Organisation (WHO) redefined traditional medicine (TM) as comprising ‘therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern scientific medicine and are still in use today’ (Sofowora 2009). Traditional medicine remedies are mostly compounded from natural products, for this reason, it has been claimed that there is a greater likelihood of their being accepted by the human body than substances which have been synthesized in a laboratory. Also the concentration of active principles in the plant is usually small and it is further diluted when a decoction for traditional use is prepared. It is believed that an aqueous decoction of a drug has a greater bio-availability to the body than many synthetic drug formulations in the market today. (Sofowora 2008).

Development of resistance to synthetic chemo-therapeutic agents is known to occur in orthodox medicine, for example, the resistance to chloroquine by some strains of the malaria parasite (*Plasmodium falciparum* or *Plasmodium vivax*) or resistance to some antibiotics by certain strains of micro organisms. Also, the traditional practitioners’ remedies often come in multi-component preparations aimed at healing several ailments simultaneously. This is more convenient for the average man than taking several different tablets or mixtures in addition to an injection (Sofowora 2008).

In the past and present decades, it is known that major diseases, such as AIDS, cancer, diabetes, hypertension, malaria, Parkinson’s disease, sickle cell anaemia,

remain incurable and paradoxically, scientific progress continues with ever increasing acceleration. The creation of large research and development (R and D) organizations continues and drug discovery process has become longer and more complex. This is due to increased understanding of biological processes at molecular level and a desire to characterize new drugs fully and also, due to increased complexity and length of regulatory review processes with increased R and D costs. The focus is now on finding breakthrough drugs because of both the medical implications and economic consequences (Olaniyi 2005).

Drug research and development encompasses four stages: basic research, feasibility studies, non clinical development and initial clinical development. Feasibility studies involve the identification of a “lead” using an interdisciplinary group to identify a new biochemical process or receptor of relevance to the disease pathology, along with identification of a new structural class of chemicals that can modulate the effect of the receptor or biochemical pathway i.e. a ‘lead’ (Olaniyi 2005). ‘Lead’ compounds are still a *sine qua non* of drug design. Natural products have been and still are an inexhaustible source of drug ‘leads’ as well as drugs. Natural products have also been utilized as chemical models or templates for the design and synthesis of many other important drugs. ‘Lead’ compounds can be obtained through isolation, identification and evaluation of bioactive substances from plants, animals or micro organisms. The ‘lead’ concept in drug design usually involves improvement of the ‘lead’. (Olaniyi 2005).

Examples are:

- a. The antimalarial drug quinine, obtained from *Cinchona officinalis* bark, served as ‘lead’ to the synthetic 8 -amino and 4 -amino *quinoline antimalarials*.

- b. Benzylpenicillin from *Penicillium notatum* or *P. chrysogenum* led to the semipenicilline of improved stability (phenoxymethyl penicillin), broad antimicrobial spectrum (ampicillin) and improved pharmacological efficiency (cloxacillin).
- c. The plant steroids, diosgenin and hecogenin, remain key precursors for the synthesis of many modern contraceptive drugs (progestins such as progesterone and its derivatives norgestrel, norethindrone) and other pharmaceuticals including betamethasone and betamethasone).

In the above examples, nature has supplied the first effective agents for further structural modification by synthesis (Olaniyi 2005). Nigeria, classified as a developing country has been talking of self reliance in many aspects since her independence and much still needs to be done. As a nation, we must reduce unwarranted importation of drugs, only very essential drugs should be imported. We should utilize locally available medicinal plants as substitutes for other drugs as well as encourage large scale cultivation of medicinal plants such that any excess can be converted into drug products for exportation. In actual fact, our rain forests are fast disappearing giving way to construction of buildings, road networks and farmland (Sofowora 2008).

In 1977, it was estimated that the drug import bill for Nigeria alone was about N120 million. It can be seen that there is the need for production of medicinal drugs from the many herbs that are used in traditional medicine in Nigeria (Sofowora 2008). It is also observed that there is an alarming increasing microbial resistance to many important drugs which has resulted in the search for leads from new organic molecules from plants with established antimicrobial properties. Medicinal plants that

are in common use in African traditional medicine, some have been observed to grow near houses and they are easily overlooked especially by urban settlers (Sofowora 2008).

Our high standards of living as well as the stressful nature of survival are making regular consumption of multivitamins and supplements, a must for many people particularly in the urban areas. In any case, our eyes have been opened to the issue of free radicals in the body which are causative agents of tumors and cancer, as well as advantages of healthy living. Some of these supplements and multivitamins are marketed under trade names such as 'Forever Living Products', 'Swissgrade', 'GNLD', 'Vitabiotics- Wellwoman and Wellman', 'Trevo', etc.

Reading their labels, one will discover that these supplements and multivitamins are largely from medicinal/natural plants from Asian countries like China and India, South America, few from some Arabian countries like Egypt and some European countries. Countries in Africa like Rwanda, Mali and Egypt now cultivate medicinal plants (Sofowora 2009). It is very possible that we also have medicinal plants in our rainforests as well as our immediate environments that can very well serve as supplements and multivitamins. As it is, these plants are getting extinct and little is known about them or that we do not have adequate documentation about them (Sofowora 2008).

1.2 Aim and Objectives

The aim of this study is to carry out the phytochemical, antimicrobial, proximate and elementary studies of the leaves of *Tetracarpidium conophorum*. The aim will be achieved through the following objectives:

- i. Collection, botanical identification and pulverization of the plant;

- ii Solvent extraction of the leaves of *Tetracarpidium conophorum* ;
- iii Phytochemical screening of the extracts ;
- iv Antimicrobial screening of the crude extracts;
- v. Purification of the crude extracts using chromatographic techniques.;
- vi Characterization of isolated compounds by FTIR, NMR and MS;
- vii Elemental analysis of the leaves of *Teteracarpidium conophorum*;
- viii Proximate analysis of the leaves of *Teteracarpidium conophorum*.

1.3 Justification

Tetracarpidium conophorum is commonly used in traditional medicine for the treatment of dysentery, abdominal pains and enteric fever. There is the need to scientifically validate the claims and uses of this plant in the treatment of diseases.

CHAPTER TWO

LITERATURE REVIEW

2.1 General Botanical Characteristics of the Family *EUPHORBIACEAE*

Walnut, common name for small flowering plants are important for the nuts and timber, most of them produce and for its representative genus. Walnut comprises such families as *Juglandaceae* (English walnut), *Euphorbiaceae* (African walnut), and *Olacaceae* (African walnut). Each family has its own peculiar characteristics, but they have some things in common such as the nuts (Ayoola *et al.*, 2011). The nuts are highly concentrated food containing high levels of essential fatty acids, vitamin B₆, trace elements such as zinc, copper and manganese, etc. (Khashayar 2008, Ayoola *et al.*, 2011).

2.1.1 Botanical Characteristics of *Juglans regia* L.

Juglandaceae is mostly found in the south east Europe, Japan, China and more widely in the new world (North America). *Juglans regia* L is the walnut tree of the botanical family *Juglandaceae* (Retrieved from wikipedia.org/wiki/Juglans_regia).

Synonyms: Persian walnut, English walnut and Common walnut (especially in Great Britain). *Juglans regia* is a large, deciduous tree attaining heights of 25 – 35 m, and a trunk up to 2 m diameter, common with a short trunk and broad crown, though taller and narrower in dense forest competition. It is a light demanding species, requiring full sun to grow well. The bark is smooth and olive-brown in colour when young. It assumes a silver grey colour as it ages, with scattered broad fissures and a rougher texture. Like all walnuts, the pith of the twigs contains air spaces; this chambered pith is brownish in colour. The leaves are alternately arranged, 25 - 40 cm long, odd - pinnate with 5 - 9 leaflets paired alternately with

terminal leaflet. The largest leaflets are the three at the apex; 10 - 18 cm long and 6 - 8 cm broad, the basal pair of the leaflets are much smaller, 5-8 cm long with the margins of the leaflets entire. The male flowers are in droppings, catkins 5-10 cm long and the female flowers are terminal clusters of two to five, ripening in the autumn into a fruit with a green, semi fleshy husk and a brown, corrugated nut. The whole fruit including the husk falls in autumn, the seed is large with a rich relatively thin seed and edible with a rich flavor (Ajaiyeoba *and Fadare* 2006, Burkill 1985).



Plate I: *Juglans regia* L. Tree
 (Retrieved from [http://www.en.m.wikipedia.org/wiki/
Juglans_regia L.\)](http://www.en.m.wikipedia.org/wiki/Juglans_regia_L.)



Plate II: Leaves of *Juglans regia* L. (Retrieved from en.m.wikipedia.org/wiki/Juglans_regia).



Plate III: Fruits of *Juglans regia* L. (Retrieved from www.en.m.wikipedia.org/wiki/Juglans_regia)

2.1.2 Botanical Characteristics of *Coula edulis* (Family: *Olacaceae*)

Coula edulis is a tree in the genus *Coula*, native to tropical West Africa from Sierra Leone to Angola. It is in abundance in the Democratic Republic of Congo, Nigeria and Sierra Leone. It prefers tropical regions and is tolerant of light shade. It can be found in the top canopy of forest as well as the lower story and had no special soil requirement. It is an evergreen tree growing to a height of 25 -28 m, and a dense crown that can cast deep shade. The leaves are arranged alternatively, simple, 10-30 cm long and 4 cm broad with an entire margin and an acuminate apex. The flowers are produced from April to June and are greenish yellow, with either four or five petals (Tamokou *et al.*, 2011).

The nut is an elipsoidal drupe available from August to January, 3 - 4 cm long, with flesh surrounding the kernel, 5- 6 mm thick, smooth in texture and can be red or green. The kernel shell is extremely hard and makes germination difficult. The nuts are usually found under the mother trees (retrieved from [en.m.wikipedia.org./wiki/Jugla-regia](http://en.m.wikipedia.org/wiki/Jugla-regia)) Synonyms; Gabon nut, African walnut, Congo wood and Tiger wood. It is not related to the walnut, being so named because its nut bears a superficial resemblance to the walnut (Tamokou *et al.*2011, Nelson *et al.*,2000).



Plate IV: *Coula edulis* tree (retrieved from [http://www.en.m.wikipedia.org/wiki/Coula -edulis](http://www.en.m.wikipedia.org/wiki/Coula_-_edulis))



Plate V: Leaves of *Coulaedulis*
(Retrieved from www.en.m.wikipedia.org/Coula__edulis)



Plate VI: Fruits of *Coula edulis* (Retrieved from wikipedia.org/wiki/Coula_edulis)

2.2 Botanical Characteristics of *Tetracarpidium conophorum* (Family: *Euphorbiaceae*)

Tetracarpidium conophorum also known as *conophor* is found in rainforest of Nigeria, in the bushy Savanna in Sierra Leone, Benin, Cameroun, Gabon, The Democratic Republic of Equatorial Guinea, and extending to Zaire. Its presence in Sierra Leone may be due to returning slaves for it is known to the Krios by its Yoruba (Nigerian) name-awusa (Burkill 1985).

Alternate scientific names of *Tetracarpidium conophorum* are:

Plukenetia conophora

Mallotus preusii

Tetracarpidium staudtii

Cleidion manni.

Table 2.1: Taxonomy of *Tetracarpidium conophorum*

Phylum	<i>Magnoliophyta</i> (flowering plants)
Class	<i>Magnoliopsida</i> (dicotyledons)
Order	<i>Euphorbiales</i> (spurges, boxwood, jojobas)
Family	<i>Euphorbiaceae</i> (spurge family)
Genus	<i>Tetracarpidium</i> (<i>tetracarpidium</i>)
Species	<i>Tetracarpidium conophorum</i> (<i>tetracarpidium</i>)

(The PLANTS Database. Retrieved from www.forestryimages.org/)



Plate VII: *Tetracarpidium conophorum* on a private cocoa plantation in Itapaji, Ekiti State, Nigeria as a climber



Plate VIII: Leaves of *Tetracarpidium conophorum* on a private cocoa plantation in Itapaji, Ekiti State, Nigeria



Plate IX: Pods of *Tetracarpidium conophorum* containing the walnuts
(Retrieved
from [www.medicinalplantsinnigeria.com/Tetracarpidiumconophorum\(awusa\)](http://www.medicinalplantsinnigeria.com/Tetracarpidiumconophorum(awusa)))



Plate X: Walnuts (nuts) purchased at Abeokuta, Ogun State, Nigeria.

Table 2.2: Local names of *Tetracarpidium conophorum*

Tribe	<i>Local name(s)</i>
Yoruba	<i>Awusa or asala (walnut)</i>
Igbo	<i>Ụkpa or okpa (walnut)</i>
Igbo (Owerri)	<i>Oke okpokirinya (male string leaf)</i>
Igbo (Uburubu)	<i>Okumu from Umu (children ie babies call babies)</i>
Efiks and Ibibios	<i>Ekporo</i>
Edo	<i>Okhue or Owke (walnut)</i>
Hausa	<i>Geda – yorubawa</i>

(Burkill 1985)

Other African names:

Cameroun: casu, kaso

Sierra Leone: awusa (from Krio, Yoruba)

(Burkill 1985, Dalziel 1939).

Tetracarpidium conophorum (African walnut) is a perennial woody climber. It is commonly found especially in southern Nigeria in places like Ekiti, Lagos, Kogi, Abeokuta, Osun, Oyo, Akampko, Nnewi, Ajaowo, Ogbomoso, etc. It is found in moist forest zones of sub-Saharan Africa. The walnut tree is a 20- 30 meter climbing shrub with many twisting branches. Its habitat is usually large trees. The walnut is a popular farm product among peasant farmers and cocoa farmers who inherit walnut farms from their parents and pass them over to their own children. It twines round any support, especially trees in its vicinity. It is an alley farmed crop, grown alongside cocoa and kola nut and uses these trees as a support for its growth. It is grown along the African coast line and it is thought to originate in south-western Nigeria. It is planted as a minor component of the mixed cropping system, just for the family and local market consumption (Malu *et al.*, 2009, Burkill 1985, Edem *et al.*, 2009, Ayoola *et al.* 2011).

The leaves are glabrous, ovate, long and margin toothed. The bases of the leaves are broadened and rounded up to the 13 x 8 cm with slender petioles up to 5 cm long. The male flowers are deciduous leaving the females at the bases of the raceme. The fruits are four winged, ridged between wings and up to 8 cm in diameter. The fruits are greenish with four round seeds in each fruit. The fruit can be 6 - 10 cm long by 3 – 11 cm wide containing sub-globular seeds 2 - 2.5 cm long with a thin brown shell resembling the temperate walnut, hence the English name. The seeds are edible even when raw. They have a bitter taste and a tonic effect like kola. Usually, the kernels are roasted and eaten in the general diet or added to cakes. The African walnut is principally cultivated for its oil rich fruits. The shell, bark and leaves of the conophor plant have antifungal, anti-parasitic and anti-dysenteric properties and the

bark is used by local people as a mild laxative. *T. conophorum* is actually regarded as a panacea for health, stress and infertility (Burkill 1985; Malu *et al.*, 2009, Edem *et al.*, 2009).

2.3 Chemical Constituents of *Tetracarpidium conophorum*

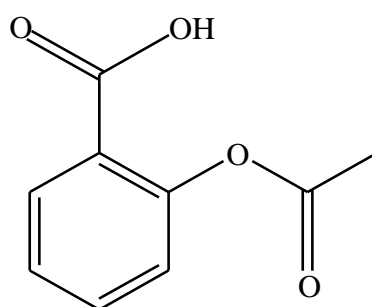
Chemical (phytochemical) constituents represent cell contents which are either food-storage products or by – products of metabolism or secondary metabolites (natural products) and these include carbohydrates, proteins, fixed oils and fats, alkaloids, purines, glycosides, volatile oils, gums, mucilages, resins, tannins, calcium oxalate, calcium carbonate and silica. Secondary metabolites are often unique to a particular plant species or group of organisms and many act as antifeedants, sex attractants or antibiotic agents, while some have no apparent biological role (Cannel 1998).

Walnut trees especially the fruits have been found to produce significant amounts of aspirin, antioxidants, essential fatty acids, phytosterols (Muanya 2011); the nuts yield a light golden colored oil composed of linolenic acid 64% , palmitic and stearic acids 15%, oleic acid 11% and linoleic acid 10% (Busson 1965); oxalates, and tannins in the raw *Tetracarpidium conophorum* nuts (Ayoola *et al.*,2011); lectins (Kuku 2012); alkaloids, saponins, flavonoids are reported in the seeds (nuts) (Malu 2009; Odoemena 2010).

2.3.1 Aspirin (Acetylsalicylic Acid)

Tetracarpidium conophorum trees have been found to produce aspirin and the leaves are considered a headache cure in south Nigeria (Dalziel 1937). Aspirin (I), also known as acetylsalicylic acid, it is a salicylate drug, often used as an analgesic to

relieve minor aches and pains, as an antipyretic to reduce fever and as anti-inflammatory medication. Aspirin also has an anti platelet effect by inhibiting the production of thromboxane which under normal circumstances binds platelet molecules together to create a patch over damaged walls of blood vessels (Lewis *et al.*, 1983).

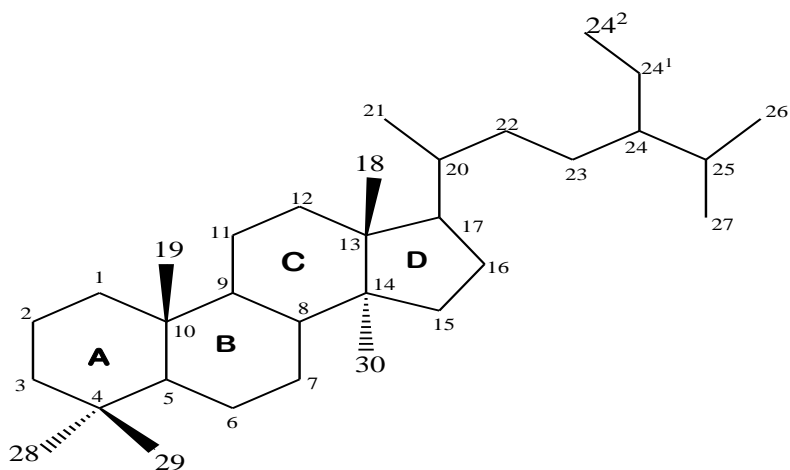


(I)
Aspirin

2.3.2: Phytosterols

Phytochemical analysis indicates that African walnuts (seeds) (*Tetracarpidium conophorum*) contain ingredients such as phytosterols that reduce breast cancer in women. The seeds are also claimed to improve endothelial functions in hypercholesteromic subjects (Odoemena *et al.*, 2010). Walnuts contain phytosterols (Muanya 2011; Obayendo 2010) which is a collective term for plant-derived sterols and stanols. Phytosterols, which compass plant sterols and stanols, are steroidal compounds (I) similar to cholesterol which occur in plants and vary only in carbon side chains and/or presence or absence of a double bond. Stanols are saturated sterols, having no double bonds in the sterol ring structure. More than 200 sterols and related

compounds have been identified (II and table 2.3). Free phytosterols extracted from oils are insoluble in water, relatively insoluble in oil and soluble in alcohols.

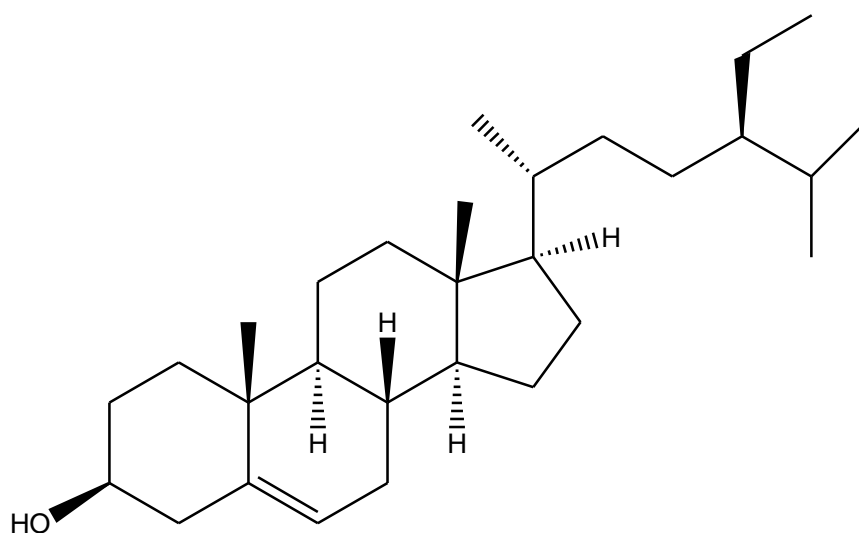


(II)

Steroidal skeleton

Table 2.3: Nomenclature for phytosterols

S/n	Process	Yield
I	Removing carbon 24 ²	Campesterol
ii	Removing carbons 24 ¹ and 24 ²	Cholesterol
iii	Hydrogenating the double bond between carbons 5 and 6	β – sitostanol
Iv	Hydrogenating the double bond between carbons 5 and 6 and removing carbon 24	Campestanol
V	Removing carbon 24 ² and hydrogens from carbons 22 and 23, and inverting the stereochemistry of C – 24	Brassicasterol (ergosta – 5, 22 dien -3 β - ol.).



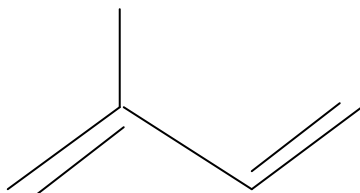
(III)
 β -Sitosterol

Nuts, which are rich in phytosterols are often eaten in smaller amounts, but can still significantly contribute to total phytosterol intake. Phytosterols inhibit the intestinal absorption of cholesterol. Nutritionists recognize two classes of phytosterol; (a) sterols, which have a double bond in the sterol ring (III); and (b) stanols, which lack a double bond in the sterol ring such as sitostanol (Gylling *et al.*, 2014).

2.3.3 Terpenes and Terpenoids.

Terpenes are a large and diverse class of organic compounds, produced by a variety of plants particularly conifers, which are often strong smelling and thus may have had a protective function. They are the major components of resin, and of turpentine produced from resin. The name “terpene” is derived from the word “turpentine”. Terpenes are major biosynthetic building blocks within nearly every living creature. When terpenes are modified chemically, such as by oxidation or rearrangement of the carbon skeleton, the resulting compounds are generally referred to as terpenoids (Cravoto *et al*, 2010).

Terpenes and terpenoids are two classes of naturally occurring compounds that are formally derived from isoprene (IV).



(IV)

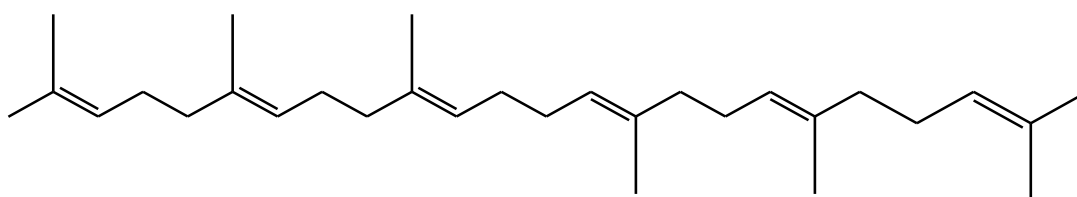
Isoprene

Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. An essential oil is a mixture of terpenes and terpenoids e.g myrcene (a constituent of bay berry oil) and menthol (a constituent of peppermint). Terpenoids can be described as modified terpenes, where methyl groups are moved or removed and oxygen atoms maybe added. All terpenoids may be defined as a group of molecules whose structure is based on a various but definite number of isoprene units (methylbuta-1, 3-diene named hemiterpene) with five carbon atoms. A rational classification of the terpenes has been established based upon the number of isoprene units incorporated in the basic molecular skeleton. The classification of terpenes are presented in table 2.4.

Table 2.4: Classification of terpenes

S/N	Terpenes	Isoprene	Carbon atoms
i	Monoterpenes	2	10
ii	Sequiterpenes	3	15
iii	Diterpenes	4	20
iv	Sesterpenes	5	25
v	Triterpenes	6	30
vi	Carotenoids	8	40
vii	Rubber	>100	>500

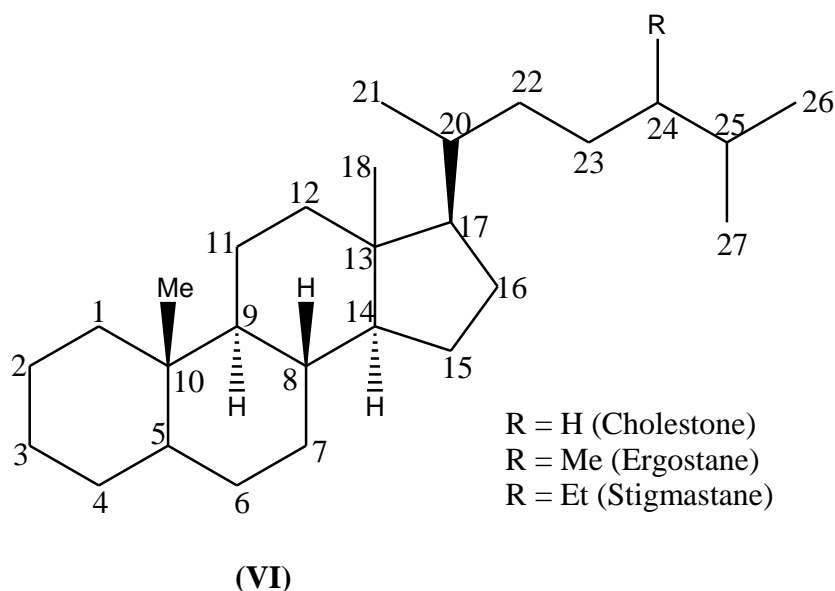
Triterpenoids form a large group of natural products which include steroids and consequently sterols; they are derived from C₃₀ precursors. Nearly 200 different triterpene skeletons are known from natural sources and represent structurally cyclization products of *squalene* (V) which is the immediate biological precursor of all triterpenoids.



(V)

Squalene

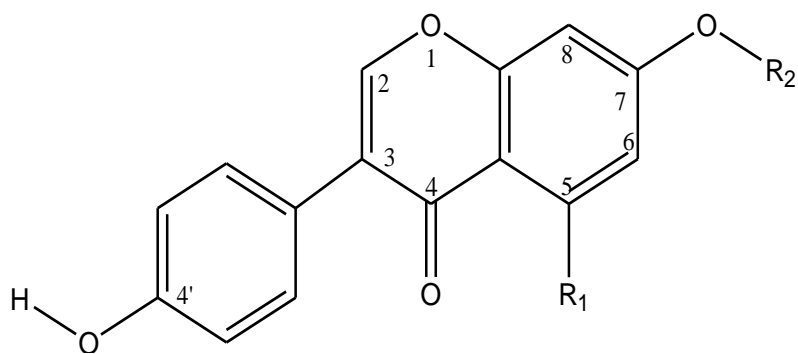
Triterpenoids are widely distributed in edible and medicinal plants and are an integral part of the human diet. They are being evaluated for use in new functional foods, drugs, cosmetics and healthcare products. Steroids are modified triterpenes which are derived also from squalene by cyclization, unsaturation and substitution. The nucleus of all steroids is the tetracyclic C₁₇ hydrocarbon 1, 2-cyclopentanoperhydrophenanthren (sterane) substituted by methyl groups at C₁₀ and C₁₃, as well as an alkyl side-chain at C₁₇ to obtain compounds such as cholestane, ergostane and stigmastane (see structure VI).



Unsaturated steroids with most of the skeleton of cholestane (VI) containing a 3 β -hydroxyl group and an aliphatic side chain of eight or more carbon atoms attached to position 17 forms the group of sterols (retrieved from www.cyberlipid.com/terpenoids).

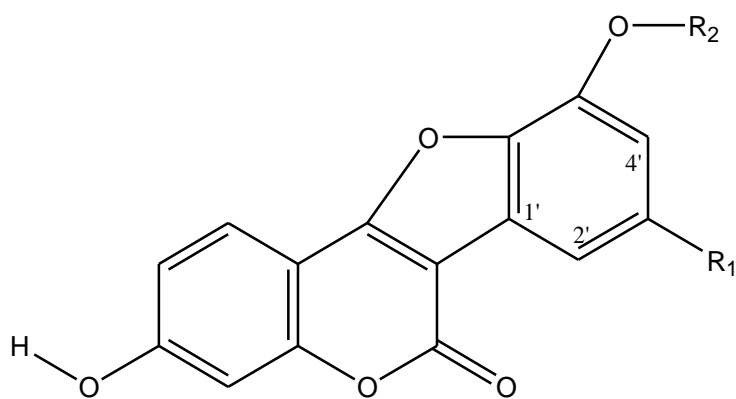
2.3.4 Phytoestrogens (Plant Estrogens)

It has been reported that walnuts (seeds) contain phytoestrogens (Moore 2011). Phytoestrogens are plant-derived xenoestrogens functioning as the primary female sex hormone not generated within the endocrine system but consumed by eating phytoestrogenic plants. Also called “dietary estrogens”, they are a diverse group of naturally occurring non-steroidal plant compounds that, because of their structure similarity with estradiol (17- β - estradiol), have the ability to cause estrogenic or antiestrogenic effects. Phytoestrogens mainly belong to a large group of substituted natural phenolic compounds: the coumestans (VIII), prenyl flavonoids and flavones (VII). These are three of the most active in estrogenic effects in this class (Yildiz 2005).



(VII)
Isoflavone

Isoflavones	R ₁	R ₂
Daidzein	H	H
Formononetin	H	CH ₃
Genistein	OH	H
Biochain A	OH	H



(VIII)

Coumestan

Coumestans	R ₁	R ₂
Coumesterol	H	H
4'-Methoxycoumesterol	H	CH ₃
Repensol	OH	H
Trifoliol	OH	CH ₃

2.3.5 Lectins

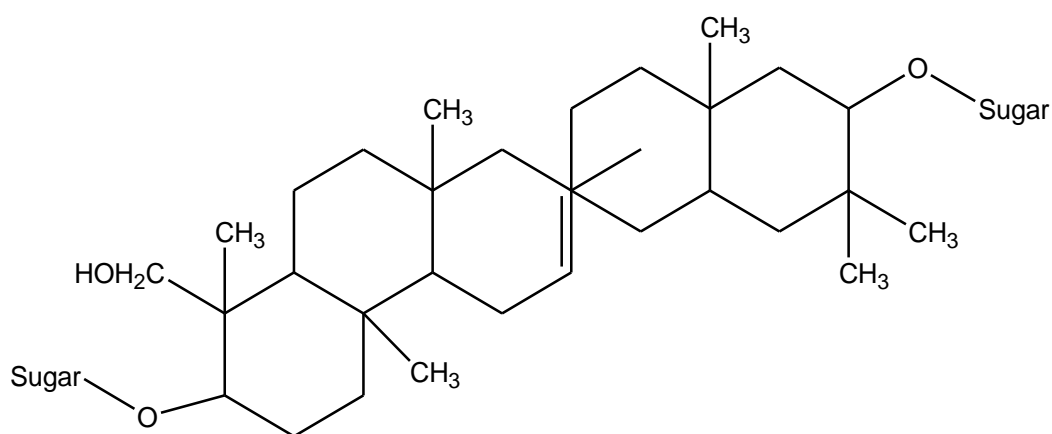
Lectins are carbohydrate-binding proteins (not to be confused with glycol proteins, which are proteins containing sugar chain or residues) that are highly specific for sugar moieties, particularly, the high specificity of plant lectins for foreign glycoconjugates (e.g. those of fungi, invertebrates and animals). They play a role in the biological recognition phenomena involving cells and proteins (Van Damme 1998; Brudner 2013).

They may bind to a soluble carbohydrate or to a carbohydrate moiety that is a part of a glycol protein or glycolipid. They typically agglutinate certain animal cell and or precipitate glycoconjugates. Lectins serve many different biological functions in animals, from the regulation of cells adhesion to glycoprotein synthesis and the control of protein levels in the blood. Lectins are also known to play important roles in the immune system by recognizing carbohydrates that are found exclusively on pathogens or that are in-accessible on host cells (Brudner 2013).

2.3.6: Saponins

Plant materials containing saponins have long been used in many parts of the world for their detergent properties. They also have haemolytic properties and when injected into the blood stream, they are highly toxic. Saponins (IX) have a high molecular weight and a high polarity. As glycosides they are hydrolysed by acids to give an aglycone (sapogenin), various sugars and related uronic acids. According to the structure of the aglycone or sapogenin, two kinds of saponin are recognized – the steroidal (commonly tetracyclic triterpenoids C_{27}) and the pentacyclic triterpenoid

types (C_{30}). Both of them have a glycosidal linkage at C-3 and have a common biogenetic origin via mevalonic acid and isoprenoid units (Evans 2009).

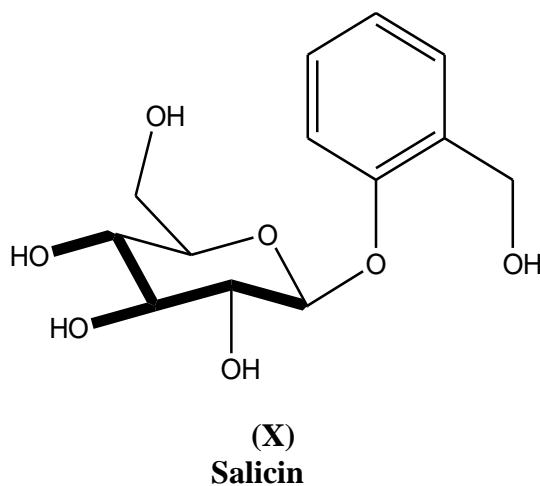


(IX)
Saponin

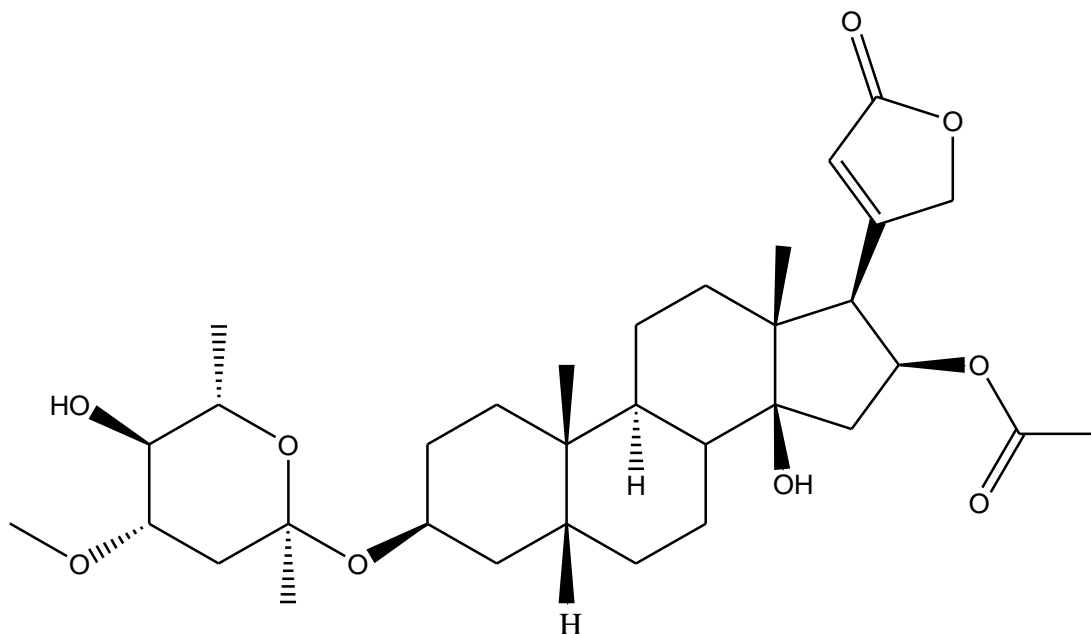
Steroidal saponins are of great pharmaceutical importance because of their relationship to compounds such as the sex hormones, cortisone, diuretic steroids, vitamin D and the cardiac glycosides. Some of them are used as starting materials for the synthesis of these compounds. Saponins seem to reduce blood cholesterol levels, reduce the risk of cancer and stimulate our immune system (Evans 2009). Pentacyclic triterpenoids are used in traditional medicine in the treatment of diabetes. They have shown multiple biological activities with apparent effects on glucose absorption, glucose uptake, insulin secretion, diabetic vascular dysfunction, retinopathy and nephropathy (Alqahtani *et al.*, 2013).

2.3.7 Glycosides

A glycoside is any of a wide variety of naturally occurring substances in which a carbohydrate portion, consisting of one or more sugars or uronic acid (i.e., a sugar acid) is combined with a hydroxyl compound (X – salicin, Evans 2009).



Many plants store chemicals in the form of (inactive) glycosides. These can be activated by enzyme hydrolysis, which causes the sugar part to be broken off, making the chemical available for use. Many such plant glycosides are used as medications, an example is Oleandrin which is a cardiac glycoside (XI) and salicin, which is a glycoside that is related to aspirin (X) (Evans 2009).



(XI)

Oleandrin

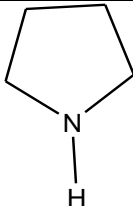
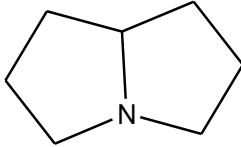
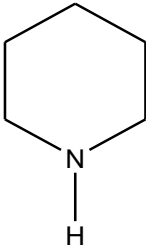
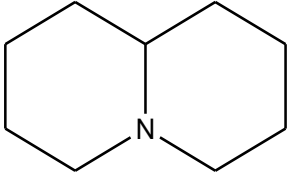
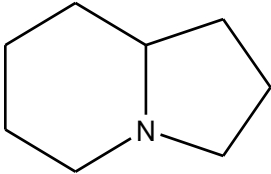
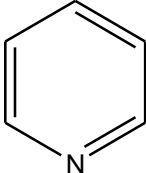
In animals and humans, poisons are (often) bond to sugar molecules as part of their elimination from the body. In formal terms, a glycoside is any molecule in which a sugar group is bonded through its anomeric carbon to another group via a glycosidic bond. Glycosides can be linked by an O-(an O- glycoside), N- (a glycosylamine), S- (a thioglycoside), or C- (a C - glycoside) glycosidic bond. Many authors require in addition that the sugar be bonded to a non-sugar for the molecule to qualify as a glycoside, thus excluding polysaccharides. The sugar group is then known as the glycone and the non-sugar as the aglycone or genin part of the glycoside. The glycone can consist of a single sugar group (monosaccharide) or several sugar groups (Oligosaccharide). Glycosides can be classified by the glycone, by the type of glycosidic bond, and by the aglycone (Yildiz 2005, retrieved from wikipedia /org/wiki/ Glycosides).

2.3.8 Alkaloids

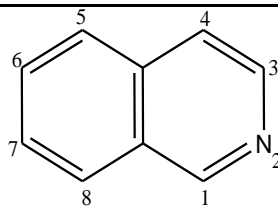
An alkaloid is a chemical substance of plant origin composed of carbon, hydrogen, nitrogen and (usually) oxygen. Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic properties. The alkaloids are organic bases similar to the alkalis (inorganic bases): the name means alkali-like. An example of an alkaloid is carpine (XII) which is formed by the process of lactonization. In addition to carbon, oxygen, hydrogen and nitrogen, alkaloids may also contain sulphur and more rarely other elements such as chlorine, bromine and phosphorous (Evans 2008).

Many alkaloids are toxic to some organisms. They often have pharmacological effects and are used as medications, as recreational drugs, or in entheogenic rituals. Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke bitter taste. Many alkaloids are used as drugs. Compared with most other classes of natural compounds, alkaloids are characterized by a great structural diversity and there is no uniform classification of alkaloids (Retrieved from [en.wikipedia.org / wiki /Alkaloids](http://en.wikipedia.org/wiki/Alkaloids)). The main classes of alkaloids are presented in table 2.5.

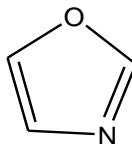
Table 2.5: Main classes of monomeric alkaloids

S/No	Class	Structure
I	Pyrrolidine derivative	
II	Pyrrolizidine derivative	
III	Piperidine derivative	
Iv	Quinolizidine derivative	
V	Indolizidine derivative	
VI	Pyridine derivative	

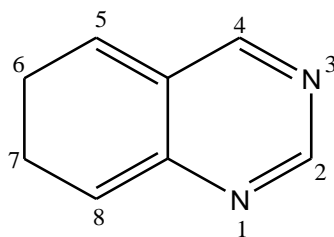
VII Isoquinoline derivatives and related
alkaloids



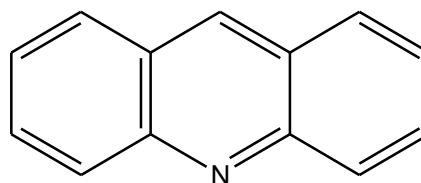
VIII Oxazole derivative

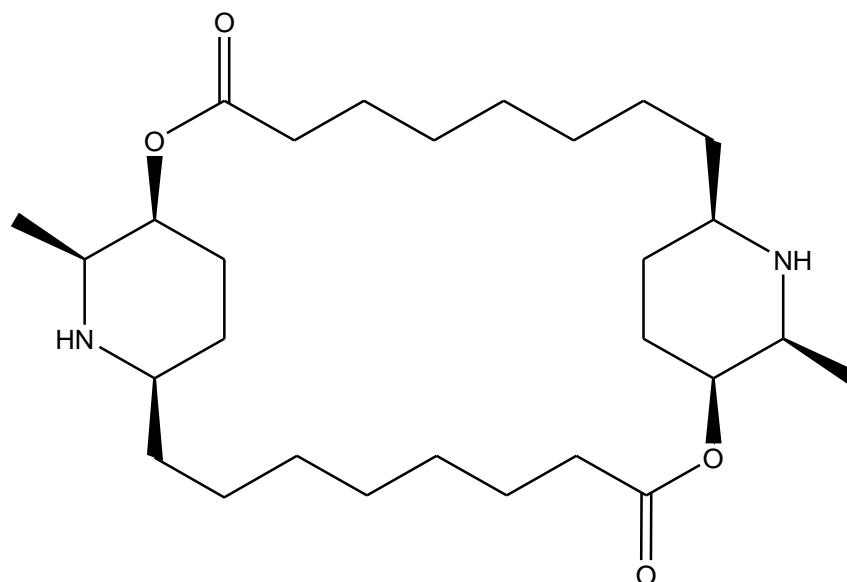


IX Quinazoline derivative



X Acridine derivative



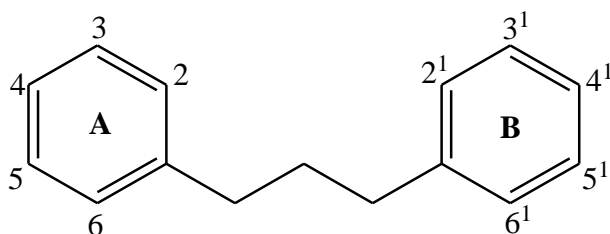


(XII)

Carpaine

2.3.9 Flavonoids

Flavonoids or bioflavonoids (from the Latin word *flavus* meaning yellow colour in nature) are a class of plant secondary metabolites. The flavonoids are polyphenolic compounds possessing 15 carbon atoms: two benzene rings joined by a linear three carbon chain (Evans 2009).



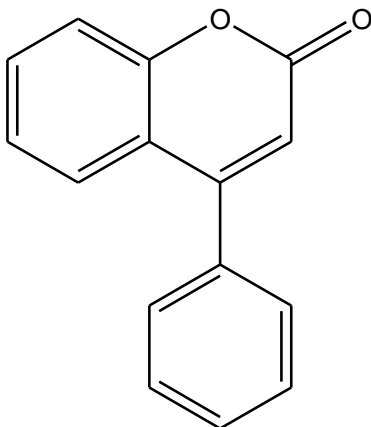
The skeleton above can be represented as the $C_6-C_3-C_6$ system. According to the IUPAC nomenclature, flavanoids can be classified into:

- i Flavonoids or bioflavonoids;
- ii Isoflavonoids, derived from 3-phenyl chromen-4-one(3-phenyl-1,4 benzo pyrone) structure (XIII);

iii Neoflavonoids, derived from 4- phenyl –coumarine (4- phenyl-1,2-benzopyrone)
 . structure (XIV);

Iv The three flavonoid classes are all ketone-containing compounds and as such,
 are anthoxanthins (flavones and flavonoids).

(XIII)
Isoflavonoid



(XIV)
Neoflavonoid

The three cycles or heterocycles in the flavonoid backbone are generally called ring A, B and C. Ring A usually shows a phloroglucinol substitution pattern.

2.3.9 Other Compounds found in *Tetracarpidium conophorum*

The nuts are oil-bearing yielding 48-60% of light golden coloured oil with a taste resembling oil. Composition is linolenic acid 64%, palmitic and stearic acids 15%, oleic acid 11% and linoleic acid 10 %. This is conophor oil (Burkill 1985).

Table 2.6: Essential Fatty Acids in walnuts

Name	Structure and Melting point
Palmitic acid (Hexadecanoic acid)	$\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$, 63°C
Stearic acid (Octadecanoic acid)	$\text{CH}_3(\text{CH}_2)_{16}\text{COOH}$, 27°C
Oleic acid (Octadec-9-enoic acid)	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$, 13°C
Linoleic acid(cis,cis 9, 12- octadecanoic acid)	$\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2\text{CHCH}(\text{CH}_2)_7\text{COOH}$, 5°C
Linolenic acid(cis,cis,cis-9,12,15 Octadecatrienoic acid)	$\text{CH}_3\text{CH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7$, 9.5°C

Two agglutinins have been reported in *Tetracarpidium conophorum* seeds (Animashaun *et al.*, 1994). An agglutinin is a substance that causes particles to coagulate to form a thickened mass. Agglutinins can be antibodies that cause antigens to aggregate by binding to the antigen binding sites of antibodies. Agglutinins can also be any substance other than antibodies such as sugar-binding protein lectins. A galactose - specific lectin was reported from the seeds of *Tetracarpidium conophorum* (Kuku *et al.*, 2012). Ayoola (2011) carried out an investigation on the root of *Tetracarpidium conophorum* and determined the vitamin composition on a dry weight

base expressed as mg/g. The result revealed that *Tetracarpidium conophorum* root could be a potential source of drug formulations (Ayoola *et al.*, 2011).

The nut of *Tetracarpidium conophorum* was found to be very rich in ascorbic acid and carbohydrate while it has a moderate amount of protein with very low ash content (Edem *et al.*, 2009). Evaluation of chelating ability of aqueous extracts of *Tetracarpidium conophorum in vitro*, there could possibly be iron chelators which will be of clinical relevance in the treatment of iron – overload disorders such as thalassemia, a group of genetically inherited blood disorders characterized by defective globin chain of haemoglobin and iron overload (Olabinri *et al.*, 2010).

2.4 Medicinal and Economic Uses of the Three Walnuts

Juglans regia, the English walnut is native to Great Britain, Europe, Asia, Australia, New Zealand, North America and Mexico. It is considered to be an astringent, anti-fungi and antiseptic in folk medicine. The walnut tree has a very long history of being used to treat a wide range of complaints (Ayoola *et al.*, 2011). Certain extracts of the walnuts have *in vitro* antioxidant and antiproliferative activity due to a high phenolic content (Negi 2011). *In vitro* tests of the walnut extract have shown a high antiatherogenic potential and osteoblastic activity, suggesting a potential biological effect of a walnut-enriched diet on cardio-protection and bone loss (Papoutsis 2008).

The ethanolic extract from the leaves of *J.regia* has anti diabetic effects on diabetes-induced rats (Asgary *et al.*, 2008). Bark and leaf crude extracts of *J.regia* showed *in vitro* activity against *Mycobacterium tuberculosis* (Cruz-Vega 2008). Other uses of *Juglans regia* are presented in table 2.6

Table 2.7: Uses of *Juglans regia*

No	Plant part	Uses
I	Leaves	Treatment of diarrhea
II	Leaves, husks	Juglone (natural herbicide)
III	Green husks	Methyl palmitate (insecticide)
IV	Dried leaves	Help in preventing hair loss
V	Green husks	Yellow dye
VI	Leaves	Brown dye
VII	Leaves (used internally)	Constipation, chronic coughs, depurative asthma, diarrhea, dyspepsia, strumous sores, anthelmintic, anti-inflammatory, purify blood, astringent, depurative and as tea.
VIII	Cotyledons	Cancer
IX	Male inflorescences	Coughs, vertigo
X	Sap	To make a sugar syrup
XI	Hull	Blood purifying agent, treat digestive tract swelling
XII	Dried green husks	As vitamin supplement as it contains 2.5 – 5% ascorbic acid
XIII	Oil from nuts	Treatment of tapeworms, used in salads.

(Negi *et al.*, 2011; Papoutsiet *al.*, 2008; Asgary *et al.*, 2008; Cruz Vega *et al.*, 2008).

Coula edulis is a tree in the genus *Coula*, native to tropical western Africa from Sierra Leone to Angola. It is not related to the walnut, being so named because its nuts bear a superficial resemblance to the walnut. Every part of the tree is used in both raw and finished states. Its timber and nuts are used extensively (retrieved from www.wikipedia.org/wiki/Coula_edulis).

Table 2.8: Uses of *Coula edulis*

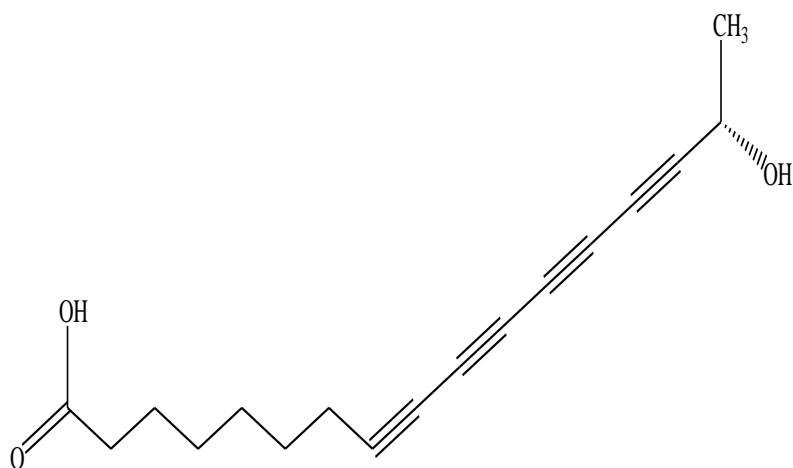
S/No	Part of the plant used	Usage
I	Bark	To produce rinses or enemas for loin pains or kidney problems.
II	Bark decoction	Dysentery, stimulate appetite, counteract anaemia
III	Dried bark	Anticancer activity
IV	Bark powder	Dressing sores.
V	Wood	Furniture, cabinet work, construction, Decorative veneers, paneling, fixtures and joinery, flooring, fuel, piling for bridges and railway ties.
VI	Nuts	Used in recipes, Mixed with meat, a source of cooking oil and ground flour, cosmetic excipient, oleic acid source, fatty acid source, used as snacks and for flavoring.
VII	Stem bark	Anti-plasmodia activity

(Adebayo-Tayo *et al.*, 2008, Tamokou *et al.*,2011).

Medicinal plants play a key role in malaria control in Africa, especially in remote areas where health facilities are limited. Anti plasmodia activity was exhibited by *Coula edulis* leaves (Zofou *et al.*, 2011). Crude ethanolic extracts of leaves, stem bark, roots and fruits of *C. edulis* were analyzed phytochemically and evaluated for their antibacterial and anti-fungal activities against five (5) clinically isolated pathogenic micro-organisms; – *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Candida albicans*. The activities of the extracts on the micro organisms justified the use of *C. edulis* commonly in West Africa folk medicine in the treatment of stomach, skin disease and also used as tonifacts, cosmetics, etc (Adebayo – Tayo *et al.*, 2008).

Antidermatophytic activity of the dichloromethane – methanol (1:1) extracts of *C. edulis* stem bark, positive activities were observed with the isolation of some fractions –a known compound 3-O- β -D-glucopyranoside of sitosterol, stigmasterol and n-hexane decanoic acid. The data obtained suggest that the CH₂Cl₂-MeOH (1:1) extracts of *C. edulis* stem bark possesses antidermatophytic properties. They also show that at high doses (=200 mg/kg BW), the extract has significant hepatotoxic and nephrotoxic activities (Tamokou *et al.*, 2011).

Minquartynoic acid (XV, with molecular formula C₁₈H₂₀O₃) was isolated from the bark of *Coula edulis*. Minquartynoic acid is (-) -17-hydroxy 9, 11, 13, 15-octadecatraynoic acid, it has anti - HIV activity and it effectively inhibited human lymphoblastoid cell killing HIV- 1 (Fort *et al.*, 2000).



(XV)
Miquartynoic acid

Tetracarpidium conophorum is cultivated principally for the nuts which are cooked and consumed as snacks. Recent studies suggest that eating walnuts may help to reduce the risk of developing breast cancer, improve cognitive skills in the elderly and beat drug resistant microorganisms. Previous studies had shown that African walnut prevents heart diseases. They suggested eating walnuts at the end of a meal might help cut the damage that fatty food can do to the arteries. It is thought that the nuts are rich in compounds that reduce hardening of the arteries and keep them flexible. Phytochemical analysis indicates that African walnuts contain ingredients such as omega-3- fatty acids, antioxidants and phytosterols that may all reduce the risk of the disease (Muanya 2011). In southern Nigerian ethnomedicine, African walnut is used as a male fertility agent; the leaves are used for the treatment of dysentery and to improve fertility in males (Ajaiyeoba *et al.*, 2006). The oil from the nut has found use in the formulation of wood varnishes, stand oil, vulcanized oil for rubber and leather substitute (Muanya 2011).

Table 2.9: Other Uses of *Tetracarpidium conophorum*.

	Part of walnut tree used	Uses	References
I	Fruits	To enhance male fertility An anti- diabetic agent Reduce risk of developing breast cancer in women Claim to improve endothelial function in hypercholesteromic subjects For the development of newer hyperglycaemia agents To tonify kidneys Strengthen the back and knees To cure asthma -Relieve pain -Increases sperm count -Enhance sexual performance in males -As a nerve tonic in ethno medicine -Antidepressant-like activity Prevent and control high blood pressure. Restores endothelial functions. Reduce harmful cell adhesion molecules - atherosclerosis. Improve age-related motor and cognitive short falls.	Odoemena <i>et al.</i> , 2012 Odoemena <i>et al.</i> , 2012 Odoemena <i>et al.</i> , 2012 Odoemena <i>et al.</i> , 2012 Odoemena <i>et al.</i> , 2012 Odoemena <i>et al.</i> , 2012 Odoemana <i>et al.</i> , 2012 Odoemena <i>et al.</i> , 2012 Aladeokin <i>et al.</i> , 2011 Muanya 2002 Muanya 2002
II	Tannins	Treatment of hemorrhoids.	Igboko 1983
III	Leaves	Treatment of dysentery.	Odoemena <i>et al.</i> ,2010
IV	Bark	Used in tea as laxative and chewed for tooth ache.	Ayoola <i>et al.</i> , 20011

2.5 Microorganisms

2.5.1 As causative agents of diseases and sicknesses

The microorganisms that normally live in or on humans usually cause no ill effects to their host. However, the majority of the pathogens responsible for human disease are also derived from these organisms (Murray *et al.*, 1999). A microorganism is a very small living organism which cannot be viewed unaided (Madigan and Martinko 2006). Microorganisms are very diverse and include all bacteria, archaea, algae, some fungi, almost all protozoa, etc (Rybicki 1990). A small portion of the microorganisms are pathogenic and these are the causative agents of diseases such as dysentery, typhoid fever, candidiasis, etc (Eckbury *et al.*, 2003). Some of the pathogenic microorganisms include; *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Vibrio cholerae*, *Candida albicans* and MRSA (Murray 1999).

Staphylococcus aureus is a gram- positive cocci bacterium and it is documented as a human opportunistic pathogen. It is wide spread in nature. Serious infections caused by *S.aureus* are bacteremia, pneumonia, meningitis and scalded skin syndrome. *E. coli* is part of the bowel flora of healthy individuals. It is a gram-negative rod. Urinary tract infections, bacteremia, meningitis and diarrheal disease are most of the most common clinical syndromes of *E. coli*. *Shigella dysenteriae* is a gram- negative, non motile bacterium rod. It is the causative agent of bloody diarrhea (dysentery) and non-bloody diarrhea. Infection with *S.dysenteriae* is associated with high rates of morbidity and mortality in developing countries, particularly when antimicrobial resistance or its misdiagnosis as amoebiasis makes appropriate treatment problematic (Murray *et al.*, 1999).

2.5.2 Culture media for microorganisms

A growth medium or culture is a liquid or gel designed to support the growth of microorganisms or cells (Madigan and Martinko 2005). There are two types of growth media for microorganisms, nutrient broth and agar plate (Schlegel 1993).

2.6 Proximate Analysis of the leaves of *Tetracarpidium conophorum*

Proximate analysis is a method for the quantitative analysis of the different macro nutrients in a feed (FAO 1994). It is also known as the Weende analysis. It was developed in 1860 by Hennberg and Stohmann in Germany (FAO 1994). Proximate analysis is a partitioning of compounds into six categories based on the chemical properties of the compounds; moisture, ash, crude protein or Kjeldahl protein (CP), crude fiber (CF) and nitrogen free extracts i.e. digestible carbohydrates (NFE).

This analysis was an attempt to duplicate animal digestion. In the proximate analysis, crude protein, oil or ether extracts, crude fiber and ash are determined chemically. Nitrogen free extract is a calculation based on the determination of the four others. Proximate should nearly always add up to 100 %, which displays the resolution of the chemical test. The system of proximate analysis still forms the basis for statutory declaration of the composition of foods in Europe (Ayoola *et al.*, 2011). Carbohydrate or nitrogen free extracts (NFE) are the human body's principal source of starch. Crude fiber (CF) is the plant cellulose in food and is called roughage. It is an essential ingredient of our food as it helps our body to get rid of indigested food. Protein is the basic material of life and makes up $\frac{3}{4}$ of our body tissue. Without dietary protein, all body functions would not take place. Proteins can be broken into

complete and incomplete proteins and complete proteins supply a sufficient amount of the nine essential amino acids. Fats and oil are essential for the proper functioning of the body at a particular proportion at a specific age. We need to consume some fats (like omega 3, 6, 9 fatty acids). Water is essential to human life and half of our weight is water. It shall be determined as moisture content. Ash is considered as the total mineral or inorganic content of the sample. Minerals act as nutrients and are essential to many processes in the body including the functioning of the heart, digestive system and bone formation. Dry matter is the solid component of plant tissue or other materials essentially what is left when all the water is removed and mainly consists of soluble carbohydrates such as fructose, sucrose, etc (AOAC 2000).

2.7 Elemental Analysis of the leaves of *Tetracarpidium conophorum*

Elemental analysis is a process whereby a sample of some material (e.g. soil, waste or drinking water, body fluids, minerals, chemical compounds) is analyzed for its elemental and sometimes isotopic composition (Eby 2007). Elemental analysis can be qualitative (determining what elements are present, characterized as alkaline - earth metals, alkali metals, trace metals, non-metals, etc) and it can be quantitative i.e determining how much of each was present (Okoh *et al.*, 2013, Nelson 2007).

Instrumental Neutron Activation Analysis (INAA) is the method of choice for the elemental analysis of the leaves of *Tetracarpidium conophorum* (Okoh 2013). INAA is a quantitative and qualitative method of high efficiency for the precise determination of a number of main components and trace elements in different types of samples. INAA is based on the nuclear reaction between neutrons and target nuclei and it is a useful method for the simultaneous determination of about 25-30 major,

minor and trace elements of geological and biological samples in parts per billion-parts per million (ppb-ppm) range without or with chemical separation.(Molnar2004).

In INAA, samples are activated by neutrons.During irradiation the naturally occurring stable isotopes of most elements that constitute the mineral samples or biological materials are transformed into radioactive isotopes by neutron capture. The activated nucleus decays according to a characteristic half-life; some nuclides emit particles only, but most nuclides emit gamma-quanta, too, with specific energies. The quantity of radioactive nuclides is determined by measuring the intensity of the characteristic gamma-ray lines in the spectra (Molnar 2004).

After the irradiation the sample is allowed to decay and then it is “counted” using High Purity Germanium detectors looking for gamma rays. The resulting gamma-ray spectra looks something like a gas chromatograph spectra with “peaks” at different “retention times” the position of each peak determines the energy of the gamma ray (identifying the responsible element) and the area under the peak is proportional to its concentration (retrieved from CID- 2284496 : Chemical analysis).

Elemental analysis entails analyzing for essential mineral elements,which are usually classified into two main groups according to their concentration in the animal or human body..These are macroelements (these are required in relatively large quantities for the normal pgsiological processes of the body such as carbon, calcium, magnesium etc, RDA is greater than 100 mg) and micro elements or micro nutrients or trace elements (these are required in small quantities such as zinc, nitrogen, cobalt, manganese etc.).Retrieved from www.major.differences.com.

CHAPTER THREE

MATERIALS AND METHODS

3.1 List of Materials

3.1.1 Reagents

The solvents that were used are of analytical grade, Sigma-Aldrich and JHD brand. They were redistilled before use. They included; petroleum ether (60-80 °C), chloroform, ethyl acetate, methanol and n-hexane.

3.1.2 Chromatographic Analyses

Pre-coated silica gel 60 F₂₅₄ TLC aluminium sheets (20 by 20 cm) produced by Merck (Germany) with a thickness of 0.2 cm were used for TLC and PTLC. BDH London, silica gel, 30 – 120 mesh was used for the column chromatography. Also for TLC and PTLC, silica gel by Kieslegel 60G (Merck Art.7731) with CaSO₄ binder and fluorescent indicator was used. For TLC, silica gel by PS Park Scientific Limited, Northampton, UK with 12 % CaSO₄ .5H₂O was also used..

3.1.3 Microbiological Media

I Mueller Hinton agar.

II Sabouraud dextrose agar.

3.2 Instrumentations

3.2.1 Fourier Transform – Infra Red (FT-IR)

The FT-IR analyses were run on Shimadzu FT-IR -8400s at National Research Institute for Chemical Technology, Bassawa, Zaria, Nigeria. The samples were analysed neat.

3.2.2 Mass Spectroscopy (MS)

The Electron – Impact Mass Spectroscopy (EI-MS) was performed on Agilent Technologies 1200 series Binary SL at the School of Chemistry, University of Kwazulu-Natal, South Africa. The identification of the isolated compounds was done by comparison with a Library search of the Mass Spectral of authenticated compounds.

3.2.3 Nuclear Magnetic Resonance (NMR)

The NMR spectral (1D and 2D) were recorded on Bruker- Avance 600 MHz NMR Spectrometers operating at 600 MHz. NMR spectral were recorded on NMR spectrophotometer using CDCl₃ as solvents with tetramethylsilane as an internal reference. This was conducted at the School of Chemistry, University of Kwazulu-Natal, South Africa.

3.2.4 Melting Point

Ernst Leitz Wetzlar melting point apparatus was used to determine the melting points of the two isolated compounds, as they occur as solid compounds.

3.3 Test Organisms for Antimicrobial Screening

- i. *Methicillin Resistance Staphylococcus Aureus* (MRSA)
- ii. *Staphylococcus aureus*
- iii. *Streptococcus pyogenes*
- iv. *Escherichia coli*
- v. *Pseudomonas aeruginosa*
- vi. *Salmonella typhi*
- vii. *Candida krusei*
- viii. *Aspergillus fumigatus*

- ix. *Aspergillus niger*
- x. *Corynebacterium ulcerans*
- xi. *Corynebacterium diphtheria*
- xii. *Bacillus subtilis*
- xiii. *Listeria monocytogenes*
- xiv. *Shigella dysenteriae*
- xv. *Campylobacter fetus*
- xvi. *Klebsiella pneumoniae*
- xvii. *Candida albicans*
- xviii. *Streptococcus pneumoniae*
- xix. *Vibro cholerae*
- xx. *Helicobacter pylori*

3.4 Sample Collection and Preparation

The leaves of *Tetracarpidium conophorum* were collected in September 2011 and September 2012 on a private cocoa plantation belonging to Oba Azeez Adebayo, Olu of Itapaji. It is located close to the border between Kwara and Ekiti States, in *Itapaji* town, in Ikole Local Government Area, Ekiti State, south west Nigeria. The plant was identified and given a voucher specimen number 2144 by Malam Mohammed Musa at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria. The leaves were air dried, pulverized and stored in clean dry jars in a dry and cool place.

3.5 Extraction of Plant Material

The Soxhlet extractor was employed in the extraction of the leaves of *Tetracarpidium conophorum* continuously and successively. The solvents used were

purchased from Sigma Aldrich; - Petroleum ether (60-80 °C), ethyl acetate, chloroform and methanol. The solvents were all redistilled before use. Pulverized leaves (300 g) were weighed out and packed into the thimble of a Soxhlet extractor with 1.50 liters of petroleum ether. The extraction was assumed to be completed when the extracting solvent in the Soxhlet extractor appeared colourless. After the hot extraction exhaustively with petroleum ether, it was followed by extraction with ethyl acetate, chloroform and methanol.

After extraction, the solvent in each extract was concentrated using rotary evaporator at 40°C. The extracts were air-dried to get a constant weight and stored in the desiccator. In table 4.4 the weight and percentage yield of each extract were recorded (Silva *et al.*, 1998; Harborne 1984).

3.6 Preliminary Phytochemical Screening

The phytochemical screenings were carried out using standard procedures (Evans 2009; Silva *et al.*, 1998, Sofowora 2008).

3.6.1 Tests for Carbohydrates

The crude extract (1.0 g) was added to 10.0 cm³ distilled water and boiled for two minutes. The mixture was filtered hot and the filtrate was allowed to cool and this sample solution was used for the following tests.

3.6.1.1 Molisch's Test

To 2 cm³ of the sample solution was added three drops of solution I (1% α -naphthol in 80% ethanoic acid) and two drops of concentrated sulphuric acid were added gently without mixing to form an upper phase. There was the appearance of a purple ring in the interphase as a result of the reaction between α -naphthol and furfural and hydroxymethyl furfural aldehydes produced by dehydration of

sacharrides. This was noticed for chloroform, ethyl acetate and methanol extracts. Pronounced purple colour was observed in the ethyl acetate extract. There was no purple coloration in the petroleum ether extract. Therefore, carbohydrate is absent in the petroleum ether extract (see table 4.4).

3.6.1.2 Seliwanoff's Test (for ketones)

A crystal of resorcinol was added to 2 cm³ concentrated hydrochloric acid and 2 cm³ of sample solution and the test tube was placed on a water bath for 5 minutes. A red colour was noticed immediately for the crude extracts with the exception of petroleum ether extract. The presence of ketose was confirmed, this is represented in table 4.4.

3.6.1.3 Barfoed's Test (General Test for Monosacharrides)

The sample solution of the crude extract (1cm³) was taken and 1cm³ of the Barfoed's reagent was added to it in a test tube and heated in a water bath. There was no formation of a red precipitate of cuprous oxide in all the four extracts. Therefore, there is the absence of monosaccharaides (see table 4.4).

3.6.1.4 Test for Pentoses

The sample solution (2cm³) was taken and there was an addition of an equal volume of hydrochloric acid containing a little phloroglucinol. All the extract sample solutions gave a red colour on heating with the exception of petroleum ether. This is indicative of the presence of pentose in ethyl acetate, chloroform and methanol crude extracts. The results are presented in table 4.4.

3.6.1.5 Fehling's Test (Standard Test for Free Reducing Sugars)

Each sample solution (2cm³) was treated with equal volume of Fehling's solution A and B and warmed on a water bath. A brick red precipitate was obtained,

indicating the presence of reducing sugars in chloroform, ethyl acetate and methanol crude extracts sample solutions with the exception of petroleum ether extract. The results are presented in table 4.4.

3.6.1.6 Test for Starch (To Distinguish Between Starch and Starch Products.)

To a portion of the sample solution (2 cm^3) of each extract was added 5 cm^3 of 5% potassium hydroxide and then mixed thoroughly. This was boiled in the water-bath for 3 minutes. A dark brown precipitate was produced on heating which indicates the presence of dextrin in ethyl acetate, chloroform and methanol crude extracts with the exception of petroleum ether extract. The results are presented in table 4.4.

3.6.1.7 Test for Agar

A drop of N/50 – Iodine solution was added to 1 cm^3 of each sample solution. The sample solution gradually changed to brown but the chloroform extract gave an olive green colouration (see table 4.4).

3.6.1.8 Test for Tragacanth

A portion of each sample solution was taken (2 cm^3) and 2% sodium hydroxide solution (2 cm^3) was added. In all the extracts there was the development of a yellow solution which is indicative of the presence of tragacanth. Four drops of a dilute solution of lead acetate was then added to 1 cm^3 of each extract sample solution. There was the formation of a flocculent white precipitate in each sample solution (see 4.4).

3.6.2 Test for Glycosides

3.6.2.1 By Dilute Mineral Acid

A portion of each extract (1.0 g) in a test tube was added 2.5 cm^3 of sulphuric acid and boiled in a water bath for 15 minutes. It was cooled and neutralized with few

drops of 20% potassium hydroxide. A mixture of Fehling's solutions A and B (5 cm^3) were added and boiled for a few minutes. There was the presence of a brick-red precipitate in all the extracts. This confirmed the presence of reducing sugars as a result of the hydrolysis of the glycosides. Results are presented in table 4.4.

3.6.2.2 Bortrager's Test (Test for Anthracene Derivatives)

To a portion of each extract (2 cm^3) was added 5 cm^3 of chloroform, this was then shaken by the hand and filtered. To the filtrate obtained was added 5 cm^3 of 10% ammonia solution and shaken. The layers were allowed to separate. A yellow colour was seen in the upper aqueous layers in petroleum ether, ethyl acetate and methanol extracts while the chloroform extract was colourless (see table 4.4).

3.6.2.3 Test for Steroidal Aglycone

A portion of the crude extract (2 cm^3) was transferred into a beaker, to which was added 10 cm^3 of ethanol (50 %) and 5 cm^3 of 15 % lead acetate solution. The beaker was placed in a boiling water bath for 3 minutes. It was allowed to cool, filtered into a separatory funnel and the filtrate was extracted twice with 5 cm^3 of chloroform and the lower chloroform layer was retained.

The chloroform layer was evaporated to dryness on a water bath, to the residue was added 1 cm^3 of 1 % (in ethanol) solution of 3, 5-dinitrobenzoic acid and 0.5 cm^3 of 1N- sodium hydroxide solution. An orange colour was observed for all the extracts, indicating the presence of steroidal aglycone in all the extracts (table 4.4).

3.6.2.4 Test for Saponin Glycosides

A portion of each crude extract (1g) was boiled with 10 cm^3 of distilled water for 2 minutes. It was filtered while hot and the filtrate was allowed to cool.

- a) To the filtrate (2.5 cm^3) was added a mixture of Fehling's solutions A and B (2.5 cm^3) and boiled for two minutes on a water – bath. All the extracts exhibited a brick – red precipitate indicating the presence of saponin glycosides(table 4.4).
- b) To a portion of the filtrate (5cm^3) was added 2.5 cm^3 of dilute sulphuric acid in a test tube and boiled for 15 minutes on a water bath. It was cooled and filtered through a Buchner funnel. The filtrate was made alkaline with the addition of a few drops of sodium hydroxide solution. A mixture of Fehling's solutions A and B (2.5cm^3) was added to the filtrate and boiled. All the extracts showed a brick-red precipitate, this is indicative of the presence of saponin glycosides.The results are presented in table 4.4
- c) A portion of the filtrate (2.5 cm^3) was diluted with 10 cm^3 of distilled water and shaken vigorously for 2 minutes. Frothing was observed in the crude extracts of ethyl acetate, chloroform and methanol extracts with the exception of petroleum ether extract. There is the presence of saponin glycosides in the three extracts (see table 4.4).

3.6.2.5 Keller-Kiliani Test (For Cardiac Glycosides)

To a portion of each extract (0.5 g) was added distilled water (5cm^3) in a test tube and 3 drops of of lead sub- acetate solution. It was mixed thoroughly and filtered. The filtrate was transferred into a separatory funnel with 5 cm^3 of chloroform. The lower layer was run into a small evaporating dish and the solvent was slowly and carefully removed by heating in a water bath. Residue was allowed to cool and then dissolved in 1 cm^3 of glacial acetic acid containing a trace of FeCl_3 solution. The solution was carefully poured on the surface of 1 cm^3 sulphuric acid in another test tube, so as to form a separate layer.

The acetic acid layer became green (due to the presence of deoxy sugar). For chloroform and ethyl acetate extracts, the acetic acid layer turned blue green due to

steroid nucleus and a red ring was formed at the interface due to deoxy sugars indicating the presence of cardiac glycosides. For methanol extract, the acetic acid layer did not change but a red ring was formed at the interface showing the presence of cardiac glycosides. The results are depicted in table 4.4.

3.6.2.6 Kedde Reagent Test

A portion of the crude extract (1 g) was dissolved in 10 cm³ distilled water, then warmed in a water bath and filtered to get sample solution. One drop of solution I (2 % of 3, 5 - dinitrobenzoic acid in methanol) and one drop of solution II (5.7 % potassium hydroxide in water) were added to 0.4 cm³ of the sample solution. A purple solution was observed within 5 minutes. The test is positive indicating the presence of cardiac glycosides (see table 4.4)

3.6.2.7 Baljet Reagent Test

Solution I (1.0 g picric acid in 100 cm³ ethanol) was measured as 2 cm³ and added to 2cm³ of solution II (10 g sodium hydroxide in 100 cm³ distilled water), these were combined and three drops of the combined solutions were added to sample solution of the crude extract. A positive result was obtained for all the crude extracts, which is indicative of the presence of cardenolides. The results are presented in table 4.4.

3.6.3 Test for Alkaloids

3.6.3.1 Using Mayer's Reagent

To HgCl₂ (1.36g) was added 60cm³ distilled water and 5 g KI in 10 cm³ distilled water. Both solutions were combined and made up with distilled water to 100 cm³. Two drops of dilute sulphuric acid were added to each extract sample solution

and three drops of the combined solutions. Yellow precipitate was observed in all extracts showing the presence of alkaloids. The results are presented in table 4.4.

3.6.3.2 Using Dragendorff's Reagent

$\text{Bi}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ (8.0 g) was dissolved in 30 % w/v nitric acid and 27.2 g KI in 50 cm³ of water. The two solutions were combined and made to stand for 24 hrs, filtered and made up with water to 100 cm³. Two drops of the above solution was added to 2 cm³ of sample solution (acidified by addition of two drops of acetic acid) of each extract. There was the formation of an orange red precipitate in each of the extract which showed the presence of alkaloids. The results are presented in table 4.4.

3.6.3.3 Using Wagner's Reagent

Iodine (1.27g) and potassium iodide (2g) were dissolved in 20 cm³ distilled water and made up to 100 cm³ distilled water. To 2 cm³ of each sample solution of the extracts were added a drop of dilute sulphuric acid and two drops of the above solution. A brown turbid solution was observed in all the extracts showing the presence of alkaloids. The results are depicted in table 4.4.

3.6.4 Test for Flavonoids

3.6.4.1 Shinoda test

A portion of each crude extract (0.5g) was dissolved in 1 cm³ distilled water and methanol (1:1) to get an alcoholic solution of the sample to which was added a little of magnesium powder and four drops of concentrated hydrochloric acid. An orange colour was observed to gradually develop in petroleum ether, chloroform and ethyl acetate extract. For the methanol extract colour change was observed to be immediate. This indicates the presence of flavones, flavonols, the corresponding 2, 3 – dihydro-derivatives and or xanthenes. The results are presented in table 4.4.

3.6.4.2 Sulphuric Acid Test

To an alcoholic solution of the sample, four drops of concentrated sulphuric acid were added. A red colour was observed at the interphase, indicative of the presence of chalcones and aurones in all the crude extracts. This colour change was observed for petroleum ether, ethyl acetate, chloroform and methanol crude extracts. The results are depicted in table 4.4

3.6.4.3 Ferric Chloride Test

From the stock solution of each extract, 2 cm³ was taken and 5 drops of freshly prepared ferric chloride (5%) were added. A greenish-brown solution was observed in all the crude extracts which indicate the presence of phenolic nucleus in petroleum ether, ethyl acetate, chloroform and methanol crude extracts. The results are presented in table 4.4 (Sofowora 2008).

3.6.4.4 Test for Plant Phenols (Specific Test for Chlorogenic Acid)

To a portion of the sample solution (1 cm³) of each crude extract was added 4 drops of 10 % ammonia solution. There was formation of a dark brown turbid solution, which means there is the presence of a phenolic compound in the four extracts.

3.6.5 Test for Sterols

3.6.5.1 Liebermann- Buchard Test

A portion of the crude extract (0.5 g) was transferred into a test tube, anhydrous acetic acid (1 cm³) and chloroform (1 cm³) were added. Then it was cooled to 0°C and one drop of concentrated sulphuric acid was added. There was the formation of a blue – green colour in petroleum ether, ethyl acetate, chloroform and methanol extracts. The colour was most intense in petroleum ether and methanol

extracts. This indicates the presence of sterols in the crude extracts and the results are presented in table 4.4.

3.6.5.2 Salkowski Reaction

A portion of each of the extract (1 mg) was dissolved in chloroform(1cm^3) and few drops of concentrated sulphuric acid (1cm^3) were added. There was a reddish-brown ring at the interphase of the extracts of petroleum ether, ethyl acetate chloroform and methanol. Therefore this showed the presence of sterols and methylated sterols in the four extracts. The results are presented in table 4.4.

3.6.6 Test for Proteins

3.6.6.1 Millon's Reagent (Freshly Prepared Mercuric Nitrate Solution)

To a portion of sample solution (2cm^3) was added a drop of Millon's reagent. A brown colouration was produced on heating for the petroleum ether, ethyl acetate, chloroform and methanol extracts. This colouration is indicative of a phenolic compound in the extracts as Millon's reagent is not specific for proteins. It also indicates the presence of tyrosine residue which occurs in nearly all proteins. The results are presented in table 4.4.

3.6.6.2 Reaction with solution of Picric acid and Tannic Acids

To a portion of sample solution (2cm^3) was added 2 drops of solutions of picric and tannic acids. There was the formation of white flocculent precipitate in petroleum ether, chloroform, ethyl acetate and methanol extracts. This indicates the presence of the presence of amino acids, the building blocks of proteins in the four crude extracts and the results are depicted in table 4.4

3.6.6.3 Xanthoprotein Test

To a portion of sample solution (2 cm^3) was added few drops of concentrated nitric acid followed by an excess of ammonia solution. An orange colour was observed in methanol extract on addition of ammonia solution while a yellow colour was produced in petroleum ether, ethyl acetate and chloroform extracts. This test is indicative of the presence of phenyl group (C_6H_5) in the protein molecules in the extracts. The results are presented in table 4.4.

3.6.6.4 Biuret Test

To a portion of sample solution (1cm^3) was added 10 % sodium hydroxide solution(1cm^3) followed by a drop of copper sulphate solution. Petroleum ether, chloroform and methanol extracts exhibited green colour while ethyl acetate extract showed a bluish-green colour. There is the absence of violet or pink colour in all the extracts, this indicates the absence of peptide bonds which can make Biuret reagent turn purple.

3.6.7 Test for Saponins

3.6.7.1 Frothing Test

To an aqueous solution of the sample (2 cm^3) was added distilled water (5 cm^3) in a test tube and was shaken for 4 minutes by hand. It was observed that there was frothing which lasted for approximately 15 minutes for petroleum ether, chloroform and ethyl acetate extracts. While 20 minutes frothing was observed for methanol extract. This shows the presence of saponins in all the four extracts. Saponins of both the steroid and triterpenoid groups respond to the above test. This indicates the presence of steroid and triterpenoid groups in the four extracts. And the results are presented in table 4.4.

3.7 Antimicrobial Sensitivity Studies

The extracts of *Tetracarpidium conophorum* leaves are being investigated based on some ethnomedicinal uses in traditional medicine (Burkill 1985; Obayendo 2010; Odoemena 2010). Antimicrobial screening was carried out on extracts of petroleum ether, chloroform, ethyl acetate and methanol extracts using some human pathogens. The microbes are clinical strains obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. All the microbes were checked for purity and viability (Tripathi 2008). The bacteria organisms were kept in slants of nutrient (Mueller Hinton) agar while those of fungi were kept in slants of Sabouraud dextrose agar (Murray *et al.*, 1999).

The crude extracts of petroleum ether, chloroform, ethyl acetate and methanol were tested on the following twenty microorganisms. *MRSA*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Candida albicans*, *Candida krusei*, *Aspergillus fumigatus*, *Aspergillus niger*, *Streptococcus pneumoniae*, *Corynebacterium ulcerans*, *Corynebacterium diphtheriae*, *Bacillus subtilis*, *Listeria monocytogenes*, *Shigella dysenteriae*, *Campylobacter fetus*, *Klebsiella pneumoniae*, *Vibrio cholerae* and *Helicobacter pylori*. This was to provide scientific evidence of the inhibitory effects of the plant extracts on the growth of the microorganisms which are the causative agents of some diseases as acclaimed in ethnomedical practices (Muanya 2011; Burkill 1985; Malu *et al.*, 2009). The results of the antimicrobial screening of the four extracts and the control (drugs) are presented in table 4.5. The zone of inhibition of the extracts' activities on the twenty microorganisms were recorded (see table 4.6). Also conducted was the determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal /

Fungicidal Concentration of the four extracts' activities on the twenty microorganisms (Tripathi 2008; Purohit *et al.*,2008). These are presented in table 4.7..

From the results of the antimicrobial analyses of the four extracts, petroleum ether extract was chosen as the first extract to elucidate pure compounds by chromatographic techniques. Three fractions namely TCPE I, TCPE II and TCPE III were obtained by chromatographic techniques (TLC, CC and PTLC). These fractions occur as yellow solids. Antimicrobial screening of the three fractions was conducted on ten (10) microorganisms: *MRSA*, *Staphylococcus aureus*,, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Candida albicans*, *Candida krusei*, *Aspergillus fumigatus* and *Aspergillus niger*. In addition, three (3) established drugs were used as control, sparfloracin, fluconazole and fulcin (table 4.7).

More extensive chromatographic techniques were further carried out on the crude extract of petroleum ether and two compounds were isolated as white (crystalline) solids. The isolated compounds (TC 1 and TC 2) were tested on all twenty (20) microorganisms. Five drugs (control) currently in use in the treatment of some illnesses were used as control. They included; fulcin, fluconazole, tetracycline, sparfloracin and ciprofloxacin. The results are presented in tables 4.11, 4.12 and 4.13 (Burkill 1985; Murray 1999;Tripathi 2008). These microbial analyses were conducted to confirm if the inhibitory properties of the crude extract of petroleum ether were still present in the isolated compounds after extensive chromatographic technique applications on the crude petroleum ether extract.

3.7.1 Media Preparation

The Mueller Hinton agar base (from Oxoid) and the Sabouraud dextrose agar were the nutrient media used for the preparation of growth media of bacteria and fungi respectively (Bauser 1966; Atlas 2004). The media were prepared according to the manufacturer's instructions. For the preparation of the nutrient agar, the nutrient agar powder (38 g) was suspended in 1 litre of distilled water. It was brought to boiling to dissolve completely. Then it was sterilized at 121 °C for 15 minutes. The media were cooled to 45 °C and 20 cm³ of each medium was poured into sterile petri dishes, covered and allowed to solidify by cooling (Atlas 2004).

3.7.2 Detection of Antimicrobial Activity

Diffusion method was the choice of method used for the screening of the extracts. A portion of the crude extract (0.4 g) was weighted and dissolved in 10cm³ DMSO in order to obtain a concentration of 40 mg/cm³. This was the initial concentration of the extract used to determine the antimicrobial activities of the plant. The sterilized medium was seeded with 0.1 cm³ of the standard inoculums of the test microbes. The inoculum was spread evenly over the surface of the medium with the use of a sterile swab (Atlas 2004; Sandven and Lassen 1999). Using a standard cork borer of 6 mm in diameter, a well was made at the centre of each inoculated medium. A portion of the sample solution of the extract (0.1cm³) of the concentration of 40 mg/cm³ was introduced into each well of the medium. The inoculated media were incubated at 37 °C for 24 hours for the bacteria and at 30 °C for 1-7 days for the fungi. After which each plate was observed for the zone of inhibition of growth. The zone was measured with a transparent metre rule and the results were recorded in millimeters. The results are presented in tables 4.5 and 4.6.

3.7.3 Determination of Minimum Inhibitory Concentration (MIC)

MIC of the extracts was carried out on the test microorganisms and this was done using the broth dilution method. Mueller Hinton broth and Sabouraud dextrose broth were prepared according to the manufacturer's instructions, boiled to dissolve and 10 cm³ of the broth was dispensed into test tubes. The test tubes have been sterilized at 121 °C for 15 minutes. The broth in the test tubes was allowed to cool. Mc- Farland's Turbidity Standard scale number 0.5 was prepared to give a turbid solution. Normal saline was prepared and it was used to make suspensions of the test microbes by dispensing 10 cm³ into the sterile test tubes. The test tubes were then inoculated and incubated at 37 °C for 6 hours. Dilution of the test microbe in the normal saline was done until the turbidity matched that of the Mc-Farland's scale by visual comparison. At this point, the microorganism had a concentration of about 1.5×10^8 cfu/cm³. Two fold serial dilution of each extract in the sterilized broth was made to obtain concentrations of 40 mg/cm³, 20 mg/cm³, 10 mg/cm³, 5 mg/cm³, 2.5 mg/cm³ and 1.25 mg/cm³. The initial concentration was obtained by dissolving 0.2 g of the extracts in 10 cm³ of the sterile broth. Having obtained the different concentrations of the extract in the broth, 0.1 cm³ of the standard inoculum of the test microorganism in the normal saline was then inoculated into the different concentrations. Incubation was done at 37 °C for 24 hrs after which each test tube was observed for turbidity (growth). The lowest concentration of the extract in the broth which showed no turbidity was recorded as the Minimum Inhibitory Concentration (MIC) (Atlas 2004; Bauser *et al.*, 1966).

3.7.4 Determination of Minimum Bactericidal/Fungicidal Concentration (MBC/ MFC)

The MBC and MFC were carried out to determine whether the test organisms were killed or whether only their growths were inhibited by the plant extracts. Mueller Hinton agar was prepared in accordance to the manufacturer's instruction, sterilized at 121 °C for 15 minutes then poured into sterile petri dishes and was allowed to solidify by cooling. This is the growth medium for bacteria while the manufacturer's instructions were also followed for the preparation of the Sabouraud dextrose agar for fungi. The contents of the MIC in the Serial dilution were then subcultured onto the agar media. Incubation was made at 37 °C for 24 hours for bacterial plates and at 30 °C for 46 hours for fungi plates. After which the plates were observed for colony growth. The MBC and MFC were the plates with the lowest concentration of the extract without any visible colony growth (Bauser *et al.*, 1966, Murray *et al.*, 1999). The results are presented in table 4.7.

3.8.1 Chromatographic Techniques Employed in the Purification and Isolation of the Crude Extract

3.8.1 Thin Layer Chromatography (TLC)

Thin layer chromatography was used to determine the presence of chemical components in the four crude extracts. TLC was also performed to obtain a most suitable mobile phase for good resolution. This was carried out using pre coated silica gel analytical (20 x 20 cm) plates which were cut with a pair of scissors. Pre-coated silica gel TLC plates were used to obtain a solvent mixture for the column chromatography with many pilot tests. Such as petroleum ether: ethyl acetate solvent mixture as 1:1, 1:2, 3:7 and 1:9. Solvent mixture of 1:9 was settled for.

3.8.2 Column Chromatography and Preparative TLC

For the column chromatography, the column used has a length of 45 cm and diameter was 2 cm. The lower end of the column was blocked with glass wool. The column was first eluted with 100 % 100 cm³ of petroleum ether and was allowed to pass through the glass wool with the elimination of air bubbles by using a glass rod to steady the glass wool. Additional 50 cm³ of petroleum ether was added. Silica gel for chromatography (50 g) was made into slurry with 200 cm³ petroleum ether (60-80 °C) and was poured gently into the column with tapping of the side of the column with a glass rod to allow the adsorbent (as stationary phase) settle evenly without air bubbles. Filter paper disc was placed on top of the packed column which has been cut to size.

The tap at the bottom of the column was released to allow the liquid to run out until it just covers the top of the medium. The crude extract (2 g) of the petroleum ether was dissolved in 2 cm³ petroleum ether (60-80 °C) and this was applied on top of the filter paper disc with a dropping pipette and more petroleum ether added. The column was eluted using the mobile phase starting with absolute petroleum ether. This was followed by using petroleum ether (60-80 °C); ethyl acetate (9:1) made to 25 cm³ and this was repeated several times. Then increasing the polarity by 1 % until absolute ethyl acetate and methanol were used (25 cm³) five times to wash the column. Twenty fractions were collected in 20 cm³ aliquots in 50 cm³ beakers. The various eluates were monitored by spotting on precoated silica gel TLC sheets and developed using various solvent mixtures. Fractions with similar TLC behaviour (with the same R_f values) were pooled together and concentrated at reduced pressure and dried under high vacuum. The 30 fractions were combined in to 6 fractions. TLC

was carried out on the combined fractions labeled BPE I, BPE II, BPE III, BPE IV, BPE V and BPE VI. Then pre-TLC was carried out on BPE I to obtain a pure compound.

The quickfit plate leveller and six glass plates (20 cm by 20 cm) were washed, dried and wiped with cotton wool with acetone to clean and remove contaminants. To 30 g alumina in a conical flask (500 cm³) was added 40 cm³ distilled water, stoppered and shaken vigorously for about 30 seconds to ensure homogenous slurry was produced. The slurry was poured immediately into the hand applicator (0.50 mm) which was run smoothly over the glass plates. The coated plates were allowed to air dry over 2 hrs and activated in an oven for 1 hour. When the plates were cooled, spotting of BPE I was done and developed by ascending technique petroleum ether (60-80°C): ethyl acetate (39:1). Development time was 40 minutes. After drying the plates were viewed under the UV lamp (366 nm) and three bands were observed to fluoresce brightly. The R_f values were determined and found to be 0.30 for TCPE I, 0.67 for TCPE II and 0.83 for TCPE III. The three bands were scraped from the plates separately into three 250 cm³ beakers, dissolved in 100 cm³ of methanol and filtered. Three fractions were collected as a yellow oily gum (TCPE I, TCPE II and TCPE III) which gave way to yellow crystals over a period of many days (Touchstone 1992; Bobbitt *et al.*, 1968).

3.8.3 Additional Purification of Crude Petroleum Ether Extract

A portion of the petroleum ether extract (0.5 g) was dissolved in 2 cm³ petroleum ether (60-80°C) and the sample solution was spotted on TLC silica pre-coated aluminum sheets. The spotted TLC plates were developed in various solvent

systems and viewed using 10 % sulphuric acid. Eventually the best solvent system n-hexane: ethyl acetate (8:2) was obtained.

This was followed by column chromatography. The length of the column was 30 cm and 3 cm in diameter. A portion of petroleum ether extract (5.0 g) was weighed, pre-adsorbed on 10 g silica gel (60-120 mesh) , mixed to get an even mixture and allowed to dry. The column was packed with silica gel (60-120 mesh) as slurry with n-hexane. Glass wool was placed on top of the packed column and the sample was added on top as a thin uniform layer and 200 cm³ of n- hexane was added.

Elution was done by gradient system of using n-hexane and ethyl acetate (Srivastave and Srivastave, 1987) by the addition of 200 cm³ to the column at a time, and at each time an elution of 100 cm³ was collected. A total of 30 fractions were collected using solvent mixture of n-hexane: ethyl acetate (95:05). Each eluate was monitored by TLC. After the development of the TLC plates, they were sprayed with 10 % sulphuric acid and dried in an oven for 2 minutes. Finally the column was washed with absolute methanol Using TLC, fractions no. 1 – 8 were pooled together as A1 and the next fractions 9 -14 were also pooled together as A2 because of the similarity in their profiles in various solvent mixtures on the TLC. A1 and A2 were concentrated under vacuum to remove the solvent. A1 and A2 exhibited presence of two prominent spots on TLC, so they were subjected to prep TLC using precasted TLC plates of silica gel on aluminum sheets and solvent mixture of n-hexane: ethyl acetate (85:15) as the mobile phase to develop. This gave rise to a pure compound 'TC 1'. TC 1 was a white crystalline compound, it was weighed (5.31 mg) and the melting point was determined.

A2 was subjected to prep TLC on precoated TLC plates of silica gel on aluminum sheets using a solvent mixture of n-hexane: ethyl acetate (8:2) and this gave rise to another pure compound, 'TC 2' which occurred as a white powdery compound. It was weighed (4.20 mg) and the melting point determined (Bobbitt *et al.*, 1968; Touchstone 1992).

3.9 Proximate Analysis of the Leaves of *Tetracarpidium conophorum*

This was carried out in the Animal Science Laboratory, Department of Animal Science, Faculty of Agricultural Sciences, Ahmadu Bello University, Zaria. The dried leaves of *T. conophorum* (100 g) were milled into very fine powder using Christy milling machine (Christy and Norris Limited, England).

3.9.1 Determination of Crude Protein (CP)

The grinded powder of the plant sample (1.2 g) was placed in the digestion flask and there was the addition of sodium sulphate (15 g), 1 g of copper sulphate, two selenized boiling granules and 25 cm³ of concentrated sulphuric acid sequentially. This was digested for 2 hours, cooled and 200 cm³ water was added. Into a 500 cm³ Erlenmeyer flask was pipetted 100 cm³ of 0.1 N hydrochloric acid and 1 cm³ of Conway indicator (mixture of methyl red and methylene blue in the ratio 1:1 in 50 % ethanol) was added. To the Kjeldahl flask containing the digested sample was added slowly 100 cm³ of 50 % sodium hydroxide down the side of the flask so that it forms a layer underneath the digestion mixture. Immediately the flask was connected to the distilling bulb of the distillation apparatus. The flask was mechanically rotated thoroughly while being heated until all the ammonia (by conversion of amino nitrogen in the plant sample) has passed over into the standard acid. Next, 150 cm³ was collected and the Kjeldahl flask was removed immediately. The tip of the flask was washed and the content was titrated with standard HCl and standard NaOH solutions

(retrieved from www.aquaculture). The percentage of protein was then determined by:-
% Protein = % Nitrogen x 6.25.

3.9.2 Determination of Crude Lipid

A grinded sample of the plant (10 g) was weighed into a thimble and it was extracted with petroleum ether (200 cm³) for 8 hours. This dissolves fats, oils, pigments and other fat soluble substances. After extraction, the petroleum ether was allowed to drain out of the thimble. The ether was then evaporated from the fat solution, allowed to dry for 30 minutes and cool in a desiccator and weighed. The resulting residue is weighed and referred to as ether extract or crude fat or crude lipid (retrieved from www.aquaculture.ugent).

3.9.3 Determination of Nitrogen Free Extract (NFE) or Digestible Carbohydrates

NFE supposedly represents the soluble carbohydrate of the feed or sample such as starch and sugar. Crude fiber represents insoluble carbohydrates. The only component in proximate analysis which is not determined analytically but is calculated by difference is NFE. Therefore, NFE accumulates all of the errors that exist in another proximate analysis components.

$$\% \text{ NFE} = \% \text{ DM} - (\% \text{ EE} + \% \text{ CP} + \% \text{ ash} + \% \text{ CF})$$

Where, DM is dry matter, EE is ether extract or crude lipid and CF is crude fiber (retrieved from www.aquaculture.ugent).

3.9.4 Determination of Ash

The ash fraction contains all the mineral elements jumbled together. A portion of the milled sample of the plant (20 g) was weighed into a crucible and the crucible was placed into a drying oven at 100 °C for 24 hours. After which the crucible was transferred to cool muffle furnace and the temperature was increased step – wise to 650 C . This temperature was maintained for 4 hours until all the carbon has been removed. The residue is the ash. The

crucible was then removed to a desiccator, it was allowed to cool and weighed (retrieved from www.aquaculture.ugent).

3.9.5 Determination of Crude Fiber

The grinded sample was weighed (2 g) into a beaker and 1.25 % sulphuric acid (100 cm³) was added plus 2 drops of n- octanol as antifoaming agent. This was boiled for 30 minutes on Labconco machine. After which this was filtered and washed with deionized water. To the residue were added 100 cm³ of 1.25 % sodium hydroxide solution and 2 drops of n- octanol. This was boiled for 30 minutes, then filtered and washed with deionized water. The residue was placed in a crucible and placed in an oven at 105 °C for an hour to dry. This was then cool in a desiccator (www.aquaculture.ugent).

3.9.6 Determination of Dry Matter (DM)

Dry matter is a measurement of total solids in a given sample when all the water is removed. Dry matter of food/ sample would include carbohydrates, fats, proteins, vitamins, minerals, anti-oxidants etc. Percent moisture content is measured as the weight lost during drying and is expressed as a percentage of the (as received) wet sample (Ayoola *et al.*, 2011).

$$\% \text{ moisture} = 100 - \% \text{ Dry Matter} = 100 - 91.8 = 8.14$$

3.10 Determination of Elemental Analysis of the Leaves of *Tetracarpidium conophorum*

The pulverized leaves of *Tetracarpidium conophorum* was weighed (0.25g) using Mettler AE 240 analytical balance. The sample was sealed in polythene bag that had been pre-cleaned with distilled water and dilute hydrochloric acid. The polythene bag containing the sample was secured inside a sample vial (that was rinsed with distilled water and allowed to dry in an oven at a maintained temperature of 60°C for two hours), with sterilized cotton wool, covered, cello-taped and set for irradiation (Okoh 2013).

The sample was irradiated using the Nigeria Research Reactor-1 (NIRR-1) which is located at the Centre for Energy Research and Training, Ahmadu Bello University, Zaria, Nigeria. The irradiation lasted for 6 hours. After the irradiation, gamma-ray measurements were performed at sample - detector geometry of 1cm for 60 minutes after a waiting period of 10 days. Counting of induced gamma rays in the activation products were carried out using High - Purity Germanium (HPGe) detector of relative efficiency of 10% at 133.5keV, the MAESTRO emulation software compatible with the ADCAM Multi – channel analyzer (MCA) card, associated electronic modules and a personal computer. Identification of gamma – ray fingerprint of product radio - nuclides through their energies and quantitative analysis procedure for determination of their concentrations were achieved using the gamma ray analysis software WINSPAN 2004 (Okoh 2013).

CHAPTER FOUR

RESULTS

4.1 Extraction of the leaves

The pulverized plant material (300 g) was extracted successively with four solvents. The weight and percentage yield of each crude extract are depicted below in table 4.1

Table 4.1: Extraction and percentage recovery of extracts from *Tetracarpidium conophorum* leaves

Extracts	Mass recovered (grams)	Percentage recovered
Petroleum ether	18.82	6.27
Ethyl acetate	12.86	2.29
Chloroform	4.36	1.45
Methanol	27.60	9.20

4.2 Proximate Analysis of the leaves of *Tetracarpidium conophorum*

The proximate analysis of the leaves of *Tetracarpidium conophorum* showed the analysis of the leaves into its major constituents namely: crude protein, crude fibre, nitrogen- free extractive, ether extract, ash content and dry matter. They are presented in table 4.2.

Table 4.2: Proximate Analysis of leaves of *Tetracarpidium conophorum*

Category	Composition (%)
Crude protein (CP)	17.68
Crude fiber (CF)	15.53
Crude ether	1.89
Ash	13.38
Nitrogen free extract (NFE)	51.52
Dry matter (DM)	91.86

4.3 Elemental Analysis by INAA of the leaves of *Tetracarpidium conophorum*

The analysis by INAA revealed the presence of thirteen elements in the leaves of *Tetracarpidium conophorum* in their various concentrations (see table 4.3). These occur as major elements (such as calcium, magnesium etc) and trace elements (such as cobalt and manganese). And they play vital roles in the human bodies (Burkill 1985, Dalziel 1937, Ayoola 2011).

Table 4.3: Elemental Analysis of *Tetracarpidium conophorum* leaves

Element	Composition (mg/kg)
Magnesium (Mg)	4756 ± 304
Aluminum (Al)	161 ± 21
Chlorine (Cl)	200 ± 16
Calcium (Ca)	33460 ± 903
Strontium (Sr)	130 ± 17
Vanadium (V)	BDL
Manganese (Mn)	262.6 ± 0.5
Dysprosium (Dy)	BDL
Sodium (Na)	29 ± 1
Potassium (K)	19390 ± 175
Arsenic (As)	BDL
Bromine (Br)	3.56 ± 0.18
Lanthanum (La)	6.6 ± 0.1
Samarium (Sm)	0.41 ± 0.01
Uranium (U)	BDL
Scandium (Sc)	BDL
Chromium (Cr)	BDL
Iron (Fe)	BDL
Cobalt (Co)	0.11 ± 0.02
Zinc (Zn)	24.3 ± 2.8
Rubidium (Rb)	BDL
Antimony (Sb)	BDL
Barium (Ba)	558 ± 22
Cerium (Ce)	BDL
Neodymium (Nd)	BDL
Europium (Eu)	BDL
Terbium (Tb)	BDL
Ytterbium (Yb)	BDL
Lutetium (Lu)	BDL
Gold (Au)	BDL
Thorium (Th)	BDL

KEY: BDL = Below Detection Limit

4.4 Phytochemical Screening of the Crude Extracts

The preliminary phytochemical screening of the four extracts of the leaves of *Tetracarpidium conophorum* showed the presence of glycosides, alkaloids, flavonoids, sterols and saponins. There is the absence of carbohydrates (table 4.4). It is observed that similar classes of phytochemicals are present in all the four extracts such as steroidal aglycones, saponin glycosides, cardiac glycosides and cardenolides. As well as the presence of sterols and methylated sterols (Evans 2009, Silva *et al.*, 1998). The frothing test was positive for all the four extracts (Evans 2009). The test for the presence of phenyl group in the protein molecules by Xanthoprotein test was positive but the test for the presence of peptide bonds using Biuret test was negative for all the four extracts. These are depicted in table 4.4.

Table 4.4: Phytochemical screening of the crude extracts of *T. conophorum*

Phytochemical test	PE	CH	EA	ME
CARBOHYDRATES				
Molisch's test	-	+	+	+
Seliwanoff's test (for ketones)	-	+	+	+
Barfoed's test (general test for monosaccharide)	-	-	-	-
Test for pentoses	-	+	+	+
Fehling's test (for free reducing sugars)	-	+	+	+
Test for starch	-	+	+	+
Test for Agar	+	+	+	+
Test for Tragacanth	+	+	+	+
Test for free anthracene derivatives	-	-	-	-
GLYCOSIDES				
Kedde reagent's test	+	+	+	+
Baljet reagent test	+	+	+	+
By dilute mineral acid	+	+	+	+
Borntrager test	-	-	-	-
Test for steroidal aglycone	+	+	+	+
Saponin glycosides	+	+	+	+
Keller Killiani test	+	+	+	+
Test for chlorogenic acids	+	+	+	+
ALKALOIDS				
Mayer's reagent	-	+	+	+
Dragendorff's reagent	-	+	+	+
Wagner's reagent	-	+	+	+
FLAVONOIDS				
Shinoda test	+	+	+	+
Sulphuric acid test	+	+	+	+
Ferric chloride test	+	+	+	+
STEROLS				
Liebermann-Buchard test	+	+	+	+
Salkowski's test	+	+	+	+
PROTEINS				
Millon's reagent	+	+	+	+
Solutions of picric and tannic acids	+	+	+	+
Xanthoprotein test	+	+	+	+
Biuret test	-	-	-	-
Frothing test	+	+	+	+

KEY: + = positive, - = negative

4.5 Antimicrobial Screening of the Crude Extracts and the Control

The antimicrobial screening of the crude extracts of the leaves of *Tetracarpidium conophorum* exhibited their inhibitory effects on the growth of gram –positive bacteria such as *MRSA*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus subtilis* and *Listeria monocytogenes*; and gram-negative bacteria such as *E.coli*, *Shigella dysenteriae*, *Campylobacter fetus*, *Klebsiella pneumoniae*, *Vibro cholerae* and *Helicobacter pylori*. The moulds (*Asper. fumigatus* and *Asper.niger*) resisted the inhibitory effects of the four crude extracts. The four extracts inhibited the growth of fungi- *Candida albicans* and *Candida krusei* (table 4.5). Malu (2009) reported that the plant relieves abdominal pains and fever. And phytochemicals like sterols and glycosides are known to have pharmaceutical properties (Evans 2009).

The antimicrobial screening of the crude extracts and the control are presented in table 4.5. Since the disc-diffusion method was used, the zones of inhibition were measured in millimeters (see table 4.6). The MIC and MBC/MFC were determined and presented in table 4.7. Other tables from 4.8 to 4.13 presented various antimicrobial activities of fractions petroleum ether extract (TCPEI, TCPEII and TCPEIII) as well as the two isolated compounds from petroleum ether extract (TC1 and TC 2).

Table 4.5: Antimicrobial Screening of leaves of T.conophorum and the control

Test organisms	PE	CH	EA	ME	Sp	Cp
MRSA	S	S	S	S	S	R
<i>Staphylococcus aureus</i>	S	S	S	S	S	S
<i>Streptococcus pyogenes</i>	R	R	R	R	S	S
<i>Escherichia coli</i>	S	S	S	S	S	S
<i>Pseudomonas aeruginosa</i>	R	R	R	R	R	S
<i>Salmonella typhi</i>	S	S	S	S	S	S
<i>Candida albicans</i>	S	S	S	S	R	R
<i>Candida krusei</i>	S	S	S	S	R	R
<i>Aspergillus fumigatus</i>	R	R	R	R	R	R
<i>Aspergillus niger</i>	R	R	R	R	R	R
<i>Streptococcus pneumoniae</i>	S	S	S	S	S	S
<i>Corynebacterium ulcerans</i>	R	R	R	S	S	S
<i>Corynebacterium diphtheria</i>	R	R	R	S	S	S
<i>Bacillus subtilis</i>	S	S	S	S	S	S
<i>Listeria monocytogenes</i>	S	S	S	R	R	S
<i>Shigella dysenteriae</i>	S	R	S	S	S	S
<i>Campylobacter fetus</i>	S	S	S	R	R	S
<i>Klebsiella pneumoniae</i>	S	S	S	S	S	S
<i>Vibrio cholera</i>	R	R	R	R	R	S
<i>Helicobacter pylori</i>	S	S	S	S	S	R

KEY: S = Sensitive to effect of crude extract
R = Resistance to effect of crude extract
PE = Petroleum ether extract
CH = Chloroform extract
EA = Ethyl acetate extract
ME = Methanol extract.
Sp = Sparfloxacin
Cp = Ciprofloxacin

Table 4.6: Zones of Inhibition of the extracts (mm) against the test microorganisms and control

Test organisms	PE	CH	EA	ME	Sp	Cp
MRSA	24	24	24	24	39	00
<i>Staphylococcus aureus</i>	15	23	27	34	37	35
<i>Escherichia coli</i>	14	27	24	22	36	38
<i>Salmonella typhi</i>	14	23	20	22	32	37
<i>Candida albicans</i>	14	20	19	20	00	00
<i>Candida krusei</i>	19	23	19	19	00	00
<i>Streptococcus pneumoniae</i>	20	24	29	23	37	35
<i>Bacillus subtilis</i>	22	27	32	35	42	40
<i>Listeria monocytogenes</i>	23	22	27	19	30	00
<i>Shigella dysenteriae</i>	20	25	27	24	40	44
<i>Campylobacter fetus</i>	21	27	30	27	00	35
<i>Klebsiella pneumoniae</i>	20	25	30	26	41	40
<i>Helicobacter pylori</i>	18	23	25	25	32	00

KEY: S = Sensitive
R = Resistance
PE = Petroleum ether extract
CH = Chloroform extract
EA = Ethyl acetate extract
ME = Methanol extract.
Sp = Sparfloxacin
Cp = Ciprofloxacin

Table 4.7: Minimum Inhibitory Concentration (MIC) and MBC/MFC (mg/cm³)

Test organisms	MIC				MBC/MFC			
	PE	CH	EA	ME	PE	CH	EA	ME
MRSA	10	10	10	10	20	20	40	40
<i>Staphylococcus aureus</i>	20	10	10	10	40	20	20	20
<i>Escherichia coli</i>	20	05	10	10	40	20	40	40
<i>Salmonella typhi</i>	20	10	10	10	40	40	40	40
<i>Candida albicans</i>	20	10	20	10	40	40	40	40
<i>Candida krusei</i>	20	10	20	20	40	40	40	40
<i>Streptococcus pneumoniae</i>	10	10	05	10	40	20	10	20
<i>Bacillus subtilis</i>	10	05	05	10	40	05	10	20
<i>Listeria monocytogenes</i>	20	10	05	10	40	40	20	40
<i>Shigella dysenteriae</i>	10	10	05	10	40	20	20	20
<i>Campylobacter fetus</i>	10	05	05	05	40	20	20	20
<i>Klebsiella pneumoniae</i>	10	10	05	10	40	20	10	20
<i>Helicobacter pylori</i>	20	10	10	10	40	20	20	20

KEY: MIC = Minimum Inhibitory Concentration

MBC/MFC = Minimum Bactericidal / Fungicidal Concentration

S = Sensitive

R = Resistance

PE = Petroleum ether extract

CH = Chloroform extract

EA = Ethyl acetate extract

ME = Methanol extract

Table 4.8: The Antimicrobial screening of fractions of petroleum ether extract– TCPE I, TCPE II, TCPE III and the control

Test organisms	I	II	III	Sp	Fl	Fu
MRSA	S	S	S	S	R	R
<i>Staphylococcus aureus</i>	S	S	S	S	R	R
<i>Streptococcus pyogenes</i>	S	R	S	S	R	R
<i>Escherichia coli</i>	S	S	S	S	R	R
<i>Pseudomonas aeruginosa</i>	R	R	R	R	R	R
<i>Salmonella typhi</i>	S	S	S	S	R	R
<i>Candida albicans</i>	S	S	S	R	S	S
<i>Candida krusei</i>	S	R	S	R	S	S
<i>Aspergillus fumigates</i>	R	R	R	R	R	S
<i>Aspergillus niger</i>	R	R	R	R	R	R

KEY: R = Resisted inhibitory effect of crude extract

S = Sensitive to the inhibitory effects of crude extract

Sp = Sparfloxacin

Fl = Fluconazole

Fu = Fulcin

Table 4.9: Zones of Inhibition of petroleum ether extract – TCPE I, II and III and control

Test organisms	I	II	III	Sp	Fl	Fu
MRSA	32	20	31	39	00	00
<i>Staphylococcus aureus</i>	35	21	30	35	00	00
<i>Streptococcus pyogenes</i>	31	00	30	41	00	00
<i>Escherichia coli</i>	27	22	25	30	00	00
<i>Salmonella typhi</i>	27	21	22	30	00	00
<i>Candida albicans</i>	27	20	26	00	29	27
<i>Candida krusei</i>	24	00	25	00	30	29

KEY:

Sp = Sparfloxacin

Fl = Fluconazole

Fu = Fulcin

Table 4.10: MIC and MBC/MFC of fractions of petroleum ether extract – TCPE I, TCPE II and TCPE III (mg/cm³)

Test organisms	MIC			MBC/MFC		
	I	II	III	I	II	III
MRSA	2.5	05	2.5	10	20	10
<i>Staphylococcus aureus</i>	2.5	05	2.5	05	20	10
<i>Streptococcus pyogenes</i>	2.5	-	2.5	10	-	20
<i>Escherichia coli</i>	05	05	05	20	20	20
<i>Salmonella typhi</i>	05	05	05	10	20	20
<i>Candida albicans</i>	05	05	05	20	20	20
<i>Candida krusei</i>	05	-	05	20	-	20

KEY: MIC = Minimum Inhibitory Concentration

MBC/MFC = Minimum Bactericidal / Fungicidal Concentration

Table 4.11: The Antimicrobial Screening of Isolated Compounds TC 1, TC 2 and the control

Test organisms	TC1	TC2	Sp	Cp	Te	Fl	Fu
MRSA	S	S	S	R	R	R	R
<i>Staphylococcus aureus</i>	S	S	S	S	R	R	R
<i>Streptococcus pyogenes</i>	R	R	S	S	R	R	R
<i>Escherichia coli</i>	S	S	S	S	S	R	R
<i>Pseudomonas aeruginosa</i>	R	R	R	S	S	R	R
<i>Salmonella typhi</i>	S	S	S	S	S	R	R
<i>Corynebacterium diphtheriae</i>	R	R	S	R	R	R	R
<i>Corynebacterium ulcerans</i>	R	R	R	S	S	R	R
<i>Bacillus subtilis</i>	S	S	S	S	S	R	R
<i>Listeria monocytogenes</i>	S	S	S	R	S	R	R
<i>Shigella dysenteriae</i>	S	S	S	S	S	R	R
<i>Campylobacter fetus</i>	S	S	R	S	R	R	R
<i>Klebsiella pneumoniae</i>	S	S	S	S	R	R	R
<i>Streptococcus pneumoniae</i>	S	S	S	S	R	R	R
<i>Vibrio cholerae</i>	R	R	S	S	S	R	R
<i>Candida albicans</i>	S	S	R	R	R	S	R
<i>Candida krusei</i>	S	S	R	R	R	S	R
<i>Helicobacter pylori</i>	S	S	R	R	S	R	R
<i>Aspergillus niger</i>	R	R	R	R	R	R	S
<i>Aspergillus fumigatus</i>	R	R	R	R	R	R	S

KEY: R = Resisted inhibitory effects of crude extracts
 S = Sensitive to inhibitory effects of crude extracts
 Sp = Sparfloxacin
 Te = Tetracycline
 Fl = Fluconazole
 Fu = Fulcin

Table 4.12: Zones of Inhibition of TC 1, TC 2 and the control (mm)

Test organisms	TC 1	TC 2	Sp	Cp	Tc	Fl	Fu
MRSA	27	26	35	00	00	00	00
<i>Staphylococcus aureus</i>	20	25	37	35	00	00	00
<i>Escherichia coli</i>	24	28	37	38	30	00	00
<i>Salmonella typhi</i>	22	29	30	40	00	00	00
<i>Bacillus subtilis</i>	32	30	42	40	35	00	00
<i>Listeria monocytogenes</i>	27	26	32	00	30	00	00
<i>Shigella dysenteriae</i>	35	32	40	44	37	00	00
<i>Campylobacter fetus</i>	28	27	42	32	00	00	00
<i>Klebsiella pneumoniae</i>	30	28	37	39	00	00	00
<i>Streptococcus pneumoniae</i>	20	22	32	35	00	00	00
<i>Candida albicans</i>	22	20	00	00	00	36	00
<i>Candida krusei</i>	20	23	00	00	00	34	00
<i>Helicobacter pylori</i>	21	24	00	00	34	00	00

KEY: Control (drugs)

Sp = Sparfloxacin

Cp = Ciprofloxacin

Te = Tetracycline

Fl = Fluconazole

Fu = Fulcin

Table 4.13: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal or Fungicidal Concentration (MBC/MFC) in mg/cm³

Test organisms	MIC		MBC/MFC	
	TC 1	TC 2	TC 1	TC 2
MRSA	6.25	12.5	25.5	25.0
<i>Staphylococcus aureus</i>	6.25	12.5	12.5	25.0
<i>Escherichia coli</i>	12.5	6.25	25.0	12.5
<i>Salmonella typhi</i>	12.5	6.25	50.0	12.5
<i>Bacillus subtilis</i>	6.25	6.25	6.25	6.25
<i>Listeria monocytogenes</i>	6.25	12.5	25.0	25.0
<i>Shigella dysenteriae</i>	6.25	6.25	12.5	6.25
<i>Campylobacter fetus</i>	6.25	6.25	6.25	25.0
<i>Klebsiella pneumoniae</i>	6.25	6.25	12.5	12.5
<i>Streptococcus pneumoniae</i>	12.5	12.5	50.0	50.0
<i>Candida albicans</i>	12.5	12.5	50.0	50.0
<i>Candida krusei</i>	12.5	12.5	50.0	25.0
<i>Helicobacter pylori</i>	12.5	12.5	50.0	25.0

4.6 Determination of the melting point of isolated compounds (TC 1 and TC 2)

The melting point was determined using Ernst Leitz Wetzlar melting point apparatus for TC 1, which occurred as white crystalline needle-like substance as 144-146 °C. The melting point of TC 2 was also determined as 217-273 °C. TC 2 occurred as white amorphous powder (Gohari *et al.*, 2009).

4.7 The IR Spectral of TC 1 (Stigmasterol) and TC 2 (Oleanolic acid)

The infra red spectral of the two isolated pure compounds (TC 1 and TC 2) were determined using Fourier Transform – Infra Red spectroscopy to give an idea of the functional groups present in the compounds (see table 4.14 and 4.17). The results obtained were compared with cited literatures.

Table 4.14: Infrared spectrum result of TC 1 (Stigmasterol)

Frequency cm^{-1}	Bond	Functional group
3426.7	O-H stretching vibration , H- bonded	Alcohol
2926.1	CH_3 stretching vibration	Alkane
1632.8	C=C	Alkene
1458.2	CH_2	Alkane
1051.2	C-O stretching	Alcohol
434	CH_2	Alkane

(Koay *et al*, 2011, Rajput and Rajput, 2012)

Table 4.15: Comparing the ^{13}C NMR and ^1H NMR spectra data of TC 1(Stigmasterol) with that obtained from the cited literature.

Position	^{13}C (TC 1)	^{13}C References	^1H (TC 1)	^1H (References)
1	37.20	37.20	1.86	1.86
2	31.70	31.60	1.82	1.84
3	71.80	71.80	3.53	3.52
4	42.20	42.27		
5	140.70	140.70	5.35	5.35
6	121.70	121.70	1.96	1.96
7	31.70	31.70	1.47	1.47
8	31.80	31.80	1.48	1.50
9	50.10	50.10	0.89	0.93
10	36.50	36.50	-	-
11	21.04	21.06	1.50	1.50
12	39.70	39.70	2.00	2.00
13	42.30	42.19		-
14	56.80	56.80	1.00	1.04
15	24.30	24.40	1.50	1.56
16	28.80	28.90	1.72	1.72
17	56.00	55.90		
18	12.05	12.04	0.70	0.70
19	19.40	19.40	1.01	1.01
20	40.40	40.40	2.04	2.06
21	21.10	21.09	1.22	1.22
22	138.30	138.30	5.15	5.15
23	129.30	129.20	5.02	5.02
24	12.10	12.10	1.53	1.53
25	51.20	51.20	1.44	1.44
26	31.70	31.90	0.90	0.92
27	21.10	21.10	0.81	0.81
28	25.40	25.40	1.56	1.56
29	12.20	12.20	0.83	0.83

(Ragasa and Cayne 2004; Koay *et al.*, 2013)

TC1- Stigmasterol

Table 4.16: Mass spectrum readings of TC 1(Stigmasterol)

Molecular Ion peak (m/z)	Abundance (%)
412	15
394	20
351	18
300	10
271	25
255	60
229	10
213	20
159	40
145	40
107	40
83	90
55	95
41	50

Table 4.17: Infra-red spectrum result of TC 2 (oleanolic acid)

Frequency cm^{-1}	Bond	Functional group
3429.6	O -H stretching vibration, H - bonded	Alcohol
2932.9	CH_3 stretching vibration	Alkane
1642.4	C=O stretching vibration	Carboxylic acid
1454.4	CH_2 , C-H bending vibration	Alkane
1381.1	CH_3 bending vibration	Alkane
1039.7	C-O stretching vibration	Carboxylic acid
448.5	C-C bending vibration	Alkane

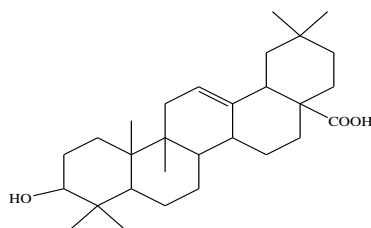
(Silverstein *et al.*, 2005, Habila *et al.*, 2013)

Table 4.18: Comparing the ^{13}C NMR and ^1H NMR spectral data of TC 2 (Oleanolic acid) with that obtained from the cited literatures.

Carbon	δC	δC (Reference)	δH	δH (Reference)
1	39.00	39.00	1.50	1.57
2	28.00	28.10	1.82	1.82
3	79.10	79.20	3.25	3.44
4	38.80	38.40	2.33	-
5	55.60	55.50	0.88	0.88
6	18.80	18.80	1.55	1.58
7	33.30	33.40	1.40	1.39
8	39.60	39.80		
9	48.00	48.20	1.40	1.72
10	37.60	37.40		
11	21.30	21.80		
12	116.90	116.60	5.40	5.49
13	158.10	158.20		
14	41.40	42.20		
15	28.20	28.40		1.22
16	23.60	23.80		2.12
17	46.60	46.70		
18	42.00	42.10	3.30	3.30
19	46.40	46.60	1.32	1.32
20	31.00	31.00	-	-
21	34.20	34.30		
22	33.20	33.20	0.89	0.77
23	28.80	28.80	0.76	0.77
24	26.50	26.60	0.78	0.80
25	15.40	15.60	0.90	0.92
26	17.50	17.50	1.00	1.04
27	27.10	26.20	1.13	1.30
28	178.90	180.00		
29	33.30	33.40		
30	23.70	23.80	0.96	

(Seebacher *et al.*, 2003; Gohari *et al.*, 2009, Uddin *et al.*, 2011)

TC2 – Oleanolic acid



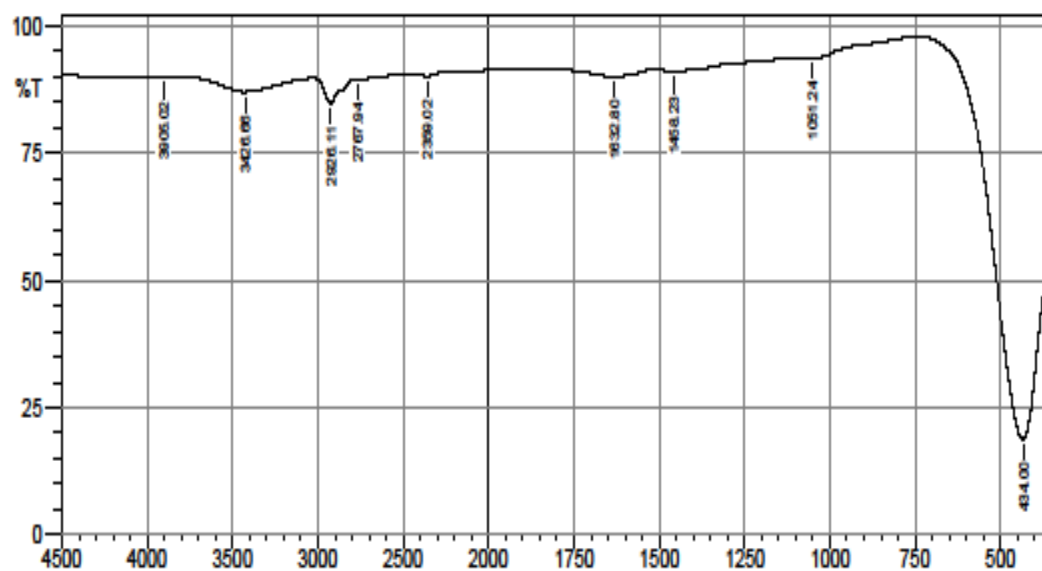


Figure 4.1: FT-IR spectrum of TC 1 (Stigmasterol)

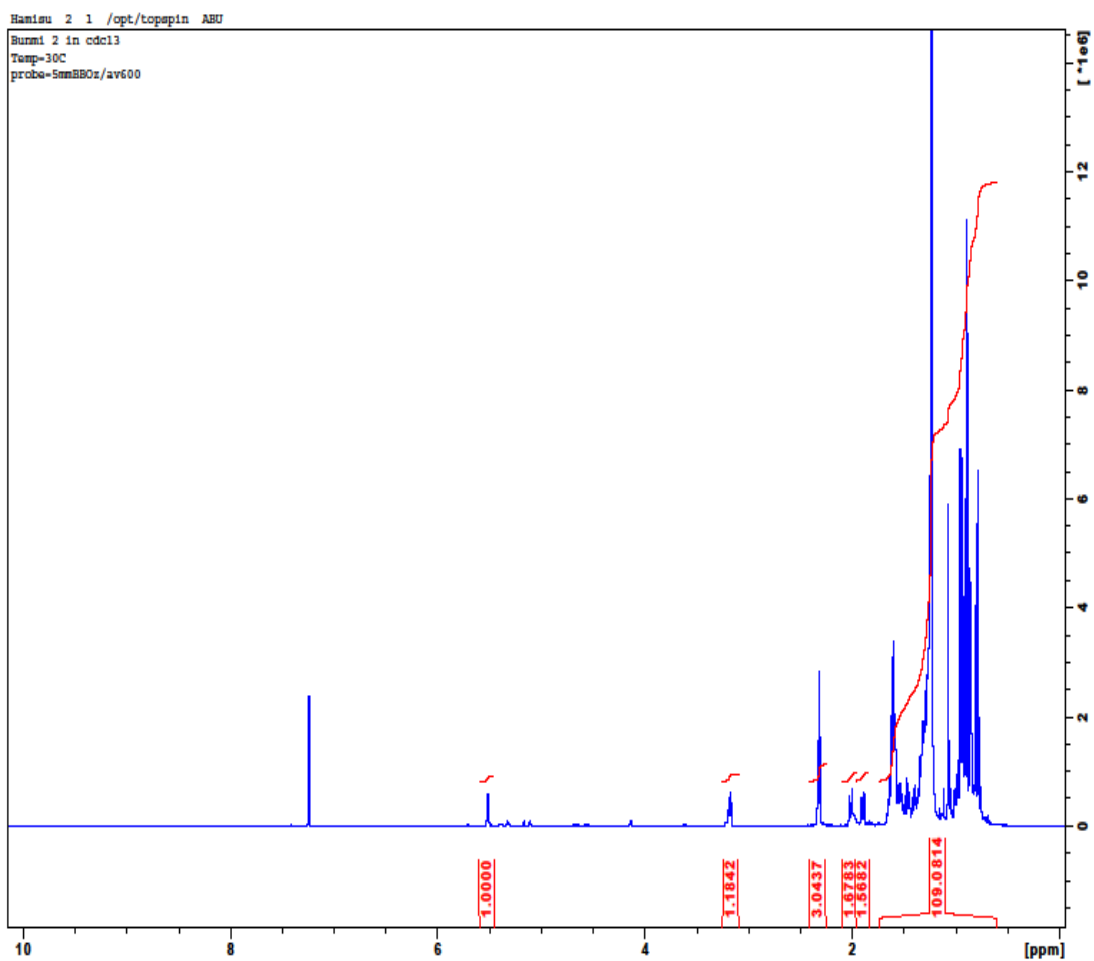


Figure 4.2: ^1H NMR of TC 1 (stigmasterol)

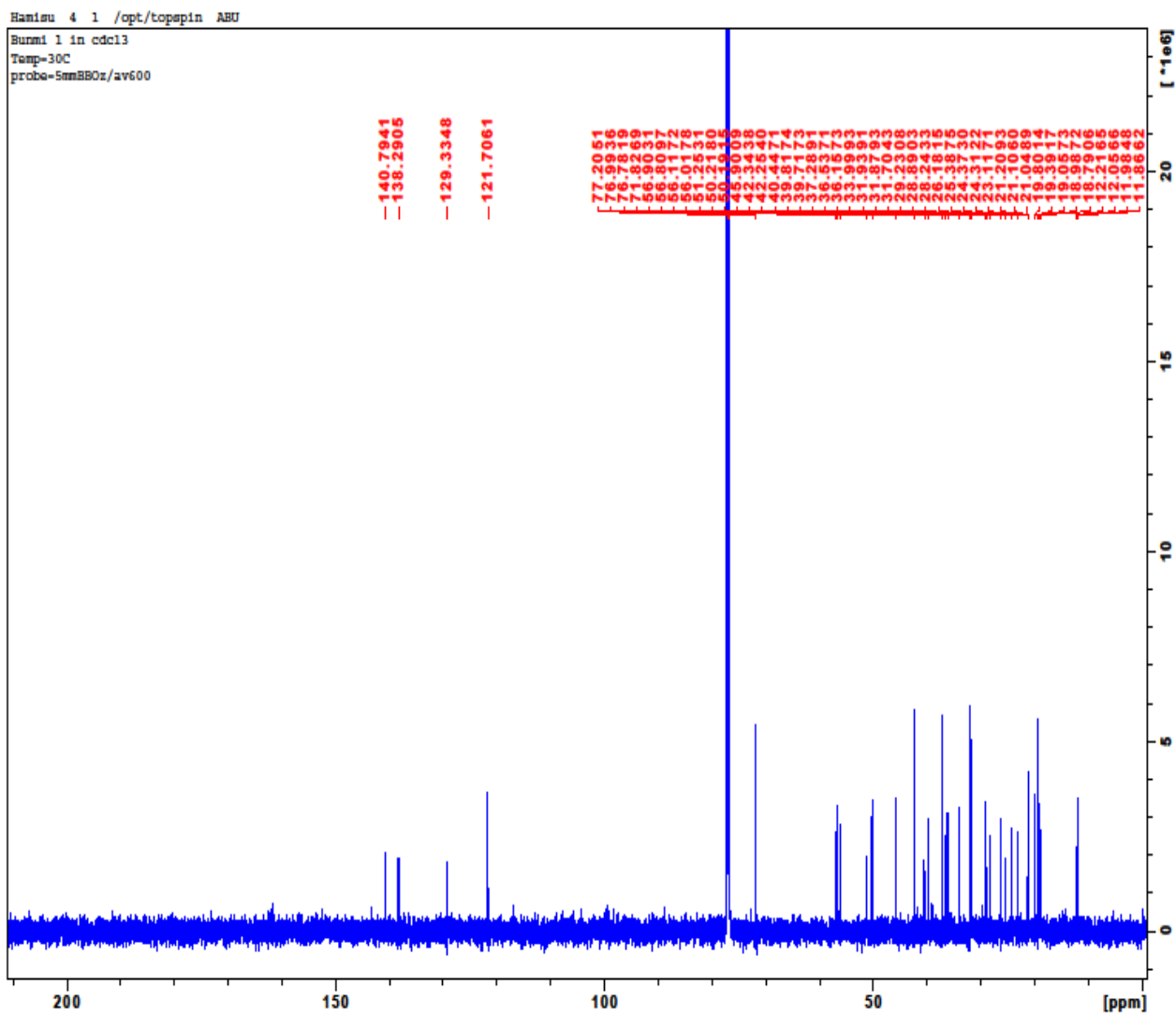


Figure 4.3: ^{13}C NMR of TC 1 (Stigmasterol)

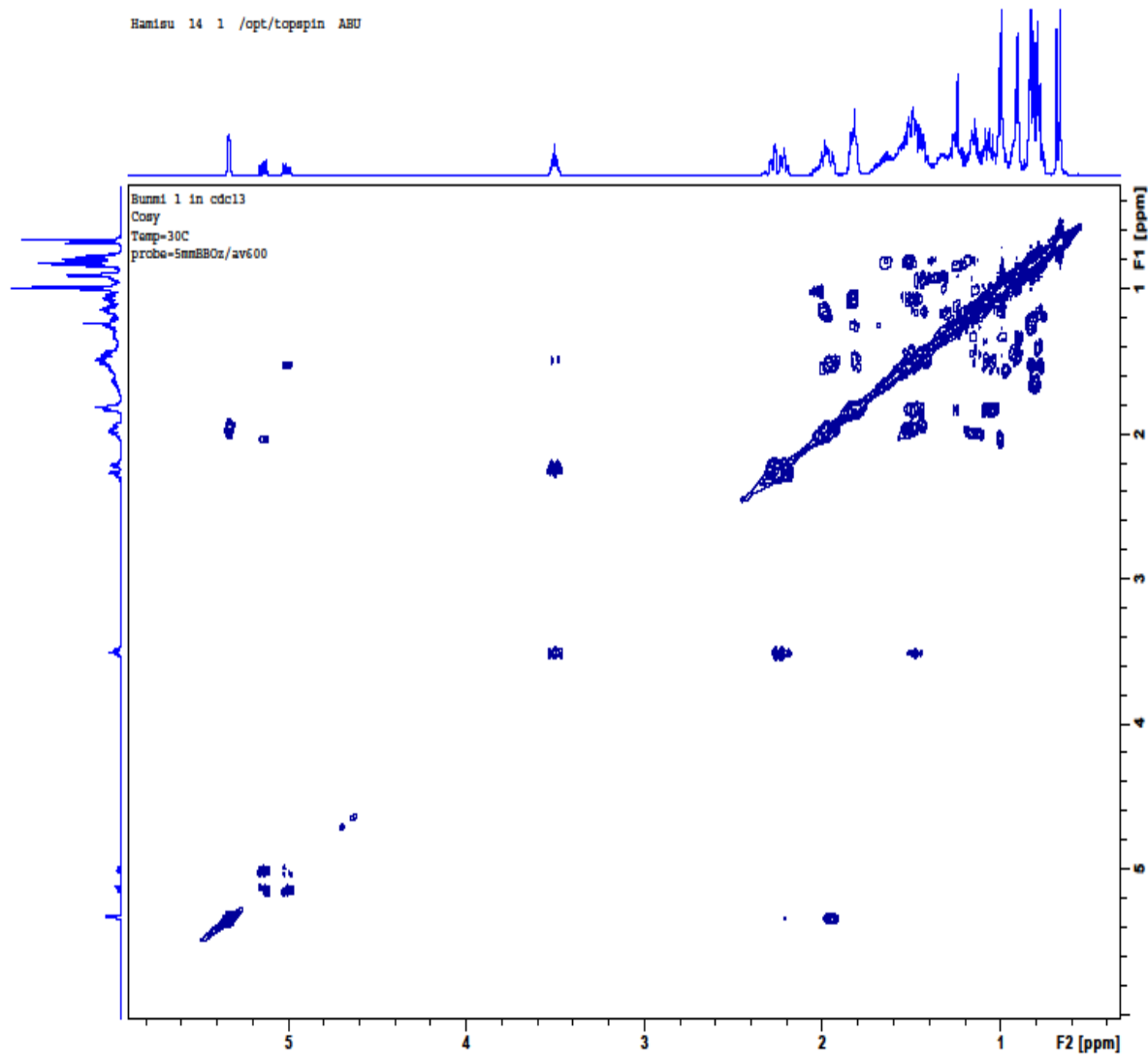


Figure 4.4: COSY of TC 1 (Stigmasterol)

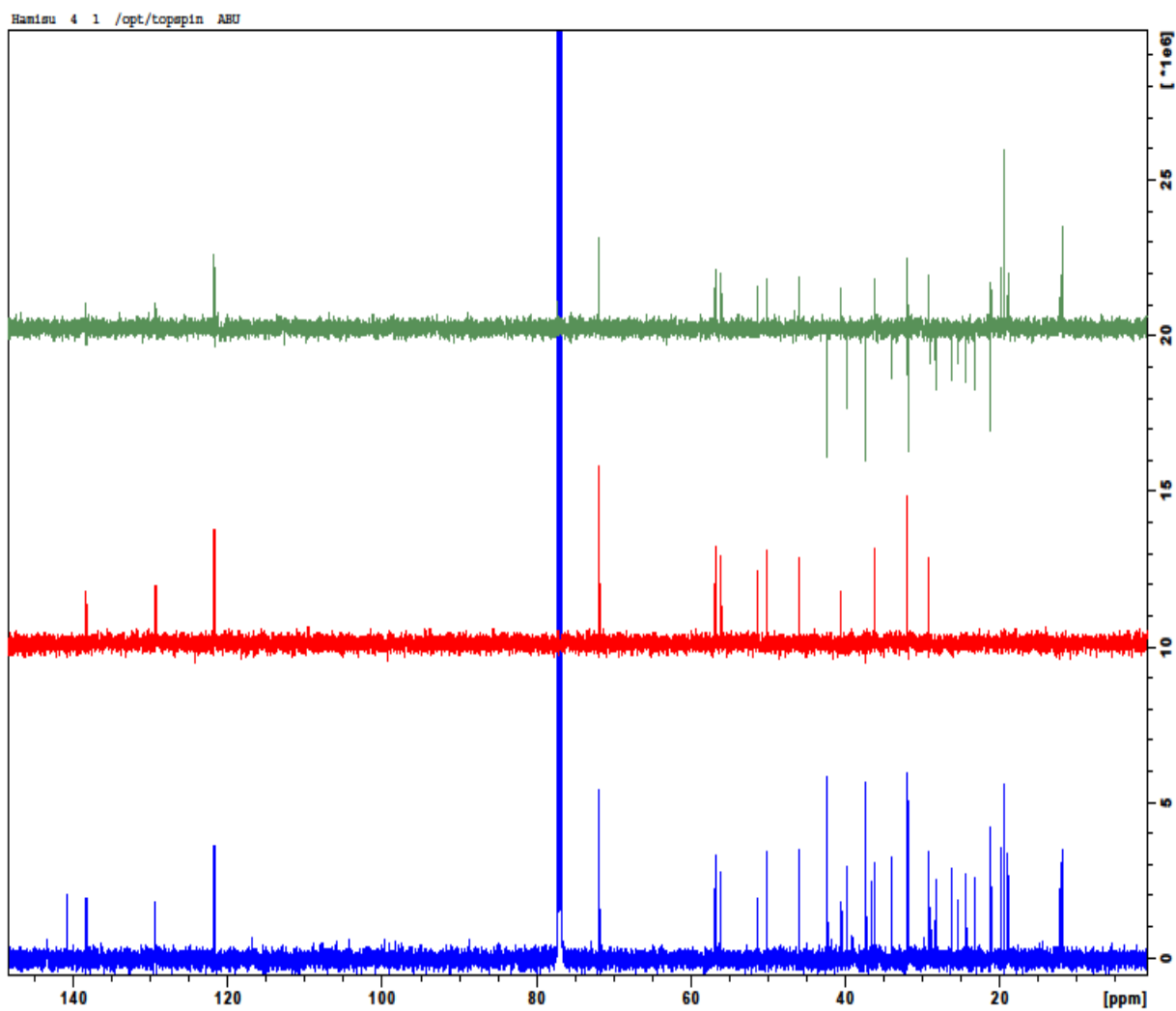


Figure 4.5: DEPT of TC 1 (Stigmasterol)

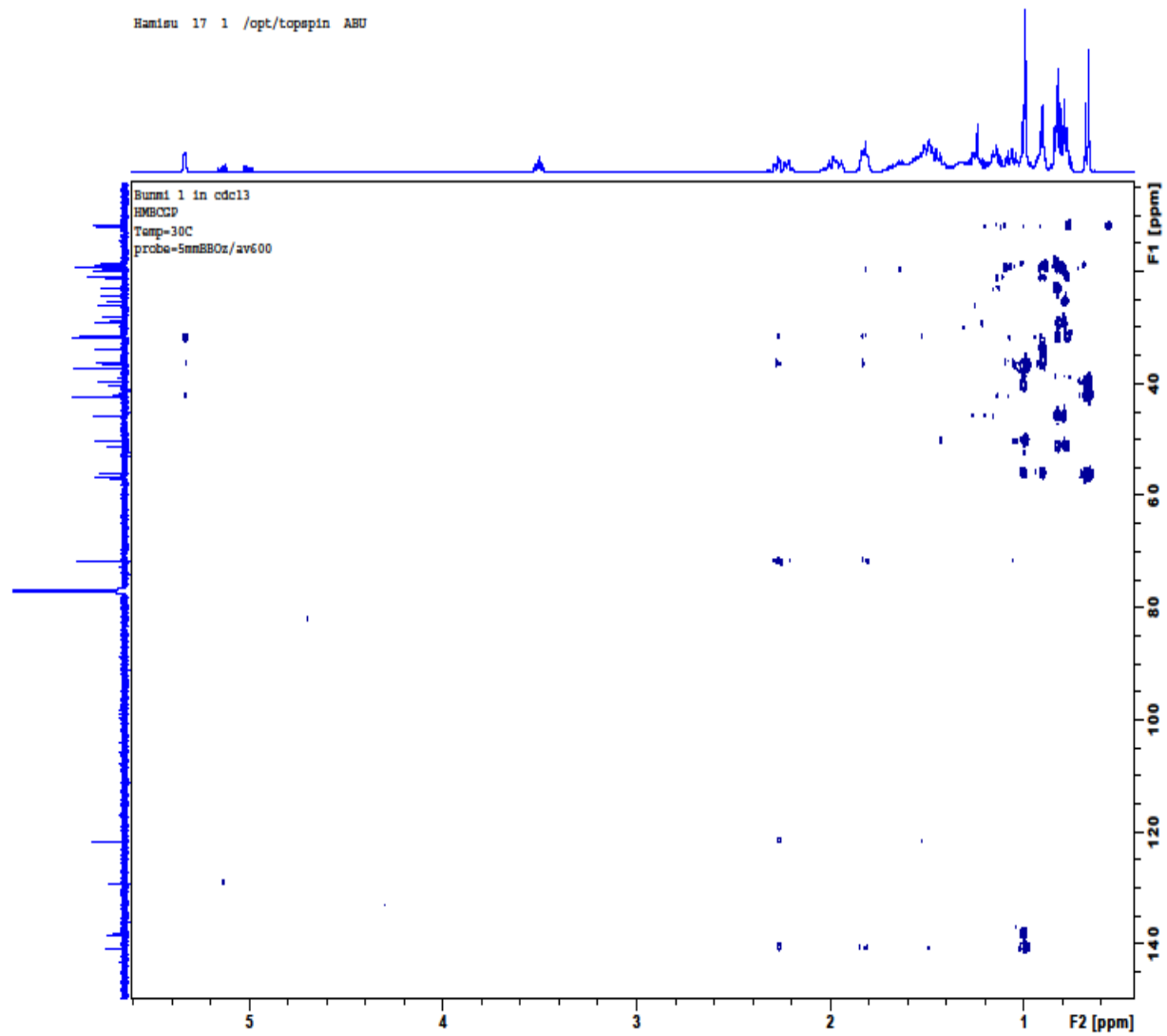


Figure 4.6: HMBC of TC 1 (stigmasterol)

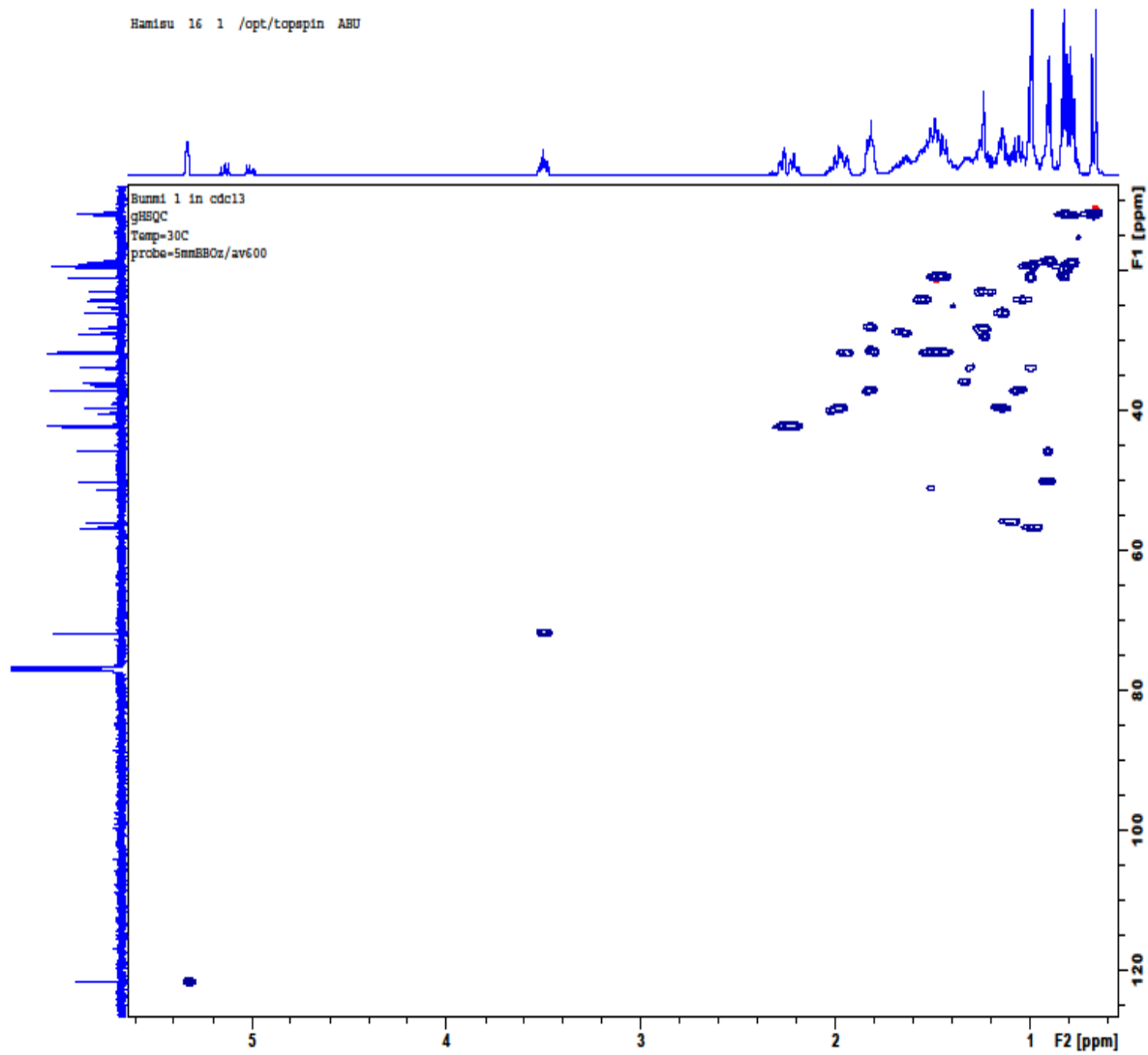


Figure 4.7: HSQC of TC 1 (Stigmasterol)

Hamisu 15 1 /opt/topspin ABU

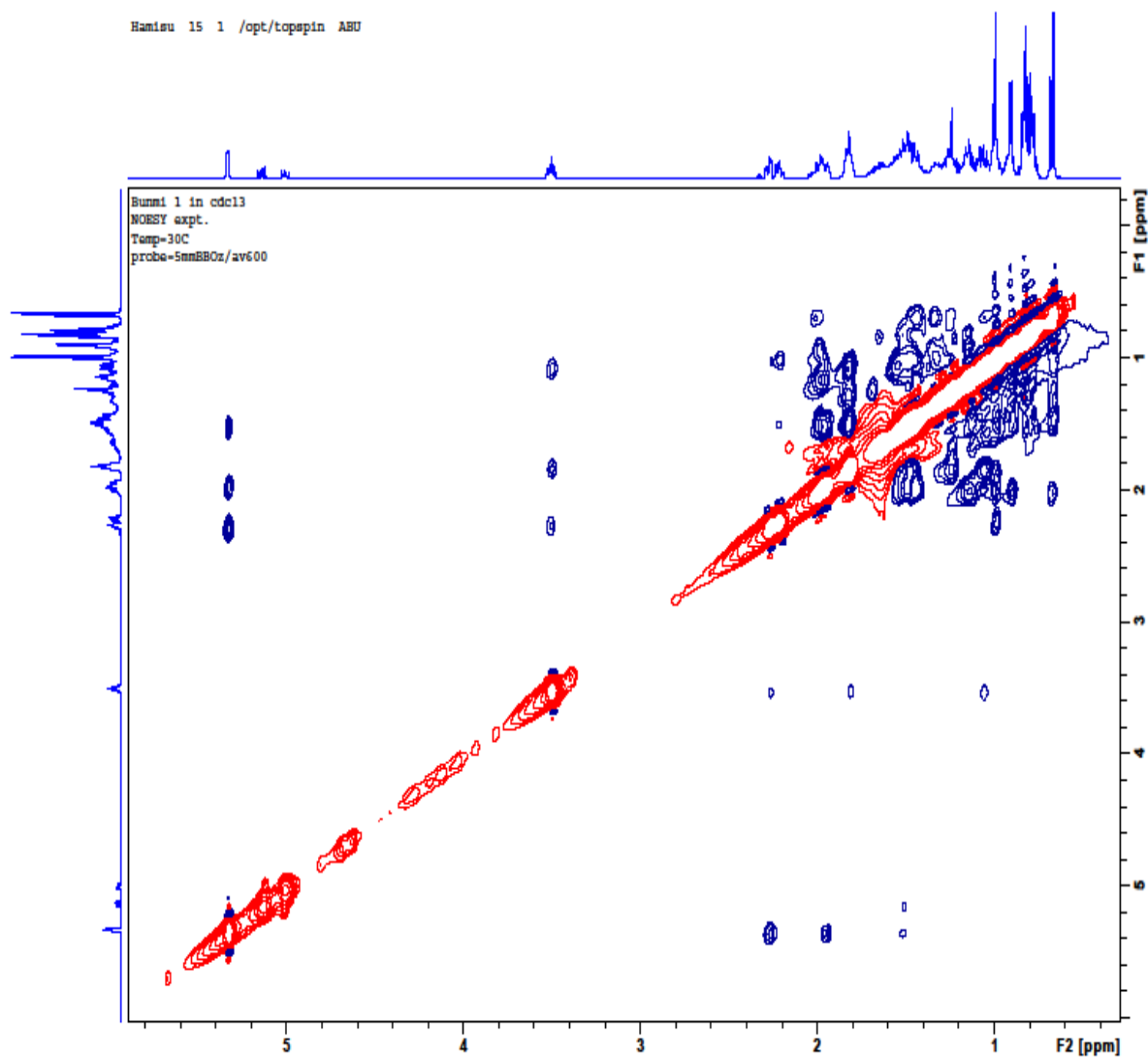


Figure 4.8: NOESY of TC 1 (Stigmasterol)

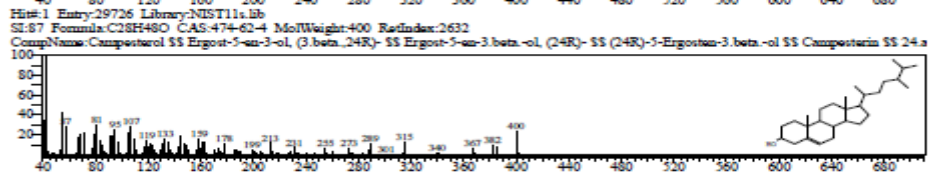
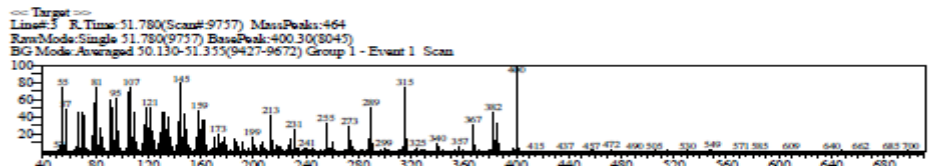
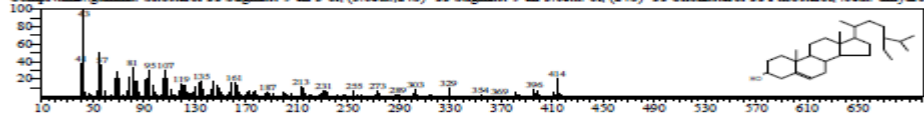
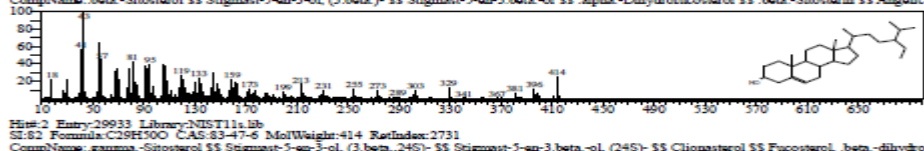
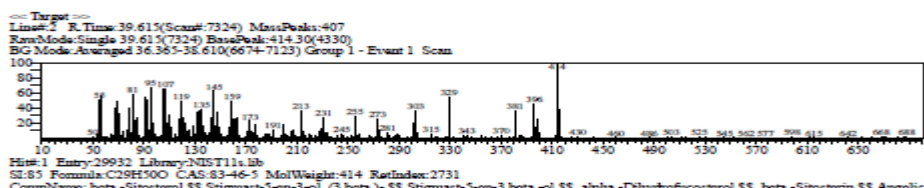
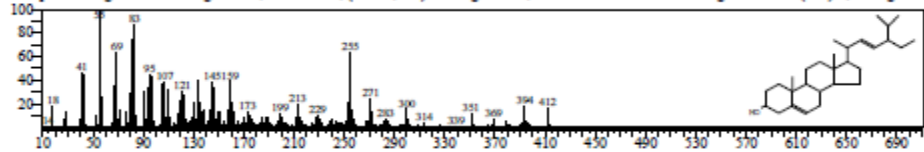
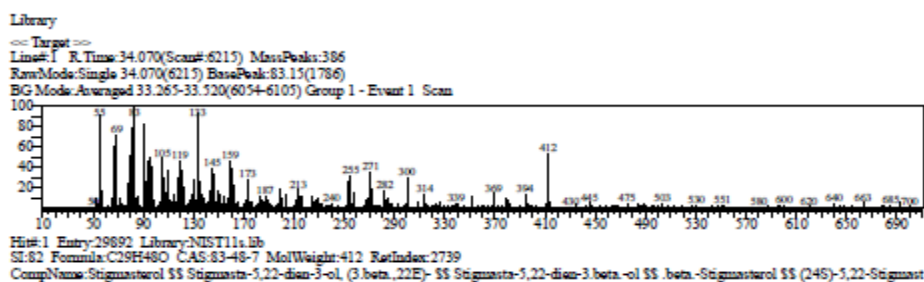
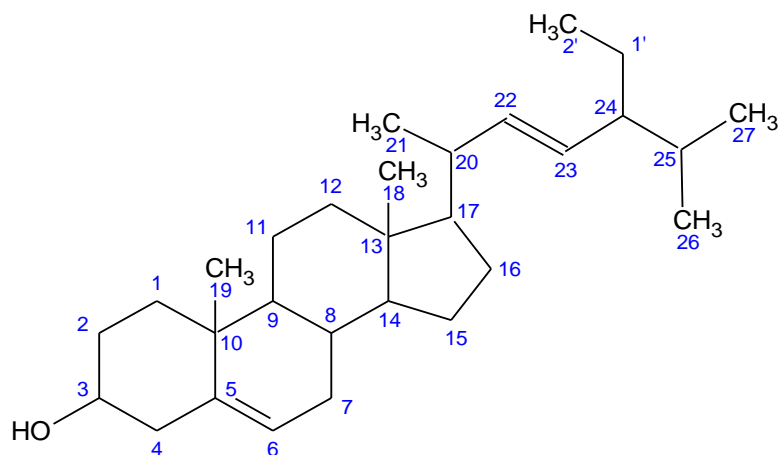


Figure 4.9: MS spectral of TC 1 (stigmaterol)



(XVI)

TC 1

3 β , 22E-Sigmasta-5, 22-dien-3-ol (Stigmasterol)

Molecular formula: C₂₉H₄₈O
Molecular weight: 412.7 g/mol
Melting point: 144-146 °C.

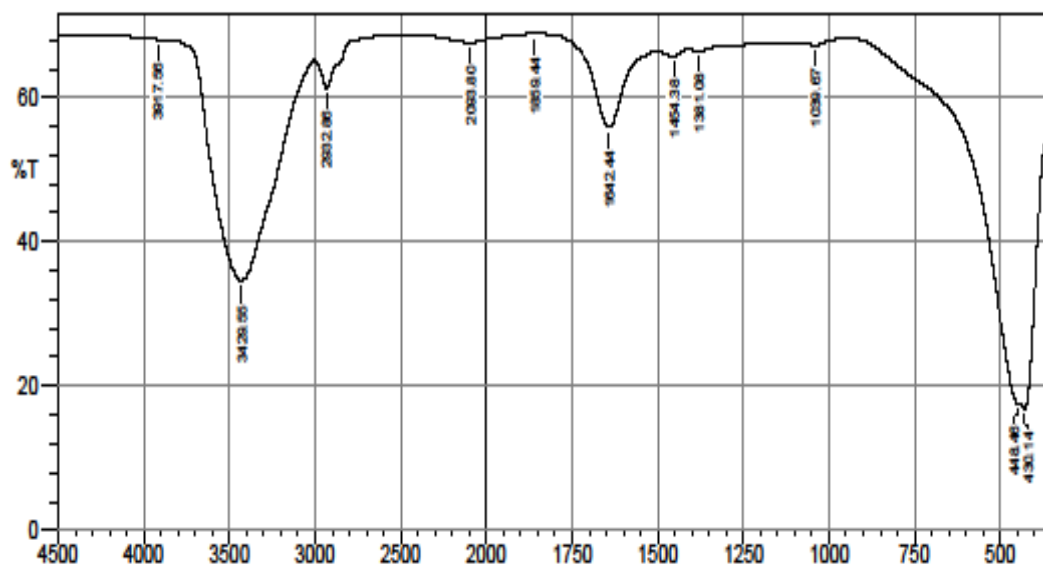


Figure 4.11: FT-IR of TC 2 (Oleanolic acid)

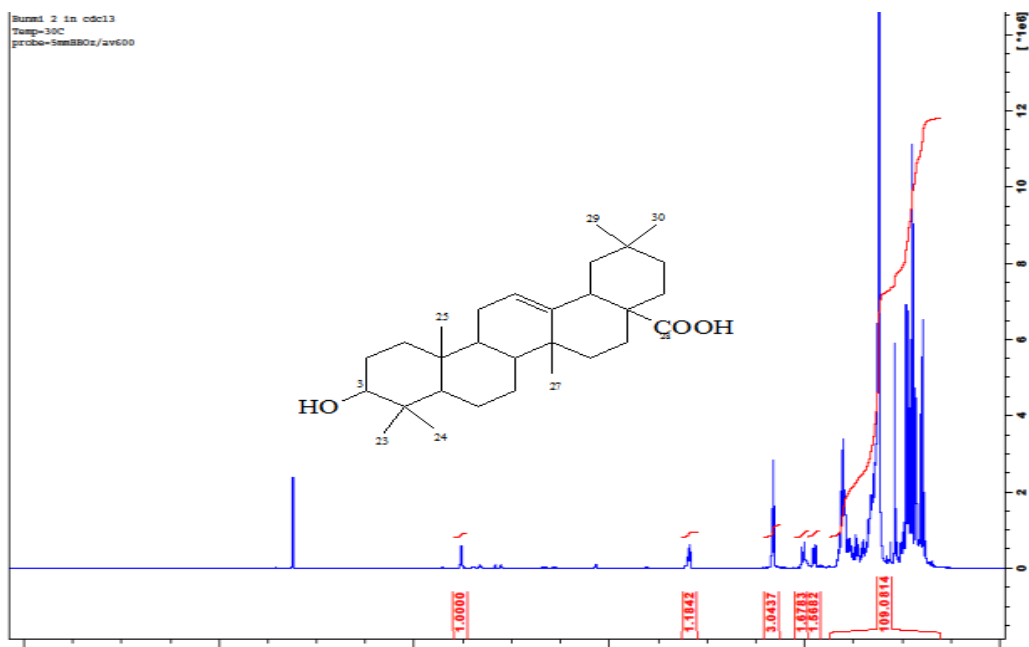


Figure 4.12: ^1H NMR of TC 2 (Oleanolic acid)

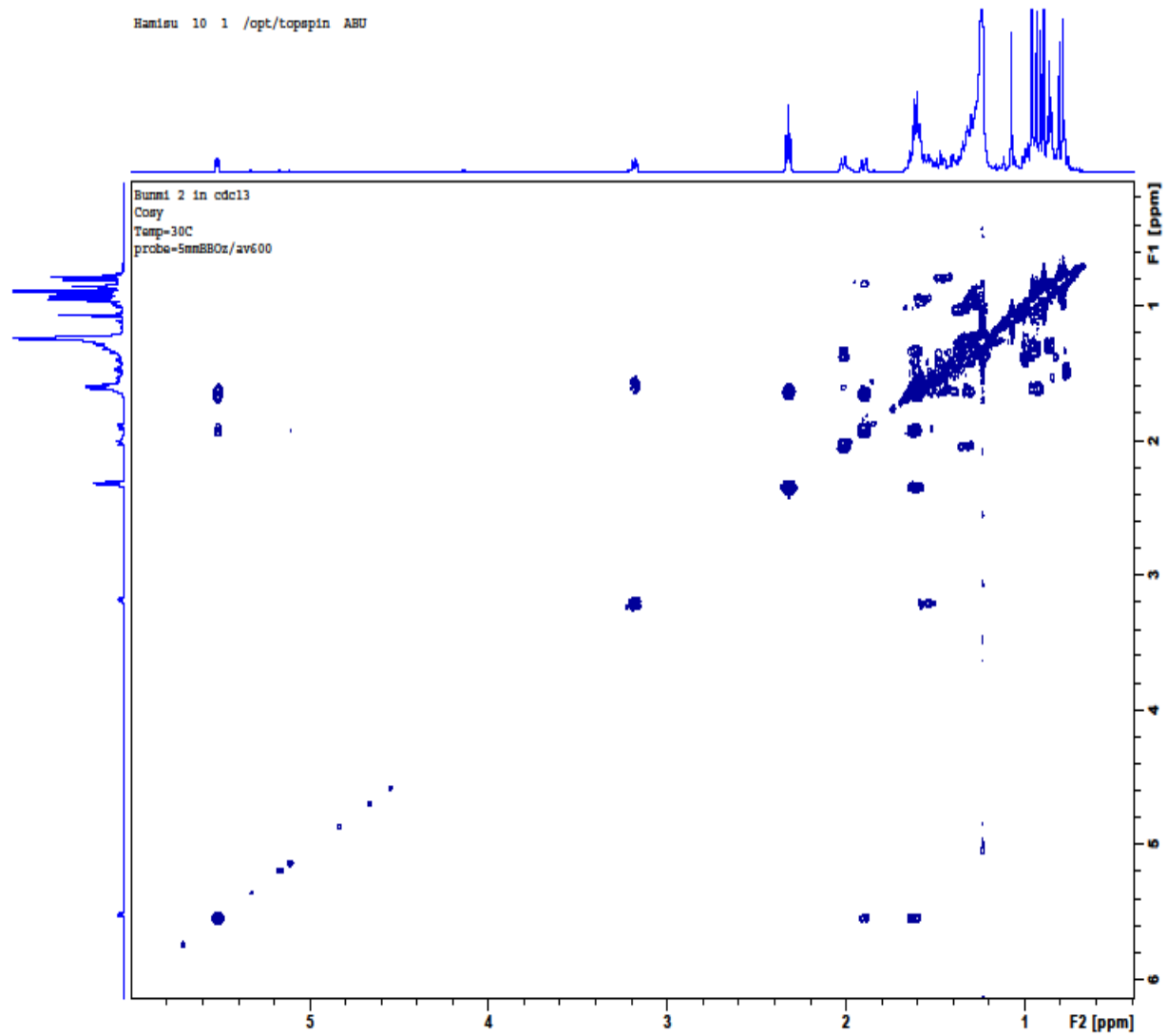


Figure 4.14: COSY of TC 2 (Oleanolic acid)

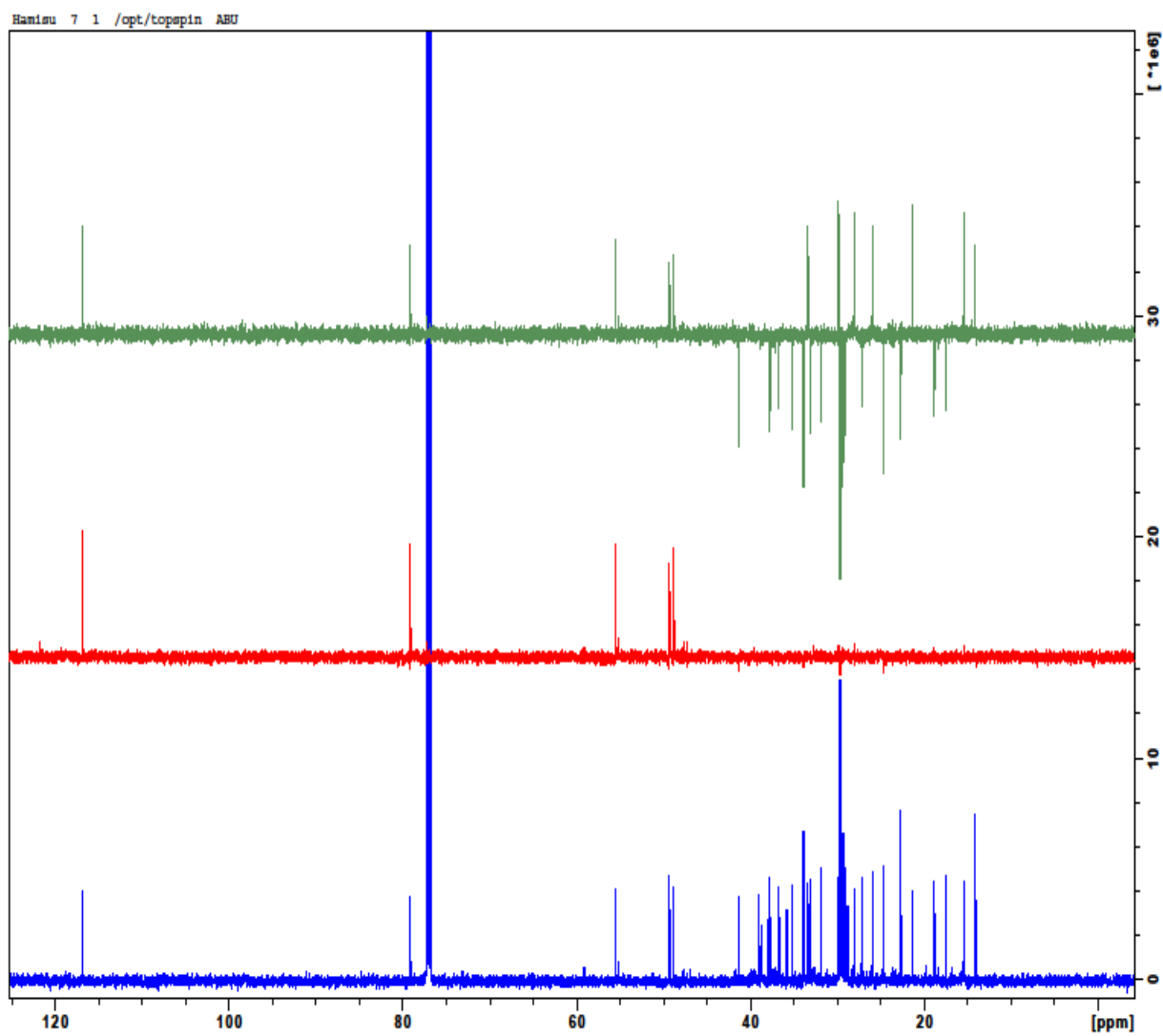


Figure 4.15: DEPT of TC 2 (Oleanolic acid)

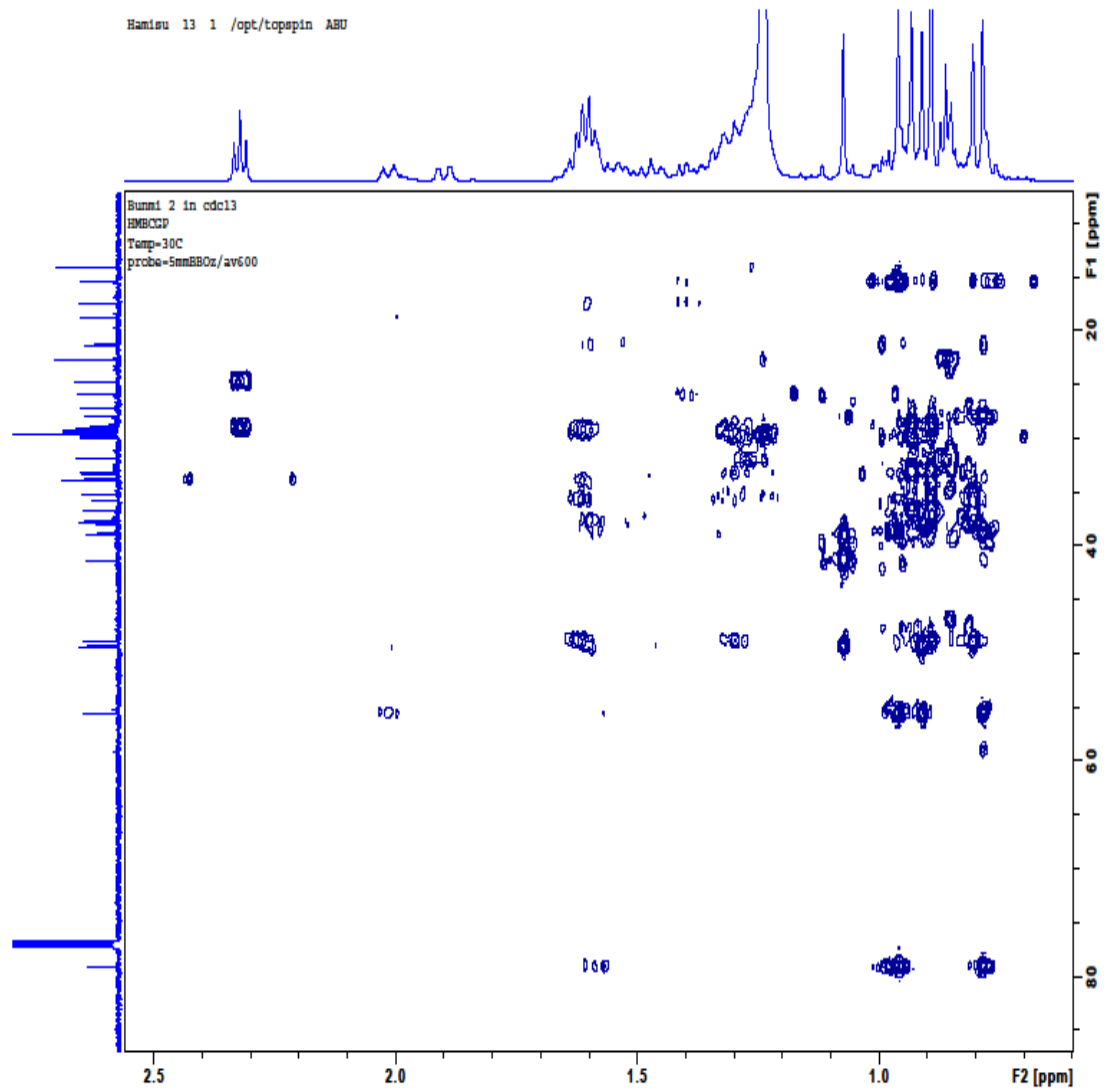


Figure 4.16: HMBC of TC 2 (oleanolic acid)

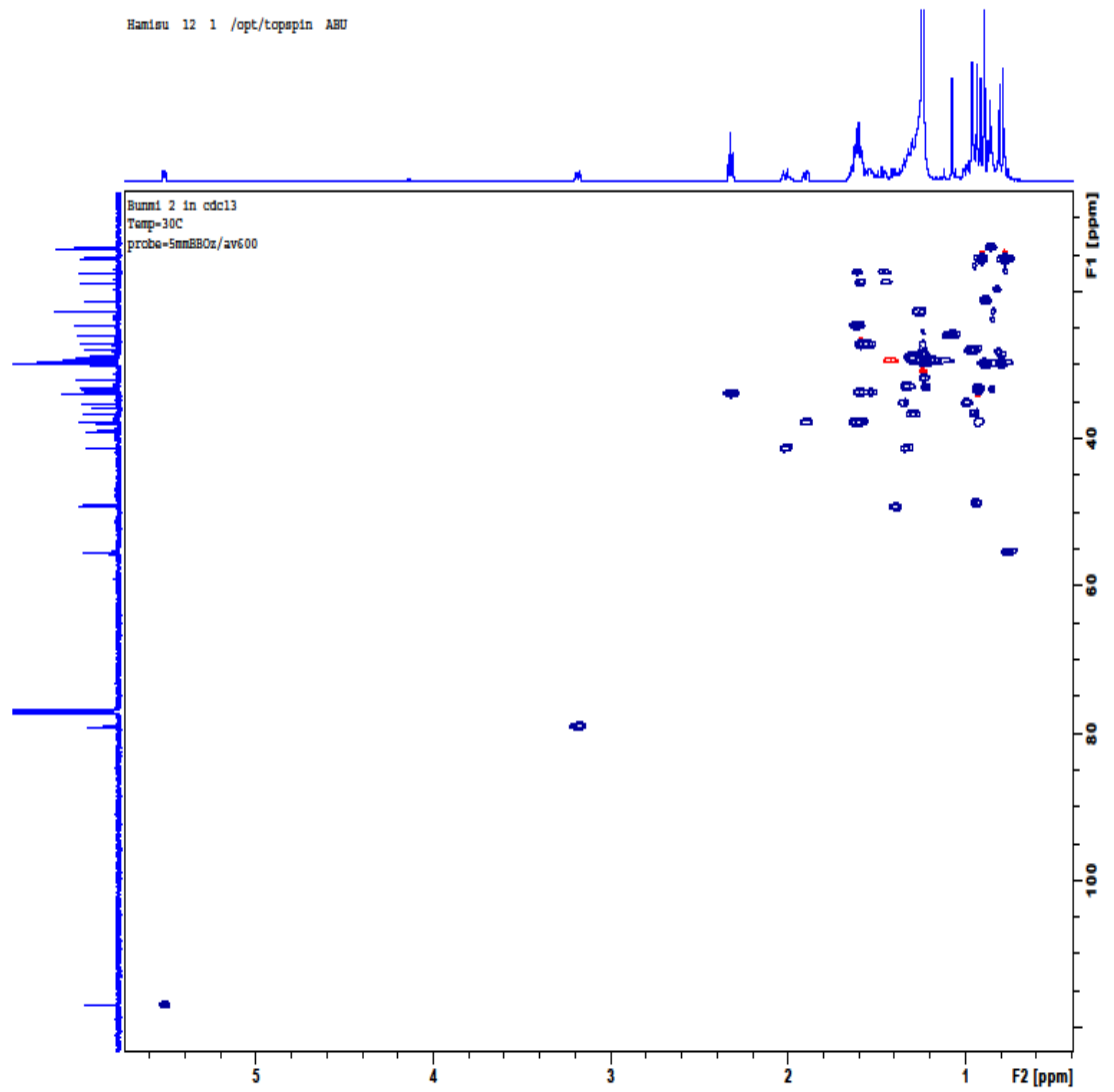


Figure 4.17: HSQC of TC 2 (Oleanolic acid)

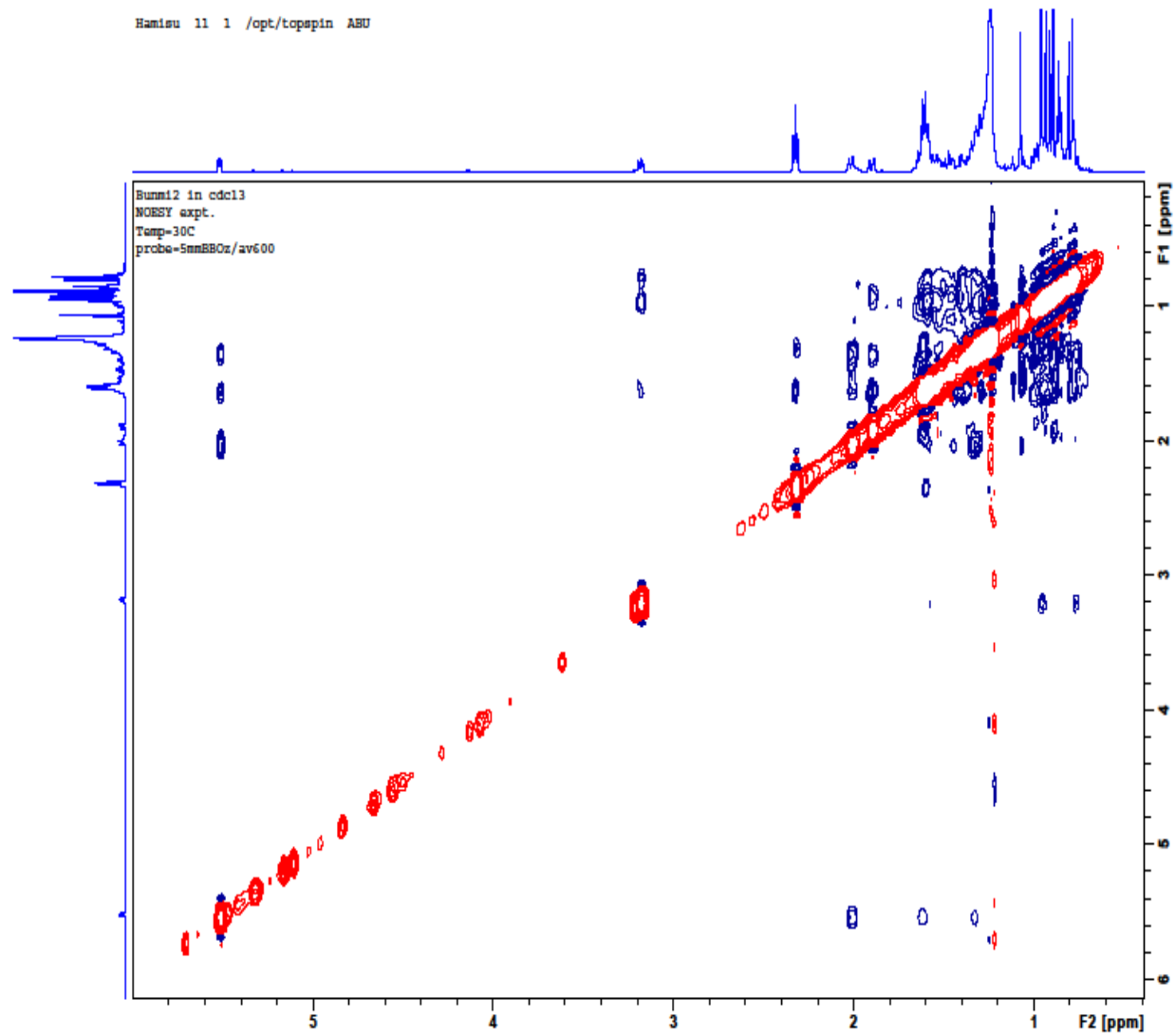
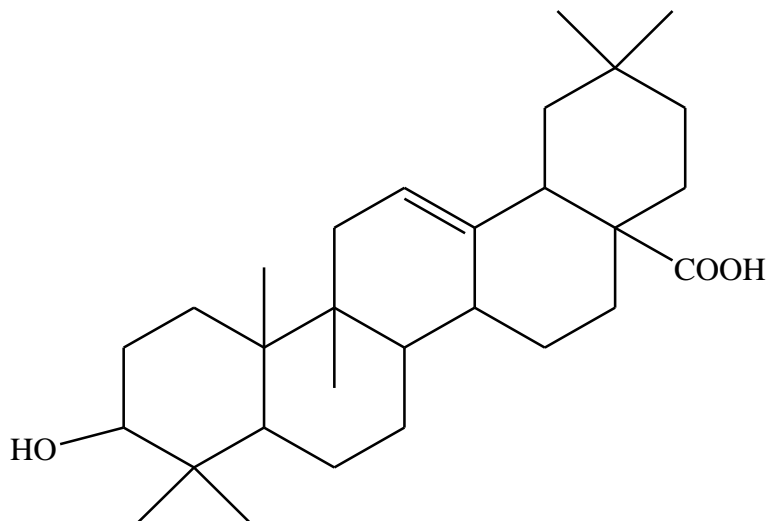


Figure 4.18: NOESY of TC 2 (Oleanolic acid)



(XVII)

TC 2

3β-hydroxyolean-12-en-28-oic acid (Oleanolic acid)

Molecular formula: $C_{30}H_{48}O_3$
Molecularweight: 456.7 g/mol
Meltingpoint: 271-273 °C.

CHAPTER FIVE

DISCUSSIONS

5.1 Extraction

The extraction of any plant material with an organic solvent or even water will yield an extract of different compounds, such as insoluble proteins, fats, alkaloids, glycosides, acids, tannins, pigments, etc (Cannel 1998). The extraction using a Soxhlet apparatus was carried out using four (4) solvents in order of increasing polarity (see table 4.1). Petroleum ether is used majorly as a non-polar solvent, it defatted the plant material, removing fatty acids, oils, waxes etc and gave a yield of 6.27 %. Methanol, the most polar was expected to efficiently penetrate cell membranes; permitting the extraction of high amount of endocellular compounds gave the highest yield of 9.20 %. Ethyl acetate gave a yield of 2.29 % and extraction with chloroform gave a yield of 1.45 %. It is expected that different compounds are extracted into each of the crude extract based on their relative solubilities in the different solvents used (Silva *et al.*, 1998; Cannel 1998).

5.2 Elemental Analysis

In order to investigate the particular type of element or mineral present in the leaves of *T. conophorum* as well as the quantity, elemental analysis was carried out. In southern Nigeria ethnomedicine, the leaves of *T. conophorum* are used to treat dysentery, improve fertility in males and considered to be a headache cure (Burkill 1985). On these bases, the leaves were analyzed by Instrumental Neutron Activation Analysis (INAA) to determine the presence and concentration of elements that play vital roles in ethnomedical practices or their absence otherwise.

The INAA conducted entails the analysis for thirty one (31) elements. Out of which fourteen (14) elements were found to be present in *Tetracarpidium conophorum* leaves and their approximate quantities. The results are depicted in table 4.3.

The analysis revealed the presence of macro elements such as Cl, Ca, Mg, Na, and K, as well as the presence of trace elements (also called micro nutrients) such as Mn, and Co. In *T. conophorum* leaves, some elements are present that play essential roles in the total wellbeing of a man. These elements are in *T. conophorum* leaves in quantities higher than the Recommended Dietary Allowance (RDA) values such as Mg (4756 mg), Ca (262 mg), Mn (262 mg), K (19390 mg), Zn (24.3 mg) and Co (0.11 mg). Of great importance are elements like Ca, Zn and Co with vital roles such as being component of the bone and cartilages, aids sexual maturity and essential for red blood cell formation respectively. Elements like barium, lanthanum, samarium, bromine, strontium and aluminum which have no established or accepted biological roles and therefore they are non-essential for the human body. They are absent in the leaves.

The consumption of *Tetracarpidium conophorum* leaves is recommended. Therefore the leaves may be playing vital biological roles in the human body as acclaimed in the ethno medicinal practices (FAO 1994; WHO 2014).

5.3 Proximate Analysis

Proximate analysis entails the analysis of biological materials as a decomposition of a human consumable good into its major constituents (FAO, 1994).

Proximate analysis is expected to always add up to 100 % and this displays the resolution of the chemical test. Looking at table 4.2 items I to V, added up to 100 %.

This means that *T. conophorum* leaves have the five major constituents expected for human consumption. From literature, the leaves and young shoots are edible and are eaten, often with rice (Burkill, 1985). The analysis reveals crude ether (1.89 %) which is favorably low, crude protein (17.68 %), nitrogen free extract (51.52%) and crude fiber (15.53 %). And this analysis justifies the consumption of *T. conophorum* leaves and they can be added to some food products in our markets to boost the protein and fibre content. The moisture content is 8.14 % as the dry matter is 91.86%.

5.4 Biological Functions of the Elements Present in the leaves of *Tetracarpidium conophorum* (Elemental Analysis)

Thirteen elements were discovered by the method of INAA conducted on the leaves of *Tetracarpidium conophorum* (see table 4.3). In the human body are macro and micro nutrients. The ones present in the leaves of *Tetracarpidium conophorum* are Ca, K, Na, Cl, and Mg (as macro nutrients) while Co and Mn are micro nutrients. Bromine, lanthanum, samarium and barium have no established biological roles in the human body and their presence constitute no hazardous or adverse effects.

The various biological functions of the ten elements found in the leaves of *Tetracarpidium conophorum* are as follows: Mg is said to help keep the heart beat steady, regulates the blood glucose levels as well as helps to maintain normal nerve and muscle function(RDA 420 mg); Al maybe involued in the action of enzymes such as succinic dehydrogenase and aminolevulinate dehydrase (involved in porphyrin synthesis); Ca is very essential for nerve transmission, muscle contraction, glandular secretions, contraction and dilation of the blood vessels, it is an essential component of bone and cartilage, an activator for key enzymes and essential for normal blood clotting; Cl is said to play a specific role in the transport of oxygen and

carbon(iv)oxide in the blood (RDA 2300 mg); Sr enhances the utilization of calcium in the human body; Mn is an essential element for bone formation and is needed for regeneration of the red blood cells, carbohydrate metabolism and reproductive cycle (RDA 2.3 mg); Na is connected with the regulation of osmotic pressure and maintenance of acid-base balance (RDA 1500 mg); K is required for glycogen and protein syntheses as well as for glucose metabolic breakdown (RDA 4700 mg); Co is an integral component of cyanocobalamin (B₁₂) and as such is essential for the red blood cell formation (RDA 5.8 ug) and Zn aids sexual maturation, immune system ,normal growth, production of sperms and DNA regulation , Zn has a function in normal fetal development and plays a vital role in lipid, protein and carbohydrate metabolism (RDA 11 mg).(Schirber, 2009).

5.5 Preliminary Phytochemical Screening of the Crude Extracts of Leaves of *Tetracarpidium conophorum*

The phytochemical screening of the four crude extracts was carried out to reveal the presence or absence of active principles (natural products). The results are depicted in table 4.4. The general test for carbohydrates was positive for all the four extracts as well as specific test for ketones, pentoses, starch, free and reducing sugars, agar and tragacanth but absent for monosaccharides.

The test for the presence of glycosides using Fehling's solutions A and B confirmed the presence of reducing sugars as a result of the glycosides, presence of steroidal aglycone, saponin glycosides and cardiac glycoside in all the four crude extracts. Absence of anthracene derivatives in all the crude extracts (Silva *et al.*, 1998). Test for presence of alkaloids were found to be positive with Mayer's reagent, Dragendorff's reagent and Wagner's reagent (Evans 2009; Silva *et al.*, 1998).

Presence of flavonoids were confirmed using Shinoda test which gave an orange colour in all the extracts. This orange colour in all the four extracts is indicative of the presence of flavonoids, flavones and or xanthenes. Sulphuric acid test gave a red colour at the interphase, this is indicative of the presence of chalcones and aurones. Ferric chloride test gave a turbid greenish- brownish solution in all the extracts but the colour was more intensive in the methanol extract. This should be expected with methanol extract being the most polar and it was expected to penetrate the plant material the most. The presence of chlorogenic acid as a residue in the crude extract was confirmed positive. This was also the test for the presence of plant phenols which was positive by the formation of a pale yellow colour in the extracts and this is indicative of flavonol glycoside (Evans 2009; Silva *et al.*, 1998).

The presence of sterols was confirmed by carrying out Liebermann- Buchard test which gave a blue green colouration in all the extracts. By using Salkowski reaction there was the formation of a reddish-brown ring at the interphase. This is indicative of the presence of sterols and methylated sterols (Trease and Evans, 2009; Silva *et al.*, 1998). The presence of proteins was confirmed positive using Millon's reagent, Xanthoprotein test and reaction with solutions of picric and tannic acids. The test was negative for Biuret test. Substances which have two – CONH groups joined to the same nitrogen atom usually respond to Biuret test. The result reveals absence of the –CONH groups joined to the same nitrogen atom as well as absence of peptides (Evans 2009).

In general, carbohydrates have been established to show pharmacological activities such as anti-tumour and anti-inflammatory agents. Chlorogenic acid is an example of pseudo tannin which acts as anti - diarrhea. Flavonoids are said to have

anti-inflammatory and anti-allergic effects as well as been vasoprotective, antiplasmodic, anti-tumor, anti-bacterial and anti-fungal properties. Related flavonoids and glycosides are known to act as sex hormones that are phyto-estrogens having positive effects in the prevention of cancer, heart disease and post-menopausal symptoms. Some alkaloids are known to have anti-cancer, anti-aging and antiviral properties (Evans 2009).

The presence of these active constituents in the leaves of *Tetracarpidium conophorum* justifies the consumption of the fresh leaves as food, its use in ethno medicinal practices, preventive medicine as well as in the development of natural antioxidants from plants (Burkill 1994; Malu 2009).

5.6 Antimicrobial Screening

Microorganisms are the cause of many infectious diseases. The organisms involve pathogenic bacteria causing diseases like dysentery and also fungi causing diseases like candidiasis (Eckburg et al., 2003).

Tables 4.5 and 4.6 revealed that the four (4) crude extracts exhibited various activities (i.e.sensitivity) against MRSA, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Salmonella typhi*, *Candida albicans*, *Candida krusei*, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Listeria monocytogenes*, *Shigella dysenteriae*, *Campylobacter fetus*, *Klebsiella pneumoniae*, and *Helicobacter pylori*. Zone of inhibition of the extracts against the test microorganisms ranged from 14.0 – 35.0 mm (table 4.6). The highest zone of inhibition was recorded against *Staphylococcus aureus* as 30.0 mm using methanol extract as an antimicrobial. All the extracts exhibited 24.0 mm as zone of inhibition against MRSA (table 4.6).

Each microorganism has a level of antimicrobial activity which will inhibit growth but not kill the microorganism, which is called Minimum Inhibitory Concentration (MIC). Petroleum ether extract showed MIC of 10 mg/cm³ against MRSA, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Shigella dysenteriae*, *Campylobacter fetus* and *Klebsiella pneumoniae*. Lowest MBC/ MFC for petroleum ether extract were 20 mg/cm³ for *S. aureus*, *E. coli*, *Sal. typhi*, *Candida albicans*, *C. krusei*, *List.monocytogenes*, *Kleb. pneumoniae* and *Helicobacter pylori* (table 4.7). Extracts of ethyl acetate, chloroform and methanol exhibited 5.0 mg/cm³ against *Campylobacter fetus* which is a causative agent of bacteremia, meningitis, septic abortions and extra intestinal infection (table 4.7). Lowest MIC for ethyl acetate extract was 5.0 mg/cm³ recorded against *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Shigella dysenteriae*, *Bacillus subtilis*, *Campylobacter fetus* and *Vibrio cholerae* (see table4.7)

Minimum Bactericidal Concentration (MBC) or Minimum Fungicidal Concentration (MFC) is the concentration that will kill the organisms (Purohit *etal.*,2008). Chloroform extract exhibited lowest MIC of 5.0 mg/cm³ against *Escherichia coli*, *Bacillus subtilis* and *Campylobacter fetus* with corresponding MBC/MFC in mg/cm³ of 20.0, 5.0 and 20.0 respectively.

Methanol extract showed lowest MIC against *Campylobacter fetus* (5.0 mg/cm³) and highest MIC of 20.0 mg/cm³ against *Candida krusei*. MIC value of 10.0 mg/cm³ was exhibited against MRSA, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Candida albicans*, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Listeria monocytogenes*, *Shigella dysentriae*, *Klebsiella pneumoniae* and *Helicobacter*

pylori with corresponding MBC/MFC values of 20.0 mg/cm³ and 40.0 mg/cm³ with methanol extract (see table 4.7).

Control was conducted. A drug is a chemical administered in an attempt to prevent, treat or diagnose disease. Natural substances are considered to be drugs when they are administered for such purposes e.g. herbal medicines (Waller *et al.*, 2010). Sparfloxacin (it has enhanced activity against gram-positive bacteria) and ciprofloxacin (it has activity against gram negative bacteria) were used as control on the same twenty (20) microorganisms (Tripathi, 2009). Their activities are depicted in table 4.6.

TCPE I, TCPE II and TCPE III which are three (3) fractions isolated from crude petroleum ether extract, they exhibited inhibitory activities against MRSA, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Salmonella typhi*, *Candida albicans* and *Candida krusei*. TCPE I showed highest zone of inhibition of 35.0 mm against *Staphylococcus aureus*. TCPE I and TCPE III showed lowest MIC of 2.5 mg/cm³ against MRSA, *Staphylococcus aureus* and *Streptococcus pyogenes*. Lowest MBC/MFC was 5 mg/cm³ by TCPE I against *Staphylococcus aureus*. Control drugs were sparfloxacin, which have inhibitory effects on bacteria; fulcin and fluconazole which are anti-fungal drugs exhibited inhibitory effects on *Candida albicans*, *Candida krusei* and *Aspergillus fumigatus*. The results are depicted in tables 4.8, 4.9 and 4.10.

The four (4) crude extracts were active against thirteen (13) microorganisms as well as TCPE I, TCPE II and TCPE III. Work was concentrated on petroleum ether extract from which two pure compounds TC I and TC 2 were isolated. They exhibited activity against the same thirteen (13) microbes (tables 4.11, 4.12 and 4.13).

The activity of the control (drugs) - sparfloxacin, ciprofloxacin, tetracycline, fluconazole and fulcin are shown in tables 4.11 - 4.13.

The thirteen (13) microbes have the following effects on man -*Candida albicans* and *Candida krusei* are responsible for candidiasis; *Staphylococcus aureus* causes meningitis, scalded skin syndrome; *Escherichia coli* is responsible for diarrhea, bacteremia etc.; *Salmonella typhi* is the causative agent for typhoid fever; *Streptococcus pneumoniae* causes pneumonia, bacteremia; *Corynebacterium ulcerans* is responsible for respiratory diphtheria; *Bacillus subtilis* is causative agent for fatal pneumonia and bacteremia; *Listeria monocytogenes* is responsible for abortion, still born or premature birth if untreated in pregnant women; *Shigella dysenteriae* is responsible for dysentery and diarrhea; *Campylobacter fetus* is associated with septic abortions, arthritic, thrombophlebitis and pelvic inflammatory disease; *Klebsiella pneumoniae* causes infections of the intestine, urinary and respiratory tracts; MRSA is responsible for difficult to treat infections in humans and *Helicobacter pylori* which is an important causative agent of gastritis and peptic ulcer (Murray *et al.*, 1999 ; Tripathi *et al.*, 2008).

The tables (4.8 - 4.13) revealed that the inhibition of the extracts, fractions (TCPE I, TCPE II and TCPE III) on the thirteen stated microorganisms and concentrations of these compounds (TC I and TC II) are less than the reference drugs (control). Zones of inhibition of TC 1 and TC 2 are comparably similar. For instance MIC of TC 1 and TC 2 on MRSA are 27.0 mm and 28.0 mm respectively (Table 4.12). Their inhibitory effects are similar on *Candida albicans* (27.0 mm and 28.0 mm respectively). TC 1 exhibited 6.27 mg/cm³ MIC on MRSA, *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogene*, *Shigella dysenteriae*, *Campylobacter*

fetus and *Klebsiella pneumoniae* (tables 4.12 - 4.13). Based on the findings above, it is evident that the extracts of *Tetracarpidium conophorum* leaves justify its uses in ethno medicinal practices and folklore medicine.

5.7.1 FT-IR spectrum of TC 1 (Stigmasterol)

The IR spectrum of TC 1 showed an intense broad band at 3426.66 cm^{-1} , characteristic of a free O–H bond stretching of an alcohol and H-bond to the –OH (see figure 4.1). At 2926.11 cm^{-1} is a sharp signal of methyl (CH_3) stretching vibration. It is of a saturated hydrocarbon. Corresponding $\text{C}=\text{C}$ - stretching vibration of a cyclic compound was noticed around 1632.80 cm^{-1} as a broad weak band.

The bending vibration of a methylene ($-\text{CH}_2$) at 1458.2 cm^{-1} was shown as a medium band and vibration due to methylene (of alkanes) was shown as an intense sharp band at 434 cm^{-1} . The C-O stretching vibration of alcohol was shown at 1051.24 cm^{-1} as a broad weak band. These readings are presented in table 4.14. These assignments are in good agreements with reported values (Kamboj *et al.*, 2010, Rajput and Rajput 2012).

5.7.2 The FT-IR spectrum of TC 2 (Oleanolic acid)

From figure 4.11 and table 4.17, the result of IR spectrum of TC 2 revealed a strong broad signal of O-H (of a free hydroxyl) stretching vibration at 3429.55 cm^{-1} . Strong band at 2932.86 cm^{-1} is of a CH_3 of a stretching vibration of a saturated alkane. At 1642.44 cm^{-1} is a strong signal of $\text{C}=\text{O}$ stretching vibration of a carboxylic acid ($-\text{COOH}$). A medium signal of $-\text{CH}_2/\text{CH}_3$ of an alkane at 1454.38 cm^{-1} . A weak signal of CH_3 bending vibration at 1381.08 cm^{-1}

C-O stretching vibration of a carboxylic acid at 109.67 cm^{-1} as a weak signal. C-C bending vibration of an alkane which may be a doublet at 448.46 cm^{-1} and

430.14 cm^{-1} as a sharp, deep signal (Silverstein *et al*, 2005, Williams *et al* , 4th edition). These assignments are in good agreement with reported values (Habila *et al.*,2012 ; Narendra *et al.*, 2014).

5.8 NMR spectral of TC 1 (Stigmasterol)

5.8.1 The ^1H NMR spectrum of TC 1

In the figure 4.2, from the 600 MHz ^1H spectrum, the total integration adds up to 29 protons. One proton signal observed at 3.53 ppm which is characteristic of the proton attached to the carbon carrying – OH functional group which is a splitting pattern. Moving from 0 - 2.0 ppm are signals due to overlapping of methine, methylene and methyl carbons.

Six methyl groups gave rise to tall, sharp peaks in the up feild region of the ^1H spectrum between 0.6 -1.0 ppm. Two of the methyls should be attached to quaternary carbons, so they appear as singlets. A signal at 3.5 ppm is characteristic of oxymethine proton (H-C-OH) it can be described as a triplet. The signals downfield at 5.0 - 5.5 ppm are characteristics of the proton of olefinic functional group (Jacobsen, 2007). These assignments were confirmed by comparism with spectral analysis data reported in cited literatures. The result is presented in table 4.15 (Ragasa and Cayne 2004; Koay *et al.*, 2013).

5.8.2 The ^{13}C NMR spectrum of TC1 (Stigmasterol)

In figure 4.3, 29 carbon signals can be counted in the 600 MHz NMR of ^{13}C . From the upfeild (10.0 – 58.0 ppm) are signals that correspond to the methane, methylene and methyl carbons. Basically the molecule can be described as a saturated hydrocarbon with the following functionalities of a sterol with olefinic carbon and an alcohol. There are four olefinic methine protons at 129.7 ppm, 141.0 ppm, 138.7 ppm

and 121.8 ppm. Carbonyl protons at 71.8 ppm were observed. There are six methyl carbons at 12.05 ppm, 12.21 ppm, 18.98 ppm, 19.40 ppm, 21.05 ppm and 21.20 ppm. Nine methylene carbons at 21.05 ppm, 24.37 ppm, 25.38 ppm, 28.89 ppm, 31.70 ppm, 31.93 ppm, 37.28 ppm, 39.71 ppm and 42.25 ppm (table 4.15). These assignments were confirmed by comparison with spectral analysis data in cited literatures (Ragasa and Cayme 2003).

5.8.3 The COSY spectrum of TC 1 (Stigmasterol)

COSY spectrum correlates one proton to another which may be 2-bond, 3-bond or 5-bond. Cross peaks were noted between proton signal at 3.50 ppm and 1.50, 2.30 and 3.50 ppm while diagonal peaks were observed at 2.30, 3.50 and 5.50 ppm (see figure 4.4).

5.8.4 The DEPT spectral of TC1 (Stigmasterol)

The study of the figure 4.5 reveals three DEPT spectral of TC 1 in CDCl₃. DEPT – 90 spectrum reveals there are nine methine (CH) carbons. In DEPT - 135 the negative peaks correspond to ten methylene (CH₂) carbons. Peaks missing from DEPT- 90 and positive in DEPT -135 are methyl (CH₃) carbons and they are the six most up field positive peaks. Four quaternary carbons in TC1 are shown as peaks in DEPT – 45 between 122 -141 ppm. These assignments were in good agreement with reported values (Ragasa and Cayne 2004; Jacobsen 2007; Koay *et al.*, 2013).

5.8.5 The HSQC spectrum of TC 1 (Stigmasterol)

This experiment permits to obtain a 2D heteronuclear chemical shift correlation between directly – bonded ¹H and ¹³C. Some important correlations are observed. Such as that of carbon signal at 71.80 ppm (with the hydroxyl group at C 3) correlates with the proton signal at 3.50 ppm, carbon signal at 121.70 ppm correlates

with proton signal (F1) at 5.50 ppm, carbon signal (F2) at 21.04 correlates with proton signal (F1) at 1.50 ppm (see figure 4.7, Koay *et al.*, 2013).

5.8.6 HMBC of TC 1 (Stigmasterol)

The HBMC of TC 1 shows correlation between carbon signal(F1) and proton signal (F2) that are separated by longer ranges between 2 – 4 bonds as depicted in figure 4.6. There are correlations between carbon signal at 36.50 ppm and various proton signals at 1.10, 1.55, 1.80, 2.20 and 5.30 ppm. Correlations between carbon signal at 140.7 ppm and the proton signals at 1.00 ppm and 2.20 ppm (Silverstein *et al.*, 2005; Chen and Sheng 2009).

5.8.7 Mass Spectroscopy (MS) of TC 1

The Mass Spectroscopy revealed molecular ion peak as 412 which corresponds to the molecular formula $C_{29}H_{48}O$. Ion peaks were observed at mass - to - charge (m/z) values and the interpretation was conducted using the database of National Institute Standard and Technology (NIST) chart library (see table 4.16 and figure 4.9). These are in good agreement with cited literatures (Koay *et al.*, 2013).

The molecular ion peak is m/z 412 and base peak of 55. Peaks at m/z 41, 55 and 69 are expected to correspond to the formula C_nH_{2n-1} with n = 3, 4, 5 respectively (that is; $C_3H_5^+$, $C_4H_7^+$ and $C_5H_9^+$ respectively). The presence of M – 18 is noticeable in spectra with alcohols (Silverstein *et al.*, 2005).

Therefore with the combined spectra data analyses using 1D – and 2D- NMR, as well as MS, TC 1 can be suggested to be a tetracyclic triterpene (XVI) – Stigmasta - 5, 22 - dien - 3 – ol (Stigmasterol).

5.9 NMR Spectral of TC 2 (Oleanolic acid)

5.9.1 1H NMR Spectrum of TC 2

In figure 4.12, from the 600 MHz ^1H spectrum the total integration adds up to 30 carbon signals and 48 proton signals. The signal at 3.25 ppm is characteristic of the oxymethine proton assigned to C-3 occurring as a splitting pattern, it is a methine proton. Up- field signals from 0.70 - 1.70 ppm are signals due to an overlap of peaks due to methine, methylene and methyl carbons. Signal of a splitting pattern at 5.40 ppm is proton of an olefinic functional group. Peaks at 0.76, 0.78, 0.90, 0.92, 0.96 and 1.00 ppm are of methyl groups (Gohari *et al.*, 2009) At 3.22 ppm is seen a splitting pattern of a triplet with a small coupling at 4.20 ppm. Moving from left to right, there is a two-proton multiplet at 1.80 - 2.10 ppm. The most likely assignment for these peaks would be in C-4. They are doublets. Five of the methyl groups of TC 2 (deduced from DEPT spectral in figure 4.15) gave rise to tall sharp peaks in the up-field region of ^1H spectrum (Gohari *et al.*, 2009; Garica-Granados *et al.*, 1998)

5.9.2 ^{13}C NMR Spectrum of TC 2(Oleanolic acid)

The spectrum of ^{13}C in figure 4.13 of TC 2 shows 30 carbon peaks. Basically it is a spectrum of a hydrocarbon with alcohol (C-3), olefinic(C-12 and C-13) and carboxylic functional groups (C-28) (Jacobsen 2007). Chemical shifts observed at 116.80 ppm and 158.10 ppm are indicative of the presence of a double bond in a triterpenoid (C-12 and C-13 respectively) (Gohari *et al.*, 2009). So C-13 is a quaternary carbon. This is because carbons lacking a proton relax much more slowly and give less intense peaks (Jacobsen 2007). The most down-field signal at 178.90 ppm is of a carboxylic acid at C-28. CDCl_3 peaks are seen at 76.0 ppm.

Oxygen desheilding chemical shift at 78.0 ppm is in the alcohol region (C-O), it can be assigned to C-3. The most sterically crowded carbons are the methine groups (CH) at C-3, C-5, C-9, C-12 and C-18. Up field signals on ^{13}C spectrum are at δ

28.80, δ 26.50, δ 15.40, δ 17.50, δ 26.00, δ 33.30 and δ 23.70. These were assigned to methyl (CH₃) substituents. These are sp² carbons. The most downfield signal at δ 178.90 is due to the presence of a carbonyl group (C=O) at C-28 in figure 4.13 (Vyas and Argal 2014; Gohari *et al.*, 2009).

5.9.3 COSY Spectrum of TC2 (Oleanolic acid)

The COSY spectrum in figure 4.14 displays a correlation of the proton at 2.30 ppm (F2) with the protons at 1.60 and 2.30 ppm. Also a correlation was displayed between the proton signal at 3.20 ppm with that at 1.60 ppm and 3.20 ppm. A proton signal at 2.30 ppm (F1) was observed to display a cross peak at 1.50 ppm and 2.30 ppm (Gohari *et al.*, 2009).

5.9.4 DEPT Spectral result of TC 2 (Oleanolic acid)

Figure 4.15 reveals three DEPT spectra of TC 2 in deuterated chloroform. Methyl carbons (CH₃) will be positive in DEPT 135 and absent in DEPT 90. Seven methyl (CH₃) groups are observed for TC 2 in DEPT 135. Methylene carbons (CH₂) will be negative in DEPT 135 and absent in DEPT -90 spectrum of TC 2. DEPT -135 suggest ten methylene (CH₂) groups corresponding to C-1, C-2, C-6, C-7, C-12, C-15, C-16, C-19, C-21 and C-22. Methine carbons (CH) will be positive in DEPT 90 and in DEPT 135. TC 2 DEPT spectra suggest 5 methine carbons (CH) at δ 79.10, 55.60, 48.00, 116.80 and 42.00 corresponding to C-3, C-5, C-9, C-12 and C-18 respectively. Deuterated chloroform peaks (1:1:1) triplet is at 78.0 ppm in all DEPT spectra (Jacobsen, 2007, Gohari *et al.*, 2009 and Onoja *et al.*, 2013). There are seven

quaternary carbon peaks deduced in DEPT-135 (Onaja and Ndukwe 2013). CDCl_3 peak (1:1:1) 'triplet' is displayed at 77.0 ppm in all the DEPT spectral.

5.9.5 HMBC Spectrum of TC2(Oleanolic acid)

The HMBC displays some correlations between ^1H signals and ^{13}C signals in figure 4.16. There is correlation between carbon signal at 28.0 ppm with proton signals at 0.86, 0.92, 1.20, 1.60 and 2.30 ppm. Correlations were observed between proton signal at 1.60 ppm with carbon signals at 28.0, 35.0, 48.0 and 78.0 ppm.

5.9.6 HSQC Spectrum of TC2 (Oleanolic acid)

In figure 4.17, there is one bond coupling of proton signal(F2) with carbon signal(F1) as well as two- and three-bond coupling (Silverstein *et al.*, 2005). One bond coupling of proton signal at 3.20 ppm with carbon signal at 79.0 ppm was displayed. Carbon signal at 1.5.0 ppm was observed to correlate with proton signals at 0.78 ppm and 0.85 ppm. Carbon signal at 121.0 ppm correlates with proton signal at 5.30 ppm. Signal at 34.0 ppm (F1) correlates with proton signals at 0.91, 1.40, 1.50 and 2.30 ppm. Carbon signal at 55.0 ppm correlates with one proton signal at 0.88 ppm. One carbon signal at 48.0 ppm correlates with two proton signals at 1.40 ppm and 1.00 ppm.

In conclusion, combining the spectral data analyses of 1D- NMR and 2D- NMR with the cited literatures, TC 2 is a pentacyclic triterpenoid (XVII) and suggested to be 3- β -hydroxyolean-12-en-28-oic acid (oleanolic acid) (Jacobsen 2007; Gohari *et al.*,2009)

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The phytochemical studies of the leaves of *Tetracarpidium conophorum* were carried out and were found to be positive for glycosides, alkaloids, flavonoids, sterols, proteins and tannins.

The proximate analysis reveals that the leaves of *Tetracarpidium conophorum* consist of the six basic classes of food recommended for the total well – being. The components include – crude fibre which contains cellulose and lignin (15.53 %); crude protein (17.68 %); oil or fat (1.8 9%); nitrogen – free extract(51.52 %) which consists of sugars, pectins, organic acids and pigments; and ash (13.35 %) which is considered as minerals or the inorganic content of the sample. This data reveals that the leaves maybe beneficial as acclaimed in ethnomedicinal practices in treatment of giddiness and relieves abdominal pains (Burkill 1985).

The result of elemental analysis reveals the presence of important elements like magnesium (4756 mg/kg), calcium (33460 mg/kg), manganese (263 mg/kg) and zinc (24.3 mg/kg). Zinc is noted for enhancement of sexual maturation and aids normal foetal development; magnesium keeps the heart beat steady as well as regulates blood glucose levels and calcium is essential for nerve transmission, muscle contraction and dilation of the blood vessels. The leaves of *Tetracarpidium conophorum* maybe effective as a male fertility agent and in the development of the foetus as acclaimed in ethno – medicinal practices.

The various antimicrobial screenings have revealed that all the extracts have anti-bacteria activities. The crude extracts exhibited various degrees of inhibition to

the growth of four gram-positive and seven gram-negative bacteria as well as the two fungi (*Candida albicans*, the most common human fungus and *C. krusei*). The inhibition of bacteria and fungi growth were dose dependent. *Aspergillus* species (the mould) exhibited resistant to the four extracts and isolated compounds. TC 1 and TC 2 exhibited similar activities against the thirteen micro-organisms. The zones of inhibition due to TC 1 and TC 2 are larger than those due to the crude extracts and this is expected as they were isolated as pure compounds. It can be concluded that the extracts of *Tetracarpidium conophorum* leaves could be effective against diarrhea, dysentery (bloody diarrhea), meningitis, bacteremia, pneumonia, arthritis, respiratory and urinary tract infections, threatened abortion and still birth as acclaimed by ethno medicinal practices. Since the four extracts and isolated compounds (TC 1 and TC 2) exhibited inhibitory effects on causative micro-organisms, the leaves of the plant appear to be a potential source of broad spectrum antibiotics. As well as being a good source of antioxidants.

Based on the interpretation of the spectral obtained by spectroscopic analyses (1D and 2D NMR) and FT-IR analyses of the isolated compounds, it can be suggested that the compounds isolated are 3 β , 22E-Stigmasta-5, 22-dien-3-ol (TC1) and 3 β -hydroxyolea-12-en-28-oic acid (TC2). These are phytosterols known to reduce the risk of diseases such as chronic inflammatory, breast cancer and diabetes (Obayendo 2010). They have been suggested to improve cognitive skills in the elderly and beat drug resistant micro-organisms (Muanya 2011). These two phytosterols are a group of promising secondary plant metabolites.

6.2 Conclusion

Natural products have been the 'leads' and templates for the design and synthesis of medicinal drugs. The 'lead' concept in drug design usually involves improvement of 'lead' (Olaniyi 2005). The pharmaceutical importance of triterpenoids cannot be over emphasized. It can be concluded that phytosterols (triterpenoids) - 3β , 22E- stigmasta-5, 22- dien-3- ol and 3β - hydroxyolean-12-en-28- oic acid were isolated from petroleum ether fraction of *Tetracarpidium conophorum* leaves. This fraction has a very high potential as a source of drug discovery for antimicrobial agents. This medicinal plant may become an integral part of the human diet as supplements and multi-vitamins. Thereby, giving the plant *Tetracarpidium conophorum* greater values beyond the consumption and sale of *awusa* (African walnut).

6.3 Recommendations

From the studies carried out, the extracts of the leaves of *Tetracarpidium conophorum* contain bioactive compounds such as the phytosterols. It is recommended that further studies be conducted on the isolates to test their acute and chronic toxicity, conduct clinical trials which include pharmacological processes and mechanisms of the various reactions. Based on all these tests, they may then be used as drugs.

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