

**PHYTOCHEMICAL AND SOME BIOLOGICAL STUDIES OF THE METHANOL  
LEAF EXTRACT OF *ASPILIA AFRICANA* (PERS.)  
C. D. ADAMS (ASTERACEAE)**

**BY**

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**A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES  
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**DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,  
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**NOVEMBER, 2016**

### Declaration

I declare that the work in this thesis entitled PHYTOCHEMICAL AND SOME BIOLOGICAL STUDIES OF THE METHANOL LEAFEXTRACT OF *ASPILIA AFRICANA* (PERS.) C. D. ADAMS (ASTERACEAE) has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria, under the supervision of Professor M. Ilyas, Professor A. K. Haruna and Professor M. I. Sule. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this institution or any other institution.

Ekarika Clement Johnson  
(Name of Student)

.....  
Signature

.....  
Date

## Certification

This thesis entitled “PHYTOCHEMICAL AND SOME BIOLOGICAL STUDIES OF THE METHANOL LEAF EXTRACT OF *ASPILIA AFRICANA* (PERS.) C. D. ADAMS (ASTERACEAE)” by Ekariika Clement JOHNSON meets the regulations governing the award of the degree of Doctor of Philosophy of Pharmaceutical Chemistry of Ahmadu Bello University Zaria and is approved for its contribution to knowledge and literary presentation.

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## **Dedication**

This research work is dedicated to my beloved family:

My Lovely Wife:                Julia Ekarika Johnson

My Beloved Children:        Udeme Clement Johnson

Prince Onoemem Johnson

Victor Itoro Johnson

Restoration Tryphena Johnson

Precious-Deborah Johnson

## Abstract

*Aspilia africana*, (Pers) C.D. Adams; (Asteraceae), a semi perennial woody plant, commonly known as haemorrhage plant is widely distributed across tropical Africa and all parts of Nigeria. The plant is well known in folkloric medicine for its anti-haemorrhagic uses and for the treatment of cardiovascular diseases, diabetes, malarial fever, wounds, coughs, microbial infections and gastro-intestinal disorders. This research was undertaken in order to isolate some of the bioactive compounds responsible for the anti-microbial and anti-diabetic activities of this plant. The dried powdered leaf material was subjected to cold maceration with 70% aqueous methanol to obtain the methanol extract which was partitioned with n-hexane, dichloromethane, ethyl acetate and n-butanol. The methanol extract and the fractions were screened for phytochemical constituents, anti-microbial and anti-diabetic properties and then subjected to chromatographic separation. Preliminary phytochemical screening results showed the presence of carbohydrate, cardiac glycosides, flavonoids, polyphenols, saponins, steroids, tannins and terpenoids in the methanol extract and the fractions. Chemical investigation of the methanol extract and fractions of the leaves led to the isolation of three pentacyclic triterpenic acids namely;  $3\beta$  – hydroxyolean – 12 – en – 28 – oic acid (Oleanolic acid),  $3\beta$  – hydroxyurs – 12 – en – 28 – oic acid (ursolic acid) and 2, 3 - dihydroxyurs – 12 en – 28 – oic acid (corosolic acid) from the butanol fraction and  $3\beta$ -hydroxyurs-12-en-28-oic (ursolic acid) from the dichloromethane fraction through silica gel column chromatography. The structures of the isolated compounds were elucidated based on the analysis of 1-D and 2-D NMR (including  $^1\text{H}$ ,  $^{13}\text{C}$ , HMBC, HMQC, COSY, and DEPT experiments), FTIR spectral data, physicochemical properties and comparison with authentic data of these compounds from literature. In Agar well-diffusion anti-microbial screening using isolated clinical strains of pathogens namely *Staphylococcus aureus*, *Methicillin Resistant Staphylococcus aureus (MRSA)*,

*Streptococcus pyogenes*, *Bacillus subtilis*, *Proteus vulgaris*, *Salmonella typhi*, *Shigella dysenteriae*, *Escherichia coli*, *Klebsiella pneumoniae* *Candida albicans* and *Candida stellatoidea*; the methanol extract and the butanol fraction (which was more active than other fractions) inhibited the growth of nine of the eleven bacterial strains and one of the three fungal strains with zones of inhibition ranging from 10 – 25mm. However, the activity of one of the isolated compounds (oleanolic acid) surpassed that of crude extract and n-butanol fraction; it inhibited the growth of all the bacteria and two of the fungal strains with inhibition zones ranging from 25 – 33mm compared with the standard drugs used. The MIC, MBC/MFC of the plant extracts ranged from between 5.00 and 10.00 mg/mL while that of the isolated compounds ranged between 0.0125 and 0.0500mg/mL. In the *in-vitro* anti-diabetic studies, the isolated oleanolic acid ( $IC_{50} = (25.45 \pm 0.45 \mu\text{g/mL}; 47.57 \pm 0.40 \mu\text{g/mL})$ ) was more potent in the inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase than the crude methanol extract ( $IC_{50} = 96.40 \pm 0.20 \mu\text{g/mL}; 95.10 \pm 0.20 \mu\text{g/mL}$ ), butanol fraction (the only active fraction,  $IC_{50} = 72.50 \pm 0.65 \mu\text{g/mL}; 72.65 \pm 3.2 \mu\text{g/mL}$ ) and the reference drug (acarbose) ( $IC_{50} = 46.31 \pm 0.58 \mu\text{g/mL}; 48.10 \pm 0.130 \mu\text{g/mL}$ ) for  $\alpha$ -amylase and  $\alpha$ -glucosidase respectively. The results of the anti-microbial and anti-diabetic studies have authenticated the traditional use of the plant to treat microbial infection and diabetes. The research work has also identified the oleanolic acid to be responsible for the anti-microbial and anti-diabetic activities of the plant.

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## LIST OF ABBREVIATION

%	-	Percentage
$^{\circ}\text{C}$	-	Degree Centigrade (Celcius)
$^{13}\text{C}$	-	Carbon-13
1D	-	One Dimension
$^1\text{H}$	-	Hydrogen Proton
2D	-	Two Dimensions
Abs	-	Absorbance
Aq	-	Aqueous
BUOH	-	n-butanol
CC	-	Column Chromatography
$\text{CD}_3\text{OD}$	-	Deutrated methanol
$\text{CDCl}_3$	-	Deutrated Chloroform
$\text{CH}_3\text{OH}$ , MEOH	-	Methanol
$\text{CHCl}_3$	-	Chloroform
$\text{cm}^{-1}$	-	Percentimetre
Conc.	-	Concentration
COSY	-	Correlation Spectroscopy
DCM	-	Dichloromethane
DEPT	-	Distortion Enhancement by Polarization Transfer
DM	-	Diabetic Mellitus
DMSO	-	Dimethylsulphoxide
ETOAc, EA	-	Ethyl Acetate

FeCl <sub>3</sub>	-	Ferric Chloride
FTIR	-	Fourier Transform Infra Red
g	-	Gramme
gem	-	germinal
H <sub>2</sub> SO <sub>4</sub>	-	Tetraoxosulphate VI acid
HCl	-	Hydrochloric acid
HMBC	-	Homonuclear Multiple Bond Correlation
HMQC	-	Heteronuclear Multiple Quantum Correlation
Hz	-	Hertz
I <sub>2</sub>	-	Iodine
IC	-	Inhibition Concentration
IUPAC	-	International Union of Pure and Applied Chemistry
J	-	Coupling constant
K <sub>3</sub> Fe(CN) <sub>6</sub>	-	Potassium hexacyano Ferrate III
KBr	-	Potassium Bromide
Kg	-	Kilogramme
LBR	-	Liebermann-Buchard Reagent
LD	-	Lethal Dose
MBC	-	Minimum Bacterial Concentration
Me	-	Methyl
MHz	-	Megahertz
MIC	-	Minimum Inhibitory Concentration
mL	-	millilitre

NaOH	-	Sodium Hydroxide
n-Hex	-	Normal Hexane
NMR	-	Nuclear Magnetic Resonance
ppm	-	Part per million
q	-	quaternary
R <sub>f</sub>	-	Retention factor
tert	-	tertiary
TLC	-	Thin Layer Chromatography
UV	-	Ultra Violet
v	-	volume
α	-	Alpha
β	-	Beta

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 Plants as Source of Natural Medicines

Plants have been known to play a very significant role in the treatment of diseases and this is the reason why they are being used in all major systems of medicines irrespective of the differences in cultural beliefs and heritages of the people (Trease and Evans, 2009). Traditional medical treatment in which plants are used as therapeutic agents is referred to as herbal medicine. Herbal medicine is described as the sixth field of alternative medicine which is also called unconventional medicine, that is, therapeutic practices, techniques, and beliefs that are outside the realm of mainstream Western health care. Herbal medicine is becoming more and more popular due to increased patronage in both industrialized and non-industrialized countries. Other fields of alternative medicine are: acupuncture, ayurvedic medicine, homeopathy and naturopathy (Blackman, 2015).

Medicinal plant species are so called because they are sources of well-known and medically useful secondary products as wide-ranging as painkillers like morphine (I), stimulants like cocaine (II) and anti-depressants like tryptophan (III) with high potency in the management of ailments in humans. Generally, medicinal plants are unique in their content of compounds that are end-products of long biosynthetic pathways which are usually not needed in such plants' metabolic processes. Davis and Heywood (1963) listed these compounds called secondary metabolites to include: alkaloids, flavonoids, terpenes, glycosides, essential oils and other organic constituents. These constituents are usually produced and stored in different parts of the plants like the roots, leaves, fruits, seeds stem bark and flowers (Kochhar, 1981). Ethnobotanical studies carried out throughout Africa confirm that native plants are the main constituent of

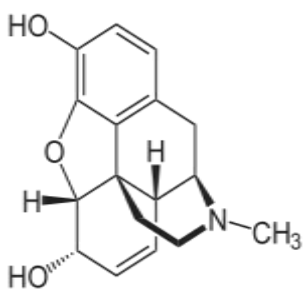
traditional African medicines (Sandhu and Heinrich, 2005; Gupta *et al.*, 2005). With 70-80% of Africa's population relying on traditional medicines, the importance of the role of medicinal plants in the healthcare system is enormous (Mander and Breton, 2006). Medicinal plants are now being given serious attention, as evidenced by the recommendation of the World Health Organization in 1970 that proven traditional remedies should be incorporated into national drug policies of all countries and also by the increased commercialization of pharmaceutical production using traditional medicinal plants with known efficacy (Last and Chavunduka, 1986, Wondergem *et al.*, 1989, Sofowora, 2008).

The approval of more than 20,000 medicinal plant species of tropical origin by the World Health Organization for search for therapeutic agents has contributed immensely to the advancement of Complementary and Alternative Medicine (CAM) (Gullece *et al.*, 2006). Currently, the identification, validation and formulation of animal management and health care policies are the focus of renewed interest in most ethno-veterinary researches (Shapovalet *et al.*, 1994; Izzo *et al.*, 1995; Souza *et al.*, 2002). These studies have extended to the list of traditional medicinal plants to include some weed species like *Taraxacum officinale* and *Stellaria media*, which were not considered before (Njoroge *et al.*, 2004; Oluyemi *et al.*, 2007). Schultes, (1994) had earlier stated that focusing attention on the plants used by indigenous people is the most effective method of identifying plants that may contain useful bioactive substances. In most rural communities in Africa, primary health care delivery services remain dependent on the use of several medicinal plants since synthetic drugs are expensive or unavailable (Ngari, 2010). The challenge remains that, studies on the efficacies of these tropical plants as new sources of natural, effective, cheaper and potentially less toxic drugs are very inadequate whereas knowledge about them and their medicinally active constituents would have made their applications in therapy possible as other

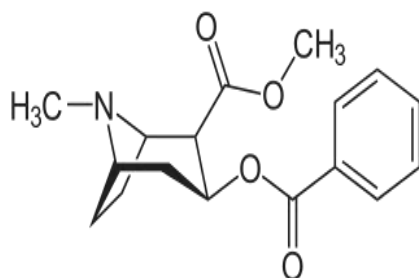
therapeutic agents described in the various pharmacopoeias(Barbieri *et al.*, 2002; Njoroge *et al.*, 2004).

According to Fabricant and Farnsworth (2012),the main objectives of using plants as sources of therapeutic agents are:

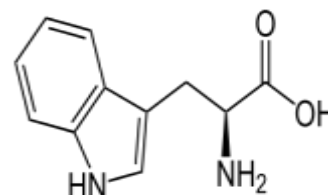
- (i) to isolate bioactive compounds for direct use as drugs, e.g. quinine (**IV**)from cinchona tree used to treat malaria and reserpine(**V**) from *Rauwolfia serpentina* used to treat high blood pressure.
- (ii) to produce bioactive compounds of novel or known structures as lead compounds for semisynthesis so as to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin (**VI**) from galegine (**VII**), taxotere (**VIII**) from taxol (**IX**)
- (iii) touse bioactive agents as pharmacological tools, e.g., naloxone (**X**), yohimbin (**XI**).
- (iv) touse the whole plant or part of it as herbal remedy, e.g., cranberry, ginger, garlic.



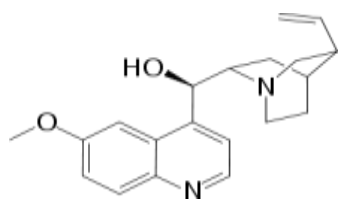
I



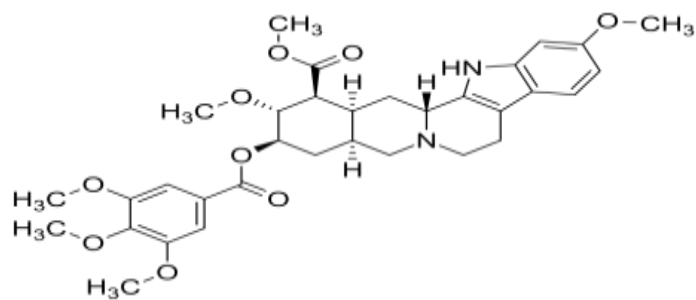
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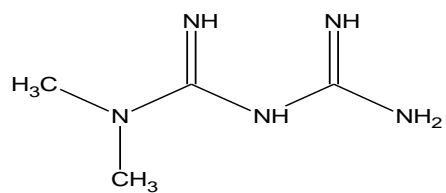
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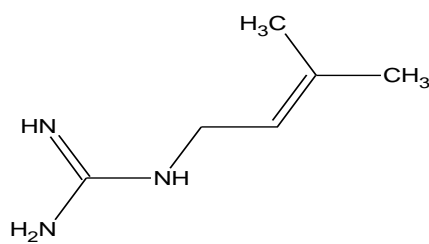
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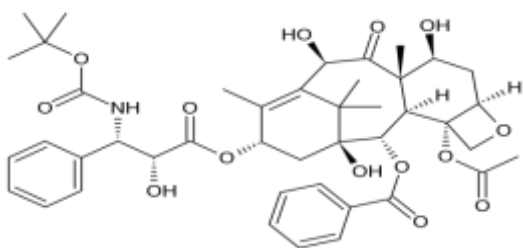
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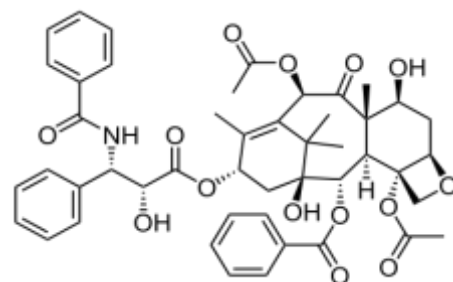
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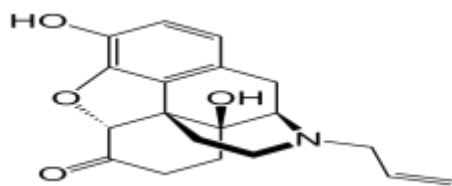
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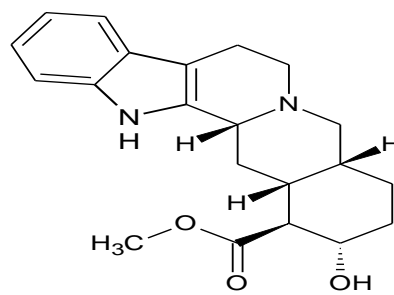
VIII



IX



X



XI

## 1.2 The Role of Secondary Metabolites

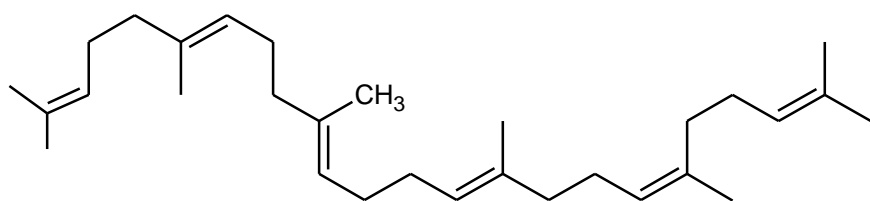
The products of secondary metabolisms are known as secondary metabolites. The majority of secondary metabolites belong to one of a number of families, each of which has particular structural characteristics arising from its biosynthesis. Some of the classes are polyketides, fatty acids, terpenoids, steroids, saponins, tannins, glycosides, phenylpropanoids and alkaloids.

Secondary metabolites are natural products that have an ecological role in regulating the interactions between plants and their environment. They can be defensive substances such as phytoalexins and phytoanticipins, antifeedants, attractants, and pheromones (Hanson, 2003). The importance of plant secondary metabolites in medicine, agriculture, and industry has led to numerous studies on the isolation, synthesis, biosynthesis and biological activity of these substances. It has been estimated that over 40% of medicines have their origins in these active natural products (Gershenzon and Kries, 1999).

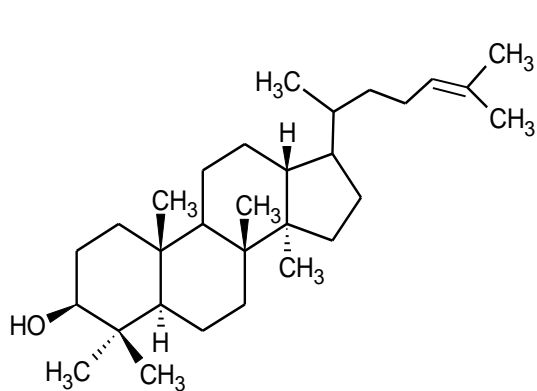
A prominent group of secondary metabolites that is attracting much attention lately is the pentacyclic triterpenoids (PCTs). PCTs are a class of  $C_{30}$  isoprenoid compounds occurring widely in plants. These compounds are produced biosynthetically by the folding and cyclization of squalene (XII) which leads to the dammarenyl ring system (XIII), which has a slightly different stereochemistry and ring structure from that of major sterols (Dewick, 2001). Biosynthetically, dammarenyl ring system undergoes ring expansion and additional cyclization to form the characteristic fifth ring found in lupeol (XIV),  $\alpha$ -amyrin (XV) and  $\beta$ -amyrin (XVI) skeletons. Ursolic acid contains the  $\alpha$ -amyrin skeleton; its  $C_{30}$  isomeroleanolic acid is  $\beta$ -amyrin.

The triterpenoids have a range of unique and potentially usable biological effects and reference to the use of plants with high saponin/triterpenoids content can be found in the first written

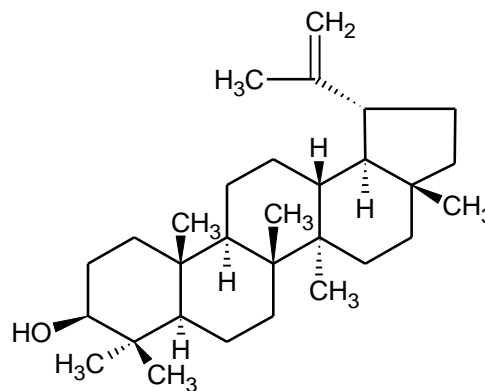
herbarial(Babalola and Shode, 2013). From biological point of view, the most important triterpenoid structures are ursane (XVII), oleanane (XVIII), lupane (XIX), and dammarane-euphane (XX)(Babalola and Shode, 2013). Ursolic and oleanolic acid have been studied for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory, diuretic, anti-spasmodic, anti-atherosclerotic, anti-tumor, anti-diabetic and anti-HIV activity (Li *et al.*, 2002; Jaki *et al.*, 2008).



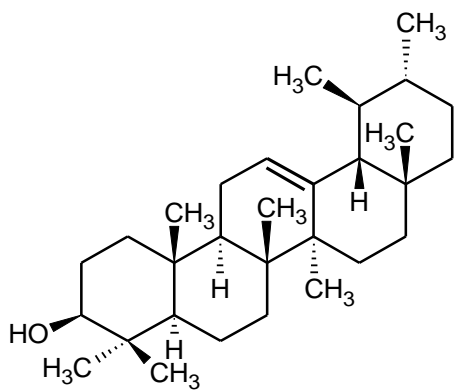
**XII**



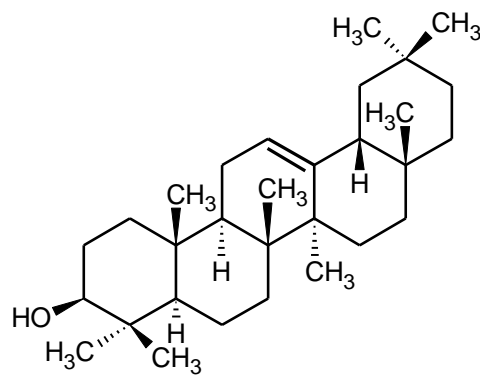
**XIII**



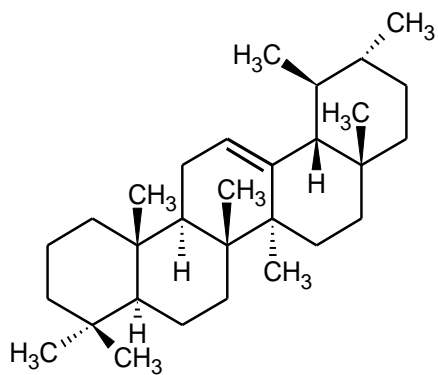
**XIV**



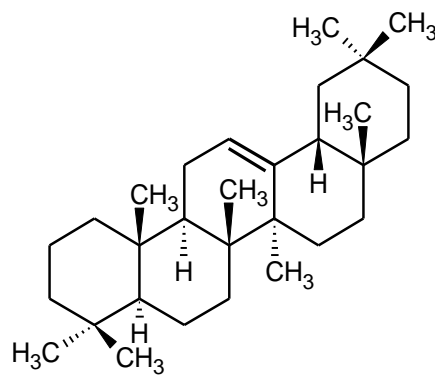
**XV**



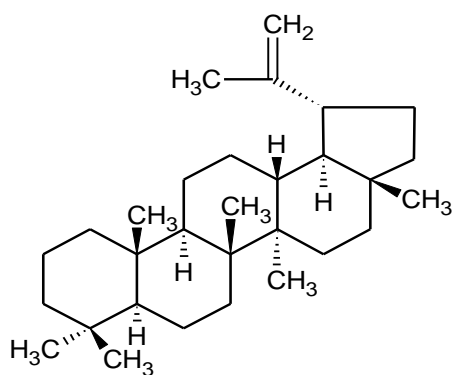
**XVI**



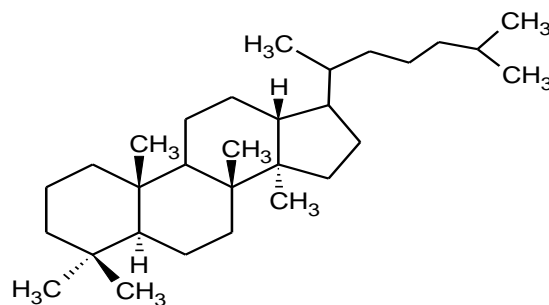
**XVII**



**XVIII**



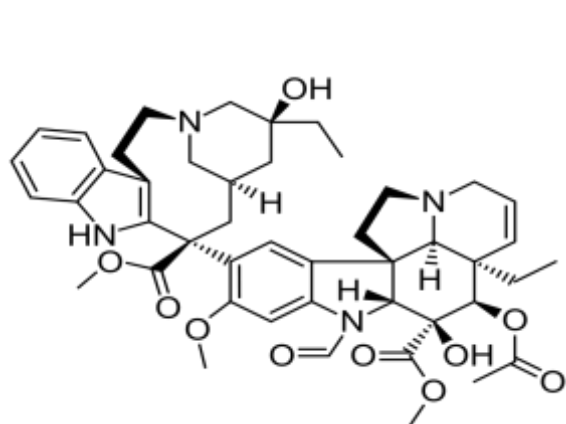
**XIX**



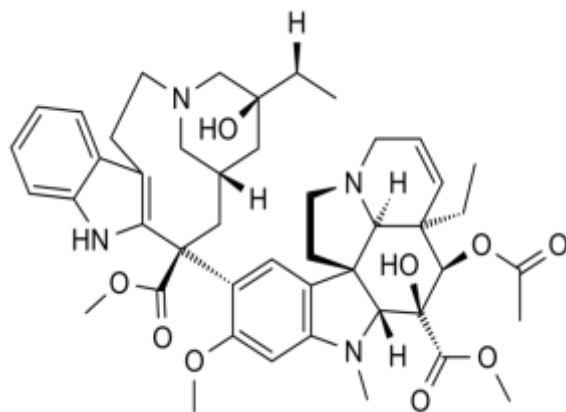
**XX**

It is universally believed that plants provide an unlimited source of novel and complex chemical structures that most likely would never be the subject of a beginning synthetic program, e.g.,

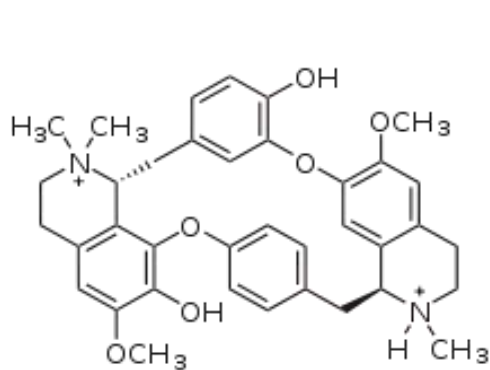
vinblastine (XXI), vincristine (XXII), *d*-tubocurarine (XXIII), digoxin (XXIV). It is important to note that most useful drugs derived from plants have been discovered by follow up of ethnomedical uses. (Fabricant and Farnsworth, 2001).



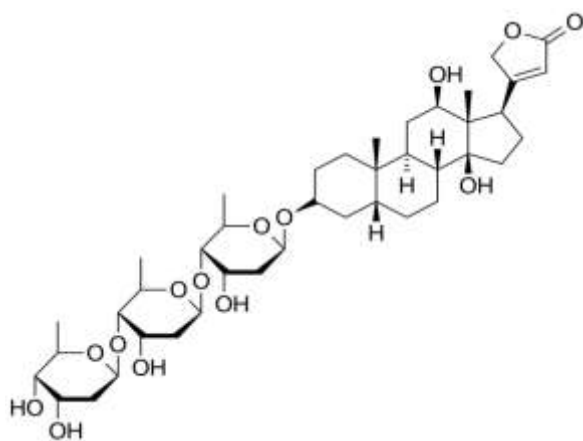
**XXI**



**XXII**



**XXIII**



**XXIV**

However, in selecting the plant of interest, the biologic targets must represent the activities that correlate best with the rationale for the ethno medical uses of plant. Thus, selection of plants based on

long-term human use (ethno medical) in conjunction with appropriate biological assays that correlate with the ethno medical uses would be most appropriate (Fabricant and Farnsworth, 2001). *Aspilia africana* is known to have many biological activities and is widely used in African traditional folkloric medicine. The leaves of *Aspilia africana* possess haemostatic, abortifacient, ecbolics, lactation stimulating, analgesic and sedative properties and is employed in the treatment of cutaneous, subcutaneous parasitic infection (including veterinary), naso-pharyngeal, skin and mucosal infections as well as venereal diseases (Burkill, 1995).

### **1.3 Anti-microbial Activity**

Anti-microbial activity is the ability of a substance to inhibit the growth of or outrightly kill microorganisms thereby eliminating their harmful effects. Antimicrobial drugs can be classified as antibacterial, antifungal and antiviral agents depending on which microorganisms are involved. These agents are called antibiotics, which may be natural or synthetic compounds produced in the laboratory. An antibiotic refers to a substance produced by microorganism or a similar substance (produced wholly or partially by chemical synthesis) which in low concentrations inhibits the growth of other microorganisms (Bowler *et al.*, 2001). Microbial infection of wounds delays healing and causes a more pronounced acute inflammatory reaction (Johnson and Yu, 1991) which can lead to further tissue injury and damage. The antimicrobial activity of the extract and fractions of *A. africana* is believed to eradicate already established infection by eliminating the source of the infection i. e. wound pathogens, thus allowing the natural tissue repair processes to start (Okoli *et al.*, 2007a).

### **1.4 Anti-diabetic Activity**

Diabetes Mellitus (DM) is a disease in which the pancreas produces insufficient amounts of insulin, or in which the body's cells fail to respond appropriately to insulin. DM is classified into two types. Type I and type II. In Type I DM, (juvenile-onset diabetes) the body does not produce insulin or produces it only in very small quantities (Nagmoti and Juvekar, 2013).

Type IIDM (adult-onset diabetes) is a more prevalent and a more worrisome disease condition that affects over 300 million (2013 estimate) people worldwide, (Simple Treatment, 2014) and this number is expected to double by 2030 if urgent action is not taken (Castellano *et al.*, 2013; Simple Treatment, 2014). It is a metabolic disorder characterized by hyperglycaemia caused by deficit or malfunction in insulin secretion and/or insulin action both of which cause the impaired metabolism of glucose, lipids and proteins (Goa *et al.*, 2010).

DM prevalence has been increased by the aging of population, socioeconomic disadvantages and lifestyles that trend towards physical inactivity and overweight/obesity (WHO, 2013). Diabetes management requires an integrated approach that includes the early intervention to prevent or delay its appearance and the use of combined therapies to control glycemia and lipidemia in its late stages. Although many drugs with different modes of action are available, novel natural anti-diabetic agents with insulin-sensitizing effects and preventive actions are highly desirable. One promising therapeutic approach to check the prevalence of hyperglycaemia is to retard or reduce the digestion and absorption of ingested carbohydrate through inhibition of carbohydrate hydrolysing enzymes such as  $\alpha$ -amylase and  $\alpha$ -glucosidase (Sandhu *et al.*, 2013). In the management of blood glucose level, the action of pancreatic  $\alpha$ -amylase and  $\alpha$ -glucosidase has to be inhibited; this is because  $\alpha$ -amylase catalyses the breaking down of starch to disaccharides and oligosaccharides and the intestinal  $\alpha$ -glucosidase catalyses the breakdown of disaccharides to release glucose which is later absorbed from small intestine into the blood circulation (Nagmoti and Juvekar 2013). Thus, retardation in digestion

and absorption of carbohydrate which in turn decreases rise in postprandial hyperglycaemia is a mechanism that should be considered in the treatment of type II diabetes (Kwon *et al.*, 2007). The application of these inhibitors can be used to formulate oral hyperglycaemic drugs for the management of type II diabetes mellitus (Kimura *et al.*, 2006).

## **1.5 Statement of Research Problems**

Preponderance of multiple drug resistant bacterial strains has become a major cause of failure in the treatment of infectious diseases by antibiotics leading high number of deaths of people suffering from diseases caused by these bacterial strains. For instance in 1993, 16.4 million, in 2002, 14.7 million and 2010 about 10 million people died worldwide while in 2012, 6812 people per million died in Africa and 3954 people per million died in Asian and Pacific countries due to acute microbial infectious diseases (Infectious Diseases Worldwide, 2012). Therefore the need for alternative natural antibiotics becomes imperative to complement the existing drugs.

Similarly, estimated 422 million adults are living with diabetes mellitus globally, according to the latest data from the World Health Organization (WHO, 2016). Diabetes prevalence is increasing rapidly; previous 2013 estimates from the International Diabetes Federation put the number at 381 million people having diabetes (Simple Treatment, 2014). The number is projected to almost double by 2030 (Wild *et al.*, 2004). Type 2 diabetes makes up about 85-90% of all cases. The WHO estimates that diabetes resulted in 1.5 million deaths in 2012, making it the 8th leading cause of death (WHO, 2016). The new global interest in the search for anti-diabetic agents from natural sources which are believed generally to be less toxic and more effective has led to the investigation of many plants. In my locality extract of *Aspilia africanais* squeezed on wound and sores and taken as

decoction to treat microbial infections and diabetes. In an attempt to contribute to the global fight against these deadly diseases this research work was undertaken on this “all important” local herb - *Aspilia africana*, to isolate the bioactive compounds that could be used to treat microbial infections and diabetes mellitus.

### **1.6 Justification of the Study**

The *Aspilia africana* is used as herb in the local communities in Nigeria to treat fresh wounds, sores, microbial infection, diabetes and other tropical diseases; biological activities of its aqueous and alcoholic extracts have been widely reported but there is no report of isolation of bioactive compounds from this plant responsible for these activities. Isolation and characterization of compounds responsible for some of these biological activities will validate the use of the plant for the treatment of the ailments.

### **1.7 Aim of the Study**

This research was undertaken to isolate some bioactive components present in *Aspilia africana* and to investigate their anti-microbial and anti-diabetic activity.

### **1.8 Objectives of the Study**

1. To conduct phytochemical investigation of the crude methanol leaf extract and its fractions.
2. To isolate some of the bioactive constituents present in the leaves and characterize the isolates using spectroscopic analysis.
3. To conduct biological studies to validate the ethno medicinal uses of leaves of *Aspilia africana* as antimicrobial and antidiabetic agents

### **1.9 Statement of Research Hypothesis**

*Aspilia africana* contains compounds with anti-microbial and anti-diabetic activity.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

The family Asteraceae or Compositae commonly referred to as the aster, daisy, composite or sunflowerfamily is an exceedingly large and widespread family of flowering plants.The family has more than 23,600 currently accepted species, spread across 1,620 genera and 13 subfamilies(Laird, 2006).

#### 2.1 Description of the Family Asteraceae

Asteraceae (Compositae) is one of the largest families of flowering plants comprising of herbs, shrubs and trees distributed throughout the world. In West Africa, the family is represented by 84 genera and 288 species (Heywood *et al.*, 2007). The Asteraceae are an economically important family, providing products such as cooking oils, lettuce, sunflower seeds, artichokes, sweetening agents, coffee substitutes and herbal teas. Several genera are of horticultural importance, including pot marigold, *Calendula officinalis*, *Echinacea* (cone flowers), various daisies, fleabane, chrysanthemums, dahlias, zinnias, and heleniums. Asteraceae are important in herbal medicine, including *Grindelia*, yarrow, and many others.The phytochemicals widely distributed in high concentration in Asteraceae include tannins, terpenes, flavonoids, steroids and triterpenes and phytosteroids such as stigmasterol and sitosterol, while the presence of alkaloids is obscure, which may be the reason most of them are susceptible to herbivores (Duke, 1992;Petacci *et al.*, 2012).

#### 2.2 Morphology of *Aspilia*

The genus *Aspilia* (Pers.) consists of 94 species most of which are herbs and shrubs. *Aspilia* is native to Africa and Latin America. The stems are scabrid; leaves lanceolate, 4–12 cm long, 0.7–3.7 cm wide, base cuneate, margins serrate, apex attenuate to acuminate, scabrid on both surfaces and often also pubescent beneath; 3-veined from base; petiole 2–4 mm long, winged. Capitula in lax terminal corymbs; stalks of individual capitula 1–5 cm long; involucre ovoid, 3-seriate, 6–14 mm long; phyllaries recurved distally, some of the outer occasionally much shorter, scabrid; paleae 7 mm long, acuminate from a wider part (almost appendiculate). Ray florets 10–12, pale to rich yellow, ray 8–12 mm long, sometimes glandular axially, tube 2 mm long, style present; disc florets 4.8–6 mm long, glabrous or nearly so; anthers with black appendage (Burkill, 1985).

### 2.3 Phylogeny of *Aspilia africana*

Kingdom	-	Plantae
Phylum	-	Spermatophyta
Class	-	Angiospermales
Order	-	Asterales
Family	-	Asteraceae
Sub Order	-	Heliantheae
Genus	-	<i>Aspilia</i>
Species	-	<i>africana</i>
Author	-	(Pers)C. D. Adams



Plate I: *Aspilia africana* (Pers.) C. D. Adams (Family, Asteraceae) in its natural habitat

#### 2.4 Common Names

The plant is commonly known as wild sunflower and haemorrhage plant in English due to its ability to stop bleeding even from severed artery. In Nigeria it is called in Ibibio and Efik -édémèèdò η° meaning sheep's tongue; infulani-fulfulde - nyarki; Hausa- ja-majina, meaning - to draw up mucus, kalankuwa, meaning - headband, toozalin-yanmaataa; Igbo - òránjílá, odedétúá – meaning - flowering grass; Nupe - èyàyaká friend of pepper; Yoruba, yúnyún meaning scratch (Burkill, 1985).

## 2.5 Description of the plant

*Aspilia africana* is a semi perennial woody herb with bristly haired stem up to 2 m high (depending on the rainfall) bearing rough, hardy, opposite leaves and yellow or white flowers. The petals are roundish and attractive. It is dispersed by wind and cultivated by seed. It flowers between September and December (Ken, 2014).

## 2.6 Distribution

*Aspilia africana* grows ubiquitously in Nigeria, in the savannah and forested zones of Africa. It is very polymorphic with at least four recognized varieties widely distributed across tropical Africa. The plant is a rapidly growing weed of cultivated land and fallows. In Nigeria it is grazed by cattle and sheep, and is much used in the Western State as a food for rabbits and hares (Burkill, 1985).

The four varieties of *Aspilia africana* viz:

- a) *Aspilia africana* (Pers) C.D. Adams. Var. *africana*. A spreading herb or scrambling shrub up to 1.8m high commonly found throughout Nigeria.
- b) *Aspilia africana* (var. *minor*) C. D. Adams. A bushy herb of higher altitudes, found in Dalaba Plateau, Fouta Djallon, Guinea Bissau, 3000 – 4000 feet and Wum, Bamenda, Cameroon, 3600 feet.
- c) *Aspilia africana* (var. *ambigua*) C.D. Adams – a weed of cleared grounds in drier forest country with golden yellow ligules found in Ruruwe – Kano State; Zaria and Birnin-Gwari – Kaduna State; Bula – Bauchi State; Ibadan, Oyo State.
- d) *Aspilia africana* var. *guinensis* (O. Hoffm. & Muschl.) C.D. Adams. This is a perennial herb with narrow leaves and yellow ligules found in Kouria to Longuery, Republic of Guinea. (Dalziel, 1955; Burkill, 1985).

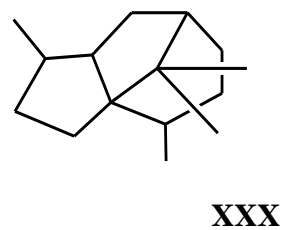
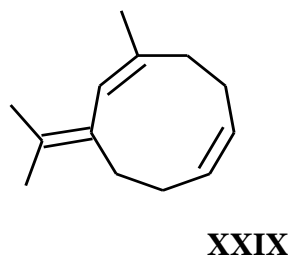
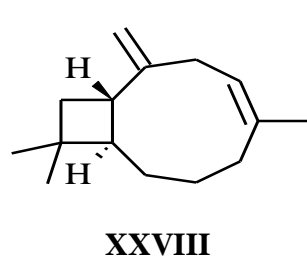
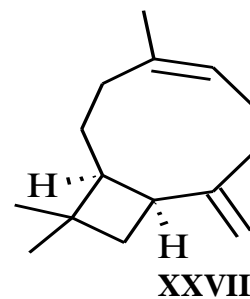
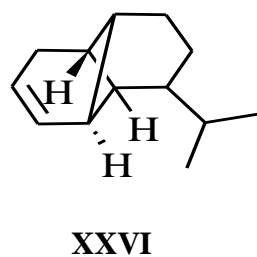
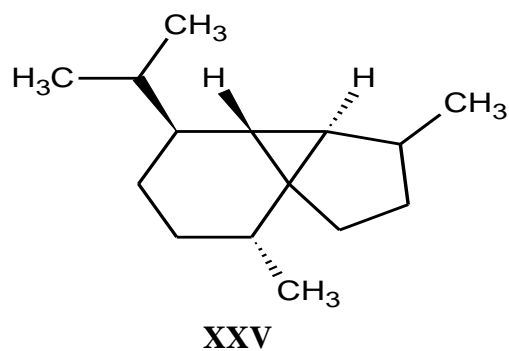
## 2.7 Ethno Medicinal Uses of *Aspilia africana*

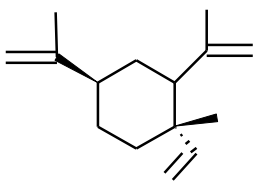
The plant *Aspilia africana* is used to treat different diseases in different ecological zones due to varying chemical composition as a result of various ecological conditions of the different places. The plant enjoys a folk reputation in Africa for its ability to stop bleeding even from severed artery as well as promote rapid healing of wounds and sores and for the management of problems related to cardiovascular diseases. The leaf decoction of *A. africana* is used by the Ibibios in the Niger Delta Region of Nigeria to treat diabetes (Okokonet *al.*, 2006b).

Besides using the fresh leaf on cuts the Ijaws of South-South Nigeria would also apply leaf-ash onto wounds and sores. A decoction has been recommended for use in treating pulmonary haemorrhage. In Tanzania, root-decoction is taken for tuberculosis and the leaf-sap is used to treat eye pains. In Ghana the leaves are used to treat cough in children and remove corneal opacities from the eye. In Uganda a leaf-concoction is taken for gonorrhoea. In Nigeria water decoction of the leaves is used as an eye-lotion for sun-blindness. The Igboland leaf-decoction is used to relieve headache and to prevent pregnancy. In Africa generally the leaf decoction is used to assist in childbirth and to promote milk flow in nursing mothers. The Ijaws of Southern Nigeria use the leaf-sap to revive a fainting person or someone suffering from an attack of nerves. The Yoruba use the plant to treat *craw-craw*, (the Yoruba name *yunyun* means 'scratch'). The plant is used in decoction for washing horses, and the leaves mixed with other materials for plastering mud floors. Amongst the Hausa, superstitious uses are prominent as a love-philtre, etc. A charm prepared from the plant and tied around the forehead attracts the 'glad eye' (*kalankuwa* means a headband); or a youth hides the plant in a maiden's house (Burkill, 1985).

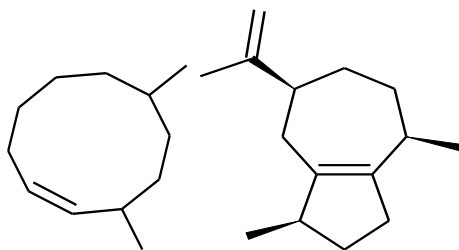
## 2.8 Phytochemistry of *Aspilia africana*

Previous phytochemical analysis of the leaves of *A. africana* led to identification of saponins and tannins as the most abundant constituents of the plant. Also present are sterols and other bioactive constituents comprising flavonoids, phenols, glycosides, resins, carbohydrates, proteins, fatty acids, essential oil and tannins (Page *et al.*, 1992; Adeniyi and Odufowora, 2000; Okoli *et al.*, 2007a). Detection of a number of terpenoids from the leaves of *A. africana* has been documented. Pulverized leaves of *A. africana* was found by Usman *et al.*, (2010) to contain oil which by GC-MS analysis gave sesquiterpenes namely; cubebene(XXV), copaene (XXVI), caryophyllene(XXVII), isocaryophyllene (XXVIII), germacrene B(XXIX), patchoulene(XXX), elemene(XXXI), germacrene D (XXXII), guaiene (XXXIII), cedrene (XXXIV) and santalol (XXXV), while the monoterpenes present were pinene(XXXVI).



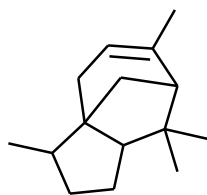


**XXXI**

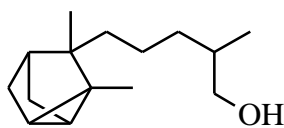


**XXXII**

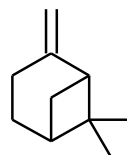
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**XXXIV**

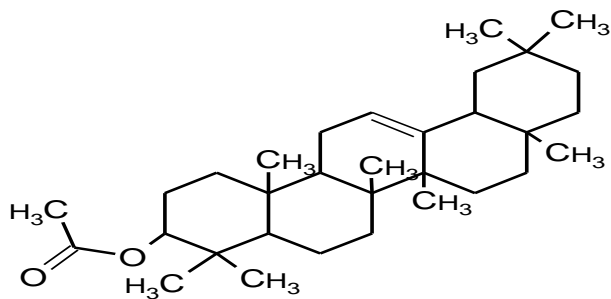


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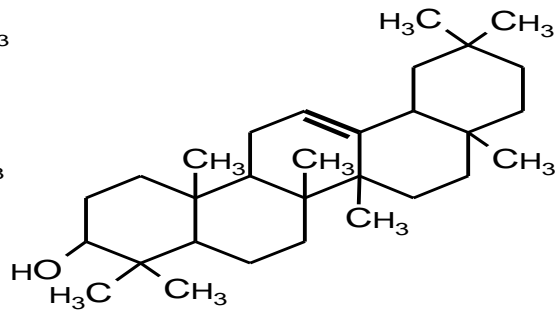


**XXXVI**

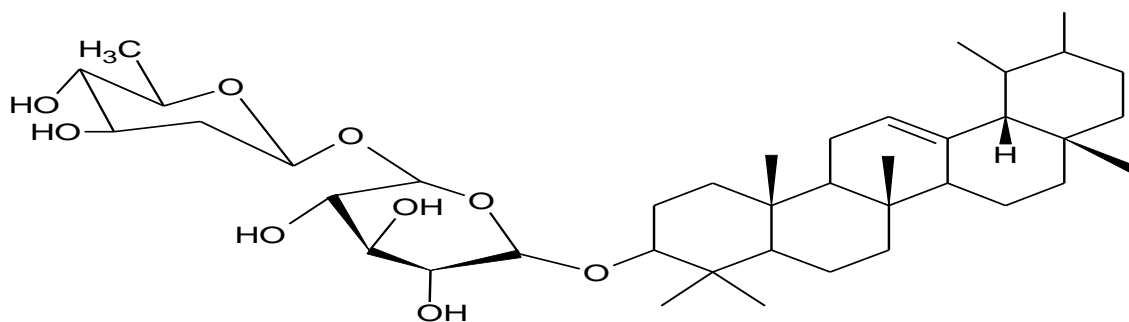
Page *et al.* (1992) isolated diterpenes- kaurenoic and grandiflorenic acids from the leaves of the plant. Faleye (2012) has also reported the isolation of three triterpenoids from the leaves of the plant namely: 3 $\beta$ -acetoxyolean-12-ene (**XXXVII**), 3 $\beta$ -Hydroxyolean-12-ene (**XXXVIII**) and 3 $\beta$ -O-[ $\alpha$ -rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -glucopyranosyl-(1 $\rightarrow$ 3)-ursan-12-ene (**XXXIX**). Inositol (**XL**) has been isolated from the ethanol leaf extract of the plant (Itaet *et al.*, 2010). Faleye and Abiodun (2012) have also reported the isolation of Trans Ethyl 3-(3, 4-dihydroxyphenyl acrylate) (**XLI**), 3-(3, 4-hydroxyphenyl-2-oxo-2H-chromene-6-carbaldehyde (**XLII**) and benzaldehyde (**XLIII**). The presence of a flavone, 5-hydroxy-3, 4, 6, 8-tetramethoxyflavone (**XLIV**) have been reported also (Kuiate *et al.*, 1999). This plant has been reported as a good source of minerals such as Ca, P, K, Mg, Na, Fe and Zn as well as vitamins such as ascorbic acid, riboflavin, thiamine, and niacin (Okwu and Josiah, 2006).



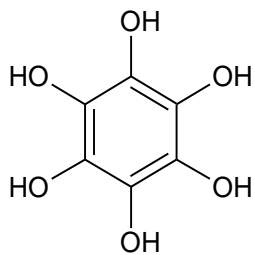
XXXVII



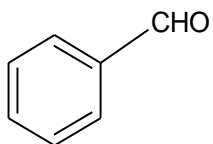
XXXVIII



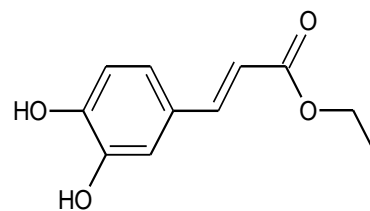
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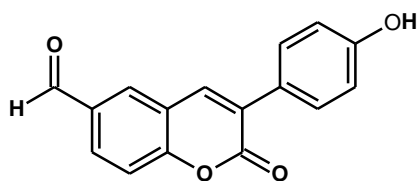
XL



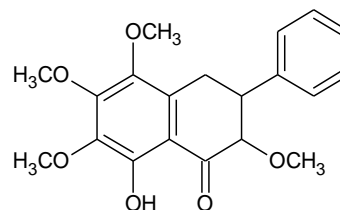
XLI



XLII



XLIII



XLIV

## 2.9 Biological Studies

*Aspilia africanaplant* has been fairly researched upon and found to have wide range of biological activities which include; antiviral, fungicide, anti-inflammatory, anti-ulcer, anticoagulant, antibacterial and membrane stabilization (Macfoy and Cline, 1990; Oyedapo *et al.*, 1998; Adeniyi and Odufowora, 2000; Okoli *et al.*, 2007b; Ubaka *et al.*, 2010). The leaf extract has also been shown to cause extracellular  $Ca^{2+}$  dependent increase in vascular tone and *in-vitro* vascular smooth muscle contractile activity (Dimo *et al.*, 2002). The ethanol leaf extract of *A. Africana* is very effective in the treatment of malaria; this was evaluated in mice with *Plasmodium berghei berghei* during early and established infection (Okokon *et al.*, 2006a). Aqueous extract of *A. africana* leaf is reported to show anti – fertility effect by altering oestrous cycle and causing a dose dependent adverse effect on ovulation in Wister strain rats (Oluyemiet *al.*, 2007).

The plant was noted to exhibit antimicrobial activities against five bacterial strains namely *Staphylococcus aureus*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* (Anibijuwonet *al.*, 2010). The median lethal dose (LD<sub>50</sub>) determined by Taziebouet *al.*, (2007) of the aqueous leaf extract using rats by oral administration was an average of 6.6g/kg body weight. The result of anti-ulcer effects of the aqueous extract showed significant ( $p < 0.05$ ) dose dependent anti-ulcer activity in the three ulcer models used (Ubaka *et al.*, 2010). Anti-diabetic and hypolipidaemic activity of ethanol leaf extract of this plant was examined by Okokon and his team of researchers (Okokon *et al.*, 2006b) and the results suggest that the leaf extract possesses anti-diabetic and hypolipidaemic properties.

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Materials/Reagents/Equipment and Analytical Procedure

##### 3.1.1 Reagents and Solvents

Distilled water, ethanol (99%, Aldrich), ferric chloride (Aldrich), bromine water (M&B), hydrochloric acid (M&B), Meyer reagent (Fischers), Dragendoff's reagent (Fischers), Ammonia (J.T. Baker), chloroform (BDH) sulfuric acid (Fischers), lead acetate (M&B), Magnesium metal (Philip Harris), glacial acetic acid (M&B) acetic anhydride (M&B), Fehling's solution A&B (Fischers), nitric acid (M&B), sodium hydroxide (M&B), silica gel (60 – 120 mesh, (BDH) dichloromethane (Sigma-Aldrich), ethyl acetate (Sigma-Aldrich), n-hexane (Nade & Dave, Engl.), Methanol (Sigma-Aldrich), ethanol (Sigma- Aldrich), n-butanol (BDH), diethyl ether (M&B), acetone (M&B).The silica gel (60-120 mesh) for column chromatography, sephadex LH-20 and the analytical grade reagents used were obtained from Sigma Aldrich.  $\alpha$ -amylase (from *Aspergillus oryzae*; CAS No 9001-19-8, Enz No 3-2-1.1) and  $\alpha$ -glucosidase (from Baker's yeast; CAS No 9001-42-47), 4-Nitrophenyl- $\alpha$ -D-glucopyranoside (CAS No 3767-28-0) were obtained from Sigma-Aldrich (USA).

##### 3.1.2 Equipment

Electronic weighing balance (Metlar, Mt 200), fume cupboard (Vultxlabline), electric water bath (Uniscope), electric oven (Gallenkamp, DHG 9101, England), 2mm sieve (Shital Scientific Industry, Bombay 400013), beakers (Pyrex), heating mantle (Bio-Tech, India, Mumbai 400016), conical flask (Pyrex and Simax), Atomic Absorption Spectrophotometer (Solar 969AA), crucible

(England), Desiccators (England), spatula, muffle furnace (Thomas Scientific), poplin cloth, dry porous thimble (England), retort stand (Unicon), glass funnel (Pyrex), maceration tank (Uniscope), syringes and needles (1mL, 5mL, Dana), cages, measuring cylinders (Pyrex), test tubes (Simax), test tube wrack, test tube holders, sintered glass column, chromatographic tank. FTIR (KBr) - 8400S Shimadzu, Japan. Electrothermal Melting Point Apparatus was used to determine the points. The UV- spectrum was recorded on Pye Unicam. TLC Silica gel 60 F<sub>254</sub> pre-coated (Aluminium) plates were obtained from Merck, Germany. The Proton NMR (<sup>1</sup>H NMR) and Carbon-13 NMR (<sup>13</sup>C NMR) spectra were run on a JEOL AS 400 (400 MHz) spectrometer (Japan) using DMSO-d<sub>6</sub>, CDCl<sub>3</sub> and CD<sub>3</sub>OD as solvents and TMS as internal standard. Solvent peaks were at 7.26 ppm for CDCl<sub>3</sub> and 2.50 ppm for DMSO-d<sub>6</sub>. The chemical shifts were reported as δ ppm relative to TMS. The samples were all run at temperature of 25°C.

### **3.1.3 Collection and Identification of Plant Materials**

The fresh plant material was collected in Wusasa, Zaria, Northern Nigeria and identified by a taxonomist Umar Gallah at Herbarium unit of the Department of Biological Science, Ahmadu Bello University, Zaria by comparing with the existing Herbarium avoucher sample (Specimen No. 1146). The plant material was rinsed in water to remove dust and other dirt and dried under shed. This dried material was pulverized and sieved. The powdered, weighed sample was stored in a labelled airtight container until when needed for use.

### **3.1.4 Extraction Procedures**

Powdered leaf material (1kg) of *A. africana* was cold-macerated in H<sub>2</sub>O-MEOH (30:70) for 72 h, filtered and concentrated *in-vacuo* to give dark-brown gummy methanol extract. The

extract(120 g) was suspended in water and the water-soluble portion was sequentially and exhaustively extracted with n-hexane, dichloromethane, ethyl acetate and n-butanol to give (after removing the solvent *in-vacuo*), n-hexane, dichloromethane, ethyl acetate and n-butanol fractions respectively. The fractions were concentrated to dryness *in-vacuo* and stored in desiccators until needed for use.

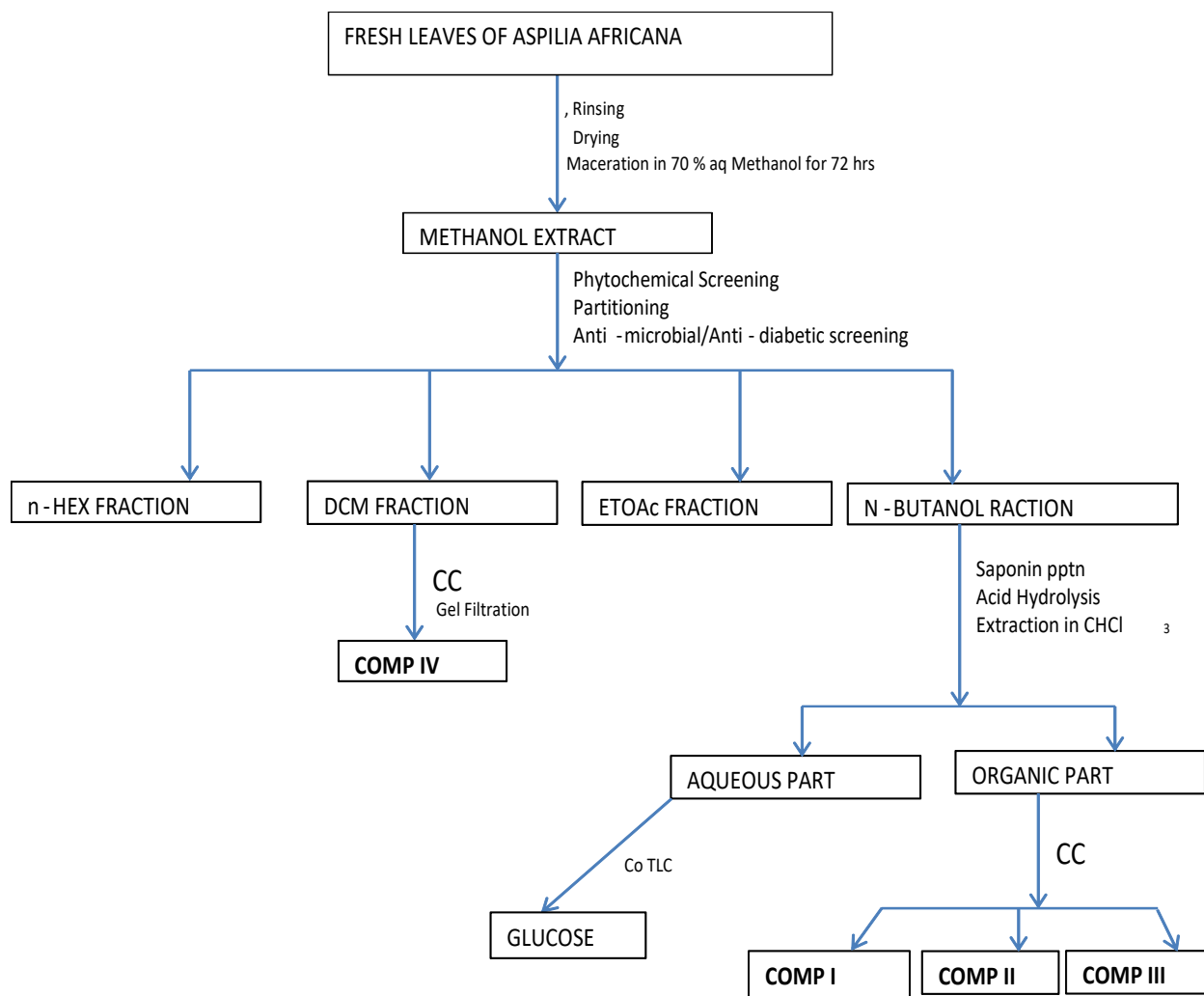


Fig. 3.1: Extraction and Isolation Flow Chart of methanol leaf extract of *Aspilia africana*

## 3.2 Preliminary Phytochemical Screening

The extract and fractions were subjected to preliminary phytochemical screening to detect the secondary metabolites present using standard methods.

### 3.2.1 Test for Alkaloids

Extract/fraction (0.5g) was stirred in a test tube with 5mL of 5% HCl on a water bath and then filtered. The filtrate was divided into three portions of 1 mL each.

- a) To the first portion, 3 drops of Dragendorff's reagent were added. Orange-red precipitate obtained indicates the presence of alkaloids.
- b) To the second portion, 3 drops of Meyer's reagent were added. A cream-coloured precipitate indicates the presence of alkaloids.
- c) To the third portion 3 drops of Wagner's reagent was added. A reddish brown precipitate was taken for the presence of alkaloids (Trease and Evans, 2002).

### 3.2.2 Test for Flavonoids

a) **Shinoda's Test:** The extract/fraction (0.1g) was dissolved in ethanol warmed and filtered. Three pieces of magnesium turnings were added to the filtrate followed by a few drops of conc. HCl. Observation of pink precipitate indicates the presence of flavonoids (Trease and Evans, 2002).

b) **Ferric Chloride Test:** Extract/fraction (0.5g) was dissolved in ethanol and boiled with a few drops of 10% FeCl<sub>3</sub> solution. A violet colouration indicates the presence of a phenolic hydroxyl group (Trease and Evans, 2002).

### 3.2.3 Test for Anthraquinones

**Borntrager's Test For Free Hydroxyanthraquinones:** Extract/fraction (0.1g) was shaken with 10mL of benzene and filtered. Few drops 10%  $\text{NH}_3$  solution were added to the filtrate and shaken again. A colour change from pink to violet in the ammoniacal (lower) phase indicates the presence of free hydroxyanthraquinones (Trease and Evans, 2002).

### 3.2.4 Test for Saponins

a) **Frothing test:** Extract/fraction (0.5g) was shaken with distilled water in a test tube and formation of foams which persisted on warming indicates the presence of saponins (Sofowora, 2008).

b) **Emulsion Test:** The result foaming mixture from 3.2.4(a) above was mixed with a small quantity of olive oil and shaken vigorously. Formation of an emulsion indicates the presence of saponins (Sofowora, 2008).

### 3.2.5 Test for Tannins

a) **Ferric Chloride Test:** The extract/fraction (0.5g) was mixed with distilled water (10mL) and filtered. 5%  $\text{FeCl}_3$  solution was added to the filtrate. A green precipitate observed indicates the presence of tannins (Trease and Evans, 2002).

b) **Bromine water Test:** The extract/fraction (0.5g) was mixed with distilled water (10mL) and bromine water added. Discoloration of the bromine water indicates the presence of tannins (Trease and Evans, 2009).

### 3.2.6 Test for Cardenolides

a) **Keller – Kiliani Test for Digitalis Glycosides:** The extract (0.5g) was dissolved in 2mL glacial acetic acid. One drop of  $\text{FeCl}_3$  was added followed by 1mL of conc.  $\text{H}_2\text{SO}_4$ . A reddish-brown ring observed at the inter-phase indicates the presence of digitoxose sugar component of cardenolides (Trease and Evans, 2002).

b) **Legal Test for Cardenolides (Aglcone):** The extract/fraction (0.5g) was dissolved in pyridine and a few drops of sodium nitroprusside together with a few drops of 20% NaOH were added. A red colour which fades to brownish yellow indicates the presence of cardenolide (Sofowora, 2008).

### 3.2.7 Test for Carbohydrates

a) **Fehling's Test for Reducing Sugars:** 0.5g of the extract was dissolved in water and a few drops of a mixture of Fehling's solution A and B were added and heated. A brick red precipitate observed indicated the presence of reducing sugars (Harborne, 1984).

b) **Molisch's Test:** The extract/fraction (0.5g) was dissolved in 2mL of distilled water and shaken. 1mL each of  $\alpha$ -Naphthol and of conc.  $\text{H}_2\text{SO}_4$  was added to the mixture and shaken. Purple ring formed at the inter-phase indicates the presence of carbohydrate (Brain and Turner, 1975).

c) **Barfoed's test for Monosaccharides:** The plant extract/fraction (0.1g) was dissolved in 2mL of distilled water, shaken and filtered. 2 mL of Barfoed's reagent was added to the filtrate and heated in a water bath for 2 minutes. A red precipitate of  $\text{Cu}_2\text{O}$  observed indicates the presence of monosaccharides (Brain and Turner, 1975).

### 3.2.8 Test for Terpenoids: Liebermann-Burchard's Test

The extract/fraction (0.5g) was dissolved in  $\text{CHCl}_3$  and 1mL of acetic anhydride added, followed by 3 drops of conc.  $\text{H}_2\text{SO}_4$ . A colour change from pink to violet indicates the presence of terpenoids (Harborne, 1984).

### 3.2.9 Test for Polyphenols: Ferric Chloride Test

The extract/fraction (0.5g) was heated with 10mL of distilled water for 30 minutes. A mixture of 1%  $\text{FeCl}_3$  and  $\text{K}_4\text{Fe}(\text{CN})_6$  was added to the extract. Deep-blue precipitate formed indicates the presence of polyphenols (Trease and Evans, 2002).

## 3.3 Chromatographic Techniques

### 3.3.1 Thin Layer Chromatography (TLC)

Thin layer chromatography of the extracts and fractions was carried out on silica gel pre-coated aluminium plates (PF<sub>254</sub>; 0.2mm)

*Technique:* one way ascending

*Spotting and development:* spots were applied manually using capillary tubes; plates were dried using air blower. The plates were developed at room temperature using Shandon chromatographic tank.

*Solvent system:* n-hexane:ETOAc/ $\text{CHCl}_3$ : MEOH solvent system in various ratios

*Visualization* UV light (254 and 366nm), iodine vapour, 10%  $\text{H}_2\text{SO}_4$  in ethanol/heat (110°C, 5-10min in oven).

### 3.3.2 Column chromatography (CC)

The following column conditions were employed to run the CC

<i>Technique:</i>	Gradient elution
<i>Column:</i>	Sintered Glass column of different dimensions
<i>Stationary phase:</i>	Silica gel, 60-120 mesh size
<i>Column packing:</i>	Wet slurry method
<i>Sample loading:</i>	Dry load method was applied – silica gel was added to the sample dissolved in a small quantity of a suitable solvent, triturated and then loaded on top of the column (Richard, 1998).
<i>Solvent for elution:</i>	Different ratios of the following solvents were used depending on the polarity of the plant materials – n-hexane, chloroform/dichloromethane, ethyl acetate, acetone and methanol.

### **3.3.3 Gel filtration Chromatographic Technique**

Gel filtration was performed using sephadex LH-20 (Sigma-Aldrich).

<i>Packing method:</i>	sephadex LH-20 was suspended in methanol for 24 hours in order to swell. It was then poured in to the column and allowed to stand overnight.
<i>Sample application:</i>	the sample was dissolved in a small quantity of the eluent and applied on to the top of the column.
<i>Solvent:</i>	n-hexane, ethyl acetate, chloroform and methanol indifferent ratios depending on polarity of the plant materials.

### 3.3.4 Preparative thin layer chromatography (PTLC)

Preparative thin layer chromatography was carried out on Fluka silica gel pre-coated glass plates 20 x 20 cm with layer 0.25 mm thick.

*Technique:* one way ascending

*Spotting and development:* the sample was dissolved in a small quantity of solvent to give a concentration of 25mg/mL. This was applied manually and uniformly to form a horizontal band 1 cm above the base of one of the edges of the plate using capillary tubes. The plates were developed at room temperature using Shandon chromatographic tank. The developed plate was dried using air blower and the position of the bands of interest were scraped off. The scraped sorbent was dissolved in chloroform and filtered. The filtrate was evaporated to obtain the compound.

*Solvent system:* n-hexane: ETOAc: CHCl<sub>3</sub>: MEOH solvent system in various ratios depending on the polarity of the samples.

## 3.4. Isolation of Compounds I, II and III

### 3.4.1 Hydrolysis of n-butanol fraction

Acid hydrolysis (Prawatet *et al.*, 1989; Alvarez *et al.*, 2003,) was performed on the n-butanol fraction, which was found to froth copiously to isolate the aglycone part of the saponins. 6g of n-butanol fraction was dissolved in 50mL methanol in a 250mL beaker and diethyl ether was added slowly by the side of the beaker until the precipitation was complete. The precipitate was centrifuged and the solid residue dried to strip of the solvent completely. This was weighed and placed in a round bottomed flask to which 50mL 4M HCl (dioxane, 1:1 H<sub>2</sub>O) was added. The set-up was refluxed on a water bath for 5 hours at 90°C. The resulting mixture was cooled and extracted with CHCl<sub>3</sub>. The CHCl<sub>3</sub> extract was evaporated to dryness *in-vacuo*. The dried mass was subjected to column chromatography. The aqueous part was tested for the presence of sugars using comparative TLC with simple monosaccharide sugars.

### **3.4.2 Column Chromatography of the hydrolysed n-Butanol Fraction**

n-Butanol fraction (2g, hydrolysed) was chromatographed over silica gel (60 – 120 mesh) packed column (3x45 cm) eluted with CHCl<sub>3</sub>-MEOH solvent system in increasing polarity starting with ratio: 99:1. 100 eluates of 10mL aliquot each were collected. Eluates with similar R<sub>f</sub> on TLC were pooled together. A total 12 major fractions were obtained (HYD1 - HYD12). Fraction HYD4 (eluates 17-26) gave a single pink spot, which when concentrated and recrystallized in methanol yielded an off-white powder which on further recrystallization afforded a white amorphous powder designated as COMPOUND I. Fraction HYD6 (eluate 32) gave a single violet spot which was concentrated and on recrystallization afforded white amorphous powder coded COMPOUND II. Fraction HYD7 (eluate 33-36) gave two spots on TLC; this was subjected to repeated gel filtration on sephadex LH-20 eluted with CHCl<sub>3</sub> 1:1 MEOH and finally purified with preparative thin layer chromatography to afford a single spot on TLC. This was concentrated and allowed to

cool in chloroform-methanol (1:1) mixture. A white amorphous powder settled at the bottom on cooling. This was designated as COMPOUND III

### **3.5 Isolation of COMPOUND IV**

DCM fraction (2g) was chromatographed over silica gel (60 – 120 mesh) packed column (3.5 x 45cm) eluted using n-hexane/ethyl acetate/methanol solvent system. 215 eluates of 10 mL aliquot were collected. Based on TLC report, fractions with similar  $R_f$  were pooled together to obtain 11 major fractions (D1 – D11). Fraction D6 (50-57) which showed 2 major spots was subjected to repeated gel filtration over sephadex LH<sub>20</sub> and eluted using n-hexane/ ethyl acetate in increasing order of polarity starting with 90:1 to afford a fraction with a single spot on TLC. Recrystallization of this fraction in CHCl<sub>3</sub>: MEOH (1:1) mixture yielded a white powder which was designated as COMPOUND IV.

### **3.6 Analyses of the Isolates**

#### **3.6.1 Physicochemical Tests**

The tests were carried out on compounds I, II and III. The yield of compound IV was too small for these tests.

##### **(a) Melting Point Determination**

The melting points were determined using Electrothermal Melting Point Apparatus. The melting point values were uncorrected.

##### **(b) Solubility Test**

The solubility of Compounds I, II and III was determined in water, methanol, chloroform and n-hexane and acetone.

(c) Acid Test:

The isolated compound (1 mg) was added to 2 mL of sodium hydrogen carbonate solution and shaken.

(d) Liebermann-Burchard's test:

The isolated compound (2 mg) was dissolved in acetic acid anhydride, heated to boiling, cooled and then 1 mL of conc.  $H_2SO_4$  added along the side of the test tube.

(e) Fehling's Test

The isolated compound(2 mg) of was dissolved in water and a few drops of a mixture of Fehling's solution A and B mixture added and heated to boiling.

### 3.6.2 Spectral Analysis of the Isolated Compounds

(a) Ultra-violet (UV) Analysis

UV was run on Spectro UV-VIS 2700 Dual beam (200 – 1100nm) Labomed, Inc. USA. About 1mg of each of isolated compound was dissolved in 5mL of chloroform and absorbance recorded at a wavelength of 200-400nm using the solvent (chloroform) as blank.

(b) Fourier Transform Infra-red (FTIR) Analysis

FTIR analysis was done using Shimadzu FTIR 8400S Fourier Transform Infrared Spectrophotometer: 1mg of each of the isolated compounds was ground into fine powder with anhydrous potassium bromide (KBr); the mixture was pressed into a transparent disc under a pressure of 10,000 psi

(c) Nuclear Magnetic Resonance (NMR)

The Proton NMR ( $^1\text{H}$  NMR) and Carbon-13 NMR ( $^{13}\text{C}$  NMR) spectra were run on a JEOL AS 400 (400 MHz) spectrometer (Japan) using  $\text{CDCl}_3$  or  $\text{DMSO-d}_6$  as solvents and TMS as internal standard. Solvent peaks were at 7.26 ppm for  $\text{CDCl}_3$  and 2.50 ppm for  $\text{DMSO-d}_6$ . The samples were all run at  $25^\circ\text{C}$ .

### 3.7 Analysis of the Hydrolysedn-Butanol Fraction

The TLC of the aqueous portion of the hydroxylate of the n-butanol fraction was carried out using pre-coated aluminium plates (0.2mm) and the solvent system -  $\text{CHCl}_3:\text{AcOH}:\text{H}_2\text{O}(6:7:1;$  v/v/v. The plates were spotted with isolated compounds and standard sugar – glucose, glucuronic acid, fructosemannose, rhamnose and xylose and developed twice in an air-tight Shandon chromatographic tank. For the first time the solvent mixture was made to rise to 12.5cm of the height of the plate. The plates were then removed from the tank and left to dry in air for 30 minutes and then placed again in the same developing solvent and allowed to rise in the same direction to the same height. The dried plates were sprayed with spraying agent (1g of diphenylamine, 1mL of aniline in 100mL of acetone, mixed with 85% orthophosphoric acid in the ratio 10:1(v/v) and heated at  $150^\circ\text{C}$  in oven for 4minutes to reveal the spots (Frag, 1978).

### 3.8 Anti-Microbial Studies

#### 3.8.1 Test Micro-organisms

The anti-microbial activity of the extracts, fractions and the isolated compounds of *A. africanus* was studied using the Agar well diffusion method (Adeniyi *et al.*, 2013) and some clinical isolates of pathogenic micro-organisms obtained from the Department of Medical Microbiology, ABUTH, Zaria. Mueller-Hinton agar medium was used for the anti-bacterial study while Sabouraud dextrose agar medium was used for antifungal study and nutrient agar medium for storing and preserving the bacterial organisms.

The bacterial species used for the study were: *Staphylococcus aureus*, *Methicillin Resistant Staphylococcus aureus (MRSA)*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Listeria monocytogenes*, *Proteus vulgaris*, *Pseudomonas fluorescense*, *Salmonella typhi*, *Shigella dysenteriae*, *Escherichia coli* and *Klebsiella pneumoniae*. The fungal species used were: *Candida albicans*, *Candida tropicalis* and *Candida stellatoidea*. The organisms were cultivated overnight in a nutrient broth (or sabouraud dextrose broth) and sustained on a slant at 4°C before use. 0.2mL of the overnight broth culture of each organism was dispensed into 20mL of the respective media and incubated for 5 hours to standardize the culture to 10<sup>6</sup>cfu/mL (Gramer, 1976; Murray *et al.*, 1995)

#### 3.8.2 Antimicrobial Susceptibility Test

The 25mL of the sterilized medium was then seeded with 0.1mL of the standard inoculums of the test microbe in the plate aseptically. The inoculum was spread evenly over the surface of the medium with a sterile swab. The plates were allowed to solidify on the laboratory bench. By means of a standard sterile cork borer (6mm in diameter), a hole (well) was made at the centre of

each of the plates. 0.1mL of the solution of the extracts and fractions of concentration of 40mg/mL was then introduced into each the holes. This procedure was also carried out for the isolated compounds (concentration = 200µg/mL) and standard drugs used as control, namely ciprofloxacin(for the bacterial strain) and fluconazole (for the fungal strains) (concentration = 50µg/mL). The plates were incubated at 37°C for 24 hours, after which each plate was observed for zone of inhibition of growth. The diameter of the hole was measured using a transparent ruler and recorded in millimetres. The antimicrobial activity was expressed in terms of the average diameter of zones of inhibition (Adeniyi *et al.*, 2013).

### **3.8.3 Minimum Inhibitory Concentration of the Extracts and isolates**

The minimum inhibition concentrations of the extracts were determined using the broth dilution method. 10mL of Mueller-Hinton broth prepared was dispensed into test tube and sterilized at 121°C for 15 minutes. The broth was then allowed to cool. Mc-Farland's turbidity standard scale number 0.5 was prepared to give a turbid solution. Normal saline solution was also prepared and 10mL of this was dispensed into the sterile test tube and the test microbe grown in the normal saline until the turbidity matched that of the Mc-Farland's scale by visual comparison. At this point, the test microbe has a concentration of about  $1.5 \times 10^8$  cfu/mL. 0.4g each of the extracts and fractions was dissolved in 10mL of the sterile broth and further two-fold dilution was made such that the concentrations of 40mg/mL, 20mg/mL, 10mg/mL, 5mg/mL and 2.5mg/mL were obtained. For the isolated compounds, 0.002g was dissolved in 10mL sterile broth to obtain initial stock concentration of 200µg/mL, which was further diluted to 100µg/mL, 50µg/mL, 25µg/mL, and 12.5µg/mL. To the different concentrations of the extracts and the isolated compounds in the sterile broth (DMSO) 0.1mL of the standard inoculums of the test microbe in the normal saline

was added and incubated at 37°C (24 h for bacteria and 72h for fungi) after which each of the test tubes was observed for turbidity (growth). The lowest concentration of the extract in the broth which showed no turbidity was recorded as MIC.

#### **3.8.4 Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)**

The Mueller-Hinton agar was prepared and sterilized at 121°C for 15 minutes. The medium was poured into sterile plates and allowed to cool and solidify. The contents of the tube prepared as MIC in the serial dilution were then sub-cultured onto the prepared medium. Incubation was made at 37 °C for 24 hours (72 h for fungi) after which each plate was examined for colony growth. The MBC/MFCs were the plates with lowest concentrations of the extract without colony growth ((Adeniyi *et al.*, 2013).

### **3.9 Anti-Diabetic Studies**

#### **3.9.1 Determination of *In Vitro* α-Amylase Inhibition**

This experimental procedure was carried out as reported by Sindhu *et al.*, (2013). The assay mixture comprising of 200µL of 0.02M sodium phosphate buffer, 20µL of enzyme and each of the plant products used namely; leaf MEOH extract (in concentrations of 20, 40, 60, 80 and 100mg/mL), BUOH fraction (in concentrations of 20, 40, 60, 80 and 100mg/mL) and isolated compounds (in concentrations of 20, 40, 60, 80 and 100µg/mL) were incubated separately for 10 minutes at room temperature after which 200µL of starch was added to all the test tubes. The reaction was terminated with addition of 400µL dinitrosalicylic acid reagent and placed in boiling water for 5 minutes, cooled and diluted with 15mL of distilled water and the absorbance was measured at 540nm. The percentage inhibition was calculated as:

$$\% \text{Inhibition} = \frac{\text{Abs}_{(540)\text{Control}} - \text{Abs}_{(540)\text{plant extract}} \times 100}{\text{Abs}_{(540)\text{Control}}}$$

The concentrations of MEOH leaf extract, BUOH fraction and the isolated compound required to inhibit 50% of the  $\alpha$ -Amylase activity were defined by the  $\text{IC}_{50}$  values determined from the plot of percentage inhibition versus log of inhibitor concentration and calculated by non-linear regression analysis from the mean inhibitory values. Acarbose was the reference  $\alpha$ -amylase inhibitor and all tests were performed in triplicate.

### 3.9.2 Determination of *In Vitro* $\alpha$ -Glucosidase Inhibition

*In vitro*  $\alpha$ -glucosidase inhibition assay was carried out as reported by Adefegha and Oboh, (2012). The  $\alpha$ -glucosidase was dissolved in 100 mM phosphate buffer (pH = 6.8) and was used as the enzyme extract. P-nitrophenyl- $\alpha$ -D-glucopyranoside was used as the substrate. Different concentrations of the test samples (20, 40, 60, 80 and 100 mg/mL for MEOH extract and BUOH fraction; 20, 40, 60, 80 and 100  $\mu$ g/mL for the isolated compound) were stirred separately with 320  $\mu$ L of 100 mM phosphate buffer for 5 minutes. 3 mL of 50 mM sodium hydroxide was added to the mixture and the absorbance was read at 410 nm. The control samples were prepared without the plant extracts. The percentage inhibition was calculated as:

$$\% \text{inhibition} = \frac{\text{Abs}_{(410)\text{Control}} - \text{Abs}_{(410)\text{plant extract}} \times 100}{\text{Abs}_{(410)\text{Control}}}$$

The concentration of methanol extract/fraction required to inhibit 50% of  $\alpha$ -glucosidase activity was defined by the  $\text{IC}_{50}$  value determined from the plot of percentage inhibition versus log of inhibitor concentration and were calculated by non-linear regression analysis from the mean of inhibitory values. Acarbose was used as the reference  $\alpha$ -glucosidase inhibitor and all the tests were performed in triplicate.

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.1 The Yield of the Extract and Fractions

The yield of the extract and fractions are represented in table 4.1

Table 4.1 Percentage Yield of Extract and Fractions

Extract/Fractions	Yield (g)	% Yield(w/w)	Description
Methanol	150.8	15.08	Dark brown gummy mass with shiny specks on the surface
n-Hexane	15.0	12.50	Red oil
Dichloromethane	18.5	15.41	Yellow mass
Ethyl acetate	10.7	8.91	Dark brown mass
n-Butanol	35.8	29.83	Dark mass

#### 4.2 Preliminary Phytochemical constituents of methanol leaf extract and fractions

The phytochemical screening of methanol leaf extract and fractions of *A. africana* revealed the presence of saponins, terpenoids, tannins, flavonoids, steroids glycosides and carbohydrates while alkaloids absent (Tables 4.2 – 4.5).

Table 4.2: Phytochemical Constituents of the Methanol Extract of leaves of *A. africana*

CONSTITUENTS	TEST	OBSERVATION	INFERENCE
Alkaloid	Dragendorff's	-	-
	Mayer's	-	-
	Wagner's	-	-
Anthraquinones	Borntrager's	Colour change - Pink to violet	+
Carbohydrates	Barfoed's	Red ppt	+
	Fehling's	Brick red ppt	+
	Molisch's	Red colouration	+
Cardenolides	Keller-Kelliani's	Reddish brown ring at interface	+
	Legal	Red colour faded brownish yellow	+
Flavonoids	Shinoda's	Green colouration	+
	FeCl <sub>3</sub>	Blue colouration	+
Polyphenols	FeCl <sub>3</sub>	Deep blue ppt	+
Saponins	Emulsion	Formation of emulsion	+
	Frothing	Frothing persist on warming	+
Tannins	Ferric Chloride Test	Green ppt	+
	Bromine water	Decolourized Bromine water	+
Terpenoids	Liebermann-Burchard	Pink/violet colouration	+
KEY:	-	= Absent;	+ = Present.

Table 4.3: Phytochemical Constituents of the n-Hexane Fraction of the Methanol Extract

CONSTITUENTS	TEST	OBSERVATION	INFERENCE
Alkaloid	Dragendorff's	-	-
	Mayer's	-	-
	Wagner's	-	-
Anthraquinones	Borntrager's	-	-
Carbohydrates	Barfoed's	Red ppt	+
	Fehling's	Brick red ppt	+
	Molisch's	Red colouration	+
Cardenolides	Keller-Kelliani's	-	-
	Legal	-	-
Flavonoids	Shinoda's	-	-
	FeCl <sub>3</sub>	-	-
Polyphenols	FeCl <sub>3</sub>	Deep blue ppt	+
Saponins	Emulsion	Formation of emulsion	+
	Frothing	Frothings persist on warming	+
Tannins	Ferric Chloride Test	Green ppt	+
	Bromine water	Decolourized Bromine water	+
Terpenoids	Liebermann-Burchard's	Pink/violet colouration	+
KEY:	- = Absent;	+ =	Present.

Table 4.4: Phytochemical Constituents of the ETOAc Fraction

CONSTITUENTS	TEST	OBSERVATION	INFERENCE
Alkaloid	Dragendorff's	-	-
	Mayer's	-	-
	Wagner's	-	-
Anthraquinones	Borntrager's	Colour change - Pink to violet	-
Carbohydrates	Barfoed's	Red ppt	+
	Fehling's	Brick red ppt	+
	Molisch's	Red colouration	+
Cardenolides	Keller-Kelliani's	-	-
	Legal	-	-
Flavonoids	Shinoda's	Green colouration	+
	FeCl <sub>3</sub>	Blue colouration	+
Polyphenols	FeCl <sub>3</sub>	Deep blue ppt	+
Saponins	Emulsion	Formation of emulsion	+
	Frothing	Frothing persist on warming	+
Tannins	Ferric Chloride Test	-	-
	Bromine water	-	-
Terpenoids	Liebermann-Burchard's	Violet colouration	+
KEY:	-	= Absent;	+ = Present.

Table 4.5: Phytochemical Constituents of the n-Butanol Fraction

CONSTITUENTS	TEST	OBSERVATION	INFERENCE	
Alkaloid	Dragendorff's	-	-	
	Mayer's	-	-	
	Wagner's	-	-	
Anthraquinones	Borntrager's	Colour change - Pink to violet	-	
Carbohydrates	Barfoed's	Red ppt	+	
	Fehling's	Brick red ppt	+	
	Molisch's	Red colouration	+	
Cardenolides	Keller-Kelliani's	Reddish brown at interface	+	
	Legal	Reddish brown fading to brownish yellow	+	
Flavonoids	Shinoda's	Green colouration	+	
	FeCl <sub>3</sub>	Blue colouration	+	
Polyphenols	FeCl <sub>3</sub>	Deep blue ppt	+	
Saponins	Emulsion	Formation of emulsion	+	
	Frothing	Frothing persist on warming	+	
Tannins	Ferric Chloride Test	Green ppt	+	
	Bromine water	Decolourize bromine water	+	
Terpenoids	Liebermann-Burchard's	Pink/violet colouration	+	
KEY:	-	= Absent	+	= Present.

### 4.3 Thin Layer Chromatography of the Leaf Methanol Extract

Thin layer chromatography of the crude methanol leaf extract using  $\text{CHCl}_3$ : MEOH ; 9:1, revealed 8 spots when sprayed with 10%  $\text{H}_2\text{SO}_4$  in ethanol and heated in oven at  $110^\circ\text{C}$  for 5 minutes (Table 4.2, Plate 4.1)

Table 4.6: TLC Analysis of the Methanol Extract ( $\text{CHCl}_3$ : MEOH; 9:1)

Spot	Colour (10% $\text{H}_2\text{SO}_4$ in ETOH)	$R_f$
1	brown	0.05
2	light brown	0.12
3	violet	0.19
4	brown	0.23
5	grey	0.35
6	brown	0.51
7	purplish yellow	0.62
8	green	0.72



Plate II: TLC profile of methanol leaf extract of *Aspilia africana*(CHCl<sub>3</sub>: MEOH; 9:1)

Table 4.7: TLC Profile of the DCM Fraction (n-Hex: ETOAc: MEOH ; 5:4:1)

Spot	Colour (10% H <sub>2</sub> SO <sub>4</sub> in ETOH)	R <sub>f</sub>
1	deep brown	0.21
2	brown	0.58
3	pink	0.75
4	reddish pink	0.80

Table 4.8: TLC profile of n-Butanol Fraction (CHCl<sub>3</sub>: MEOH; 9:1)

Spot	Colour (10% H <sub>2</sub> SO <sub>4</sub> in ETOH)	R <sub>f</sub>
1	dark brown	0.06
2	green	0.10
3	green	0.18
4	pink	0.23
5	pink	0.29
6	pink	0.82



Plate III: TLC profile of n-butanol fraction ( $\text{CHCl}_3$ : MEOH; 9:1; 10% $\text{H}_2\text{SO}_4$  spray reagent)

#### 4.4. Isolation of COMPOUNDS I, II and III

Table 4.9: Eluates and fractions obtained from the column chromatographic separation of hydrolyzed n-Butanol fraction of methanol leaf extract (organic extract)

S/No	Eluates	Solvent System	Spots	Code
1	1-2	CHCl <sub>3</sub> 100%	4	HYD1
2	3-11	CHCl <sub>3</sub> : MEOH 99:1	3	HYD2
3	12-16	CHCl <sub>3</sub> : MEOH 98:2	3	HYD3
<b>4</b>	<b>17-26</b>	<b>CHCl<sub>3</sub>: MEOH 97:3</b>	<b>1</b>	<b>HYD4</b>
5	27-31	CHCl <sub>3</sub> : MEOH 97:3	3	HYD5
<b>6</b>	<b>32</b>	<b>CHCl<sub>3</sub>: MEOH 97:3</b>	<b>1</b>	<b>HYD6</b>
<b>7</b>	<b>33-36</b>	<b>CHCl<sub>3</sub>: MEOH 97:3</b>	<b>2</b>	<b>HYD7</b>
8	37-45	CHCl <sub>3</sub> : MEOH 97:3	3	HYD8
9	47-55	CHCl <sub>3</sub> : MEOH 96:4	3	HYD9
10	56-69	CHCl <sub>3</sub> : MEOH 95:5	3	HYD10
11	70-80	CHCl <sub>3</sub> : MEOH 90:10	3	HYD11
12	81-100	CHCl <sub>3</sub> : MEOH 100%	3	HYD12

#### 4.5 Physicochemical Properties COMPOUND I

##### (i) Physical Tests

Physical Appearance: White powder

Yield: 225mg

Melting point: 299 - 302°C

Solubility: Insoluble in H<sub>2</sub>O, soluble in MEOH, CHCl<sub>3</sub>, n-Hexane

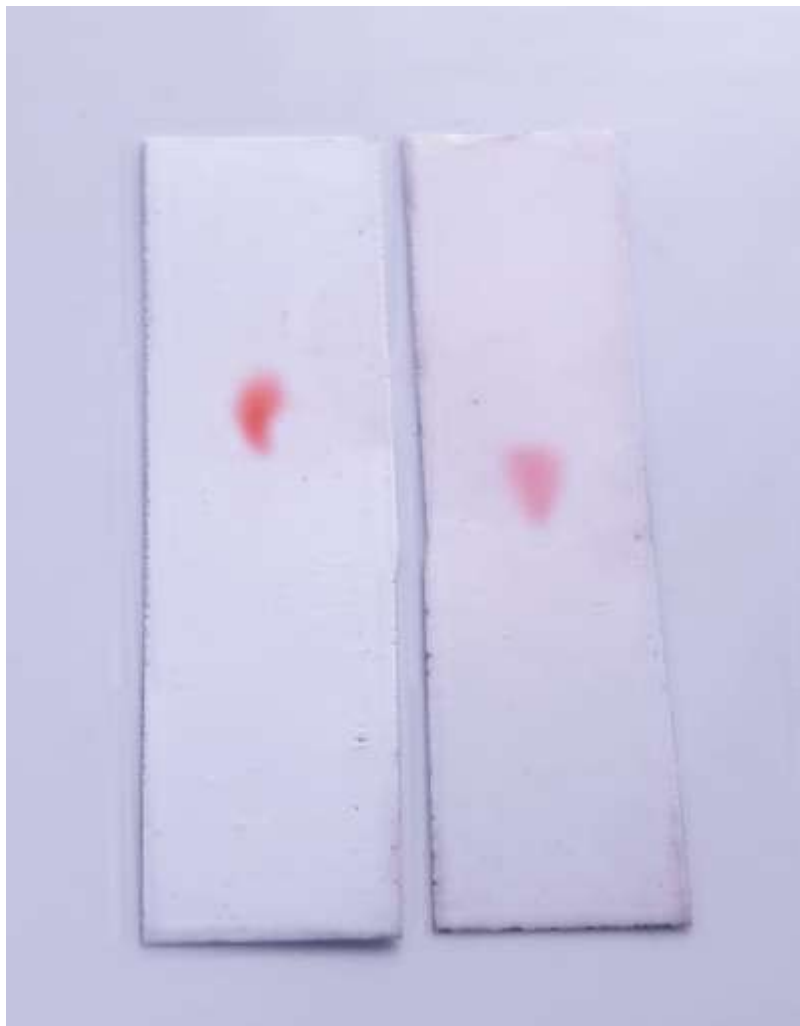
R<sub>f</sub> 0.75 (CHCl<sub>3</sub> : MEOH 9:1), 0.52 (n-Hex : ETOAc 4:1)

##### (ii) Chemical Tests

Acid/NaHCO<sub>3</sub> Test: - Acid Positive

Triterpenoid/Lieberman-Burchard's Test: - Positive (pentacyclic triterpenoid)

Fehling's Test - Negative (Reducing sugar)



A =  $\text{CHCl}_3$  : MEOH (9:1)

B = n-Hex :  $\text{CHCl}_3$  (4:1)

Plate IV: TLC profile of COMPOUND I

## 4.6 Spectral Analysis of COMPOUND I

### 4.6.1 FTIR Analysis of COMPOUND I

Figure 4.1 shows the FTIR spectrum of COMPOUND I. The characteristic absorptions are at:

3436.30  $\text{cm}^{-1}$ , 2931.90  $\text{cm}^{-1}$ , 1728.28  $\text{cm}^{-1}$ , 1532.50  $\text{cm}^{-1}$

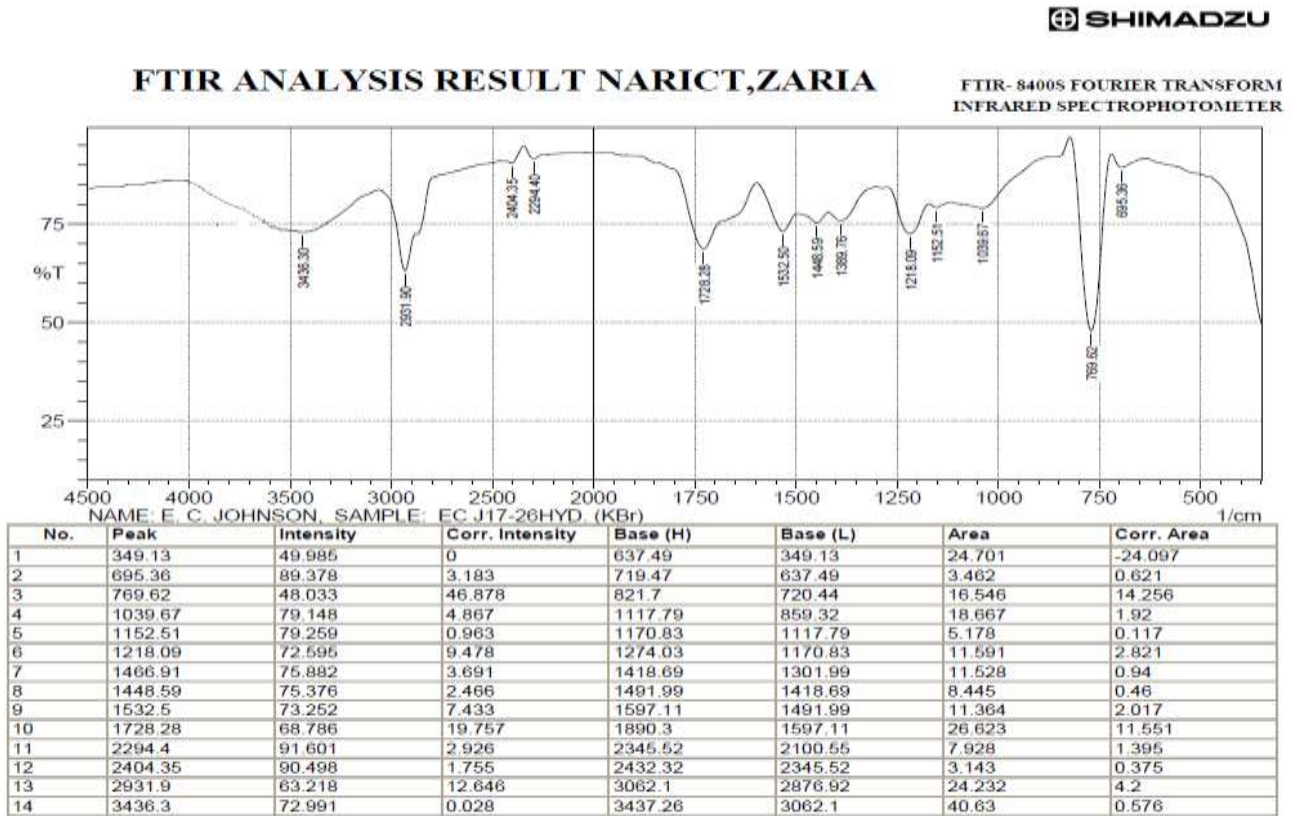


Fig. 4.1: FTIR Spectrum of COMPOUND I

Table 4.10: FTIR Spectral Data of COMPOUND I

Bands (cm <sup>-1</sup> )	Intensity (%)	Vibrations
3436	strong	OH stretch
2931	moderate	CH stretch
2404	strong	OH (COOH)
1728	moderate	C=O (stretch; acid)
1389	strong	gem-dimethyl
1218	strong	two methyls on quaternary carbon
1152	strong	tert alcohol CO stretch
1039	strong	C-O (COOH)

## 4.6.2 Proton NMR of COMPOUND I

Typical of pentacyclic triterpenoid resonances were observed at  $\delta_H$  5.39 ppm (H-12), 3.35 ppm (H-3), 2.27 ppm (H-18), 1.15 (H-30), 0.97 (H-21), 0.93 (H-24), 0.93 (H-24), 0.97 (H-23), 0.79 (H-25), 0.76 (H-26).

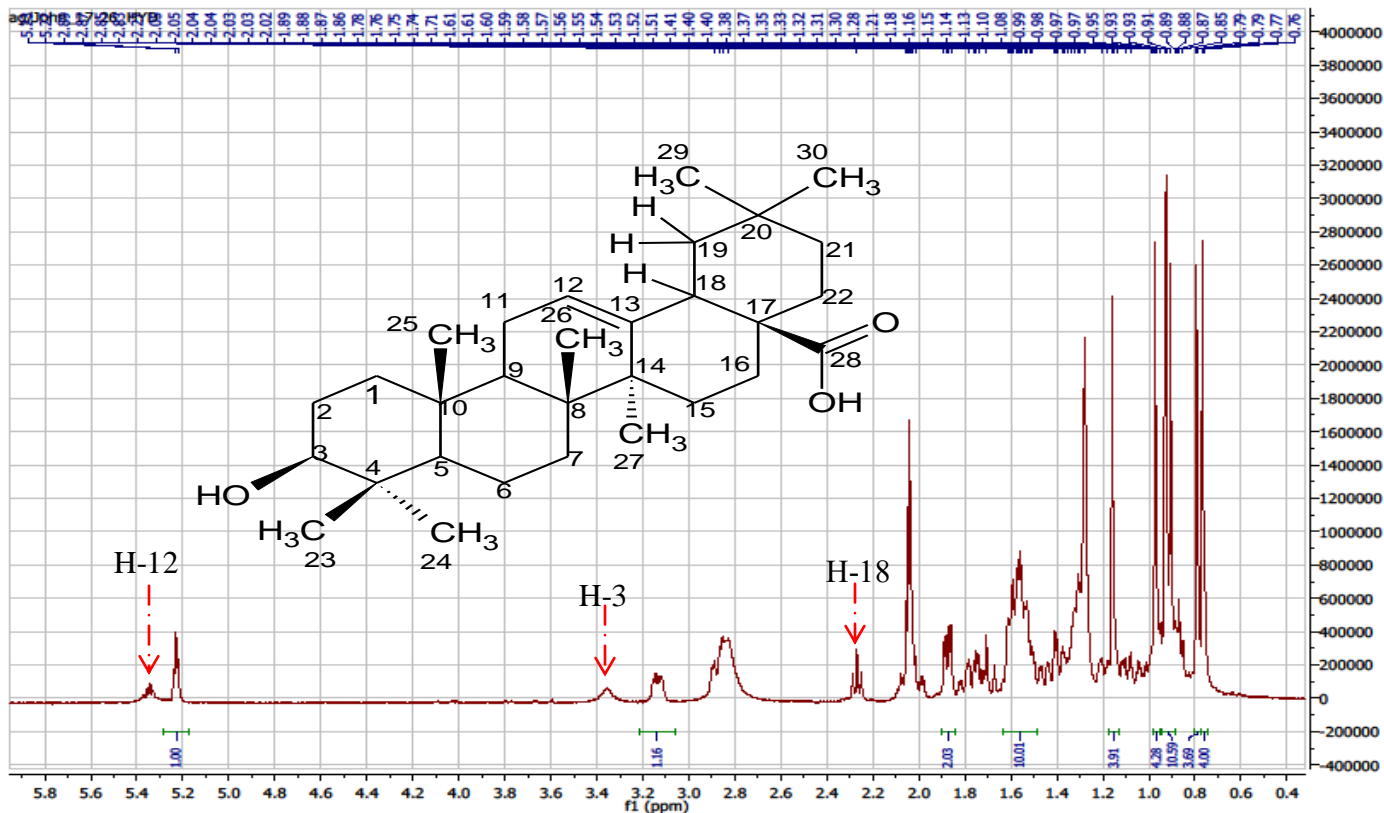


Fig. 4.2: <sup>1</sup>H NMR Spectrum of COMPOUND I

### 4.6.3 Carbon -13 NMR Spectrum of COMPOUND I

Typical pentacyclic triterpenoid peaks observed at  $\delta_C$  77.78 (C-3), 55.39 (C-5), 122.24 (C-12), 144.12 (C-13), 178.03 (C-28).

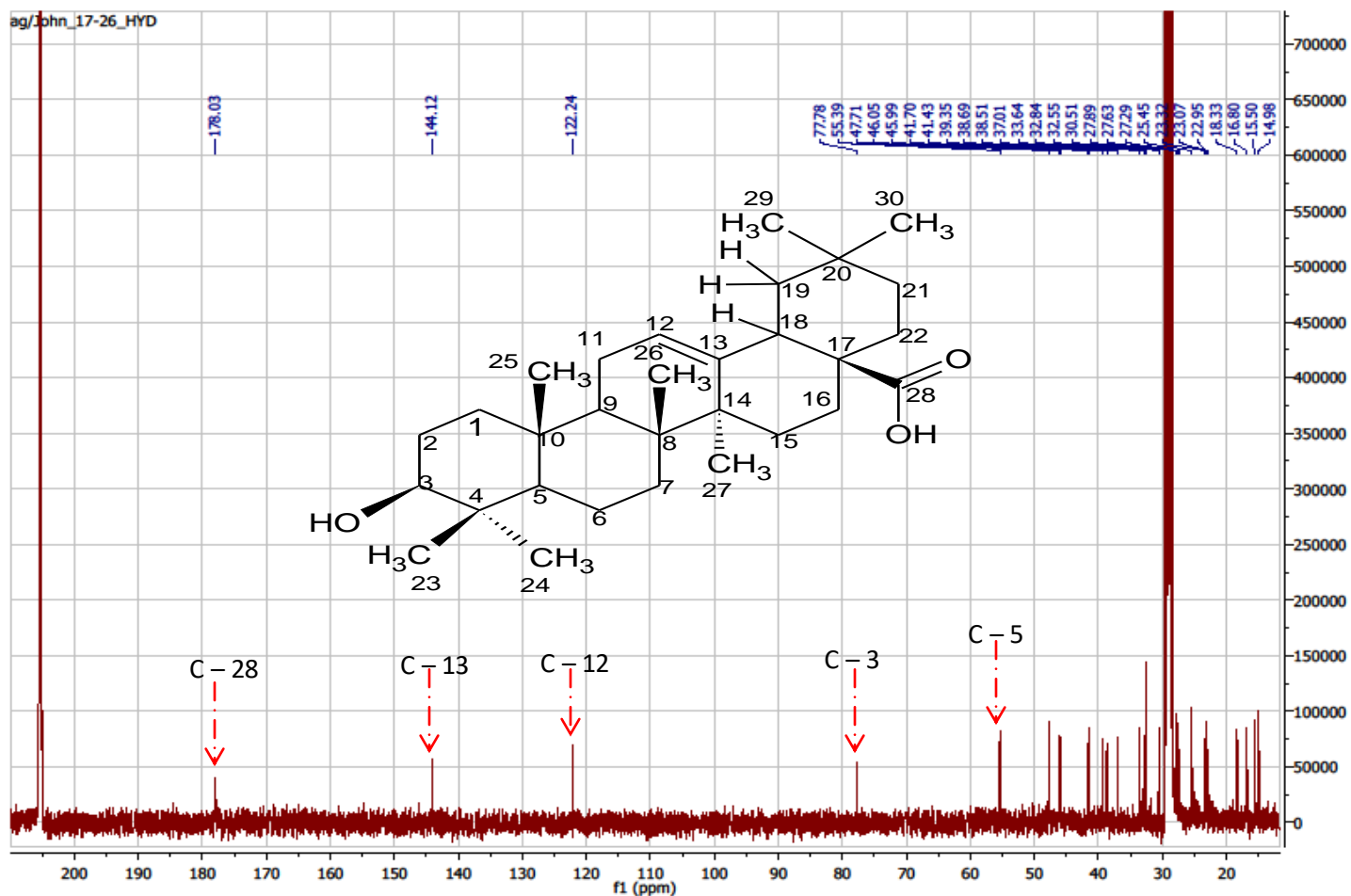


Fig. 4.3: <sup>13</sup>C NMR Spectrum of COMPOUND I

#### 4.6.4 $^1\text{H}$ $^1\text{H}$ COSY NMR Spectrum of COMPOUND I

The COSY spectrum showed the following couplings:  $\delta\text{H} 1.53$  ppm (H-1)/  $\delta\text{H} 1.10$  ppm (H-2);  $\delta\text{H} 2.27$  ppm (H-18)/  $\delta\text{H} 1.70$  ppm (H-19);  $\delta\text{H} 0.97$  (H-23)/  $\delta\text{H} 0.93$  (H-24);  $\delta\text{H} 0.76$  ppm (H-26)/  $\delta\text{H} 0.93$  ppm (H-27).

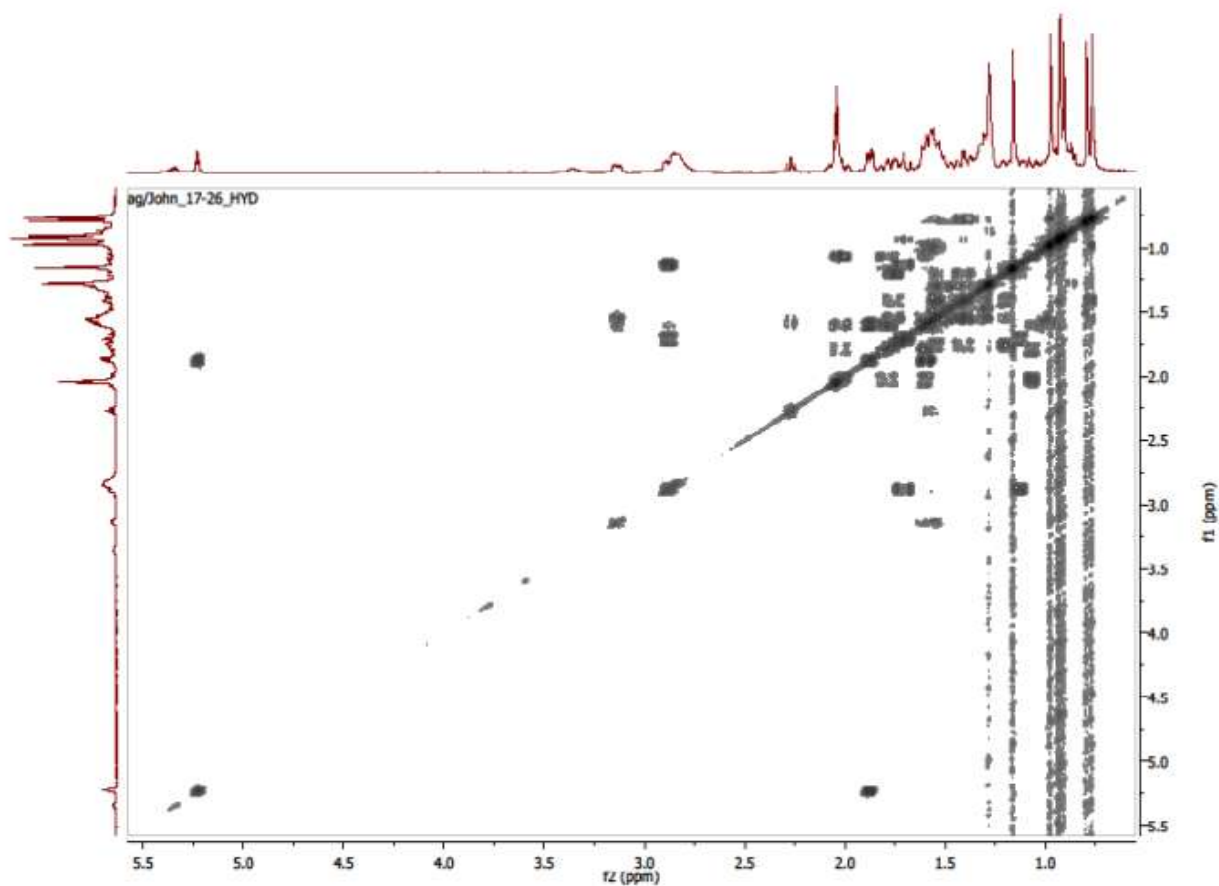


Fig. 4.4:  $^1\text{H}$   $^1\text{H}$ COSY NMR Spectrum of COMPOUND I

#### 4.6.5 Heteronuclear Multiple Quantum Correlation (HMQC) Spectrum of COMPOUND I

Figure 4.5 shows the HMQC spectrum of COMPOUND I.

Proton resonances were assigned to the respective carbons using HMQC.  $\delta$ C 78.03 ppm (C-3) correlated with  $\delta$ H 3.35 (H-3),  $\delta$ C 55.39 ppm (C-5)/  $\delta$ H 0.77 ppm (H-5);  $\delta$ C 18.33 ppm (C-6)/  $\delta$ H 1.33 ppm (H-6);  $\delta$ C 47.71 ppm (C-9)/  $\delta$ H 1.60 ppm; (H-9);  $\delta$ C 122.24 ppm (C-12)/  $\delta$ H 5.35 ppm (H-12);  $\delta$ C 41.43 ppm (C-18)/  $\delta$ H 2.27 ppm (H-18);  $\delta$ C 16.80 ppm (C-25)/  $\delta$ H 0.79 ppm (3H, H-25).

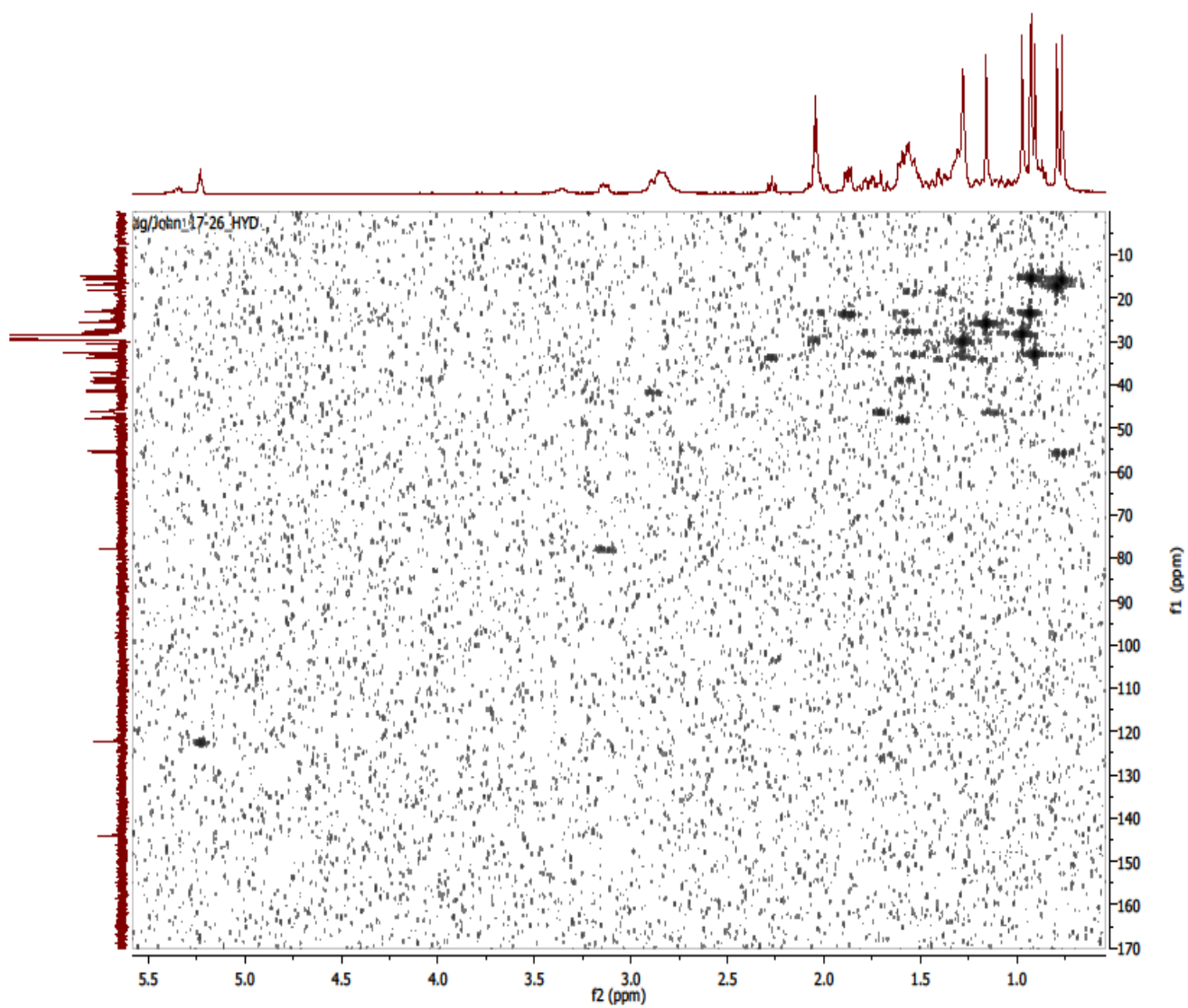


Fig. 4.5: HMQCNMR Spectrum of COMPOUND I

#### 4.6.6 Heteronuclear Multiple Bond Correlations (HMBC) of COMPOUND I

Long range correlations were observed between Me-25 and C-1, 5, 9, 10; Me-26 and C-7, 8, 9, 14; Me-23 and C-3, 4, 5, 24; Me-29 and C-19, 20, 21, 30; Me-30 and C-19, 20, 21, 29.

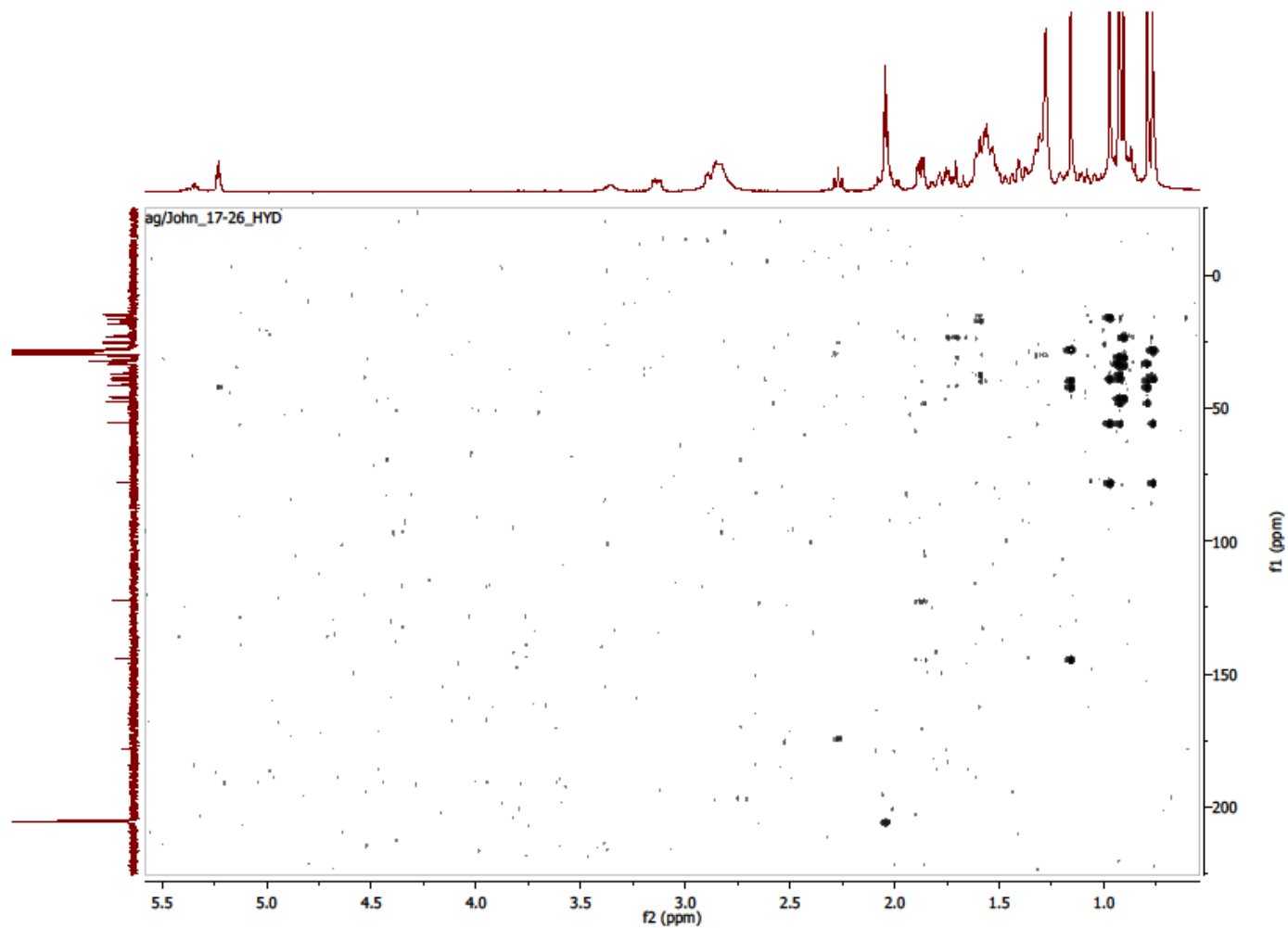


Fig. 4.6: HMBC NMR Spectrum of COMPOUND I

Table 4.11: <sup>1</sup>H and <sup>13</sup>C NMR Chemical shifts of COMPOUND I

Position of C	COMPI		COMPI		HMBC	COSY
	δC (ppm)	C-Type	δH ppm	J (Hz)		
1	39.35	CH <sub>2</sub>	1.53		25	H-3
2	27.63	CH <sub>2</sub>	1.10			
3	77.78	CH	3.35	brs	23, 24	H-1
4	38.55	C	-			
5	55.39	CH	0.77		3, 5, 23, 24	
6	18.33	CH <sub>2</sub>	1.33			
7	33.64	CH <sub>2</sub>	1.57			
8	41.70	C	-			
9	47.70	CH	1.60			
10	37.01	C	-			
11	23.32	CH <sub>2</sub>	1.86			
12	122.24	CH	5.35	t, 4		
13	144.12	C	-			
14	41.70	C	-			
15	30.51	CH <sub>2</sub>	1.38			
16	23.07	CH <sub>2</sub>	1.54			
17	45.99	C	-			
18	41.43	CH	2.27	t, 14		H-19
19	46.06	CH <sub>2</sub>	1.70		20, 21, 30	H-18
20	32.84	C	-		19, 29, 30	
21	38.69	CH <sub>2</sub>	1.21			
22	27.89	CH <sub>2</sub>	1.30			
23	27.29	CH <sub>3</sub>	0.97	s	3, 4, 5, 24	
24	14.98	CH <sub>3</sub>	0.93	s	4, 23	
25	16.80	CH <sub>3</sub>	0.79	s	1, 5, 9, 10	
26	15.50	CH <sub>3</sub>	0.76	s	7, 8, 9, 14	H-27
27	22.95	CH <sub>3</sub>	0.93	s		H-26
28	178.0	C	-			
29	32.55	CH <sub>3</sub>	0.91	s	19, 20, 21, 30	
30	25.45	CH <sub>3</sub>	1.15	s	19, 20, 21, 29	

400MHz <sup>1</sup>H, 100MHz <sup>13</sup>C CDCl<sub>3</sub>;

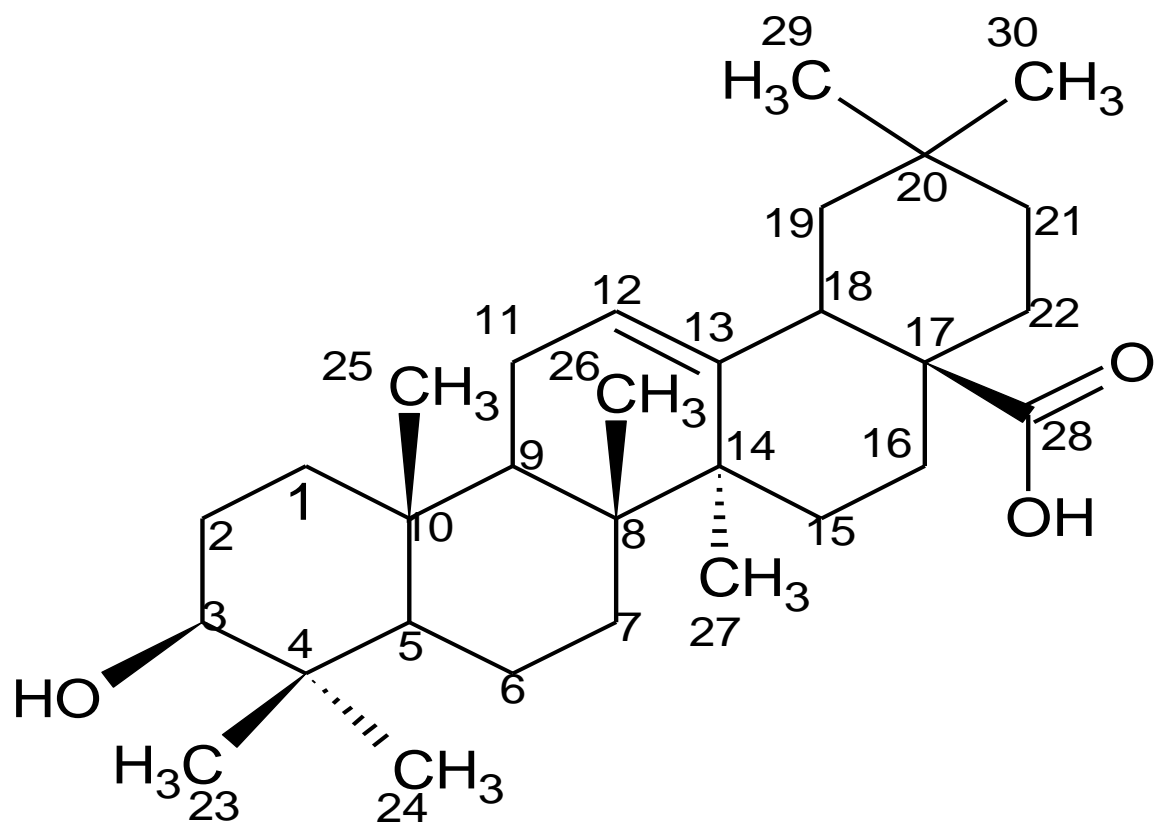


Fig. 4.7: Structure of **COMPOUND I**

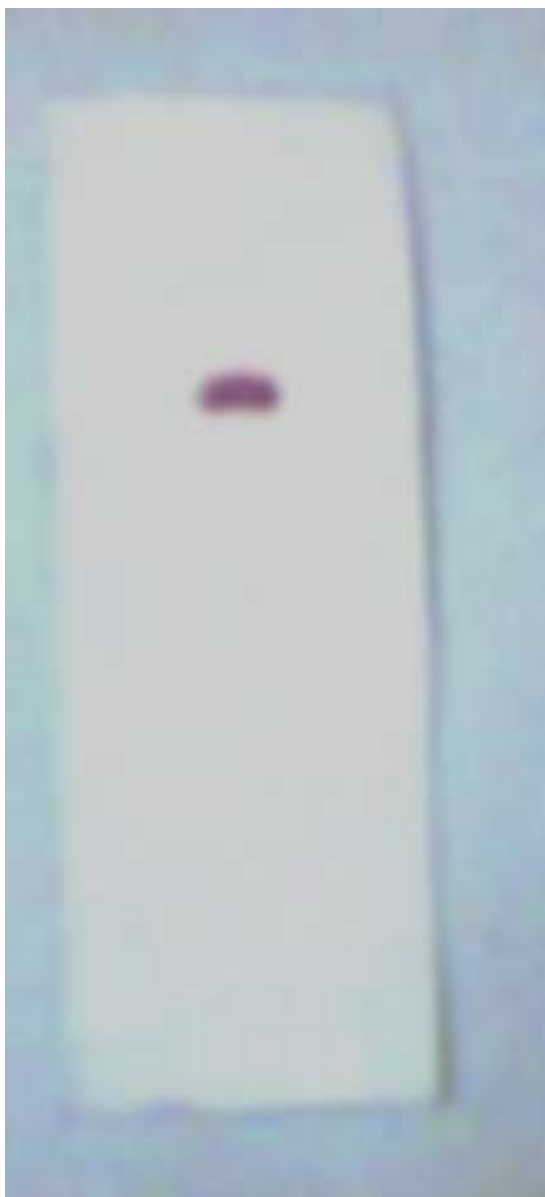
## 4.7 The Physicochemical Analysis of COMPOUND II

### Physical Characteristics

Physical Appearance	-	White amorphous powder
Yield:	-	14mg
Melting point:	-	284-287°C
Solubility:	-	soluble in CHCl <sub>3</sub> and DMSO, MEOH and acetone; insoluble in water
(R <sub>f</sub> ):	-	0.78 (CHCl <sub>3</sub> :MEOH, 9:1); 0.54 (n-Hex:ETOAc, 4:1)

### Chemical Characteristics

Acid/NaHCO <sub>3</sub> test:	-	Positive (effervescence, CO <sub>2</sub> was produced)
Lieberman-Burchard's test:		Positive (violet colour)
Fehling's Reaction:		Negative



0.78 (CHCl<sub>3</sub>:MEOH, 9:1)



0.54 (n-Hex:ETOAc, 4:1)

Plate V: TLC profile of COMPOUND II

## 4.8. Spectral Analysis of COMPOUND II

Figures 4.8 -4.10 show the spectra of FTIR, proton NMR and C-13 NMR of COMPOUND II

### 4.8.1 FITR Analysis of COMPOUND II

Figure 4.8 shows the FTIR spectrum of COMPOUND II. The characteristic absorptions are at:

$3452.70\text{ cm}^{-1}$ ,  $2931.90\text{ cm}^{-1}$ ,  $1733.10\text{ cm}^{-1}$ ,  $1647.26\text{ cm}^{-1}$

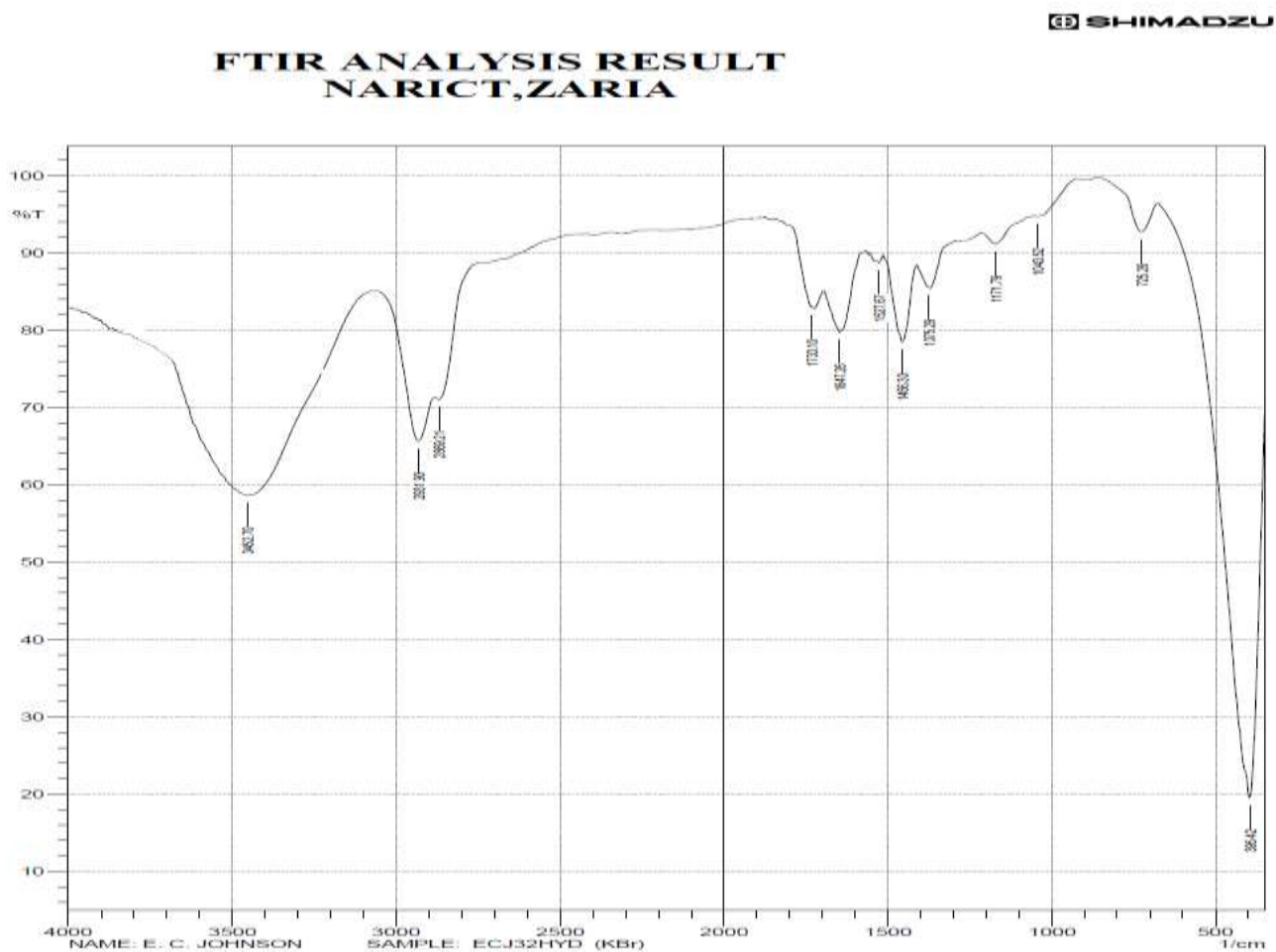


Figure 4.8: FTIR spectrum of **COMPOUND II**

Table 4.12: FTIR Spectral Data of COMPOUND II

Bands (cm <sup>-1</sup> )	Intensity (%)	Vibrations
3452.70	moderate	OH stretch
2931.90	moderate	CH stretch
1733.10	strong	C=O (stretch; acid)
1647.26	strong	C=C (stretch; alkene)
1375.29	strong	gem-dimethyl
1171.79	strong	tert alcohol CO stretch



### 4.8.3 Carbon-13 NMR of COMPOUND II

The characteristic peaks of triterpenoid were observed at  $\delta_{\text{C(ppm)}}$  80.58, (C-3), 55.26, (C-5), 121.65, (C-12), 145.19, (C-13), 173.69 (C-28).

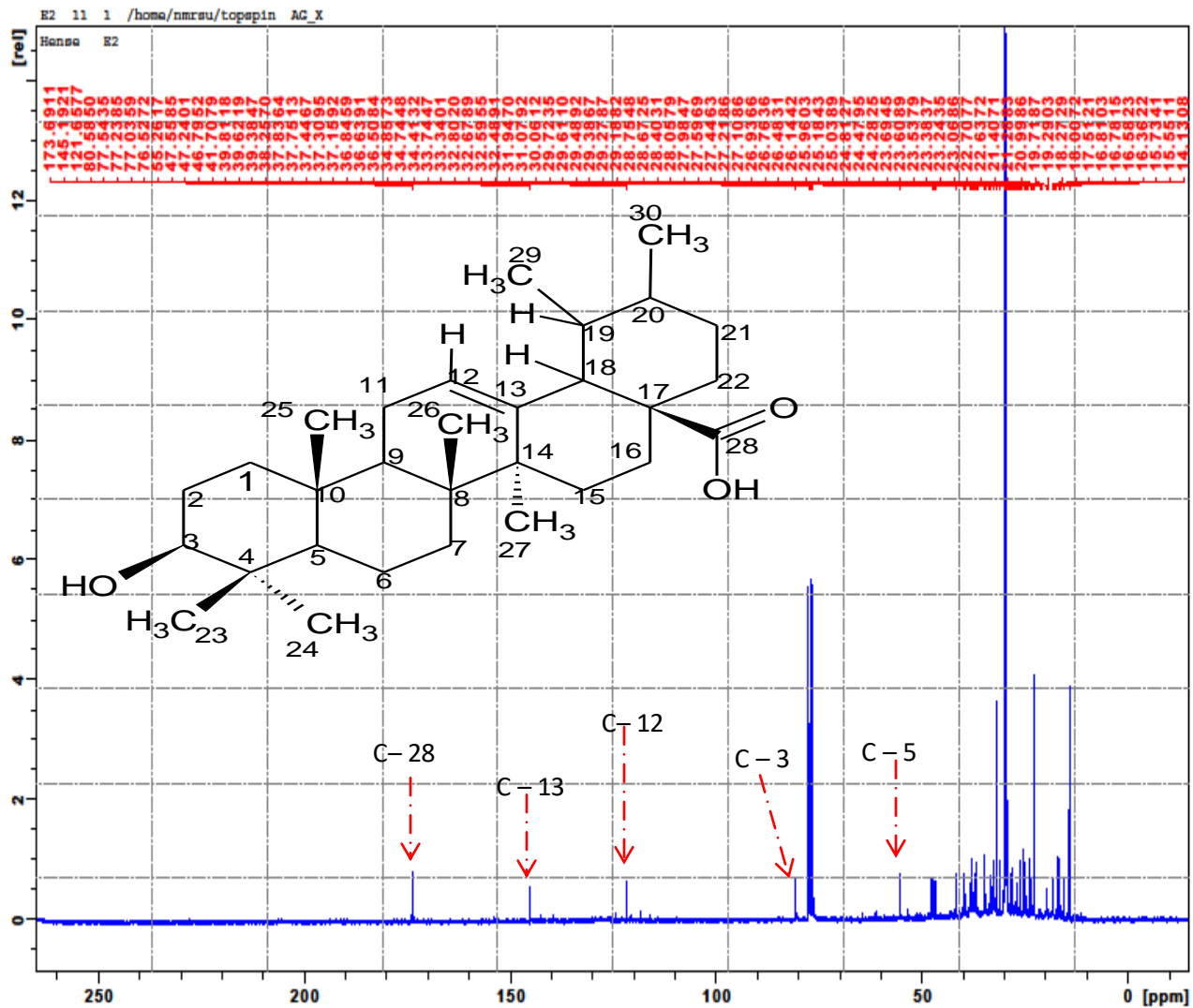


Fig 4.10:  $^{13}\text{C}$  NMR spectrum of **COMPOUND II** in  $\text{CDCl}_3$

Table 4.13:  $^1\text{H}$  and  $^{13}\text{C}$  NMR Chemical shifts of **COMPOUND II**

Position of C atom	DEPT	$\delta_{\text{C}}$ (ppm)	$\delta_{\text{H}}$ (ppm)
1	CH <sub>2</sub>	39.38	1.00
2	CH <sub>2</sub>	28.40	1.74
3	CH	80.58	3.69
4	C	38.25	-
5	CH	55.26	0.86 (d, J=3.0)
6	CH <sub>2</sub>	18.27	1.55
7	CH <sub>2</sub>	33.34	1.55
8	C	39.81	-
9	CH	47.55	1.64
10	C	37.83	-
11	CH <sub>2</sub>	23.69	1.96
12	CH	121.65	5.38 (t, J=4.5Hz)
13	C	145.19	-
14	CH <sub>2</sub>	41.70	1.16
15	CH <sub>2</sub>	29.18	2.07
16	CH <sub>2</sub>	24.28	-
17	C	47.24	1.45
18	CH	47.55	2.35 (d, J=7.5Hz)
19	CH	39.62	1.46
20	CH	39.62	1.40
21	CH <sub>2</sub>	30.06	1.51
22	CH <sub>2</sub>	37.15	-
23	CH <sub>3</sub>	28.75	0.99s
24	CH <sub>3</sub>	16.58	0.99 s
25	CH <sub>3</sub>	16.36	0.96 s
26	CH <sub>3</sub>	17.51	0.93 s
27	CH <sub>3</sub>	25.96	1.28 s
28	C	173.69	-
29	CH <sub>3</sub>	16.78	1.03 (d, J=4 Hz)
30	CH <sub>3</sub>	21.28	0.98 (d, J=6.2Hz)

250 MHz  $^1\text{H}$ , 100 MHz  $^{13}\text{C}$  in  $\text{CDCl}_3$

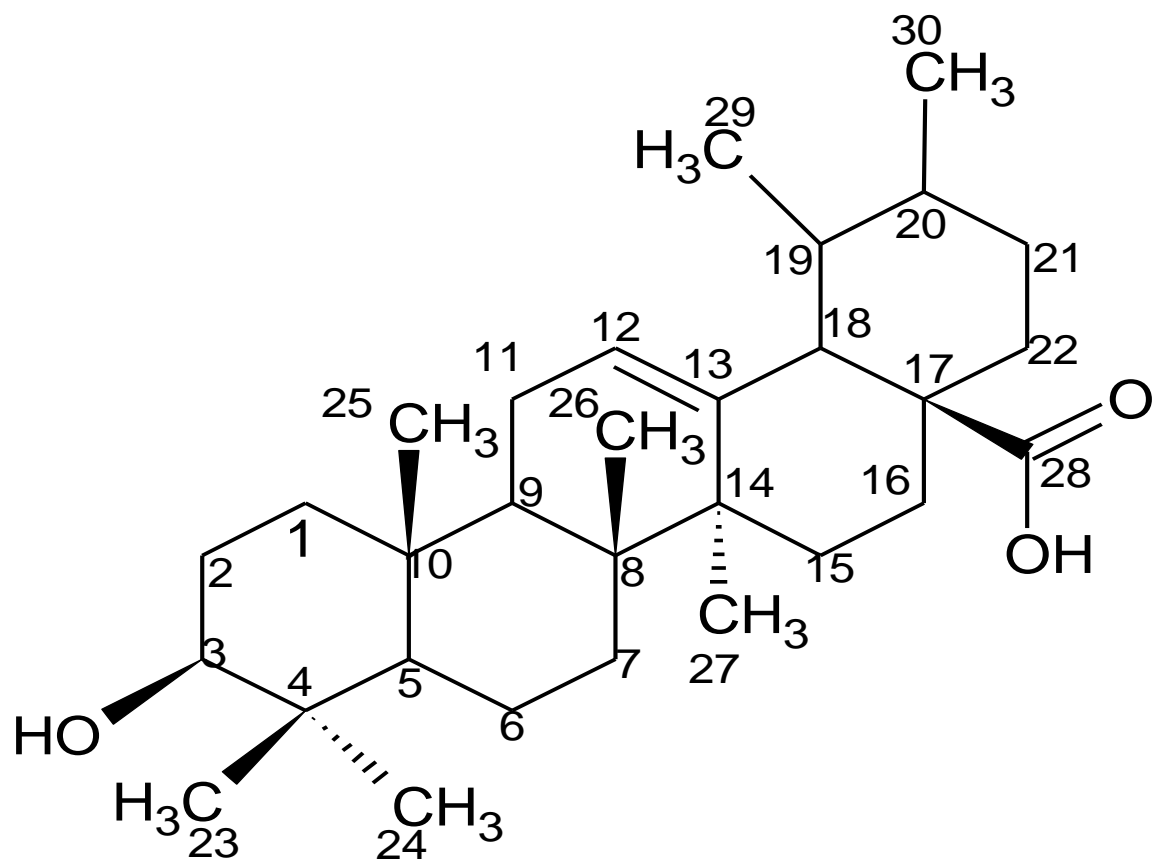


Fig. 4.11: Structure of **COMPOUND II**

#### 4.9 Physicochemical Analysis of COMPOUND III

##### The Physical Characteristics

Physical appearance:	White amorphous powder
Yield:	25 mg
Melting point:	245 - 248°C
Solubility:	soluble in CHCl <sub>3</sub> , MEOH and acetone, insoluble in water
Retention factor (R <sub>f</sub> ):	0.77 (CHCl <sub>3</sub> : MEOH, 9:1); 0.59 (n-Hex: ETOAc, 4:1)

##### The Chemical test

Acid/NaHCO <sub>3</sub> test:	Positive (effervescence, CO <sub>2</sub> was produced)
Lieberman-Burchard's test:	Positive (violet colour)
Fehling's Reaction:	Negative



n-Hex : ETOAc (4:1)

CHCl<sub>3</sub> : MEOH (9:1)

Plate VI: TLC profile of COMPOUND III

## 4.10. Spectral Analysis of COMPOUND III

### 4.10.1 FTIR Analysis of COMPOUND III

Figure 4.10.1 shows the FTIR spectrum of COMPOUND III. The characteristic absorptions are at:  $3472\text{ cm}^{-1}$ ,  $2993\text{ cm}^{-1}$ ,  $1725\text{ cm}^{-1}$

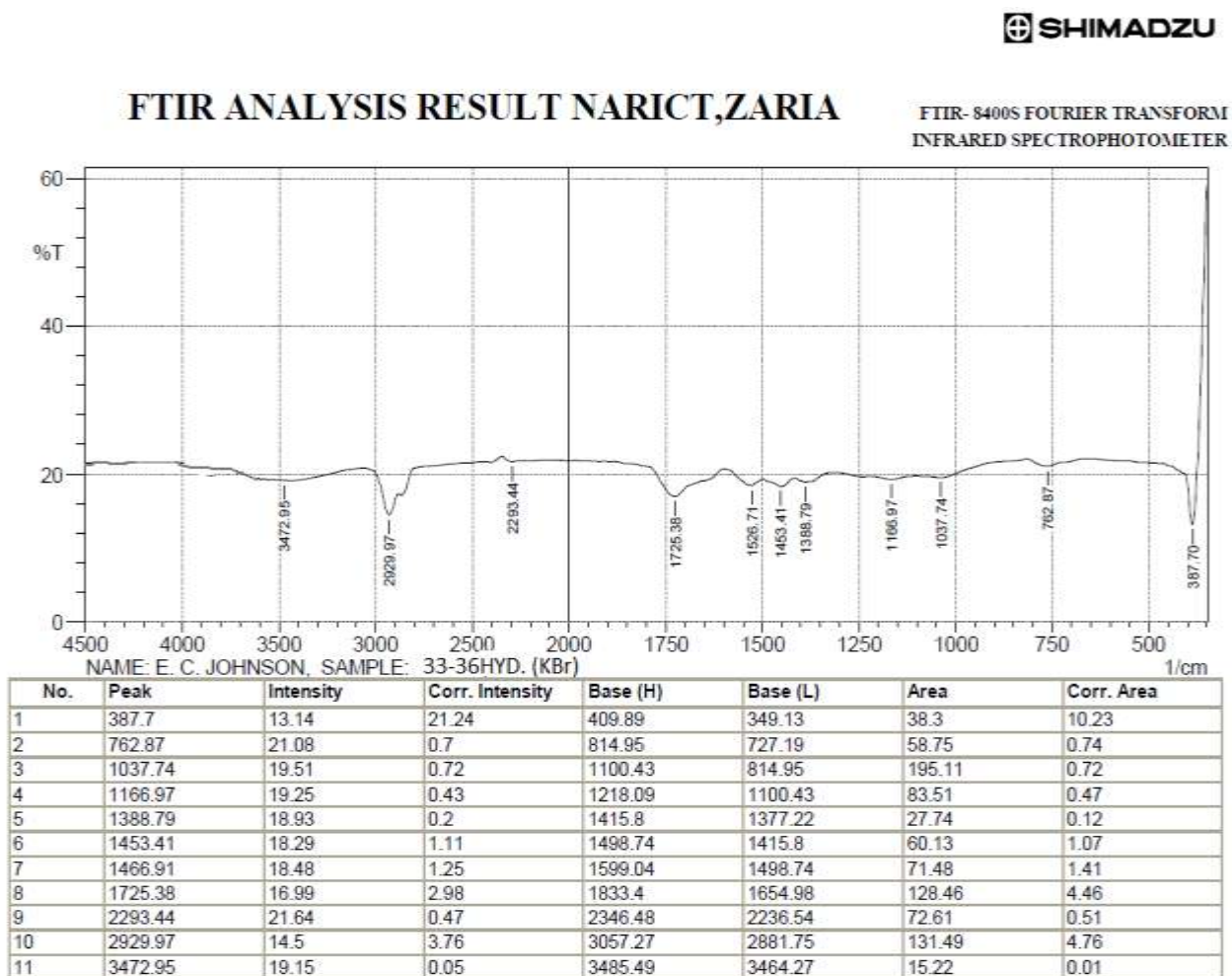


Fig 4.12: FTIR spectrum of COMPOUND III

Table 4.14: FTIR Spectral Data of COMPOUND III

Bands (cm <sup>-1</sup> )	Intensity (%)	Vibrations
3472	weak	OH stretch
2930	weak	CH stretch
2293	weak	OH (COOH)
1725	weak	C=O (stretch)
1389	weak	gem-dimethyl
1167	weak	tert alcohol CO stretch

#### 4.10.2 Proton NMR Analysis of COMPOUND III

Fig 4.13 shows the  $^1\text{H}$  NMR spectrum of Compound III. The characteristic pentacyclic triterpenoid peaks occurred at:  $\delta_{\text{H}}$  (ppm) 3.47 (H-2) 3.65 (H-3), 5.35 (H-12), 2.30 (H-18), 1.02 (H-23), 1.00 (H-24), 1.0 (H-25), 1.02 (H-26), 1.03 (H-27), 0.97 (H-29), 0.99 (H-30).

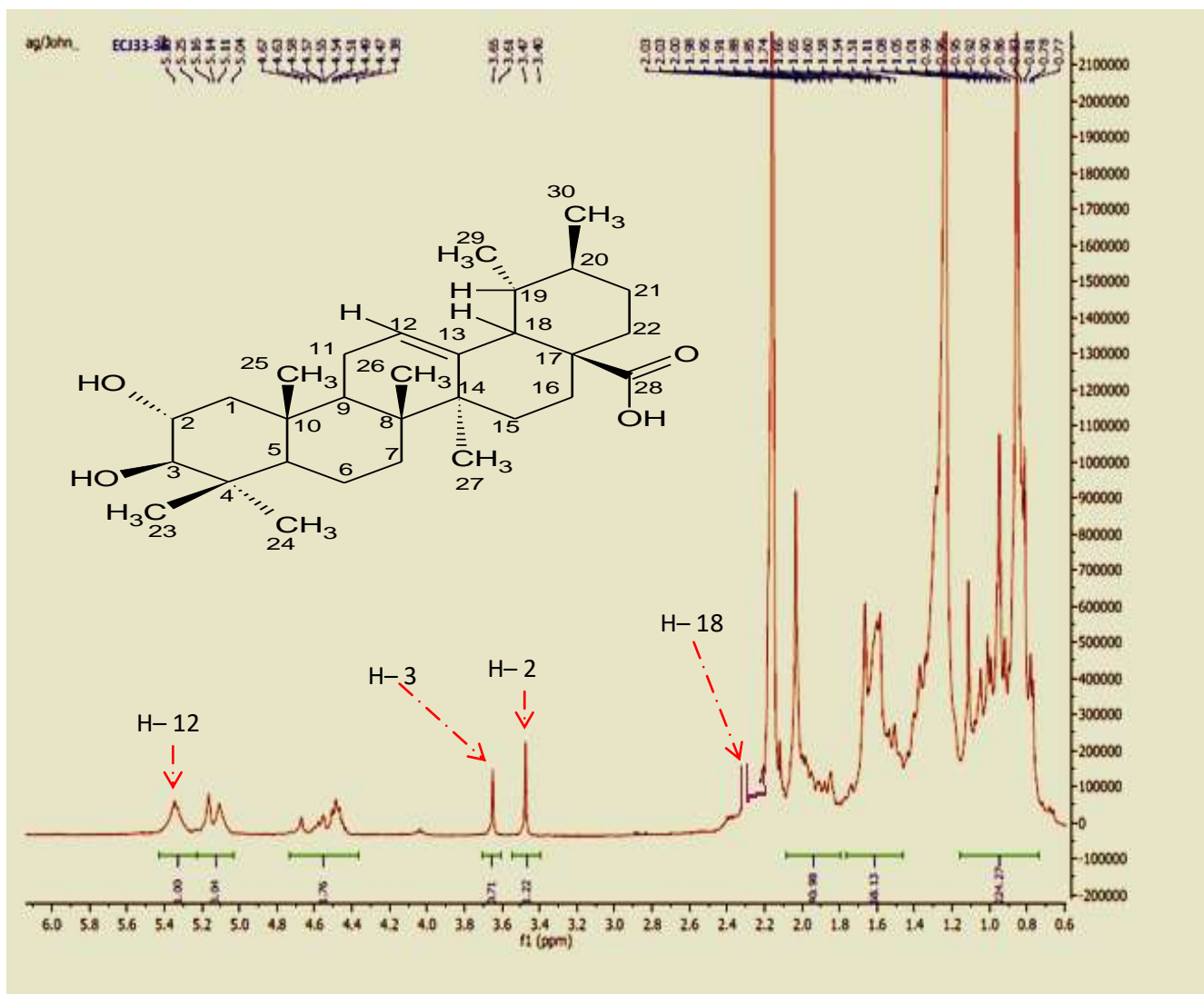


Fig 4.13:  $^1\text{H}$  NMR spectrum of COMPOUND III

### 4.10.3 $^{13}\text{C}$ NMR Analysis of COMPOUND III

The characteristic pentacyclic triterpenoid resonances at:  $\delta_{\text{C(ppm)}}$  64.41 (C-2), 80.62 (C-3), 55.26 (C-5), 121.65 (C-12), 140.00 (C-13), 173.74 (C-18)

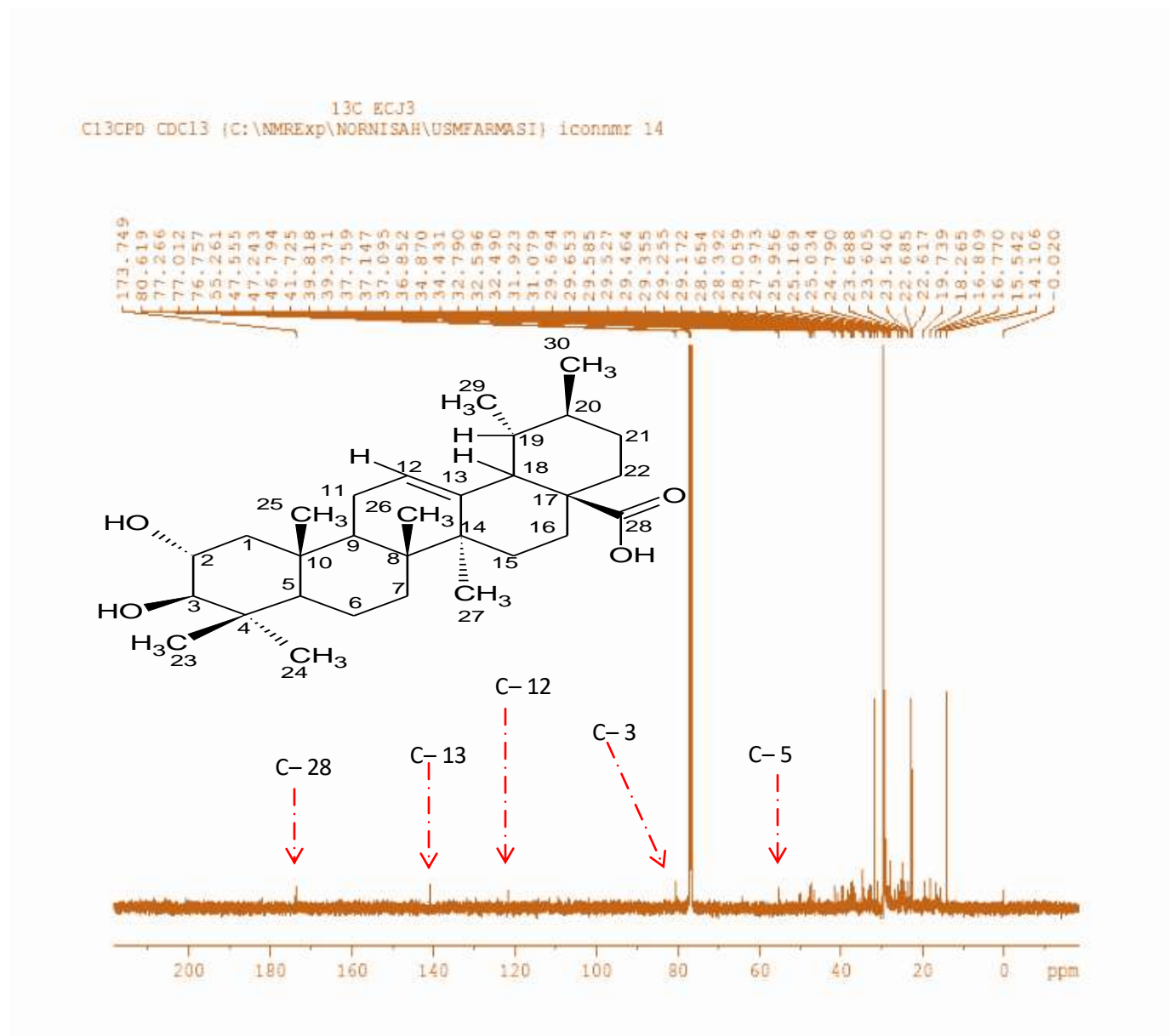


Fig 4.14:  $^{13}\text{C}$  NMR Spectrum of COMPOUND III

#### 4.10.4 DEPT-135 of COMPOUND III

DEPT-135 (Fig 4.15) Negative resonances due to  $\text{CH}_2$  observed at 46.77 ppm assigned to C-1, 34.87 ppm (C-7), 34.43 (C-22), 22.68 ppm (C-16) and 18.26 (C-6). The positive resonances due to  $\text{CH}_3$  at 28.39 ppm assigned to C-23, 24.79 ppm (C-30), 23.79 ppm (C-27), 19.02 ppm (C-26), and 16.99 ppm (C-25).

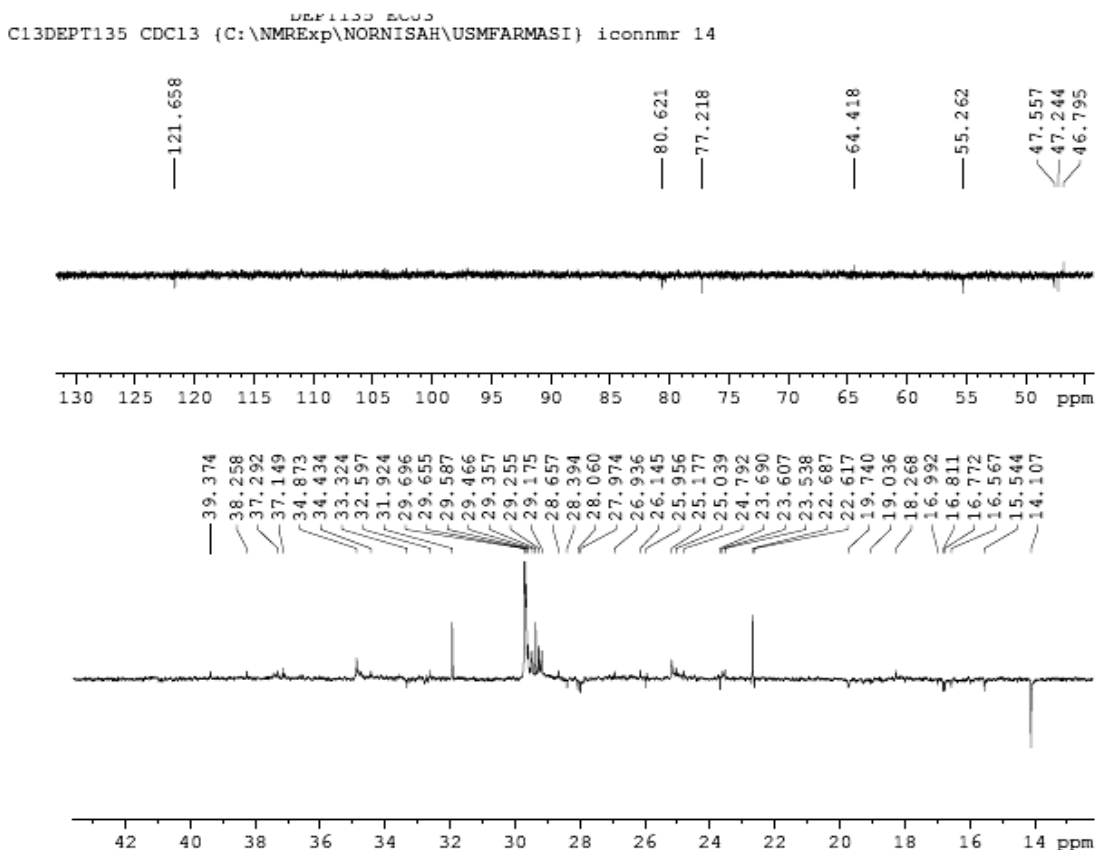


Fig 4.15: DEPT135 NMR Spectrum of COMPOUND

#### 4.10.5 DEPT-90 of COMPOUND III

Figure 4.16 shows DEPT-90 of COMPOUND III; positive resonances attributed to methine carbon (CH) observed at 121.65 ppm assigned to C-12, 80.66 ppm (C-3), 64.41 ppm (C-2), 55.26 ppm (C-5), 47.24, (C-20), 47.55 (C-9), 32.79 ppm (C-19).

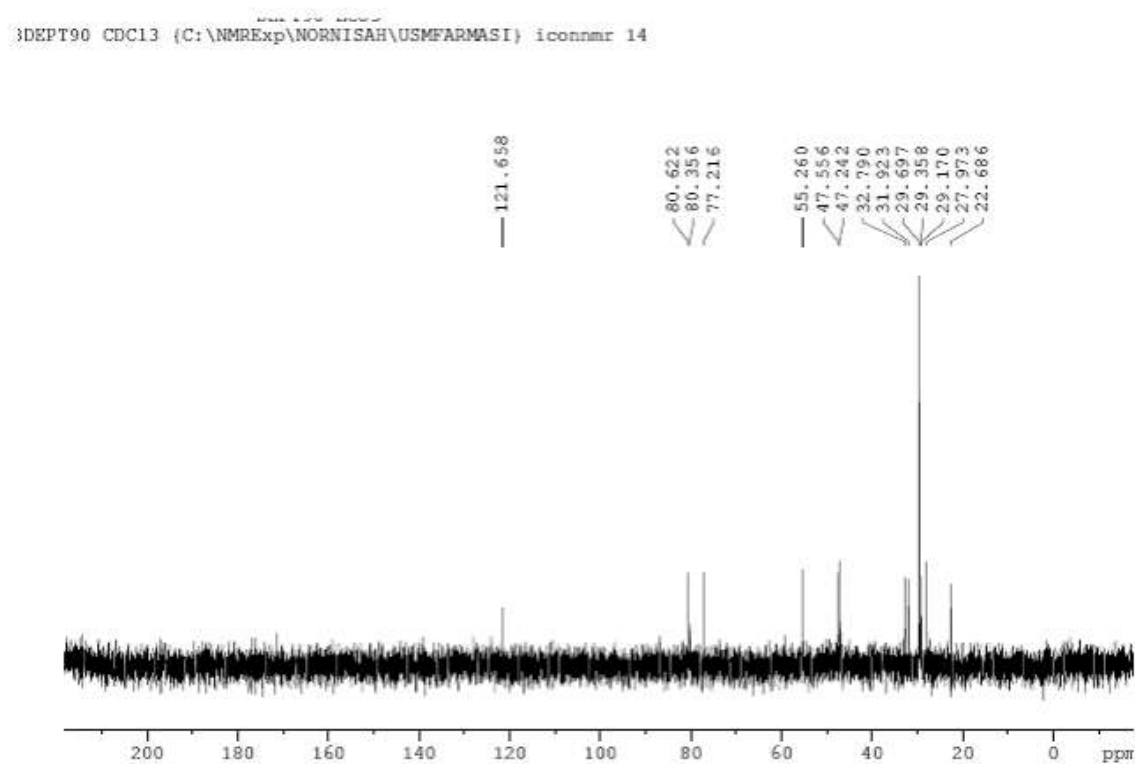


Fig. 4.16: DEPT90 NMR Spectrum of COMPOUND III

#### 4.10.6 DEPT-Q OF COMPOUND III

Figure 4.21 shows the spectrum of DEPT-q of COMPOUND III: Resonances attributed to quaternary carbon (C) appeared at 173.74 ppm assigned to C-28, 145.00 ppm (C-13), 41.72 ppm (C-17), 40.84 ppm (C-8), 39.37 ppm (C-4), 38.25 ppm (C-10), and 37.75 ppm (C-14)

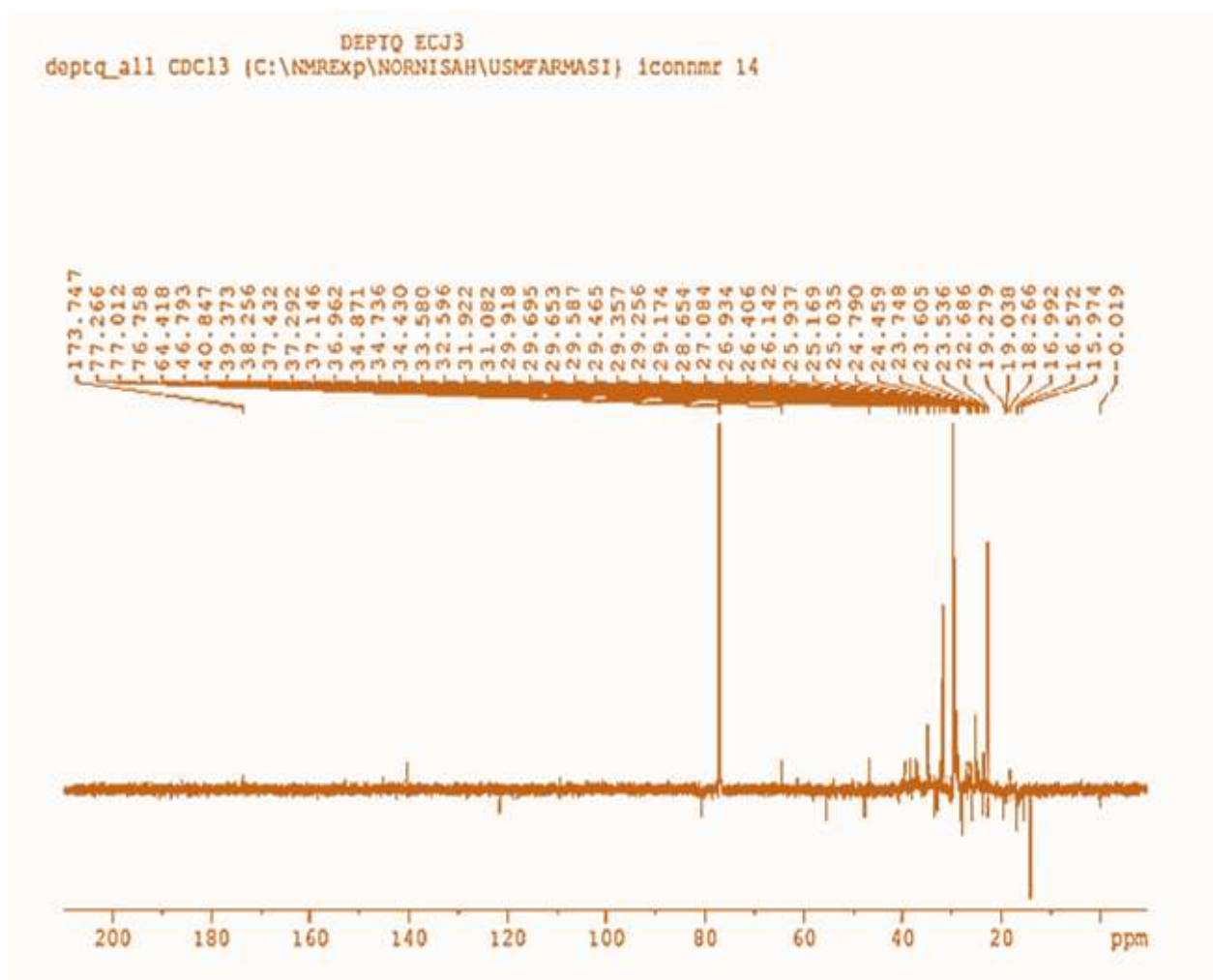


Fig. 4.17: DEPT Q(All) NMR Spectrum of COMPOUND III

Table 4.15:  $^1\text{H}$  and  $^{13}\text{C}$  NMR Chemical shifts of COMPOUND III

Position of C	C-Type	$\delta_{\text{C}}$ (ppm) COMPOUND III	$\delta_{\text{H}}$ (ppm) COMPOUND III
1	CH <sub>2</sub>	46.79	1.021
2	CH	64.41	3.47(1H,d, J= 10 Hz)
3	CH	80.62	3.65 (1H,d, J=7.5Hz)
4	C	39.81	-
5	CH	55.26	0.88
6	CH <sub>2</sub>	18.26	1.39
7	CH <sub>2</sub>	34.87	1.55
8	C	40.84	-
9	CH	47.55	1.87
10	C	38.25	-
11	CH <sub>2</sub>	23.60	1.42
12	CH	121.65	5.35
13	C	140.00	-
14	C	41.72	-
15	CH <sub>2</sub>	29.17	1.39
16	CH <sub>2</sub>	22.68	2.27
17	C	47.55	-
18	CH	55.26	2.30 (1H, d, J = 8.0 Hz)
19	CH	32.79	1.68
20	CH	47.24	-
21	CH <sub>2</sub>	29.69	1.49
22	CH <sub>2</sub>	34.43	1.56
23	CH <sub>3</sub>	28.39	1.02s
24	CH <sub>3</sub>	16.81	1.00s
25	CH <sub>3</sub>	16.99	1.00s
26	CH <sub>3</sub>	19.02	1.02s
27	CH <sub>3</sub>	23.79	1.03s
28	C	173.74	-
29	CH <sub>3</sub>	16.77	0.97d (3H, d, J=5.6 Hz)
30	CH <sub>3</sub>	24.79	0.99d (3H, J, J=5.8 Hz)

500 MHz  $^1\text{H}$ , (CDCl<sub>3</sub>) 100MHz  $^{13}\text{C}$  (CDCl<sub>3</sub>) of COMPOUND III

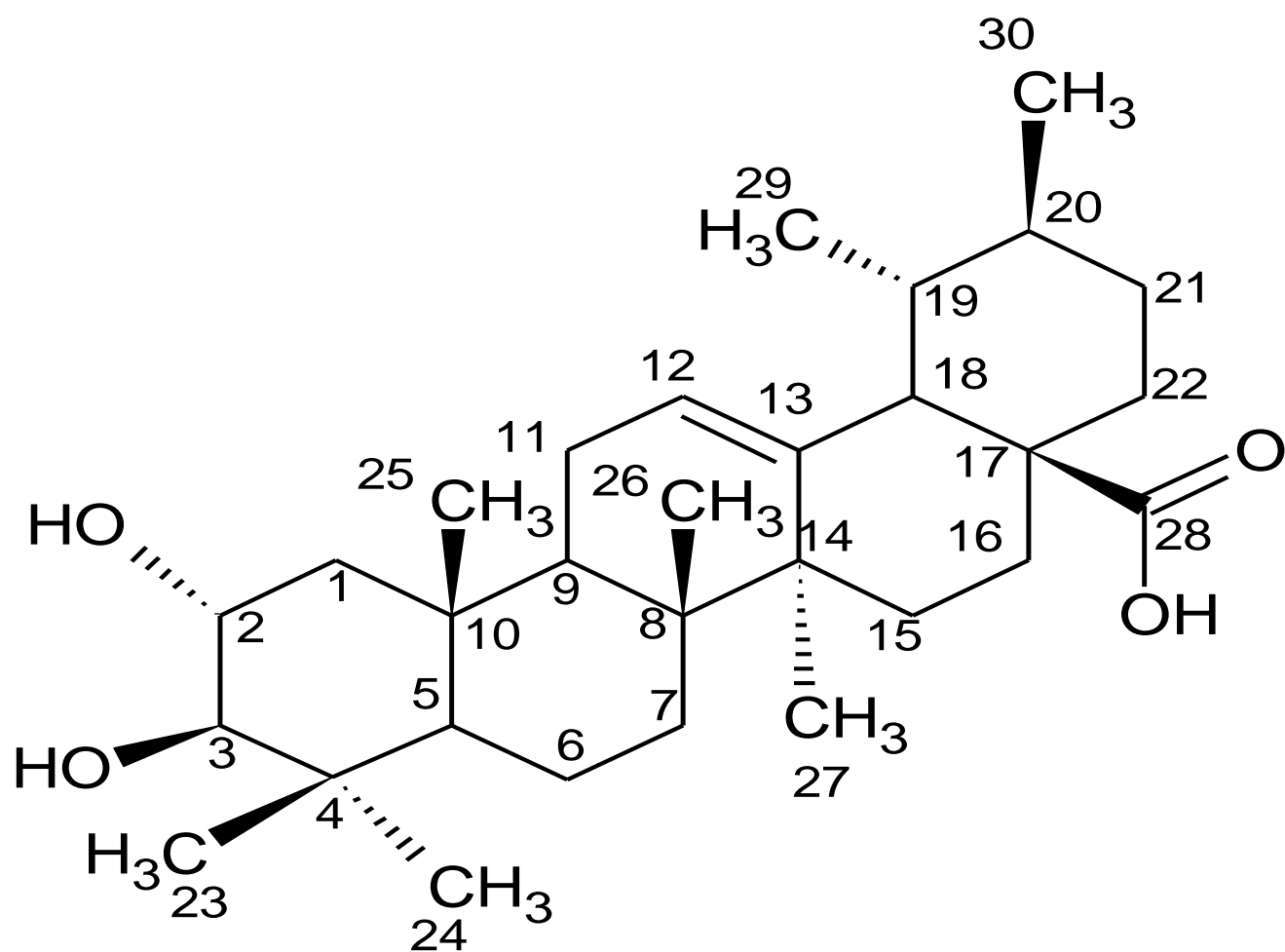


Fig. 4.18: Structure of **COMPOUND III**

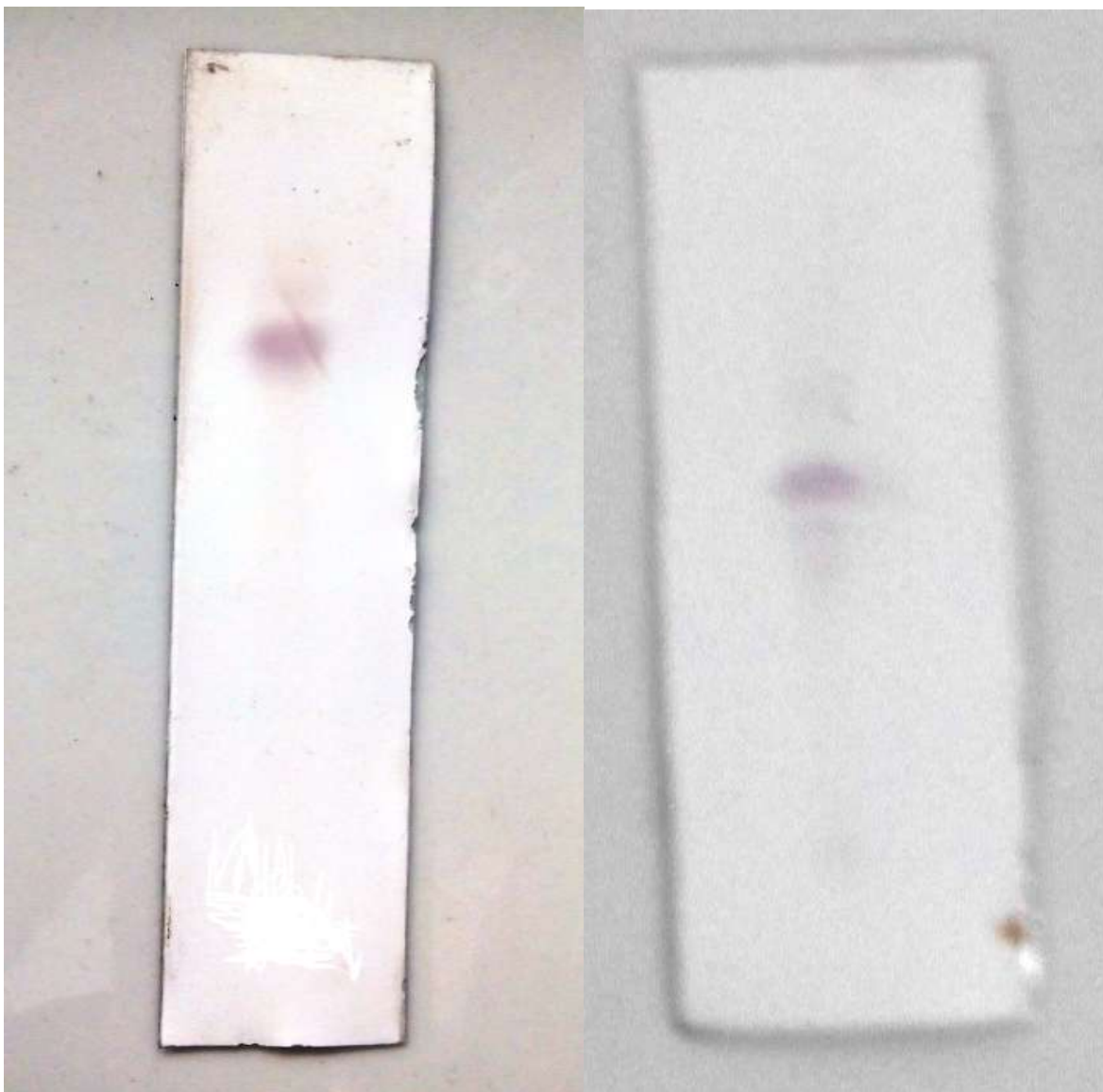
#### 4.11 Isolation of COMPOUND IV

Table 4.16: Eluates and fractions obtained from the column chromatographic separation of dichloromethane fraction of methanol leaf extract

S/No	Eluates	Solvent System	Spots	Code
1	1-20	n-HEX. (100%)	-	D1
2	21-29	n-HEX:ETOAc: (90:10)	3	D2
3	30-36	n-HEX:ETOAc (70:30)	3	D3
4	37-45	n-HEX:ETOAc (70:30)	1	D4
5	46-49	n-HEX:ETOAc (70:30)	3	D5
<b>6</b>	<b>50-57</b>	<b>n-HEX:ETOAc (50:50)</b>	<b>2</b>	<b>D6</b>
7	58-100	n-HEX:ETOAc (30:70)	3	D7
8	101-145	n-HEX:ETOAc:MEOH (30:60:10)	3	D8
9	146-174	ETOAc:MEOH (50:50)	3	D9
10	175-189	ETOAc:MEOH (40:60)	3	D10
11	175-215	MEOH (100)	3	D11

#### 4.12 Physical Tests on COMPOUND IV

- i. Physical Appearance      White amorphous powder
- ii. Yield                      7mg
- iii.  $R_f$                       0.76 ( $\text{CHCl}_3$ :MEOH 9:1) 0.53 (n-Hex : ETOAc 4:1)



0.76 ( $\text{CHCl}_3$ :MEOH 9:1)

0.53 (n-Hex : ETOAc 4:1)

Plate VII: TLC profile of COMPOUND IV

## 4.13 Spectral Analysis of COMPOUND IV

### 4.13.1 Proton NMR Analysis of COMPOUND IV

Fig. 4.1 shows the proton NMR spectrum of COMPOUND IV: The characteristic triterpenoid peaks were observed at  $\delta_{\text{H}}(\text{ppm})$ , 5.41 (H-12), 3.24 (H-3), 2.30 (H-18), 1.30 (H-23), 0.97, (H-24), 0.90 (H-25), 1.36 (H-26), 1.18 (H-27), 0.79 (H-29), 0.98 (H-30)

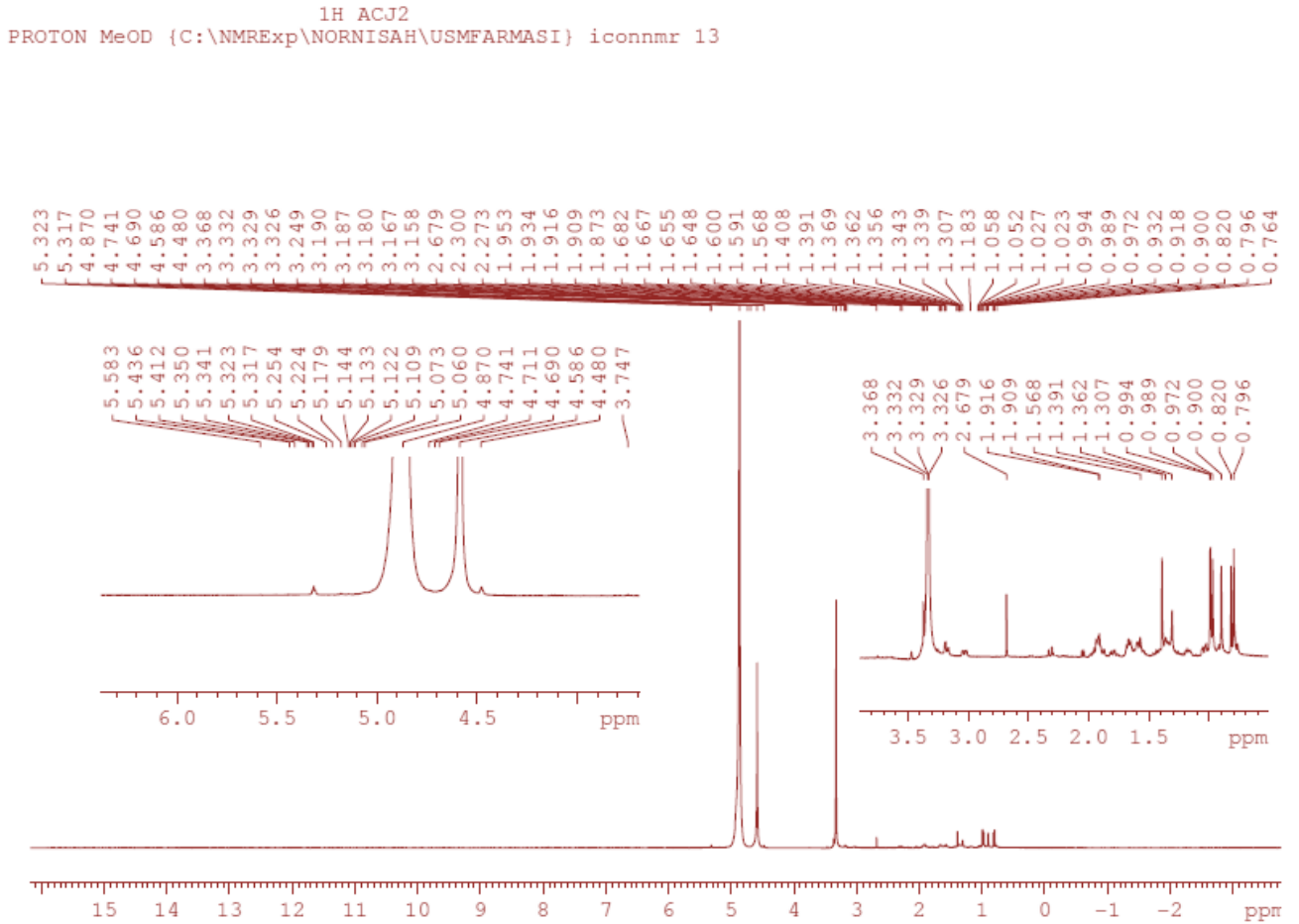


Fig. 4.19: Proton NMR Spectrum of COMPOUND IV

1H ACJ2  
PROTON MeOD {C:\NMRExp\NORNISAH\USMFARMASI} iconnmr 13

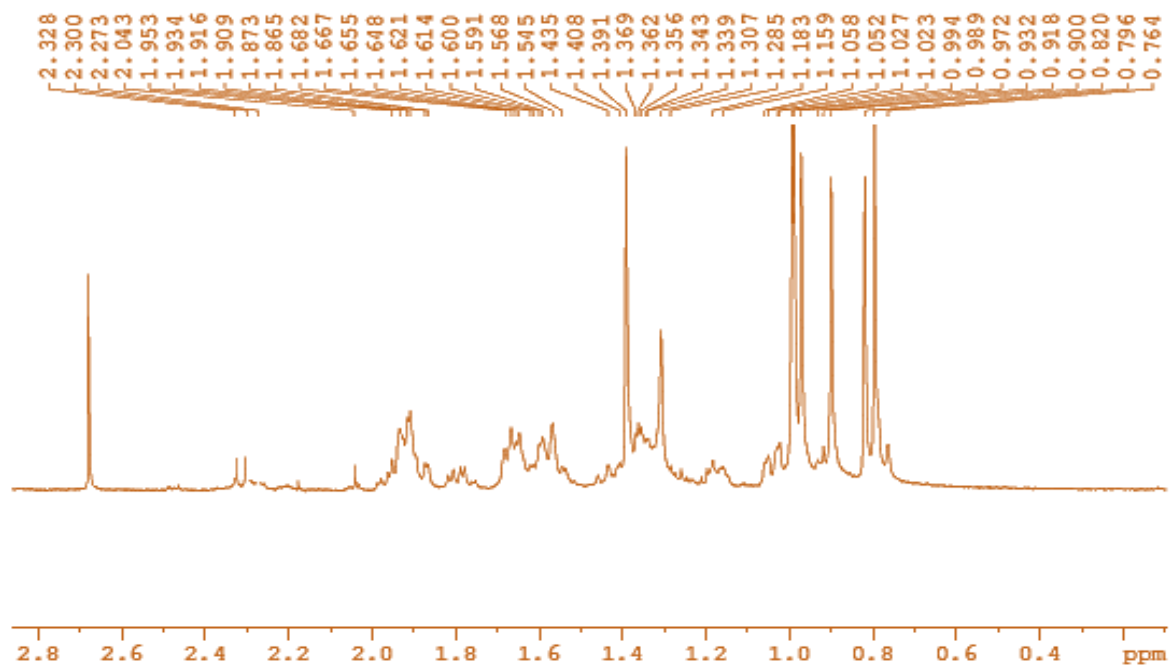


Fig. 4.20: Proton NMR of Spectrum of COMPOUND IV (expanded)

### 4.13.2 Carbon-13 NMR Analysis of COMPOUND VI

Fig 4.20 shows **Carbon-13NMR of COMPOUND IV**.The characteristic triterpenoid resonances occurred at:  $\delta_{C(ppm)}$ 78.33 (C-3), 55.46 (C-5), 122.14 (C-12), 143.93 (C-13), 180.80 (C-28).

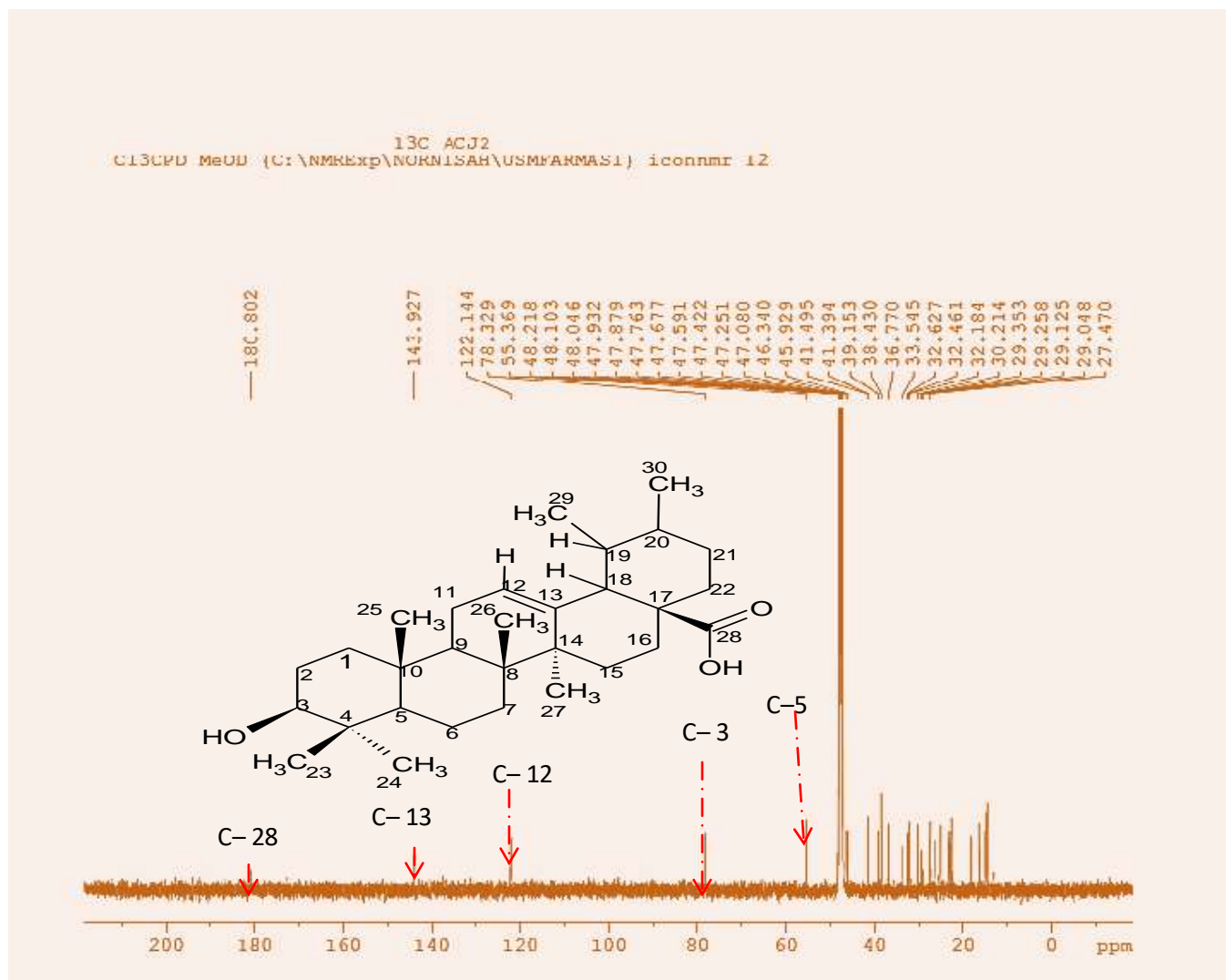


Fig. 4.21: Carbon-13 NMR Spectrum of COMPOUND IV

### 4.13.3 DEPT-135 of COMPOUND IV

DEPT-135 spectrum of COMPOUND IV showed Negative resonances due to CH<sub>2</sub> at 27.33 ppm (C-15) and 23.53 (C-16). The positive resonances due to CH<sub>3</sub> at 25.84 ppm (C-28), 16.37 ppm (C-25), 14.59 ppm (C-26).

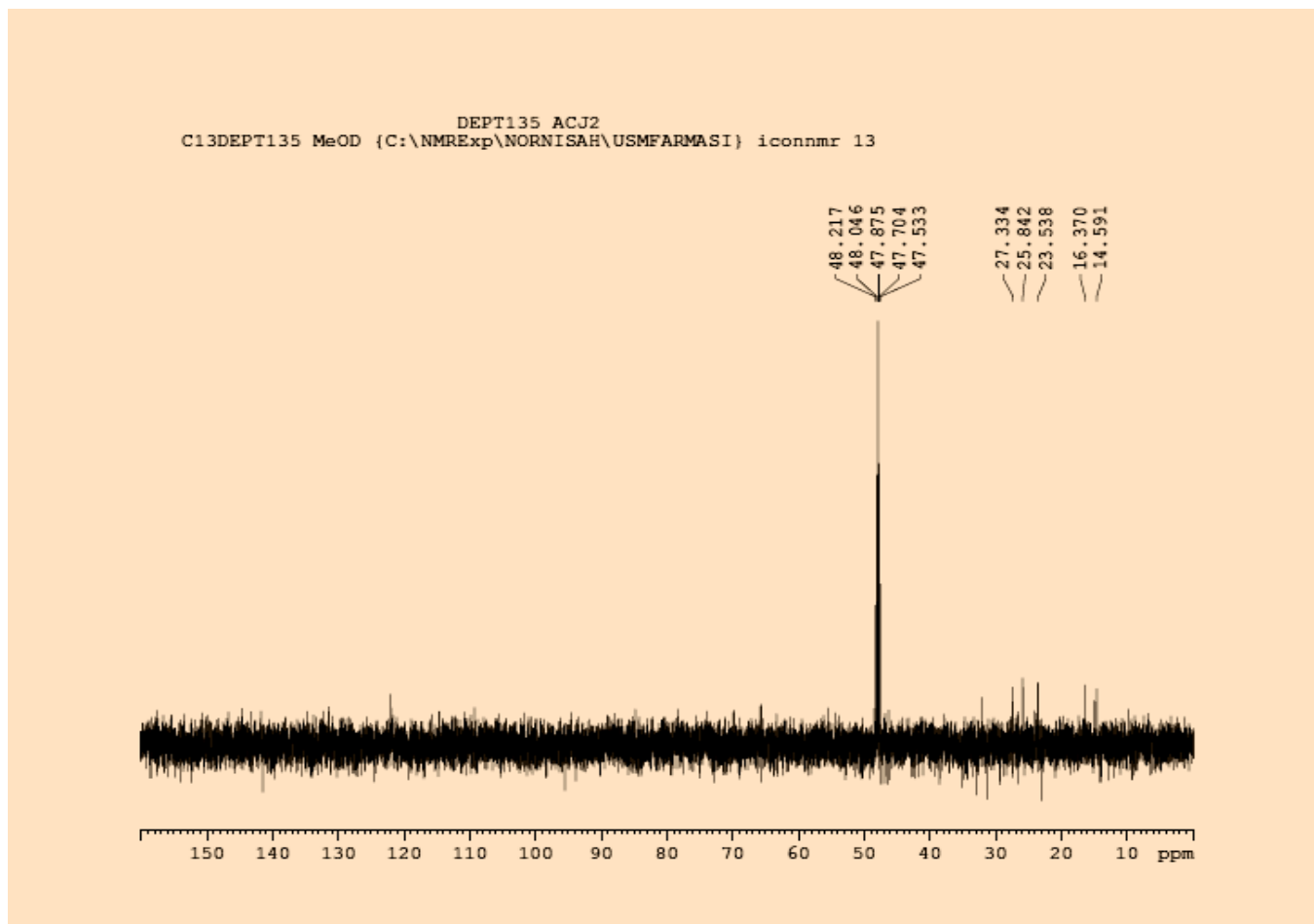


Fig. 4.22: DEPT 135 Spectrum of COMPOUND IV

#### 4.13.4 DEPT-90 of COMPOUND IV

Figure 4.23 shows DEPT-90 of COMPOUND IV; positive resonances attributed to methine carbon (CH) observed at 122.01 ppm assigned to C-12, 78.33 ppm (C-3), 55.46 ppm (C-5)

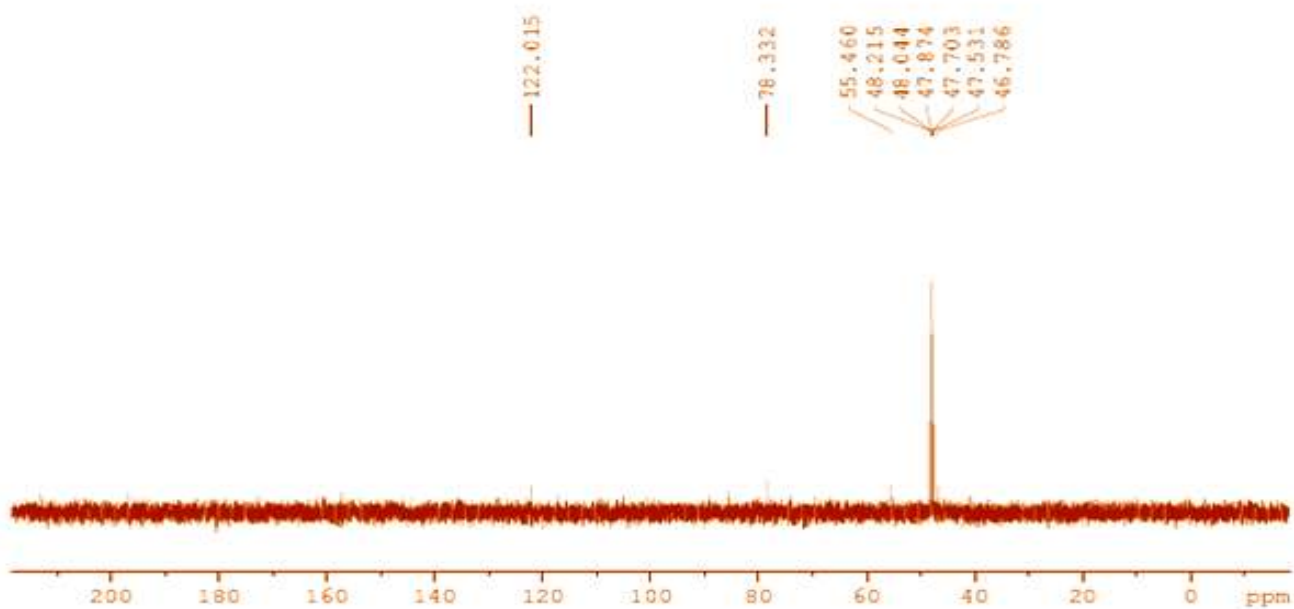


Fig. 4.23: DEPT 90 Spectrum of COMPOUND IV

Table 4.17:  $^1\text{H}$  and  $^{13}\text{C}$  NMR Chemical shifts of COMPOUND IV

Position of carbon	Type of Carbon	$\delta\text{C}$ (ppm)	$\delta\text{H}$ (ppm)	Coupling constant (J, Hz)
1	$\text{CH}_2$	36.76	1.56	
2	$\text{CH}_2$	27.33	1.68	
3	$\text{CH}$	78.33	3.24	d (J = 10.8 Hz)
4	C	39.20		
5	$\text{CH}$	55.46	0.90	
6	$\text{CH}_2$	16.36	1.59	
7	$\text{CH}_2$	33.50	1.60	
8	C	40.01		
9	$\text{CH}$	48.10	1.65	
10	C	38.05		
11	$\text{CH}_2$	23.53	1.934	
12	$\text{CH}$	122.14	5.41	brs
13	C	143.93		
14	C	42.20		
15	$\text{CH}_2$	27.33	1.39	
16	$\text{CH}_2$	23.53	2.14	
17	C	47.08		
18	$\text{CH}$	42.50	2.30	(d, J = 7.0 Hz)
19	$\text{CH}$	39.10	1.64	
20	$\text{CH}$	48.21	1.05	
21	$\text{CH}_2$	33.78	1.66	
22	$\text{CH}_2$	32.10	1.95	
23	$\text{CH}_3$	26.48	1.30	s
24	$\text{CH}_3$	16.37	0.97	s
25	$\text{CH}_3$	14.59	0.90	s
26	$\text{CH}_3$	19.58	1.36	s
27	$\text{CH}_3$	25.84	1.18	s
28	C	180.80	-	
29	$\text{CH}_3$	18.05	0.79	d (J = 6.7Hz)
30	$\text{CH}_3$	24.10	0.98	d (J = 6.4Hz)

250MHz  $^1\text{H}$ , 100MHz  $^{13}\text{C}$   $\text{CD}_3\text{OD}$

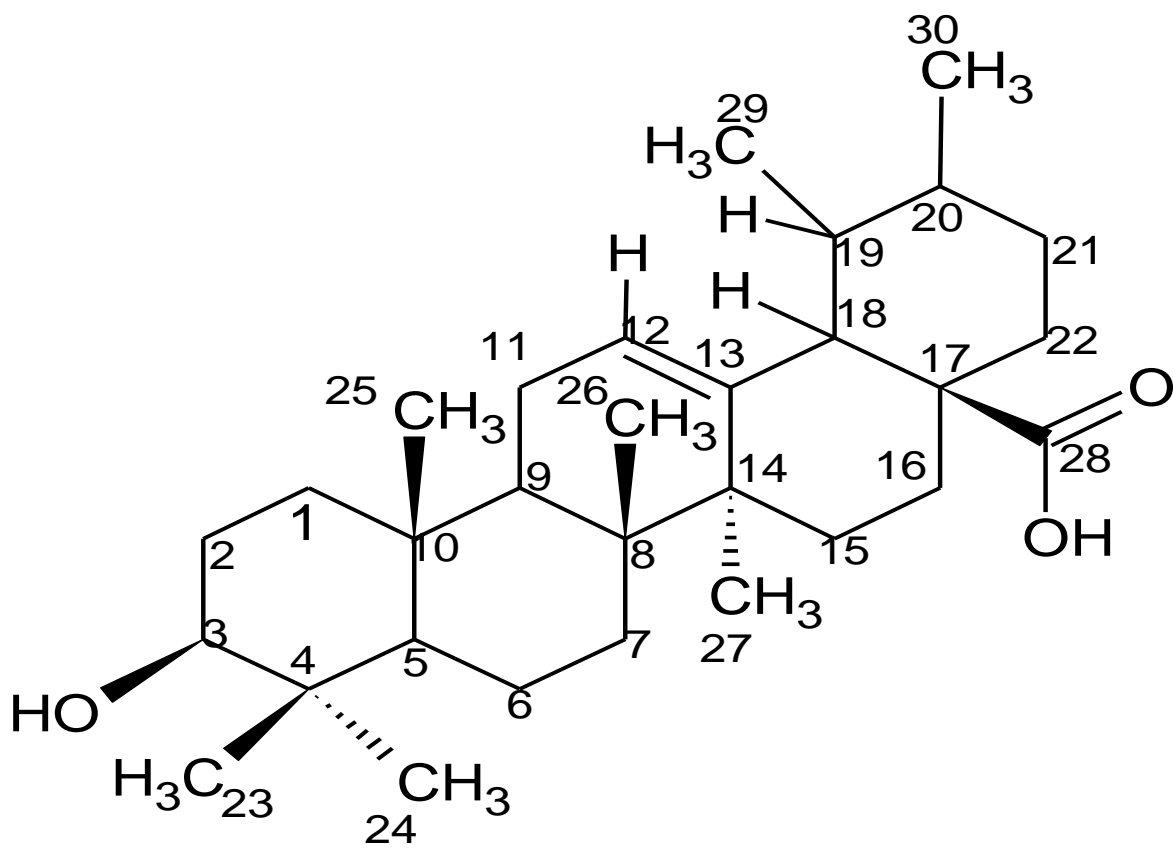


Fig 4.24 Structure of COMPOUND IV

#### 4.14 Co-TLC Analysis of n-butanol hydroxylate

Table 4.18 shows that  $R_f$  of n-butanol hydroxylate compared with the standard monosaccharide sugars.

Table: 4.18: Co-TLC Analysis of n-butanol hydroxylate with standard monosaccharide sugars

Sample	RF			
	1 <sup>st</sup> Experiment	2 <sup>nd</sup> Experiment	3 <sup>rd</sup> Experiment	Average
*Glucose	0.40	0.38	0.31	0.36
Glucuronic Acid	0.45	0.34	0.38	0.39
*n-Butanol hydroxylate	0.40	.038	0.31	0.36
Fructose	0.45	0.41	0.46	0.44
Mannose	0.39	0.44	0.39	0.41
Rhamnose	0.45	0.37	0.37	0.40
Xylose	0.25	0.27	0.24	0.25

\* $R_f$  of n-Butanol hydroxylate same as that of Glucose

#### 4.15 Antimicrobial Studies

Tables 4.19 and 4.20 show the results of anti-microbial susceptibility of the isolated compound, MEOH extract and BUOH fraction as compared to the control drugs for bacterial and fungal strains.

The results show that most of the pathogens including highly resistant bacterial strain like MRSA, *C. albicans* and *C. stellatoidea* were highly susceptible to COMPOUND 1, with diameter of zones of inhibition ranging between 25.00 and 32.00 mm. The pathogens were less susceptible to the crude methanol extract and n-butanol fraction with zones of inhibition between 10 and 27 mm. *Listeria monocytogenes* was not susceptible to any of the test samples including the control drug. Similarly *Candida tropicalis* did not respond to any of the test samples except the control drug. Crude methanol extract and n-butanol fraction did not show any activity with *Streptococcus pyogenes* and *Shingella dysenteriae* while *Pseudomonas fluorescense* did not respond to COMPOUND 1. Generally the anti-bacterial and anti-fungal activities of the plant extracts and isolated compound compared favourably with that of the standard drugs (ciprofloxacin for bacteria and fluconazole for the fungi). The MIC of the plant extracts ranged between 5.00 and 10 mg/mL, while that of the isolated compound ranged between 0.0125 and 0.0500 mg/mL. The MBC/MFC also ranged between 5.00 and 10.00 mg/mL and that of the isolated compound between 0.0125 and 0.0500 mg/mL (12.5 – 50 µg/mL).

Table 4.19: Susceptibility of bacterial and fungal strains to the isolate, extract, fraction and control drugs

Test Organism	Zone of Inhibition (mm)				
	COMP 1	ME	BE	CP	FL
<i>Staphylococcus aureus</i>	31.21±0.20 <sup>a</sup>	20.00±0.3 <sup>b</sup>	24.10±0.1 <sup>b</sup>	37.00±0.0 <sup>a</sup>	0.00
<i>MRSA</i>	30.7±0.210 <sup>a</sup>	21.50±0.3 <sup>b</sup>	22.00±0.0 <sup>b</sup>	32.00±0.1 <sup>a</sup>	0.00
<i>Streptococcus pyogenes</i>	29.02±0.15 <sup>a</sup>	0.00	0.00	38.00±0.2 <sup>b</sup>	0.00
<i>Bacillus cereus</i>	30.25±0.20 <sup>a</sup>	20.10±0.1 <sup>b</sup>	27.11±0.5 <sup>a</sup>	40.30±0.3 <sup>c</sup>	0.00
<i>Listeria monocytogenes</i>	0.00	0.00	0.00	0.00	0.00
<i>Proteus vulgaris</i>	25.22±0.80 <sup>a</sup>	20.07±0.5 <sup>a</sup>	22.20±0.8 <sup>a</sup>	0.00	0.00
<i>Pseudomonas fluorescense</i>	0.00	19.50±0.2 <sup>a</sup>	24.43±0.3 <sup>a</sup>	39.20±0.6 <sup>b</sup>	0.00
<i>Salmonella typhi</i>	28.30±0.40 <sup>a</sup>	21.00±0.0 <sup>b</sup>	24.24±0.2 <sup>b</sup>	32.20±0.5 <sup>a</sup>	0.00
<i>Shingella dysenteriae</i>	26.10±0.10 <sup>a</sup>	0.00	0.00	39.30±0.4 <sup>b</sup>	0.00
<i>Klesiella pneumoniae</i>	25.20±0.10 <sup>a</sup>	22.32±0.1 <sup>a</sup>	25.20±0.2 <sup>a</sup>	30.32±0.8 <sup>b</sup>	0.00
<i>Escherchia coli</i>	31.50±0.40 <sup>a</sup>	22.06±1.4 <sup>b</sup>	25.33±0.5 <sup>b</sup>	42.36±0.7 <sup>c</sup>	0.00
<i>Candida albicans</i>	26.02±0.20 <sup>a</sup>	20.30±0.5 <sup>b</sup>	22.10±0.1 <sup>b</sup>	0.00	40.20±0.2 <sup>c</sup>
<i>Candida stellatoidea</i>	27.22±0.20 <sup>a</sup>	10.0±0.2 <sup>b</sup>	12.0±0.2 <sup>b</sup>	0.00	34.10±0.2 <sup>c</sup>
<i>Candida tropicalis</i>	0.00	0.00	0.00	0.00	32.30±0.4

KEY: COMP 1 =COMPOUND I; ME=Methanol extract; BE= n-Butanol fraction; CP= Ciprofloxacin; FL=Fluconazole. Values are mean ± standard error of the mean, diameter of cork borer = 6 mm Values with different superscript are significantly different

Table 4.20: MIC and MBC of the isolate, extractfraction and the control drugs on the susceptible bacteria and fungi strains.

Organism		COMP 1 mg/mL	MEOH EXT. mg/mL	BUOH FR. mg/mL	CONTROL* mg/mL
<i>Staphylococcus aureus</i>	MIC	0.0250	5.00	5.00	0.003125
	MBC	0.0500	5.00	5.00	0.003125
MRSA	MIC	0.0250	5.00	5.00	0.003125
	MBC	0.0250	5.00	5.00	0.003125
<i>Streptococcus pyogenes</i>	MIC	0.0500	-	-	0.003125
	MBC	0.0500	-	-	0.003125
<i>Bacillus cereus</i>	MIC	0.0250	5.00	5.00	0.003125
	MBC	0.0250	5.00	5.00	0.003125
<i>Listeria monocytogenes</i>	MIC	-	-	-	-
	MBC	-	-	-	-
<i>Proteus vulgaris</i>	MIC	0.0500	5.00	5.00	0.003125
	MBC	0.0500	5.00	5.00	0.003125
<i>Pseudomonas fluorescences</i>	MIC	0.0250	10.00	5.00	0.003125
	MBC	0.0250	10.00	5.00	0.003125
<i>Salmonella typhi</i>	MIC	0.0250	10.00	5.00	0.003125
	MBC	0.0250	10.00	5.00	0.003125
<i>Shingella dysenteriae</i>	MIC	0.0250	5.00	5.00	0.003125
	MBC	0.0250	5.00	5.00	0.003125
<i>Klebsiella Pneumoniae</i>	MIC	0.0500	5.00	5.00	0.003125
	MBC	0.0500	5.00	5.00	0.003125
<i>Escherchia coli</i>	MIC	0.0500	10.00	10.00	0.003125
	MBC	0.0500	10.00	10.00	0.003125
<i>Candida albicans</i>	MIC	0.0500	0.00	10.00	0.003125
	MFC	0.0500	0.00	10.00	0.003125
<i>Candida stellatoidea</i>	MIC	0.0250	0.00	0.00	0.003125
	MFC	0.0250	0.00	0.00	0.003125
<i>Candida tropicalis</i>	MIC	-	-	-	-
	MFC	-	-	-	-

\*CONTROL : Ciprfloxacin for bacteria; Fluconazole for fungi

#### 4.16 Anti-Diabetic Studies

Tables 4.21 and 4.22 show the results of  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition assay of crude methanol, n- butanol fraction and COMPOUND I, compared to the control drug (acarbose). The  $IC_{50}$  of crude methanol, n- butanol fraction, COMPOUND I and Control drug in  $\alpha$ -amylase assay are respectively,  $96.40 \pm 0.2$  mg/mL,  $72.50 \pm 0.65$  mg/mL,  $25.45 \pm 0.45$   $\mu$ g/mL,  $46.31 \pm 0.58$   $\mu$ g/mL while in the  $\alpha$ -glucosidase assay the following were obtained respectively  $95.10 \pm 0.20$  mg/mL,  $72.65 \pm 3.20$  mg/mL,  $47.57 \pm 0.40$   $\mu$ g/mL,  $48.10 \pm 0.13$   $\mu$ g/mL.

Table 4.21: % Inhibition of  $\alpha$ -amylase by MEOH Extract, BUOH Fraction, COMPOUND I and Reference Drug(Acarbose) at varying concentrations

CONCENTRATION ( $\mu$ g/mL)	20	40	60	80	100	$IC_{50}$
MEOH EXTRACT %INHIBITION	30.35	37.48	40.21	44.29	51.89	$96.40 \pm 0.20^*$
BUOH FRACTION %INHIBITION	30.20	35.15	48.56	52.16	56.20	$72.50 \pm 0.65^*$
COMPOUND I %INHIBITION	47.00	58.00	72.00	73.00	85.00	$25.57 \pm 0.45^*$
ACARBOSE %INHIBITION	32.00	44.00	63.00	69.00	74.00	$46.31 \pm 0.58^*$

Data are given as mean $\pm$ SD (n=3; \* p<0.05)

Table 4.22: % Inhibition of  $\alpha$ -Glucosidase by MEOH Extract, BUOH Fraction, COMPOUND I and Reference Drug(Acarbose)at varying concentrations

CONCENTRATION ( $\mu\text{g/mL}$ )	20	40	60	80	100	IC <sub>50</sub>
MEOH EXTRACT %INHIBITION	33.34	42.11	46.03	52.03	56.14	95.10 $\pm$ 0.20*
BUOH FRACTION %INHIBITION	33.23	36.22	43.14	45.10	51.9	72.65 $\pm$ 3.20*
COMPOUND I %INHIBITION	34.00	45.00	63.00	66.00	74.00	47.57 $\pm$ 0.40*
ACARBOSE %INHIBITION	33.00	44.00	60.00	67.00	71.00	48.10 $\pm$ 0.13*

Data are given as mean $\pm$ SD (n=3; \* p<0.05)

## CHAPTER FIVE

### 5.0 Discussion

#### 5.1 Preliminary Phytochemical Investigation

The results of the preliminary phytochemical screening carried out on the methanol extract and its fractions revealed the presence of contains, anthraquinones, carbohydrates, cardiac glycosides, cardenolides, flavonoids, polyphenols, saponins, steroidal glycosides, tannins and terpenes in good quantities. These phytochemical constituents have been reported to be associated with the biological activity of the plant (Okoli *et al.*, 2007a).

##### 5.1.1 COMPOUND I

**COMPOUND I** was obtained as a white amorphous solid (225 mg) from the n-butanol fraction hydrolysis, m.pt 299-302 °C, TLC R<sub>f</sub> 0.75 (CHCl<sub>3</sub>-MeOH 9:1), 0.56 n-Hex:ETOAc 4:1). It was soluble in chloroform and methanol, and insoluble in water. It responded positively to the Liebermann-Burchard's test for pentacyclic triterpene and produced effervescence with NaHCO<sub>3</sub> suggesting the presence of triterpenic acid (Mann *et al.*, 2012). The UV( $\lambda_{max}$ ) was at 221 nm (c = 0.15, CHCl<sub>3</sub>). The FTIR spectrum showed the presence of hydroxyl group (3436 cm<sup>-1</sup>); C-H str (2931 cm<sup>-1</sup>); carbonyl group (1728 cm<sup>-1</sup>), olefinic group (769.62 cm<sup>-1</sup>) and C-O (acid) functional group (1152, 1039 and 1016 cm<sup>-1</sup>) implying the presence of unsaturation and acid carbonyl function in the molecule (Spectroscopic Data, 2015).

<sup>1</sup>H-NMR spectrum showed a downfield signal at  $\delta$  5.35 (t, J = 4 Hz) assigned to the olefinic proton at H-12 and  $\delta$ <sub>H</sub> = 3.35 (br, s) at H-3 (1H geminal to OH) are typical of an oleanane triterpenoid structure ((Mahato and Kundu, 1994; Lopes *et al.*, 1999; Choudhary *et al.*, 2008). <sup>1</sup>H-NMR spectral data also revealed seven methyl singlets peaks at  $\delta$  = 0.76, 0.79, 0.91, 0.93, 0.93,

0.97, 1.15. The fact that all the methyl proton signals are singlets suggests that the triterpenoid should have an oleanane ( $\beta$ -amyrin group) rather than ursane ( $\alpha$ -amyrin group) structure (Faleye, 2012).

From the  $^{13}\text{C}$ , and 2D NMR, i.e. COSY, HMQC and HMBC spectroscopic analysis, the presence of thirty carbon atoms was established; this consisted of eight quaternary, five tertiary, ten secondary carbons and seven methyl groups (Mann *et al.*, 2012).  $^{13}\text{C}$  NMR: the chemical shifts at  $\delta$ 178.03 ppm, 144.12 ppm and 122.24 ppm were typical characteristics peaks of oleanane type of triterpenoid skeleton assigned to C-28, C-13 and C-12 respectively. The deshielding chemical shift at  $\delta$ 78.03 was assigned to C-3 (oxymethine carbon). In the HMBC experiments there were connectivities between Me-25 and C-1, 5, 9, 10; Me-26 and C-7, 8, 9, 14; Me-23 and C-3, 4, 5, 24; Me-29 and C-19, 20, 21, 30; Me-30 and C-19, 20, 21, 29.

From the HMQC experiments, proton resonances were assigned to the respective carbons. Signals at  $\delta$ C 78.03 ppm (C-3) correlated with  $\delta$ H 3.35 (H-3). The following also correlated,  $\delta$ C 55.39 ppm (C-5)/  $\delta$ H 0.77 ppm (H-5);  $\delta$ C 18.33 ppm (C-6)/  $\delta$ H 1.33 ppm (H-6);  $\delta$ C 47.71 ppm (C-9)/  $\delta$ H 1.60 ppm; (H-9);  $\delta$ C 122.24 ppm (C-12)/  $\delta$ H 5.35 ppm (H-12);  $\delta$ C 41.43 ppm (C-18)/  $\delta$ H 2.27 ppm (H-18);  $\delta$ C 16.80 ppm (C-25)/  $\delta$ H 0.79 ppm (3H, H-25).

The COSY experiment showed the following couplings:  $\delta$ H 1.53 ppm (H-1)/  $\delta$ H 1.10 ppm (H-2);  $\delta$ H 2.27 ppm (H-18)/  $\delta$ H 1.70 ppm (H-19);  $\delta$ H 0.97 (H-23)/  $\delta$ H 0.93 (H-24);  $\delta$ H 0.76 ppm (H-26)/  $\delta$ H 0.93 ppm (H-27). A triplet observed at 2.27 ppm with a J value of 14.0 Hz indicated coupling between a single proton at H-18 and two protons at H-19. This triplet only appears if the compound is of the  $\beta$ -type -oleanane triterpene; where two protons are attached to C-19 (Bhatt, 2011).

On the basis of melting point (c. f. Litt 301- 302°C; Gohari *et al.*, 2009), spectral and chemical evidences, **COMPOUND I** was identified as 3β-hydroxyolean-12-en-28-oic acid also known as oleanolic acid with the structure shown in Fig 4.11.

The identity of the compound was finally determined by comparison of <sup>13</sup>C chemical shifts with the reported data (Seebacher *et al.*, 2003, Gohari *et al.*, 2009, Mann *et al.*, 2012).

### 5.1.2 COMPOUND II

**COMPOUND II** was obtained as a white amorphous powder (14 mg), TLC R<sub>f</sub> value, 0.78 (CHCl<sub>3</sub>-MeOH, 9:1), 0.54 (n-Hex: ETOAc 4:1). It was found to be insoluble in water but soluble in chloroform, acetone, and methanol. It reacted with NaHCO<sub>3</sub> and produced effervescence, which turned limewater milky and extinguished a glowing splint thus confirming it is an acid. It also gave a positive Liebermann-Burchard test for pentacyclic triterpene.

The FTIR spectrum of **COMPOUND II** showed the presence of hydroxyl group (3449.8 cm<sup>-1</sup>); C-H str (2930.9 cm<sup>-1</sup>); carbonyl group (1727.31 cm<sup>-1</sup>), olefinic group, C=C (725.26 cm<sup>-1</sup>) and C-O (acid) functional group (1170.0 and 1045 cm<sup>-1</sup>). The FTIR spectrum also gave absorption 1373.36 cm<sup>-1</sup> (gem-dimethyl at C-4), but the peak at 1218 cm<sup>-1</sup> was absent; showing that two methyls on quaternary carbon (C-20) atom- is absent indicating that **COMPOUND II** is an ursane triterpene.

<sup>1</sup>H-NMR spectrum showed an alkenic proton at δ<sub>H</sub> of 5.38 (t, J = 4.5 Hz), a proton geminal to OH group at δ<sub>H</sub> 3.69. Five singlet methyls at δ<sub>H</sub> 0.99, 0.99, 0.96, 0.93 and 1.28, (H-23, 24, 25, 26, 27), two doublet methyls at δ<sub>H</sub> 0.98, (J = 6.8, C-30) and 1.03 (J = 4.0 Hz, C-29), which are the main features of ursane skeleton (Ayatollahi *et al.*, 2011; Patil and Nitave, 2014). The fact that

not all the methyl proton signals are singlets suggests that the triterpenoid should have an ursane (-amyrin group) rather than oleanane ( $\beta$ -amyrin) skeleton (Faleye, 2012).

The  $^{13}\text{C}$ -NMR spectrum of **COMPOUND II**(Fig. 4.14, Table 4.12) shows 30 signals, consisting of seven quaternary carbons, seven methines, nine methylenes and seven methyls deduced from the DEPT experiments. The most downfield signal resonated at  $\delta$ 173.69 is attributed to the carboxylic acid (C-28) (Silverstein *et al.*, 2005). The appearance of signals at  $\delta$ 121.65 and 145.19 indicated the presence of a double bond in the triterpenoid structure. The  $\alpha$ -type orientation was established by appearance of a doublet at  $\delta$ H 2.35 with  $J = 7.5$  Hz assigned to H-18, (a doublet appears here because the two groups attached to C-19 are hydrogen and methyl and the coupling between a single proton at C-18 and C-19 would produce a doublet (Bhatt, 2011).

The NMR spectroscopic data of **COMPOUND II**are similar to those of **COMPOUND I**.The fundamental difference between **COMPOUND I**(the oleanane or  $\beta$ -amyrin type triterpenoid) and**COMPOUND II**(the ursane or  $\alpha$ -amyrin type triterpenoid) is at C-19 and C-20 of the ring E. In $\alpha$ -typetriterpene, the two methyl groups (C-29 and C-30) on ring E are attached vicinally to C-19 and C-20respectively whereas in  $\beta$  type triterpene (oleanane type) both methyl groups are attached geminally to C-20 only.At these positions methyls in **COMPOUND II**are tertiary (ursane type) while those **COMPOUND I**are quaternary (oleanane type)(Bhatt, 2011).

On the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra the resonances at  $\delta_{\text{H}}$  7.28ppm  $\delta_{\text{C}}$ and 77.5, 77.23, 77.03 and 76.52ppm are attributable to the solvents.

On the basis of melting point(c. f. Litt. 284-287<sup>0</sup>C; Babalola and Shode, 2013), spectral data, chemical evidences and comparison of <sup>13</sup>C-chemical shifts with the reported data (Seebacher *et al.* 2003), **COMPOUND II** was identified as 3 $\beta$ -hydroxyurs-12-en-28-oic acid or ursolic acid with structure shown in Fig. 4.15.

### 5.1.3 COMPOUND III

**COMPOUND III** crystallized in MEOH as a white amorphous powder (25 mg), mp 238-242 $^{\circ}$ C, TLC R<sub>f</sub>, 0.79 (CHCl<sub>3</sub>-MeOH, 9:1), 0.58 (n-Hex:ETOAc 4:1). **COMPOUND III** was Insoluble in water but soluble in chloroform, acetone and methanol. It produced effervescence with NaHCO<sub>3</sub> and gave a positive Liebermann-Burchard's test for pentacyclic triterpene.

The FTIR spectrum of **COMPOUND III**(Fig 4.16) showed the presence of alcoholic OH (3453.41 cm<sup>-1</sup>); C-H str (2929.97cm<sup>-1</sup>); carbonyl group (1725.38 cm<sup>-1</sup>), olefinic group (trisubstituted C=C), (762.87 cm<sup>-1</sup>) and C-O (alkoxy) functional group at (1166.97 and 1037.74 cm<sup>-1</sup>).

<sup>1</sup>H-NMR spectrum (Fig 4.17) showed an alkenic proton at  $\delta_H$  of 5.35 (br, s), a proton germinal to OH groups at  $\delta_H$  3.65(1H,d, J = 10.0, Hz, H-2) and 3.47 (1H, d, J = 7.5 Hz, H-3),  $\delta_H$  2.30 (d, J = 8.0 Hz, H-18), five singlet methyls at  $\delta_H$  1.02,1.0, 1.0, 1.02, 1.03, (H-23, 24, 25, 26, 27) and two doublet methyls at  $\delta_H$  0.97 (J = 5.6, C-30) and 0.99 (J = 5.8 Hz, C-29) suggesting a typical ursane triterpenoid structure (Park *et al.*, 2002, Faleye, 2012).

Coupling constant (J=10.0 Hz) of the H-2 in <sup>1</sup>H-NMR spectrum indicated the axial interaction with H-3. The coupling constant of H-2 showed equatorial (J= 7.5 Hz) coupling with the two protons at H-1. (Park *et al.*, 2002).The <sup>13</sup>C-NMR spectrum of **COMPOUND III** is similar to

that of **COMPOUND II** except the chemical shift at  $\delta_C$  64.42 ppm due to the second OH group in the molecule assigned to C-2. The spectrum shows 30 signals, consisting of eight quaternary carbons, seven methines, eight methylenes and seven methyls deduced from the DEPT experiments. The downfield signal at  $\delta_C$  173.74 is attributed to the carboxylic acid (C-28). The appearance of signals at  $\delta_C$  121.65 and 145.00 indicated the presence of a double bond in the structure.

In DEPT-90 (Fig 4.20), positive resonances attributed to methine carbon (CH) were observed at 121.65 ppm assigned to C-12, 80.66 ppm (C-3), 77.21 ppm (C-2), 64.41 ppm (C-18), 55.26 ppm (C-5), 47.24 ppm (C-20), 47.55 (C-9), 32.79 ppm (C-19).

In DEPT-135 (Fig 4.19), negative resonances attributed to secondary carbon ( $CH_2$ ) were observed at 46.77 ppm assigned to C-1, 34.87 ppm (C-7), 34.43 (C-22), 29.67 ppm (C-21), 29.17 ppm (C-15), 23.60 ppm, (C-11), 22.68 ppm (C-16), 18.26 (C-6). The positive resonances attributed to methyl carbon ( $CH_3$ ) appeared at 28.39 ppm assigned to C-23, 24.79 ppm (C-30), 23.79 ppm (C-27), 19.02 ppm (C-26), 16.99 ppm (C-25), 16.81 ppm (C-24) and 16.77 ppm (C-29)

In DEPT-q (Fig 4.21), resonances attributed to quaternary carbon (C) appeared at 173.74 ppm assigned to C-28, 145.00 ppm (C-13), 41.72 ppm (C-17), 40.84 ppm (C-8), 39.37 ppm (C-4), 38.25 ppm (C-10), and 37.75 ppm (C-14) (Hamed and Regents, 2009).

In comparison the  $^{13}C$  NMR data of **COMPOUND III** showed a good agreement with reported data of corosolic acid (Park *et al.*, 2002; Lee and Juang, 2005).

On the basis of melting point, spectral analysis and chemical reactions **COMPOUND III** was identified as 2, 3-dihydroxyurs-12-en-28-oic acid or corosolic acid with suggested structure in Fig 4.22. The identity of the compound was further confirmed by comparison of carbon-13 chemical shifts with the reported data (Park *et al.*, 2002).

#### 5.1.4 COMPOUND IV

COMPOUND IV was obtained as a white powder with  $R_f$  0.78 ( $\text{CHCl}_3$ : MEOH) which is typical of Ursane triterpenoid in this solvent system (Flores *et al.*, 2005).

The  $^1\text{H-NMR}$  spectrum showed an alkenic proton at 5.41 (singlet, broad) a proton geminal to –OH group 3.24 (d  $J_{\text{axax}} = 10.8$  Hz) showing its  $\alpha$ -axial orientation. The peak at 2.30 (d,  $J = 7.0$  Hz) H-18) showed coupling between 1H at H-19 with 1H at H-18; five singlet methyls at 1.36, 1.3, 1.18, 0.97, 0.90, 0.97 and 0.90, two doublet methyls at 0.98 (d,  $J=6.4\text{Hz}$ ; H-30) and 0.79 (d,  $J= 6.7$  Hz; H-29) are typical characteristics of ursane skeleton (Park *et al.*, 2002).

The  $^{13}\text{C}$  NMR spectrum and the analyses of DEPT experiments showed the presence of 30 carbon atoms consisting of seven quaternary carbon, seven tertiary, nine secondary and seven methyls carbons. The most down field signal resonated at  $\delta 180.80$  is attributed to the presence COOH (C-28) (Spectroscopy Data Tables, 2015). The appearance of signals at  $\delta 122.14$  and  $143.93$  indicated the presence of a double bond in the triterpenoid structure (Seebacher *et al.*, 2003).

The NMR spectral data of **COMPOUND II** resembled those of ursolic acid very closely as confirmed through literature data (Seebacher *et al.*, 2003).

## 5.2 Antimicrobial Studies of *Aspilia africana*

The anti-microbial activities of the extracts and one of the isolates (**COMPOUND I**) were compared to those of ciprofloxacin and fluconazole (the standard drugs).

The minimum inhibitory concentration of the methanol leaf extract was 10mg/mL for *P. Flouresence*, *S. taphii* and *E. Coli* while those of the *S. aureus*, *MRSA*, *B. cereus*, *S. dysenteriae* were 5mg/mL in each case. From the fractions of the methanol extract, n-butanol fraction was more potent with MIC of 5mg/mL for six of the bacterial strains and one of the fungal strains. This informed our choice of the n-butanol fraction for further investigation.

**COMPOUND I** had MIC of 25µg/mL for four of the pathogens and 50ug/mL for seven of the bacterial pathogens. Some of the pathogens that were susceptible to the MEOH extract, BUOH fraction and the isolated **COMPOUND I** have been implicated in wound infections; these include: *S. aureus*, *S. pyogenes*, *E. Coli* and *MRSA* (Dow *et al.*, 1999). This has validated the use of this plant to treat wounds and sores by traditional herbal medicine practitioners. The isolates and extract of leaves *Aspilia africana* have broad spectrum activity since gram positive and gram-negative bacteria were sensitive to them. These microorganisms were selected based on their established identity as wound pathogens and are always implicated in wound sepsis and sores (Hugo and Russel, 1983). The results show that all the pathogens including highly resistant bacterial strain like *MRSA*, and yeasts like *C. albicans* and *C. stellatoidea* are highly susceptible, with diameter of zones of inhibition ranging between 25.00 and 32 mm (Ibeh and Uraih, 2003). The anti-bacterial and anti-fungal activities of the plant extracts and isolated compounds compared favourably with that of the standard drugs (ciprofloxacin for bacteria and fluconazole for the fungi). The MIC of the plant extracts ranged between 5 and 10 mg/mL, while that of the

isolated compounds ranged between 0.0125 and 0.0500 mg/mL. Thus the plant product may be described as bactericidal or fungicidal in action (Ibeh and Uraih, 2003). Findings of this study indicate that the bioactive isolate is very effective and could possibly be employed in tackling the problem of resistance of the virulent microorganisms. It could be concluded that, these bioactive constituents are responsible for the anti-microbial activity of the leaves of this plant. Therapeutic uses of oleanolic acid, ursolic acid and corosolic acids such as anti-microbial, hepatoprotective anti-hyperglycaemic and anti-inflammatory have been well documented (Liu, 1995). Thus isolation of these compounds from *A. africana* lends credence to the traditional use of the plant in the treatment of these disease conditions especially those of microbial origins.

### 5.3 Anti-Diabetic Activity

The phytochemical investigation of *Aspilia africana* led to the isolation of an anti-diabetic agent (oleanolic acid) that inhibited the actions of two important enzymes,  $\alpha$ -amylase and  $\alpha$ -glucosidase implicated in the metabolism of carbohydrate in human body.  $\alpha$ -Amylase catalyses the breaking down of starch to disaccharides and oligosaccharides and the intestinal  $\alpha$ -glucosidase catalyses the breakdown of disaccharides to release glucose which is later absorbed from small intestine into the blood circulation (Nagmoti and Juvekar, 2013). This inhibition in digestion and absorption of carbohydrate which in turn decreases rise in postprandial hyperglycemia is a simple but highly effective therapeutic approach to check the prevalence of hyperglycemia (Kwon *et al.*, 2007). The isolated compound - oleanolic acid has been shown to demonstrate a powerful anti-diabetic activity in this research work through this approach. This has been corroborated by the previous work on done the compound (Castellano *et al.*, 2013). Ursolic acid, an isomer of oleanolic acid and one of the compounds isolated in this work has been severally reported as anti-diabetic agent (Liu, 1995; Sun *et al.*, 2006; Babalola and Shode, 2013).

Corosolic acid another compound isolated from *Aspilia africana* in this research work has been reported to decrease blood sugar levels within 60 minutes in human subjects (Miura *et al.*, 2012) and is well known anti-diabetic agent (Lee *et al.*, 2005, Wen *et al.*,2005; Jung *et al.*, 2006).

Isolation of three pentacyclic titerpenic acid (oleanolic acid ursolic acid and corosolic acid) from a very common and abundantly available herb like *Aspilia africana* is a great gain to the fight against diabetes mellitus.

## CHAPTER SIX

### 6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

#### 6.1 Summary

Preliminary phytochemical screening of methanol extract and fractions of the leaves of *Aspilia africana* revealed the presence of carbohydrates, cardiac glycosides, cardenolide flavonoid, polyphenols, saponins, steroids, tanins and terpenoids.

Extensive phytochemical investigation led to the isolation of three pentacyclic triterpenic acids identified by physicochemical and spectral analysis as 3 $\beta$ -hydroxyolean-12-ene-28-oic acid (oleanic acid), 3 $\beta$ -hydroxyurs-12-ene-28-oic acid (ursolic acid) and 2,3-dihydroxyurs-12-ene-28-oic acid (corosolic acid) from the dichloromethane and n-butanol fractions of methanol leaf extract. Oleanolic and ursolic acids are isomers which exist together in nature as free acid or as aglycones for oleanane and ursane- type triterpenoid saponins. In the analysis of the aqueous part of the hydrolysed saponins, precipitated from butanol fraction, glucose was identified as the glycone portion. This may have glycosylated at C-2, C-3 or C-28 positions, thus making the original saponin molecule either monodesmosides or bisdesmosides.

In the anti-microbial screening, the crude methanol leaf extract and the n-butanol fraction inhibited the growth of nine out of the eleven bacterial strains and one of the three fungal strains but the activity of the isolated oleanolic acid was more even at lower concentration inhibiting the growth of all the bacterial strains and two of the fungal strains showing that the pentacyclic triterpenoid may be responsible for the anti-microbial activity of the plant.

In the *in-vitro* antidiabetic studies, the isolated compound, oleanolic acid, was more potent in the inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase than the methanol extract and the butanol fraction of

the leaf. This confirms that the pentacyclic triterpenic acid is part of the anti-microbial agents in the leaf of *Aspilia africana*.

Oleanolic acid (OA), Ursolic acid (UA) and Corosolic acid (CA) have been reported to have biological activity including anti-microbial and anti-hyperglycemic properties (Liu, 1995; Xu, *et al.*, 2012). By our findings in this research work, the active principles responsible for the anti-microbial and anti-diabetic activity in *Aspilia africana* is the isolated Oleanolic acid (OA), and by extension Ursolic acid (UA) and Corosolic acid (CA).

## 6.2 Conclusion

This study has shown that *Aspilia africana* contains anti-microbial and anti-diabetic compounds, which have been identified as oleanolic acid and extension ursolic acid and corosolic acid. This is the first time these compounds have been isolated from *Aspilia africana*.

## 6.3 Recommendations

1. Further studies should be carried out to isolate flavonoids triterpenoids and other compounds from the flower part of the plant
2. The n-hexane fraction of the methanol leaf-extract should be analysed for less polar compounds.
3. Herbal preparation of the leaves should be standardised for use as supplement for diabetic patients

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## APPENDIX I

<sup>1</sup>H and <sup>13</sup>C NMR Chemical shifts of COMPOUND I compared with reference compound

Position of Carbon	Compd 1		Compd 1	*Oleanolic acid	*Oleanolic acid
	δC (ppm)	DEPT	δH ppm	δC (ppm)	δH ppm
1	39.35	CH <sub>2</sub>	1.53	39.00	1.02
2	27.63	CH <sub>2</sub>	1.10	28.10	1.82
3	77.78	CH	3.35	78.20	3.44
4	38.55	C	-	38.80	
5	55.39	CH	0.77	55.40	0.88
6	18.33	CH <sub>2</sub>	1.33	18.80	1.39
7	33.64	CH <sub>2</sub>	1.57	33.10	1.53
8	41.70	C	-	39.90	-
9	47.70	CH	1.60	47.70	1.71
10	37.01	C	-	37.90	-
11	23.32	CH <sub>2</sub>	1.86	23.80	1.96
12	122.24	CH	5.35	122.6	5.49
13	144.12	C	-	144.8	-
14	41.70	C	-	42.20	-
15	30.51	CH <sub>2</sub>	1.38	29.00	1.22
16	23.07	CH <sub>2</sub>	1.54	23.80	1.96
17	45.99	C	-	46.70	-
18	41.43	CH	2.27	42.10	3.30
19	46.06	CH <sub>2</sub>	1.70	46.60	1.83
20	32.84	C	-	31.00	-
21	38.69	CH <sub>2</sub>	1.21	34.30	1.23
22	27.89	CH <sub>2</sub>	1.30	37.20	1.82
23	27.27	CH <sub>3</sub>	0.97	26.40	1.24
24	14.98	CH <sub>3</sub>	0.93	14.40	1.02
25	16.80	CH <sub>3</sub>	0.79	16.20	0.93
26	15.50	CH <sub>3</sub>	0.76	17.30	1.04
27	22.95	CH <sub>3</sub>	0.93	22.20	1.30
28	178.0	C	-	180.0	-
29	32.55	CH <sub>3</sub>	0.91	32.90	0.97
30	25.45	CH <sub>3</sub>	1.15	26.20	1.02

400MHz <sup>1</sup>H, 100MHz <sup>13</sup>C CDCl<sub>3</sub>; \*Ref Cpd (Seebacher *et al.*, 2003)

## APPENDIX II

<sup>1</sup>H and <sup>13</sup>C NMR Chemical shifts of **COMPOUND II** in CD<sub>3</sub>OD compared with reference compound

Position of C atom	DEPT	COMPOUND II	COMPOUND II	Ursolic acid	Ursolic acid
		$\delta_C$ (ppm)	$\delta_H$ (ppm)	$\delta_C$ (ppm)	$\delta_H$ (ppm)
1	CH <sub>2</sub>	39.38	1.00	39.2	1.00
2	CH <sub>2</sub>	28.40	1.74	28.2	1.81
3	CH	80.58	3.69	78.2	3.44dd
4	C	38.25	-	39.6	-
5	CH	55.26	0.86 d	55.9	0.88
6	CH <sub>2</sub>	18.27	1.55	18.8	1.58
7	CH <sub>2</sub>	33.34	1.55	33.7	1.59
8	C	39.18	-	40.1	-
9	CH	47.55	1.64	48.1	1.65
10	C	37.83	-	37.5	-
11	CH <sub>2</sub>	23.69	1.96	23.7	1.96
12	CH	121.65	5.58 s	125.7	5.49
13	C	145.19	-	139.3	-
14	CH <sub>2</sub>	41.70	1.16	42.6	1.22
15	CH <sub>2</sub>	29.18	2.07	28.8	2.14
16	CH <sub>2</sub>	24.28	-	25.0	-
17	C	47.24	1.45	48.1	1.49
18	CH	47.55	2.35d	53.6	2.63d
19	CH	39.62	1.46	39.5	1.49
20	CH	39.62	1.40	39.4	1.05
21	CH <sub>2</sub>	30.06	1.51	31.1	1.40
22	CH <sub>2</sub>	37.15	-	37.15	1.97
23	CH <sub>3</sub>	28.75	0.99s	28.8	1.24s
24	CH <sub>3</sub>	16.58	0.99 s	16.5	1.02s
25	CH <sub>3</sub>	16.36	0.96 s	15.7	0.92s
26	CH <sub>3</sub>	17.51	0.93 s	17.5	1.06s
27	CH <sub>3</sub>	25.96	1.28 s	24.0	1.24s
28	C	173.69	-	179.7	-
29	CH <sub>3</sub>	16.78	1.03 d	17.5	1.02d
30	CH <sub>3</sub>	21.28	0.98 d	21.4	0.97d

250MHz <sup>1</sup>H, 100MHz <sup>13</sup>C CD<sub>3</sub>OD; Ref Cpd (Seebacher *et al.*, 2003)

### APPENDIX III

<sup>1</sup>H and <sup>13</sup>C NMR Chemical shifts of **COMPOUNDIII** compared with the reference compound

Position of C atom	DEPT	COMPOUND III	COMPOUND III	Corosolic acid
		$\delta_C$ (ppm)	$\delta_H$ (ppm)	$\delta_C$ (ppm)
1	CH <sub>2</sub>	46.79	1.02	48.4
2	CH	64.41	3.47	68.6
3	CH	80.62	3.65	83.8
4	C	39.81	-	42.2
5	CH	55.26	0.88	55.9
6	CH <sub>2</sub>	18.26	1.39	18.9
7	CH <sub>2</sub>	34.87	1.55	33.2
8	C	40.84	-	40.4
9	CH	47.56	1.87	48.1
10	C	38.258	-	38.5
11	CH <sub>2</sub>	23.607	1.42	23.9
12	CH	121.65	5.43	122.5
13	C	145.00	-	144.9
14	C	41.725	-	40.1
15	CH <sub>2</sub>	29.175	1.39	28.3
16	CH <sub>2</sub>	22.687	2.27	23.7
17	C	47.555	-	46.6
18	CH	55.26	2.30	55.9
19	CH	32.79	1.68	39.5
20	CH	47.24	-	42.0
21	CH <sub>2</sub>	29.69	1.49	30.0
22	CH <sub>2</sub>	34.43	1.56	34.2
23	CH <sub>3</sub>	28.39	1.02s	29.3
24	CH <sub>3</sub>	16.81	1.00s	16.8
25	CH <sub>3</sub>	16.992	1.00s	17.5
26	CH <sub>3</sub>	19.026	1.02s	17.7
27	CH <sub>3</sub>	23.792	1.03s	23.8
28	C	173.749	-	180.2
29	CH <sub>3</sub>	16.772	0.97d	16.9
30	CH <sub>3</sub>	24.792	0.99d	23.8

250MHz <sup>1</sup>H, 100MHz <sup>13</sup>C CDCl<sub>3</sub> of COMPOUND III

\*(Lee and Juang, 2005)

#### APPENDIX IV

The % Inhibition of A-Amylase by COMPOUND I compared with the Standard Drug (Acarbose) At Varying Concentrations

CONC ( $\mu$ /mL)	%INHIBITION BY COMPOUND I	IC <sub>50</sub> OF COMPOUND I	%INHIBITION BY ACARBOSE	IC <sub>50</sub> OF ACARBOSE
20	46.84	25.45 $\pm$ 0.450	31.50	46.31 $\pm$ 0.580
40	57.61		43.84	
20	71.92		62.94	
80	73.40		68.84	
100	85.50		74.42	

#### APPENDIX V

The % Inhibition of  $\alpha$ -Glucosidase by COMPOUND I Compared With The Standard Drug (Acarbose) At Varying Concentrations

CONC ( $\mu$ g/mL)	%INHIBITION BY COMPOUND I	IC <sub>50</sub> OF COMPOUND I	%INHIBITION BY ACARBOSE	IC <sub>50</sub> OF ACARBOSE
20	34.06	25.45 $\pm$ 0.450	32.50	48.10 $\pm$ 0.130
40	45.20		43.70	
20	62.85		59.40	
80	65.63		66.60	
100	72.14		70.60	

## APPENDIX VI

The % Inhibition of  $\alpha$ -Glucosidase By COMPOUND I At Varying Concentrations

CONCENTRATION	1 <sup>st</sup> Test	2 <sup>nd</sup> Test	3 <sup>rd</sup> Test	MEAN $\pm$ SD	INHIBITION	%INHIBITION
20 ug/mL	1.312	1.301	1.221	1.278 $\pm$ 0.041	0.110	34.06
40 ug/mL	1.019	0.968	1.070	1.019 $\pm$ 0.042	0.146	45.20
60 ug/mL	0.795	0.703	0.527	0.675 $\pm$ 0.111	0.203	62.85
80 ug/mL	0.440	0.677	0.803	0.640 $\pm$ 0.150	0.215	65.63
100 ug/mL	0.359	0.285	0.403	0.349 $\pm$ 0.049	0.233	72.14

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ABSORBANCE OF CONTROL =2.404

## APPENDIX VII

The % Inhibition of  $\alpha$ -Glucosidase by Acabose at varying Concentrations

CONC	1 <sup>st</sup> Test	2 <sup>nd</sup> Test	3 <sup>rd</sup> Test	MEAN $\pm$ SD	INHIBITION	%INHIBITION
20ug/mL	0.120	0.299	0.235	0.218 $\pm$ 0.091	0.105	32.50
40ug/mL	0.207	0.189	0.150	0.182 $\pm$ 0.025	0.141	43.70
60ug/mL	0.112	0.135	0.146	0.131 $\pm$ 0.017	0.192	59.40
80ug/mL	0.118	0.097	0.109	0.108 $\pm$ 0.011	0.215	66.60
100ug/mL	0.120	0.094	0.071	0.095 $\pm$ 0.025	0.228	70.60

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ABSORBANCE OF CONTROL =2.404

### APPENDIX VIII

The % Inhibition of A-Amylase by COMPOUND I at varying Concentrations

CONCENTRATION	1 <sup>st</sup> Test	2 <sup>nd</sup> Test	3 <sup>rd</sup> Test	MEAN $\pm$ SD	INHIBITION	%INHIBITION
20ug/mL	1.312	1.301	1.221	1.278 $\pm$ 0.050	1.126	46.84
40ug/mL	1.019	0.968	1.07	1.019 $\pm$ 0.051	1.385	57.61
60ug/mL	0.795	0.703	0.527	0.675 $\pm$ 0.14	1.729	71.92
80ug/mL	0.44	0.677	0.803	0.640 $\pm$ 0.18	1.764	73.40
100ug/mL	0.359	0.285	0.403	0.349 $\pm$ 0.06	2.055	85.50

ABSORBANCE OF CONTROL = 0.323

### APPENDIX IX

The % Inhibition of A-Amylase by Acarbose at varying Concentrations

CONC	1 <sup>st</sup> Test	2 <sup>nd</sup> Test	3 <sup>rd</sup> Test	MEAN $\pm$ SD	INHIBITION	%INHIBITION
20ug/mL	1.785	1.584	1.572	1.647 $\pm$ 0.12	0.757	31.49
40ug/mL	1.271	1.302	1.477	1.35 $\pm$ 0.11	1.054	43.84
60ug/mL	0.88	0.844	0.949	0.891 $\pm$ 0.05	1.513	62.94
80ug/mL	0.615	0.801	0.831	0.749 $\pm$ 0.12	1.655	68.84
100ug/mL	0.599	0.608	0.638	0.6150 $\pm$ 0.12	1.789	74.42

ABSORBANCE OF CONTROL = 0.323

## APPENDIX X

The Inhibition of A-Amylase by crude MEOH extracts At Varying Concentrations

CONC (mg/mL)	RAW VALUES OF ABSORBANCE			MEAN ± SD	SD	INHIBITION	%INHIBI
	1 <sup>ST</sup> TEST	2 <sup>ND</sup> TEST	3 <sup>RD</sup> TEST				
20	2.41	1.30	1.31	1.67	0.6	0.73	30.35
40	1.02	2.40	1.09	1.50	0.8	0.90	37.48
60	2.30	0.99	1.03	1.44	0.7	0.97	40.21
80	2.40	0.82	0.80	1.34	0.9	1.06	44.29
100	0.93	1.00	1.54	1.16	0.3	1.25	51.89

## APPENDIX XI

Inhibition Of  $\alpha$ -Amylase by BUOH fraction at Varying Concentrations

RAW VALUES OF ABSORBANCE			MEAN ± SD	SD	INHIBITION	%INHIBI
1 <sup>ST</sup> TEST	2 <sup>ND</sup> TEST	3 <sup>RD</sup> TEST				
1.41	2.30	1.32	1.68	0.5	0.73	30.20
2.02	1.67	0.99	1.56	0.5	0.85	35.15
1.90	1.10	1.13	1.38	0.5	1.03	42.80
1.44	1.28	1.10	1.27	0.2	1.13	47.06
1.13	1.10	1.23	1.15	0.1	1.25	52.01

Absorbance (Control) = 2.404

## APPENDIX XII

Inhibition of Alpha Glucosidase by BUOH fraction at varying concentrations

Conc. (mg/mL)	Raw values of absorbance			MEAN $\pm$ SD	INHIBITION	%INHIBITION
	1	2	3			
20	0.123	0.29	0.262	0.23 $\pm$ 0.089	0.098	30.34
40	0.211	0.208	0.142	0.19 $\pm$ 0.039	0.136	42.11
60	0.151	0.122	0.25	0.17 $\pm$ 0.067	0.14867	46.03
80	0.173	0.165	0.127	0.16 $\pm$ 0.025	0.168	52.01
100	0.158	0.136	0.131	0.14 $\pm$ 0.014	0.18133	56.14

Control = 0.323

## APPENDIX XIII

Inhibition of Alpha Glucosidase by MEOH extract at varying concentrations

Conc. (mg/mL)	Raw values of absorbance			MEAN $\pm$ SD	INHIBITION	%INHIBITION
	1	2	3			
20	0.163	0.245	0.239	0.22 $\pm$ 0.046	0.11	33.23
40	0.201	0.234	0.183	0.21 $\pm$ 0.026	0.12	36.22
60	0.181	0.132	0.238	0.18 $\pm$ 0.053	0.14	43.14
80	0.123	0.270	0.139	0.18 $\pm$ 0.081	0.15	45.10
100	0.198	0.156	0.137	0.16 $\pm$ 0.031	0.16	49.33

Control = 0.323

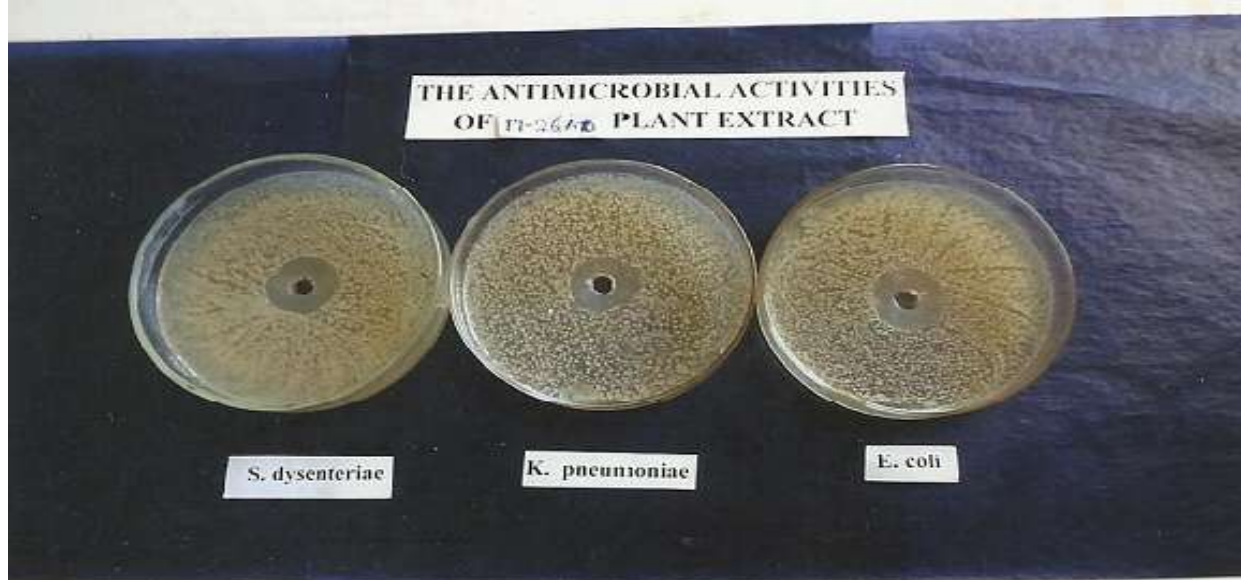
#### APPENDIX XIV

Inhibition of *S. aureus*, MRSA, *S. pyogenes* and *B. cereus* by COMPOUND I



#### APPENDIX XV

Picture of inhibition of *S. dysenteriae*, *K. pneumoniae* and *E. coli* by COMPOUND I



## APPENDIX XVI

Picture of inhibition of *E. Coli*, *P. Vulgaris*, *C. albicans* and *C. stellatoidea* by BUOH



## APPENDIX XVII

Inhibition of *S. aureus*, MRSA, *S. pyogenes* and *B. cereus* by BUOH



### APPENDIX XVIII

Inhibition of *S. aureus*, MRSA, *S. pyogenes* and *B. cereus* by MEOH extract of leaf of *Aspilia africana*



### APPENDIX XIX

Inhibition of monocytogenes, *P. fluorescense*, *S. typhi* and *S. dysenteriae* by MEOH extract of leaf of *Aspilia africana*

