ISOLATION, CHARACTERISATION AND ANTIMICROBIAL ACTIVITY OF BIOACTIVE CONSTITUENTS FROM THE LEAF EXTRACT OF *Adenodolichos paniculatus* (Hua) Hutch. & Dalz (FABACEAE)

BY

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OCTOBER, 2017

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE IN ORGANIC CHEMISTRY

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ZARIA, NIGERIA

OCTOBER, 2017

DECLARATION

I declare that the work in this dissertation entitled **"ISOLATION, CHARACTERISATION AND ANTIMICROBIAL ACTIVITY OF BIOACTIVE CONSTITUENTS FROM LEAF EXTRACT OF *Adenodolichos paniculatus* (FABACEAE)"** has been carried out by me in the Department of Chemistry under the supervision of Dr. I. A. Bello and Prof. G. I. Ndukwe. The information derived from the Literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any other University.

Ibrahim Isyaku	Signature	Date

CERTIFICATION

This dissertation entitled "**ISOLATION, CHARACTERISATION AND ANTIMICROBIAL ACTIVITY OF BIOACTIVE CONSTITUENTS FROM LEAF EXTRACT OF *Adenodolichos paniculatus* (FABACEAE)"** by Ibrahim Isyaku meets the regulations governing the award of the degree of Master of Science in Organic Chemistry of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

_____ _____

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Dean, School of Postgraduate Studies

DEDICATION

This research work is dedicated first to **GOD** Almighty for the opportunity He has given me again to achieve this level of education and to my daughter Fatima Ibrahim Isyaku

ACKNOWLEDGEMENT

I give special thanks and glory to GOD almighty for giving me the grace, wisdom and good health to complete this research work. My sincere gratitude to my supervisors Dr. I. A. Bello and Prof. G. I. Ndukwe for their commitment, constructive, intellectual advice, academic discussions and corrections, to make this work worthy despite their busy schedules. Dr. J. D. Habila, Mr. John Anyam, Mr. Tajudin Nasiru, Mr. Jonathan for their advice, professional and academic input to put the work in shape, your kind gesture is highly appreciated.

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ABSTRACT

The plant *Adenodolichos paniculatus* which is used in ethno - medicine for the treatment of blennorrhoea, diarrhea, dysentery, small pox, heart burn and wound dressing was investigated for its chemical constituents and antimicrobial activity. The leaves of this plant were subjected to microwave assisted extraction to obtain *n* – hexane, chloroform, ethyl acetate and methanol extracts. The extracts were then subjected to preliminary phytochemical screening which revealed the presence of alkaloids, anthraquinones, flavonoids, reducing sugars, saponins, tannins and steroids/terpenes. Purification through various column chromatography, the ethyl acetate extract led to the isolation of compounds X1 (mixture of stigmasterol and β – sitosterol) and X2 (nonanoic acid) after spectral analysis IR, ^{1}H NMR and ^{13}C NMR. Determination of inhibitory activity (sensitivity test) using agar well diffusion method showed activity i.e. minimum inhibitory concentration (MIC) against *Staphylococcus aureus, Salmonella typhi,* and *Candida albicans* ranging from 12 – 50 mg/ml, Minimum Bactericidal Concentration/ Minimum Fungicidal Concentration (MBC/MFC) ranging from 25 – 50 mg/ml, which is found to be bacteriostatic against *Salmonella typhi*. *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Bacillus subtili* showed no MBC (Bacteriostatic) for the methanol, ethyl acetate, chloroform and *n* –hexane extract.

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ABREVIATIONS

CNMR	Carbon Nuclear Magnetic Resonance
HNMR	Proton Nuclear Magnetic Resonance
IR	Infra-Red
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
NMR	Nuclear Magnetic Resonance
℃	Degrees Celsius
R_f	Retardation Factor
TLC	Thin Layer Chromatography
WHO	World Health Organization
EA	Ethyl acetate
CHL	Chloroform
HEX	Hexane
ME	Methanol

CHAPTER ONE

1.0 INTRODUCTION

There are a large number of medicinal plants whose scientific importance has not been explored. All over the world, plants have served as the richest source of raw materials for traditional as well as modern medicine, particularly in Africa and Asia (Tsakala *et al*., 2006). Medicinal plants contain some compounds which have definite physiological actions on animals and humans. These are called secondary metabolite, and include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids (Doge *et al.,* 2005). Medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents (Ododo *et al.,* 2016). Phytochemicals are naturally occurring in medicinal plants leaves, stem bark, fruits and roots where they serve as defense mechanism and protect from various diseases (Ahmad *et al.,* 2006). Traditional medicine by means of plant extracts continues to provide health treatment for over 80% of the world's population, especially in the developing world (WHO, 2002). The rapid increase of bacterial drug resistance is becoming a worldwide problem. There is an urgent need to develop new antimicrobial drugs with better/improved activity in order to overcome bacterial drug resistance (Afolayan *et al.,* 2008). The continual search for, and the interest in natural plant products, for use as medicines has acted as the catalyst for exploring methodologies involved in obtaining the required plant materials and hence searching into their constituents for possible medicinal usage (Ibrahim *et al*., 2013). The identification and isolation of such active compounds makes it a more effective therapeutic application. It prevents consumers from taking certain plants that have no medicinal value or are poisonous to them (Ozolua *et al.,* 2009). The use of plants remains undisputed

despite the advent of the Pharmaceutical Chemistry during the early twentieth century, which brought with it the ability to synthesize an enormous variety of drug molecules and there by allowed the treatment of previously incurable and/or life-threatening diseases (Dutta, 2013). Although chemically synthesized drug have also gained popularity and became the basis of Pharmaceutical industries. Over the years, however, synthetic drugs have been hijacked by unwanted side-effects, toxicity, high cost and inefficiency, among other problems. Also the search for new drugs against a variety of illnesses through chemical synthesis and other modern approaches has not been too successful. These factors, as well as the emergence of new infectious diseases, the proliferation of disorders such as cancer and growing multidrug resistance in pathogenic microorganisms, have prompted renewed interest in the research and discovery of potential drug molecules from medicinal plants (Arjun Patra, *et. al*, 2010).

1.2 Statement of the Problem

The rapid increase of bacterial drug resistance is becoming a worldwide problem. There is an urgent need to develop new antimicrobial drugs with better/improved activity in order to overcome bacterial drug resistance (Afolayan *et al.*, 2008). There are a large number of medicinal plants whose scientific importance has not been explored. All over the world, plants have served as the richest source of raw materials for traditional as well as modern medicine, particularly in Africa and Asia (Tsakala *et al*., 2006).

1.3 Research Justification

This work was conducted because of the need to search for newer and more potent antimicrobial agents to whom there is lesser resistance in a world where pathogenic microorganisms are building and increasing resistance to drugs. The need to scientifically

establish or otherwise comfirm the ethnomedicinal claims on the plant. The World Health Organization (WHO, 2005) estimates that 4 billion people, or 80 percent of the world's population, use herbal medicine for some aspect of primary health care. It is against this background that, *Adenodolichos paniculatus* which is a plant used extensively as herbal remedy in some parts of Nigeria and West Africa, is being investigated. To the best of our knowledge, there is no reported work on the isolation and characterization of some bioactive compounds from the plant.

1.4 Aim and Objectives of the Research

1.4.1 Aim

The aim of this project work is to isolate and characterize some bioactive compounds from the leaves extract of *Adenodolichos paniculatus* and to evaluate their biological activity

1.4.2 Objectives of the research

This aim will be achieved through the following objectives:

i. Phytochemical and antimicrobial screening of the crude extracts.
ii. Separation, purification and isolation of the bioactive constituents using chromatographic techniques (TLC, column chromatography)
iii. Spectroscopic analysis (NMR FTIR)
iv. Characterization and structural elucidation of the isolated compound(s) using spectroscopic techniques.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Family Fabaceae

The family fabaceae are one of the large plant family in the dicotyledons of about 16400 species in 657 genera. They are of cosmopolitan distribution, growing in every soil (clay, sandy and loamy) and climate. The family is divided into mimosoideae and caesalpiniodeae which are mostly tropical. And lotoideae (papiliniodeae) which are both tropical and temperate (Harbone, 2009; Burkill, 1985a)

2.1.1 The Genus *adenodolichos*

Adenodolichos is a genus of flowering plants in the legume family, Fabaceae. It belongs to the subfamily Faboideae. It grows seasonally in to tropical woodland, shrub, often appearing soon after bush burning operation. (Hutchison and Dalziel, 1958; Burkill 1985b)



Plate I: The plant *Adenodolichos paniculatus*

Botanical classification.

The botanical classification of *Adenodolichos paniculatus* is as follows:

- Kingdom: Plantae
- Phylum: Magnoliophyta
- Class: Magnoliopsida
- Order: Fabales
- Family: Fabaceae
- Genus: Adenodolichos
- *S*pecies: *Adenodolichos paniculatus* (Hutchison and Dalziel, 1958; Burkill, 1985b; Sethpakdee, 1992).

2.2. Common Names

The plant *Adenodolichos paniculatus* is known by different names in different part of Nigeria, the Birom people call it kpàrák, Fulfulde usually call it kilikainawa and Hausa people call it kwaiwa, kwííwáá, kwííyáá, waken wuta (Burkill, 1985a)

2.2.1 Uses

Different parts of the plant are used for different purposes, for example: the leaves are mostly used in dressing for burns, for toothache, and for heart burn. The root is mostly used to treat liver problem, dysentery and also used as a pain-killer, while the stem is used in the treatment of diarrhea and blennorrhoea (Burkill 1985a; Babajide and Atanda, 2008; Sani *et al*; 2010).

2.2.2 Morphology (description)

The plant *Adenodolichs paniculatus (Leguminocaea, Faba*ceae) is a shrub of 4 - 5 m high found in the savanna, bush and jungle, from Guinea to Northern Nigeria, and across to

Sudan. The Hausa name, wáákén wuta, means ‘fire bean.’ This is perhaps because the plant springs up freely after bush burning operation. The twigs are hairy, and the bark is rough. The leaves are up to 12 x 14 cm, glossy above and heavily veined and somewhat rusty haired below. It has flowers of the typical 'pea' type. These are composed of one large back petal (the standard), two side petals called wings and two lower petals fused together to form a 'keel'. Pods are indehiscent, up to 26 x 7 cm with rusty brown hairs which wears off as the pods mature, becoming somewhat concerted as they age. The pods persist on the tree but finally fall and decay on the ground to pea sized seeds. An edible pulp surrounds these seeds. The roots are one of the most easily recognizable features of this plant. It has irregular nodules on the roots which enable the plant to absorb nitrogen from the air in the soil and convert it into the nitrogen the plant needs for growth. This enables the plant to grow in poor soil. (Hutchison and Dalziel, 1958; Burkill, 1985b; Akerele *et al.*, 1991)

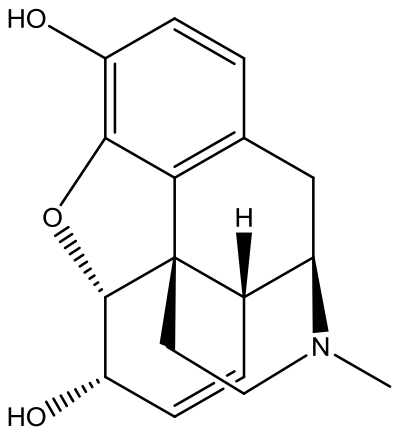
2.3 Pharmacological Products in Plants

Alkaloids, tannins, steroids, phenolic compounds, terpenoids, proteins, waxes, glycosides, carbohydrates etc. are responsible for physiological activity of plant products.

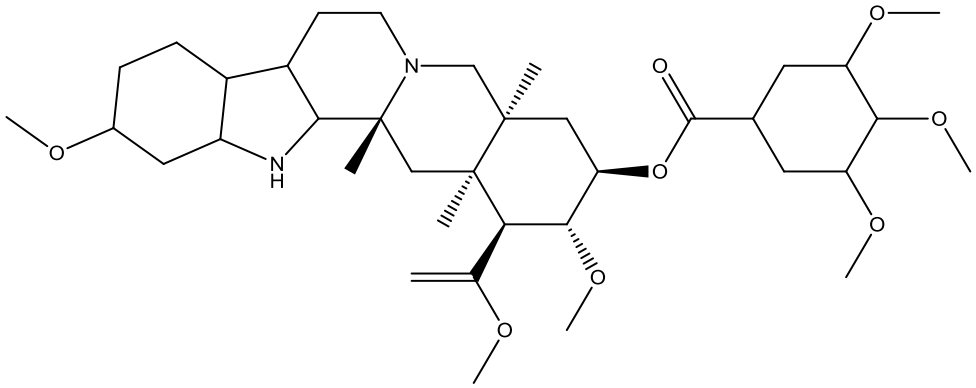
2.3.1 Alkaloids

Most alkaloids are obtained from plants. They are pharmacologically active, basic compounds derived from amino acids. They may contain one or more heterocyclic nitrogen atoms in the molecule (Cordell, 1981; Cseke *et al.*, 2006). In fact, most nitrogen-containing secondary metabolites are usually deemed alkaloids, but amines or glucosamine may be grouped differently. The word “alkaloid” originated from Arabic, *al-qali* (an early form of soda ash) (Cordell, 1981). Many alkaloids have a bitter taste, and a large number of them exhibit potent physiological effects on mammals. For example, morphine (I)

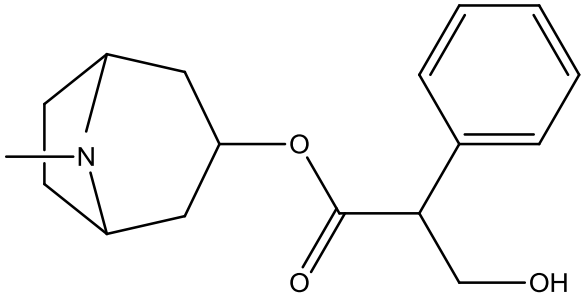
shows narcotic effects; reserpine (II) is an antihypertensive agent; atropine (III) is a smooth muscle relaxant; cocaine (IV) is a potent central nervous system stimulant; and strychnine (V) is a nerve stimulant.



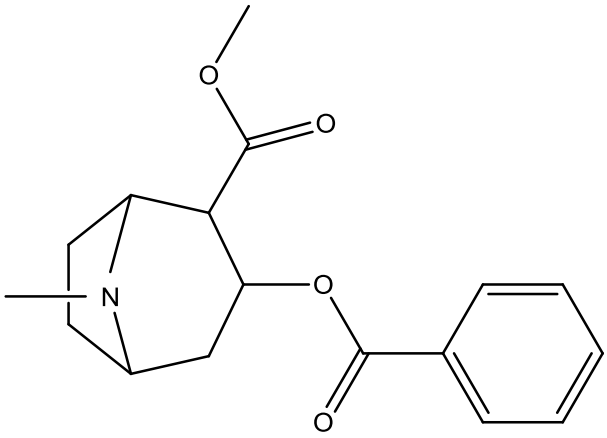
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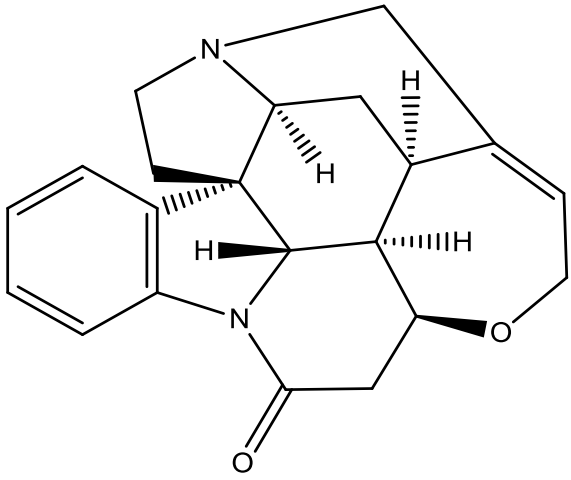
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(III)



(IV)

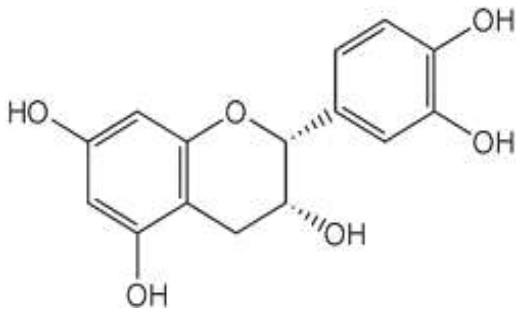


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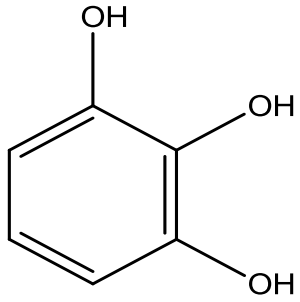
2.3.2 Tannins

Tannins are water-soluble oligomers, rich in phenolic groups, capable of binding or precipitating water soluble proteins (Hagerman and Butler, 1989). The tannins, common to vascular plants, exist primarily within woody tissues but can also be found in leaves, flowers, or seeds. Plant tissues that are high in tannin content are highly astringent and are avoided by most animals. Tannins fall into two groups: either condensed tannins,

Epicatechin (VI) or hydrolysable tannins, Pyrogallol (VII) (Notka *et al*., 2004; Cseke *et al.*, 2006).



(VI)

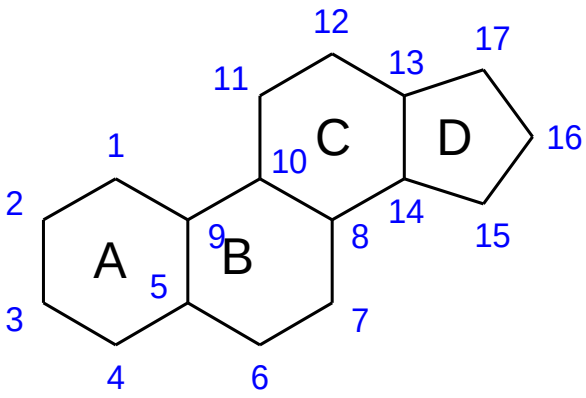


(VII)

2.3.3 Steroids

Steroids are chemical messengers. Some steroids are also hormones. All steroids are derived from the acetate biosynthetic pathway. Hundreds of distinct steroids have been identified in plants, animals and fungi, and most of them have interesting biological activities. They possess a perhydrocyclopentaphenanthrene nucleus (VIII). The four rings are lettered A, B, C and D, and the carbon atoms are numbered beginning in the A ring. These fused rings can be trans-fused or cis-fused. In steroids, the B, C and D rings are always trans-fused. In most naturally occurring steroids, rings A and B are also trans-

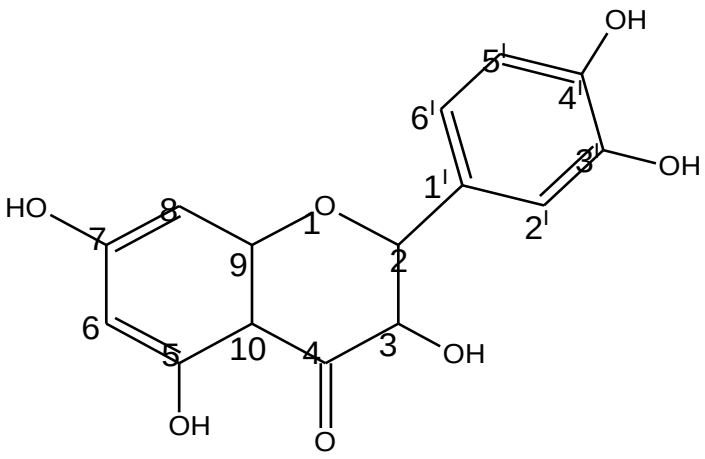
fused. Different steroids vary in the functional groups attached to these rings (Sarker and Lutfun, 2007).



(VIII)

2.3.4 Phenolic compounds

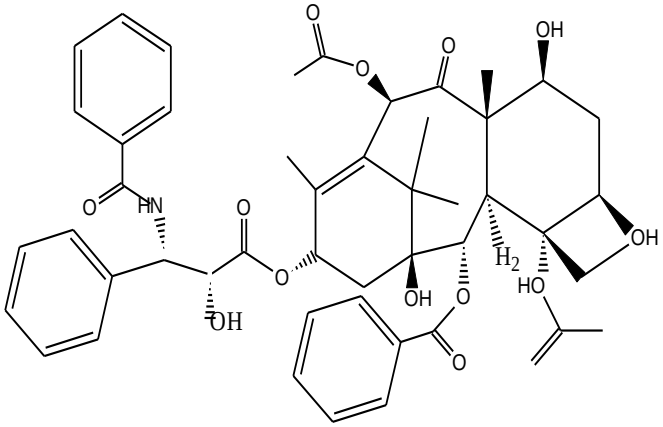
This is a large group of structurally diverse naturally occurring compounds that possess atleast a phenolic moiety in their structures. For example, quercetin (IX) is a flavonoid that has five phenolic hydroxyls at C-3, C-5, C-7, C-3' and C-4′ the phenolic group of compounds encompasses various structural types, for example: phenylpropanoids, coumarins, flavonoids and isoflavonoids, lignans, tannins, etc. (Krishnamachari *et al*., 2002; Sarker and Lutfun, 2007), Quercetin possesses various degrees of antioxidant or free radical scavenging properties (Sarker and Lutfun, 2007)



(IX)

2.3.5 Terpenoids

There are more than 23,000 known terpenoids. They are the largest class of natural products. The terpenoids find wide ranging applications in industry and beyond. For example, the antimalarial drug Artemisinin and the anticancer drug paclitaxel (Taxol®) Fig. (1.11), are two of a few terpenes with established medicinal applications (FAO, 1995). Artemisinin and its derivatives are presently the most potent drugs against *Plasmodium falciparum* (White, 1997; Vivek *et al*., 2010)



(X)

2.4 Bacterial Agents

2.4.1 *Staphylococcus*

The genus *Staphylococcus* consists of cluster-forming, gram-positive cocci. *Staphylococcus* derived from Greek *staphyle* "bunch of grapes" and *kokkos* "grain or berry" the main pathogen within the genus. *Staphylococcus aureus* is the cause of a wide range of major and minor infections in man and animals. Currently, there are some 27 different species of *Staphylococci.* These fall into two main groups on the basis of their capability to clot blood plasma by action of the enzyme coagulase. *Staphylococcus aureus* is by far the most important coagulase positive species. The coagulase-negative *Staphylococci* are skin commensals, which are now recognized as important opportunistic pathogens that can cause infections associated with prosthesis, catheters and implants *(Staphylococcus epidermidis)* and urinary tract infections *(Staphylococcus saprophyticus.).* It has not been common practice for medical microbiologists to identify coagulase-negative *Staphylococci* to the species level. However, methods are now available that facilitate identification and the 28 importance of other recently recognized coagulase-negative species is being assessed (Pfaller and Herwaldt, 1988). *Staphylococcus aureus* which is present in the nose and on the skin of a variable proportion of healthy people is an opportunistic pathogen, in that, it causes infection most commonly at sites of lowered host resistance such as damaged skin or mucous membranes or haematomas in the cancellous tissues of a long bone (Jonson *et al.*, 1990; Zakirai, 2009).

2.4.2 *Bacillus subtilis*

Bacillus subtilis belongs to the Gram-positive Firmicutes and has been investigated for more than a century. Moreover, Bacillus species have also been used in biotechnology as

industrial production strains for a long time (Torsten and Johann, 2005). *Bacillus subtilis* serves as a model organism and is considered a reference for cell differentiation and adaptation. This model status makes it one of the most extensively studied organisms in nature, with the ultimate goal of obtaining a complete understanding of physiological processes that constitute the life of a cell (Sicuia *et al.*, 2015). The *Bacillus* genus includes Gram positive, rod shaped bacterial cells of 0.3-2.2 to 1.2-7.0 cm, most of them motile, with peritrich flagella. These bacteria are chemoheterotrophes that can use various nutritional substrates.

2.4.3 *Escherichia coli*

E. coli are a large and diverse group of bacteria which is a Gram-negative (bacteria which do not retain crystal violet dye). Although most strains of *E. coli* are harmless, others can make people sick (James and Kaper, 1998). Some kinds of *E. coli* cause disease by making a toxin called Shiga toxin. *Escherichia coli* is the predominant facultative anaerobe of the human colonic flora. The organism typically colonizes the infant gastrointestinal tract within hours of life, and, thereafter, *E. coli* and the host derive mutual benefit. *E. coli* usually remains harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause infection. Moreover, even the most robust members of our species may be susceptible to infection by one of several highly adapted *E. coli* clones which together have evolved the ability to cause a broad spectrum of human diseases (James and Kaper, 1998). Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the body. Three general clinical

syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/ diarrheal disease.

2.4.4 Salmonella

Salmonellae are "Gram – Negative" widely distributed in nature. All vertebrates appear capable of harbouring *Salmonellae* in their gut. It has also been isolated in a wide range of arthropods such as flies, cockroaches, fleas, and ticks. *Salmonella typhi* and *Salmonella paratyphi* A, B and C are primary human pathogens which are rarely isolated from other animals. *Salmonellae paratyphi*, while essentially a human pathogen, is occasionally; isolated from cattle, pigs, poultry, and other animals. The cycle of transmission in these hosts has not been fully demonstrated (Christie, 1987). The ability of Salmonellae to enter and survive within macrophages and other cells particularly in the liver and kidney occasionally leads to persistent infection and the chronic carrier state where sufferers from Salmonella infection continue to excrete the organism in their stools for days or weeks after complete clinical recovery. In the treatment and clinical management of enteric fever due to Salmonellae the problem of bone marrow toxicity and widespread emergence of chloramphenicol resistance in *Salmonella typhi* in many parts of the world prompted the search for alternative drugs and among these are ampicillin and cotrimoxazole and they have been shown to be comparable in efficacy to chloramphenicol (Levine *et al.*, 1989).

2.4.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an aerobic Gram-negative bacillus considered to be an opportunistic pathogen. It is a highly versatile microorganism able to tolerate low oxygen conditions. It can survive with low levels of nutrients and grow in temperatures ranging from 4-42°C (Jonathan and Jiyoung, 2011). These characteristics allow it to attach itself

and survive on medical equipment and on other hospital surfaces, which favors the beginning of infections in immunocompromised patients *P.aeruginosa* is often preliminarily identified by its pearlescent appearance and grape-like odor *in vitro*. Definitive clinical identification of *P. aeruginosa* often includes identifying the production of both pyocyanin and fluorescein as well as its ability to grow at 42°C, In recent years a worldwide emergence of multidrug-resistant strains of *Pseudomonas aeruginosa* has been observed (Sara *et al*., 2013a). This opportunistic pathogen produces mechanisms of resistance to several antibiotics. The resistance to carbapenems in *P. aeruginosa* strains has been associated with bacterial biofilm formation favored by the presence of exopolysaccharides (EPS) embedded in an extracellular matrix and to the production of type IV pili (T4P) (Sara *et al*., 2013b).

2.4.6 *Fungi*

Fungi constitute a large diverse group of heterotrophic organisms which exist as saprophytes, parasites or commensals. Most are found as saprophytes in the soil and on decaying plant material. About 180 of the 250,000 known fungi species are recognized as capable of causing diseases (mycosis) in man and animals (Rippo, 1988). Most of these are mould fungi but there are number of pathogenic yeast fungi and many are dimorphic. Dimorphic fungi usually assume the mould form when growing as saprophytes in nature and the yeast form when causing infection. In the laboratory, the yeast form can be induced by culture at 37℃ on rich media such as blood agar, whereas the mould form develops when incubated at a lower temperature (22-27℃) on a less rich medium such as Sabouraud's agar (Rippo, 1988). Yeast infections affecting skin and nails and mucous membranes of the mouth and vagina, are usually caused by *Candida albicans* which are

found as commensals of man. Yeast infections are generally endogenous in origin but can be transmitted sexually. There are relatively few therapeutically useful antifungal agents that are available. This is due mainly to the fact that fungi and man are both eukaryotes and most substances that kill or inhibit fungal pathogens are also toxic to the host. Most superficial Candida infections respond well to topical therapy with nystatin, amphotericin B, or azoles. Superficial Candida infections occasionally occur on the penis after intercourse with females with vaginal thrush. The yeast may also infect the outer ear (Roberts *et al.,* 1984), in oral candidosis, nystatin, ampholericin *B* and miconazole may be effective in lozenge or gel form. Most cases of vaginal candidosis can be treated successfully with a single application of an azole derivative or oral therapy with fluconazole or itraconazole (Odds, 1988; Zakirai, 2009).

2.5 Infectious Diseases

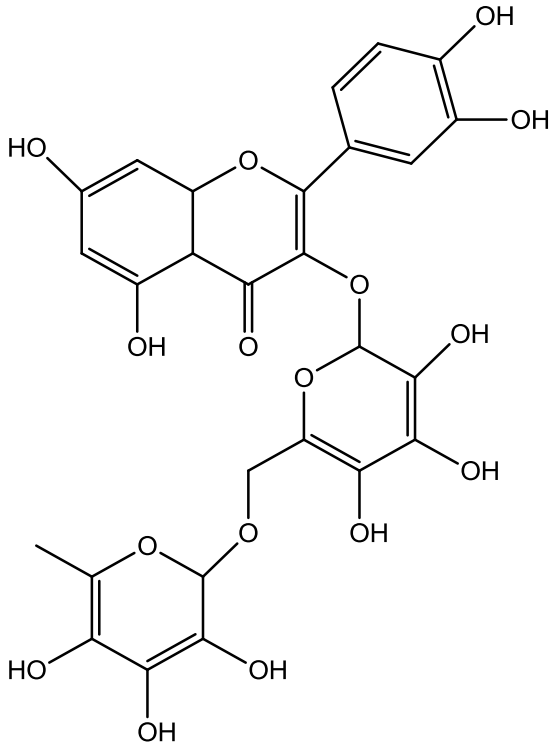
Infectious diseases account for approximately one-half of all deaths in tropical countries (Iwu *et al*., 1999). The World Health Organization estimates that nearly 50,000 people die each day throughout the world from infectious diseases (WHO, 2009). Several antibiotics exhibit inhibitory activity on different pathogenic organisms. However, the development and spread of resistance to currently available antibiotics is a worldwide concern. Antibiotics are defined as natural compounds, produced by microorganisms, with selective antibacterial activity that does not have any strong side effects on human (Chanda and Rakholiya, 2011). Their mechanism of action is either through killing the bacteria (bactericidal effect) or by inhibiting bacterial growth (bacteriostatic effect). The discovery of antibiotics had eradicated the infections that once ravaged humankind. But their indiscriminate use has led to the development of multidrug-resistant pathogens. For

example, around 90–95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin (Casal *et al*., 2005) and in most of the Asian countries 70–80% of the same strains are methicillin resistant (Chambers, 2001). An alternative therapy to treat antibiotic resistant microorganisms is the use of plant extracts. There are several reports on the antimicrobial activity of different plant extracts that were effective antimicrobics (Vaghasiya *et al*., 2008; Fisgin *et al*., 2009; Jeong *et al*., 2009; Darwish and Aburjai, 2010; Yoon *et al*., 2011). However, the problem of drug resistance is on the increase. There is an urgent need to develop still newer, useful and important antimicrobial agents (Sharma and Kumar, 2006; Negi and Dave, 2010); or new ways to treat the resistant microorganisms. An alternative approach is the use of bioactive plant extracts.

2.6 Phytochemistry of *Adenodolichos paniculatus* and other species in the genus.

Some phytochemical investigations that have been reported on *Adenodolichos paniculatus* showed that the plant contains a wide range of compounds, ranging from: alkaloids, anthraquinones, carbohydrates, flavonoids, glycosides, tannins, saponins and phenolic compounds (Sani *et al*., 2010). There was no report on isolation of any compound from any of the specie in the genus Adenodolichos but several reports indicated that there were so many isolation from the extracts of some genus from the fabaceae family. For examples, phytochemical investigation of the genus Astragalus, showed the presence of saponins, flavonoids and also neoeriocitrin (XI) was isolated (Xiaoxia *et al*., 2014). Ana *et al*. (2014), reported the characterization of aliphatic esters using mass spectrometry, infrared and NMR spectroscopy, from *Senna villosa*, among the compounds isolated was 2-(heptyloxy) pentadec-1-ene (XII). Flavones, flavonols, flavans, isoflavons, chalcons, triterpenoid, and sesquiterpens were also found to be present in the genus Tephrosia. Some

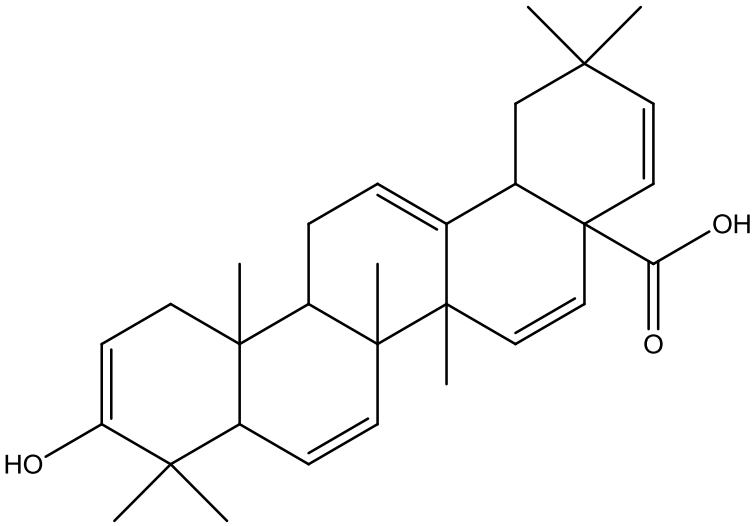
compounds were also isolated from the genus, one of which is morolic acid (XIII) (Yinning *et al.,* 2014). Lin and Mingyu, (2013), reported the isolation of two new aromatic compounds from the genus Entada; butyl- 2, 5-dihydroxyphenyl acetate (XIV) and butyl-2-O-*β*-D-glucopyranosyloxy-5-dihydroxyphenyl acetate, together with three known ones, methyl-2,5-dihydroxyphenyl acetate, ethyl-2,5-dihydroxyphenyl acetate and 2-O-*β*-D-glucopyranosyloxy-5-hydroxyphenyl acetic acid. Neoeriocitrin (Fig. 2.1), morolic acid (Fig. 2.3) and butyl - 2,5-dihydroxyphenyl acetate (Fig. 2.4) show a potent inhibitory activity against HIV-1 replication.



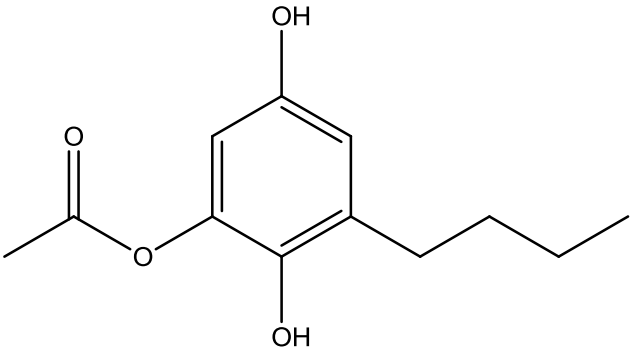
(XI)



(XIV)



(XV)



(XVI)

2.7. Biological Activity of *Adenodolichos paniculatus* and other species in the genus

Adenodolichos paniculatus possesses analgesic and anti-inflammatory activity (Sani *et al*., 2010). Also some genus from the family have anti-inflammatory, immunostimulant, anti-oxidative, antidiabetic, cardio protective, antimicrobial, antitumor, antiprotozoan, and also show a potent inhibitory activities against HIV-1 replication (Lin and Mingyu, 2013; Ana *et al*., 2014; Fabian *et al*., 2014; Xiaoxia *et al*., 2014; Yinning *et al.*, 2014).

2.8. Microwave-assisted Extraction (MAE)

Ganzler *et al*. (1990) showed that Microwave-assisted extraction for biologically active compounds has so many advantages over other conventional extraction methods. Microwave-assisted extraction method required shorter time, less solvent, provided higher extraction rates and lower costs. Numerous biologically active compounds have been extracted by application of microwave-assisted extraction (Monica *et al*., 2010). Tomaniova *et al.* (1998) have shown that MAE was more efficient than conventional extraction methods in the extraction of tea polyphenols and tea caffeine. As already mentioned, MAE is increasingly employed in the extraction of natural products as a substitute to common techniques of extraction for a number of reasons: reduced extraction time, reduced solvent consumption, environmentally friendly process as a result of increased efficiency and clean transfer of energy to the medium; improved extraction yield and product quality, because materials can be rapidly heated, and often processed at lower temperatures; up to 70% energy saving compared to conventional energy forms from the high energy densities (Afoakwah *et al.*, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals/Reagents

The chemicals and the reagents used were of high purity and analytical grade; the solvents were distilled before used. Silica gel $60F_{254}$ was used throughout for thin-layer chromatography.

3.1.2 Equipment

I .Kenwood K25MSS11 microwave oven

II. FTIR spectroscopy (Agilent Technologies, Carry 630 Fourier Transformed Infrared spectrophotometer).

III. NMR spectrophotometer gilent-NMR-vnmrs400 was used for the characterization,

IV. Stuart SMP40 digital melting point apparatus.

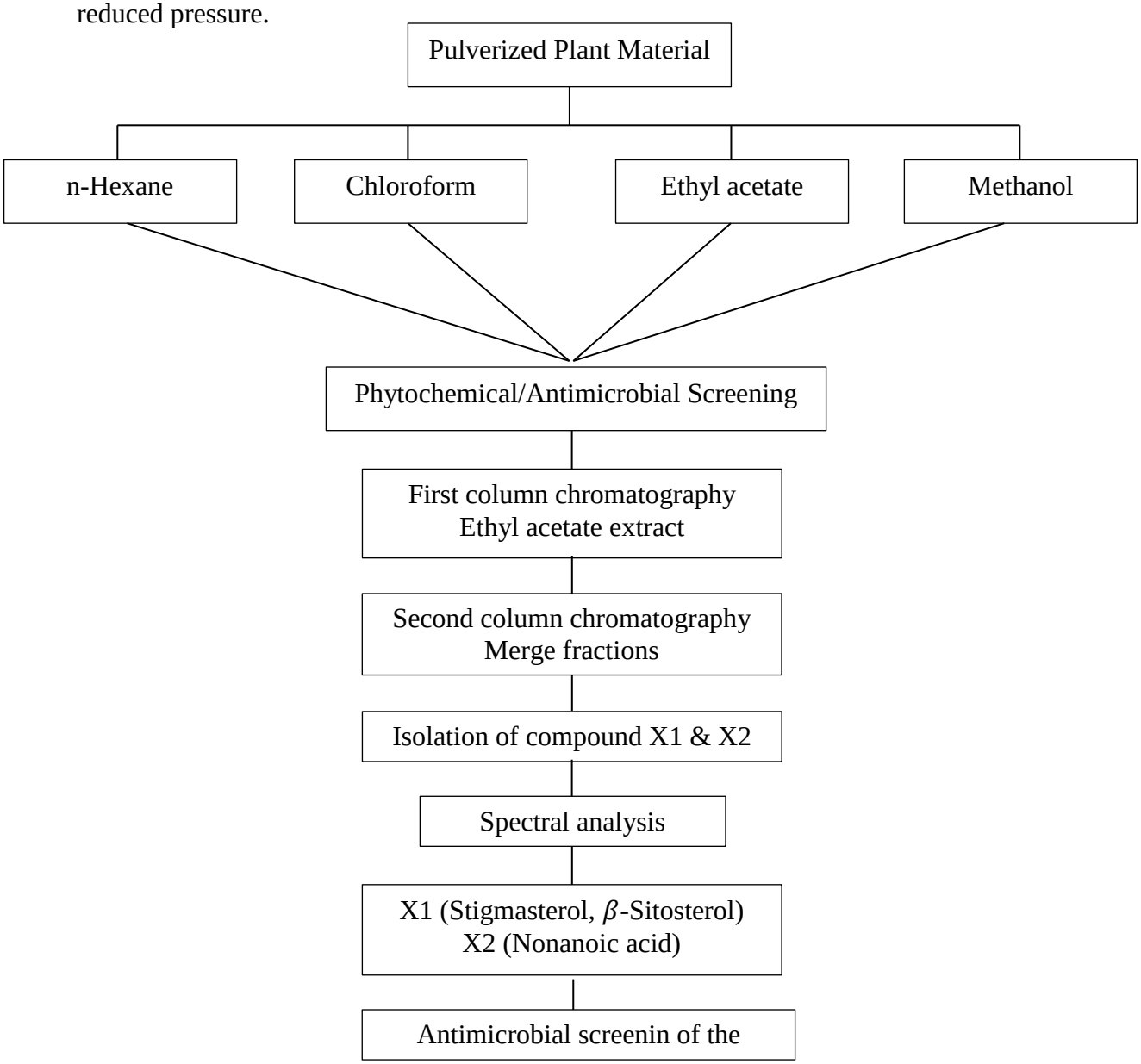
3.2 Methods

3.2.1 Plant collection

The leaves of *Adenodolichos paniculatus* were collected from Benue state, Nigeria, on 21st May, 2015. It was identified and authenticated at the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University, Zaria. as specimen with voucher number 3107. The leaves were air dried, powdered, and stored in air tight containers for laboratory analyses.

3.2.2 Extraction

The plant material (1kg) was placed into jam jars up to three-quarter full; n-hexane was added to just cover the plant material. Afterward they were placed in a microwave under low heat and microwaved at 3 minutes pulses, removed and allowed to cool. The procedure was repeated 5 times. The microwaved plant material was then extracted exhaustively using n-hexane, chloroform, ethyl acetate and methanol in increasing order of polarity, to give four extracts. A rotary evaporator was used to recover the respective solvents at reduced pressure.



3.3 Phytochemical Screening

Chemical tests were carried out on the plant extracts to identify the secondary metabolites present in the plant, using standard procedures as described by Sofowora (1982); Sofowora (1993a); Sofowora (1993b); Edeoga *et al*., (2005) and Harborne (2009).

3.3.1 Test for reducing sugar

Molisch's test (General test for Reducing Sugar): To the aqueous extract (2 ml) in a test tube was added three drops of Molisch's reagent followed by three drops of concentrated sulphuric acid by the side of the test tube, and then allowed to form a lower layer. A purple to violet coloured interface indicated the presence of Reducing Sugar. The mixture, was shaken gently and allowed to stand for two minutes, followed by dilution with water (5 ml) a dull violet precipitate indicated the presence of Reducing Sugar (Trease and Evans, 1972; Trease and Evans, 1989; Trease and Evans, 2002).

3.3.2 Test for anthraquinone glycosides.

To the extract (0.2g) benzene (10 ml) was added and shaken for 5 minutes. 10% ammonia solution (5 ml) was added and shaken. The presence of a pink, red or violet colour in the ammoniacal (lower layer) phase indicated the presence of free anthraquinones. For the anthraquinone glycosides, the extract (0.2 g) was boiled with dilute sulphuric acid (10 ml) and filtered while hot. The filtrate was shaken with benzene (5 ml). The benzene layer was separated, and 10% ammonia solution (3 ml) was added, and the mixture shaken. The layers were allowed to separate. A pink, red or violet colouration in the lower layer showed the presence of anthraquinone glycosides (Sofowora, 1993a).

3.3.3 Test for saponin glycosides

Frothing test: The extract (0.2 g) was shaken with water (5 ml) in a test tube for 30 seconds. Persistent foam for 15 minutes or when warmed on water bath indicated the presence of saponins (Sofowora, 1993b).

3.3.4 Test for steroids and terpenoids

a) *Salkowski's test for steroids:* The extract (0.5 g) was dissolved in chloroform and 2ml concentrated sulphuric acid was carefully added down the side of the test tube to form a lower layer. A reddish brown colour at the boundary indicated the presence of a steroidal ring (Sofowora, 1993a).

b) *Liebermann - Buchard test:* The extract (0.5 g) was dissolved in chloroform (2 ml) and filtered into a clean, dry test tube. Acetic anhydride (2 ml) was added to the filtrate and shaken. Two drops of concentrated sulphuric acid were added carefully down the side of the tube to form a lower layer. A brownish-red or violet ring at the zone of contact of the two liquids and the upper layer turning green indicated the presence of steroids and terpenes (Sofowora, 1993a).

3.3.5 Test for tannins

a. *Ferric chloride test*: The extract (0.2 g) was stirred with water (5 ml) and filtered. Two drops of ferric chloride solution were added to 2ml of the filtrate in a test tube. A green or greenish black precipitate indicated the presence of condensed tannins, while a blue or bluish black precipitate showed the presence of hydrolysable tannins (Trease and Evans, 1989).

b. *Lead subacetate test*: Three drops of lead subacetate solution was added to the aqueous extract (1ml). A coloured precipitate indicated the presence of tannins (Trease and Evans, 2002).

3.3.6 Test for alkaloids

General test: About 0.2 g of the extract was stirred with 5 ml of 1% hydrochloric acid on a water bath and then filtered. To 1ml of the filtrate, 2-3 drops of the following reagents were added: Wagner's reagent (solution of iodine in potassium iodide), Mayer's reagent (potassium mercuric iodide solution) and Dragendroff's reagent (potassium bismuth iodide solution). The presence of a precipitate: Light brown to brownish precipitate - Wagner's reagent, White or creamy white precipitate - Mayer's reagent, Orange or orange - yellow precipitate - Drangendroff's reagent, indicates the presence of alkaloids (Trease and Evans, 1989).

3.3.7 Test for cardiac glycosides

The extract (1.0 g) was dissolved in water (1ml). Lead sub acetate solution was added and filtered. The filtrate was divided into two for the following tests:-

(a) Keller-killiani Test (for Steroidal nucleus):

To the first portion of the filtrate, chloroform was added. The chloroform layer was separated and evaporated to dryness on a water bath. The residue was dissolved in 3.5% ferric chloride solution in glacial acetic acid (3 ml). The mixture was allowed to stand for three minute before being transferred to a test tube. Concentrated sulphuric acid (1.5 ml) was added along the side of the test tube to form a lower layer. On standing, the appearance of a brown colour at the junction of the two liquids and a pale green colour in the upper layer indicated the presence of cardiac glycosides (Brain and Turner, 1975).

(b) *Kedde's test for Cardenolides:*

To the second portion of the filtrate of 2% solution of 3, 5 – dinitrobenzoic acid in methanol (1 ml) was added followed by 5% aqueous sodium hydroxide solution (2 ml). An immediate violet colour indicated the presence of cardiac glycosides (Sofowora, 1993a).

3.3.8 Test for flavonoids

Sodium hydroxide test: The extract (0.2 g) was dissolved in water (2 ml) and filtered. 10% sodium hydroxide solution (2 ml) was added. A yellow solution indicated the presence of flavonoids. On addition of dilute hydrochloric acid, the solution became colourless (Brain and Turner, 1975).

Shinoda's test: The extract (0.2 g) was diluted with ethanol. A small piece of magnesium filing was added, followed by five drops of concentrated hydrochloric acid. A red or pink colour indicated the presence of flavonoids (Silva *et al.*, 1998).

3.4 Antimicrobial Screening

The test organisms used for this analysis were clinical isolates of bacteria and fungi obtained from the Department of Microbiology, Ahmadu Bello University, Zaria. The isolate were: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Candida albicans*, and *Pseudomonas aeroginosa*.

3.4.1 Cultivation and standardization of test organisms

A loop full of each of the test organisms were taken from the agar slant and sub cultured into test tubes containing sterile nutrient agar for the bacteria and potato dextrose agar for the fungi. The test tubes were then incubated for 24 h at 37^{o}C, while the plate of potato dextrose agar was incubated at room temperature for about 5 days for fungi. The broth culture was standardized using sterile normal saline to obtain a density of 10^{6} Cfu/ml for

the bacteria. A sporulated test fungal spore was harvested with 0.05% Tween80 in sterile normal saline and standardized to 10^6 spores/ml. (Andew 2001; Girish and Satish 2008)

3.4.2 Preparation of extracts solution for antibacterial activity assay

The solutions (30 mg/ml each) of the *n* - hexane, chloroform, ethylacetate and methanol extracts were prepared by dissolving their crude extracts in dimethylsulfoxide (DMSO) and sterile distilled water respectively. The solvents were also used as a negative control. Serial dilution of each extract was prepared in a concentration ranging from 12.5, 25, 50, and 100 mg/ml for the determination of minimum inhibitory concentration (MIC) value. The antibacterial activity of the leaf extract of *Adenodolichos paniculatus* was compared to ciprofloxacin which is a commercial synthetic antibiotic active against *S. aureus* and *E. coli* pathogens (Ghalem, 2014).

3.4.3 Determination of Inhibitory activity (Sensitivity Test) of the extract using Agar well diffusion method

The standardized inocula of both the bacterial and fungal isolates were streaked on sterilized Mueller Hinton and potato dextrose agar plates respectively with the aid of sterile swab sticks. Four wells were punched on each inoculated agar plate with a sterile cork borer. The wells were properly labeled according to different concentrations of the extract prepared which were 100, 50, 25 and 12.5 mg/ml respectively. Each well was allowed to stay on the bench for about one hour. This was to enable the extract to diffuse into the agar. The plates were then incubated at 37℃ for 24 h (Mueller Hinton agar) while the plate of potato dextrose agar were incubated at room temperature for 5 days. At the end of the incubation period, the plates were observed for any evidence of inhibition which will appear as a clear zone that was completely devoid of growth around the wells (zone of

inhibition). The diameters of the zone were measured using a transparent ruler calibrated in millimeter and the result was recorded (Mattana *et al*., 2010).

3.4.4 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the extract was determined using the serial dilution method with the Mueller Hinton broth used as diluents. The lowest concentration of the extract showing inhibition for each organism when the extract was tested during sensitivity test was serially diluted in the test tubes containing Mueller Hinton broth. The organisms were inoculated into each tube containing the broth and extract. The inoculated tubes were then incubated at 37℃ for 24 h. At the end of the incubation period, the tubes were examined/observed for the presence or absence of growth using turbidity as criterion. The lowest concentration in the series without visible sign of growth (turbidity) was considered to be the minimum inhibitory concentration (MIC) the result was also recorded (Mattana *et al*., 2010).

3.4.5 Determination of minimum bactericidal concentration/minimum fungicidal concentration (MBC/MFC)

The result from the minimum inhibitory concentration (MIC) was used to determine the minimum bactericidal concentration/Minimum Fungicidal Concentration (MBC/MFC) of the extract. A sterilized wire loop was dipped into the test tube(s) that did not show turbidity (clear) in the MIC test and a loopful was taken and streaked on sterile nutrient agar and potato dextrose agar plates. Incubation of the plates was carried out at 37℃ for 24 h for bacteria and 27℃ for 5 days for fungi after which the plates were observed for colony growth. The MBC/MFC was/were the plate(s) with lowest concentration(s) without colony growth.

3.5. Purification Techniques

3.5.1 Thin layer chromatography (TLC)

Analysis of the four extracts began with analytical thin layer chromatography (TLC) using various solvent mixtures. In each case, the developed spots were visualized under UV light (200-400nm) (Raju *et al.,* 2014) after which they were sprayed with 10% H_2SO_4. The spots were observed and their R_f values calculated and recorded.

3.5.2 Column chromatography

3.5.2.1 Column chromatography of ethyl acetate extract

The ethyl acetate extract was found to possess more activity on the tested micro-organisms; hence it was selected for further purification. The ethyl acetate extract (5.00 g) was chromatographed, using the following column conditions.

(a) Technique - Gradient elution

(b) Column - Glass column 3.80 x 50cm.

(c) Stationary phase - Silica gel, 60 – 200 mesh size (Qualikem laboratory reagent)

(d) Column packing - Wet slurry method.

(e) Sample loading - The sample was applied using dry loading method (Cannel, 2000); the Sample was dissolved in a small amount of chloroform. It was then mixed with a small quantity of silica gel, dried in a rotary evaporator, and then loaded on top of the column.

(f) Solvent for elution - different solvents systems were used based on the TLC profile of the crude extract. Gradual elution started with *n* – hexane 100%, *n* – hexane – ethyl acetate mixtures, (90:10, 85:15, 80:20, 60:40, 50:50,70:30) and finally end with ethyl acetate 100 %. The process was monitored using thin layer chromatography. Fractions were collected in 100 ml aliquots, and were pooled together based on their TLC profile to obtain ten fractions. The fractions were analyzed using TLC and viewed under UV as well as

spraying with 10% H_2SO_4. Fraction (1-10) showed single spot each with same R_f value and they were pooled together and labeled X1, and fraction (11-20) gave single spot of same R_f values and were pooled together and labeled X2. The fractions X1 and X2 crystallized on standing and were found to be soluble in chloroform. To further purify the two compounds preparative thin layer chromatography was used (Raju *et al*., 2014), using hexane-ethyl acetate 95:15 as solvent system for both X1 and X2. The two isolates X_1 and X_2 were weighed and subjected to spectral analysis.

3.5.2.2 Chemical tests on compounds X1 and X2

Test for Steroid:

Compound X1 give a red coloration of *Salkowski test* for steroidal ring (Sofowora, 1993a; Silva, *et al.,* 1998)

Compound X1 also give a brownish – red colour of *Liebermann Buchard test* for steroids and terpenes (Silva *et al.,* 1998)

Test for Fatty Acid:

Compound X2 was mixed with 5 ml of ether, and allowed to evaporate on a filter paper. The filter paper was dried. The appearance of transparency on the filter paper indicated the presence of fatty acids (Sofowora, 1993a; Silva *et al.,* 1998)

3.5.2.3 Melting point measurement

A melting point (m.p.) measurement was performed by procedure as described by Brittain (2009) on a Stuart SMP40 melting point apparatus. A few crystals of the compounds were placed in a thin walled glass capillary tube and then inserted into the side of the heating block via the hole provided in the apparatus. The compounds were then heated until they

melted. The temperature at which the solid just began to melt and that at which it was completely liquid, were recorded as the melting point range of the compounds.

3.5.2.4 Spectral analysis of compounds

The isolated compounds X_1 and X2 were subjected to FTIR spectroscopy using carry 630 FTIR, the H-NMR and 13C NMR analysis were carried out using Agilent-NMR-vnmrs400.

CHAPTER FOUR

4.0 RESULTS

4.1 Extraction

The powdered leaf (1kg) of *Adenodolichos paniculatus* after microwave-assisted extraction using 5litres *n*-hexane, chloroform, ethyl acetate and methanol respectively each yielded the following weight in grams (g) and percentage (%) shown in Table 4.1.

Table 4.1: Percentage Recovery of n–Hexane, Chloroform, Ethyl acetate and Methanol extracts of *Adenodolichos paniculatus*

Extract	Colour	Weight (g)	$^{0}/_{0}$ Recovery
N-hexane	Green	17.24	1.73
Chloroform	Green	14.41	1.41
Ethyl acetate	Dark Green	10.00	1.00
Methanol	Black	42.26	4.23

4.2 Phytochemical Screening

Phytochemical screening of the extract of *Adenodolichos paniculatus*, revealed the presence of reducing sugar, tannins, saponins, cardiac glycosides, alkaloids, flavonoids, anthraquinones and steroids as shown in Table 4.2.

Table 4.2: Phytochemical Constituents of the Leaf Extract of *Adenodolichos paniculatus*

Constituent	**Extract**			
	Methanol	Ethyl acetate	Chloroform	N-hexane
Tannins	+	+	+	+
Saponin	+	+	-	-
Cardiac glycoside	+	+	+	+
Alkaloid	+	+	-	-
Flavonoid	+	+	+	-
Anthraquinone	+	-	-	-
Steroid	-	+	+	+
Reducing sugars	+	+	+	+

Key: - = absent, + = present

The TLC profile of some fractions from the first column using the solvent system n-hexane: ethyl acetate 9: 1 is as shown in plate II.

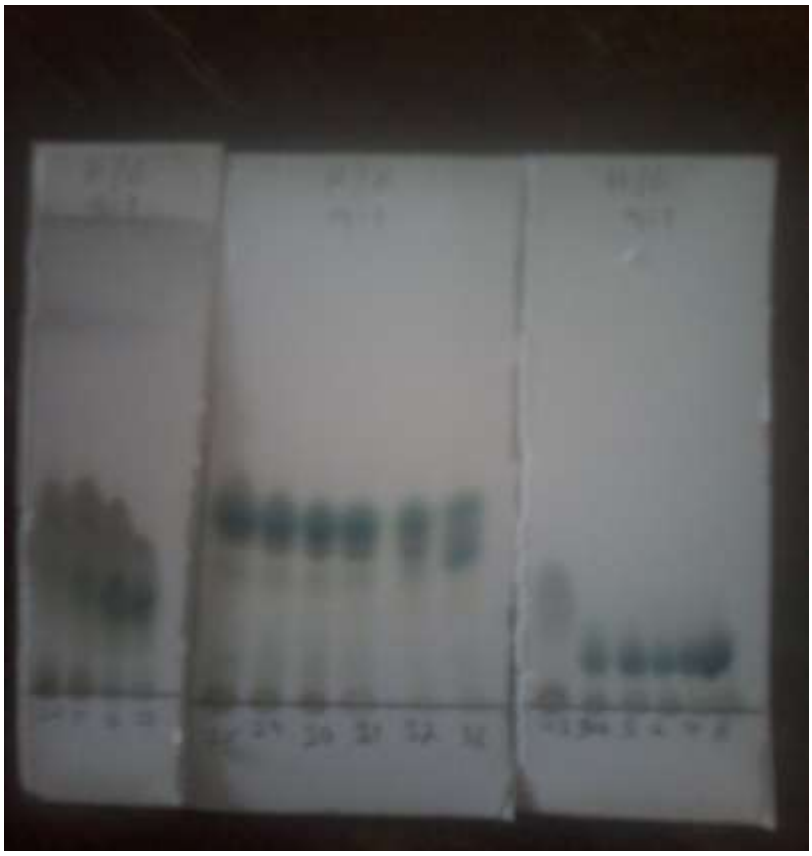


Plate II: TLC Profiles of HEX, CHL, EA and ME Extracts using HEX: EA (9:1)

HEX= Hexane CHL=Chloroform EA= Ethyl acetate ME= Methanol

The TLC profile of some fractions from the second column using the solvent system chloroform: ethyl acetate 9: 1 is as shown in plate III.



Plate III: TLC Profile of Some Column Collections CHL: EA (9: 1)

CHL= Chloroform EA= Ethyl acetate

The TLC profile of the isolated compounds X1 and X2 using the solvent system n-hexane: ethyl acetate 9: 1 is as shown in plate IV (A) and (B).



A B

Solvent system: Hexane: ethyl acetate 8:2

Plate IV: (A) and (B) the TLC of compound X1 and X2 respectively.

Table 4. 3: **Chemical Test on Compound X1 and X2 are shown in Table 4.3**

Test	Observation	Inference
Liebermann – Buchard test	Red coloured ring produced	Steroid present
Salkowski's test	A reddish brown colour was formed at the interface	Steroid present
Compound X2 mixed with 5 ml ether, and allowed to evaporate on a filter paper	There was appearance of transparency on the filter paper	Fatty Acid present

4.4 Melting point of X1 and X2

Table 4. 4: Melting point of X1 and X2

Compound	**Melting Point (°C)**	
	Experimental	Literature
X1	134.2-136.4	134–135 (Hang and Dussault, 2010; Luhata and Munkombwe; 2015; Ododo *et al.,* 2016).
X2	11.2 - 12.3°C	12.5℃ (Wahlberg and Lindberg, 2003).

4.4.1 FTIR Analysis of X1

The FTIR Spectrum of Compound X1 is shown in Figure 4.1.

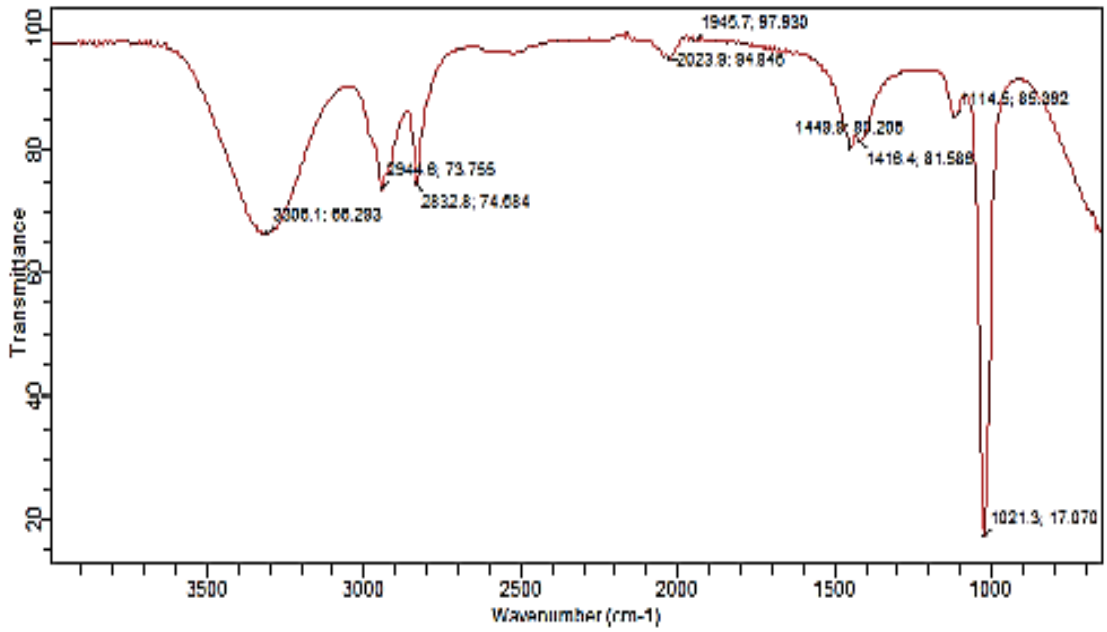


Figure 4.1: spectrum of Compound X1

4.4.2 ^{1}H NMR (δppm, 400 MHz, $CDCl_3$) of Compound X1

The proton NMR spectrum of compound X1 is shown in Figure 4. 2.

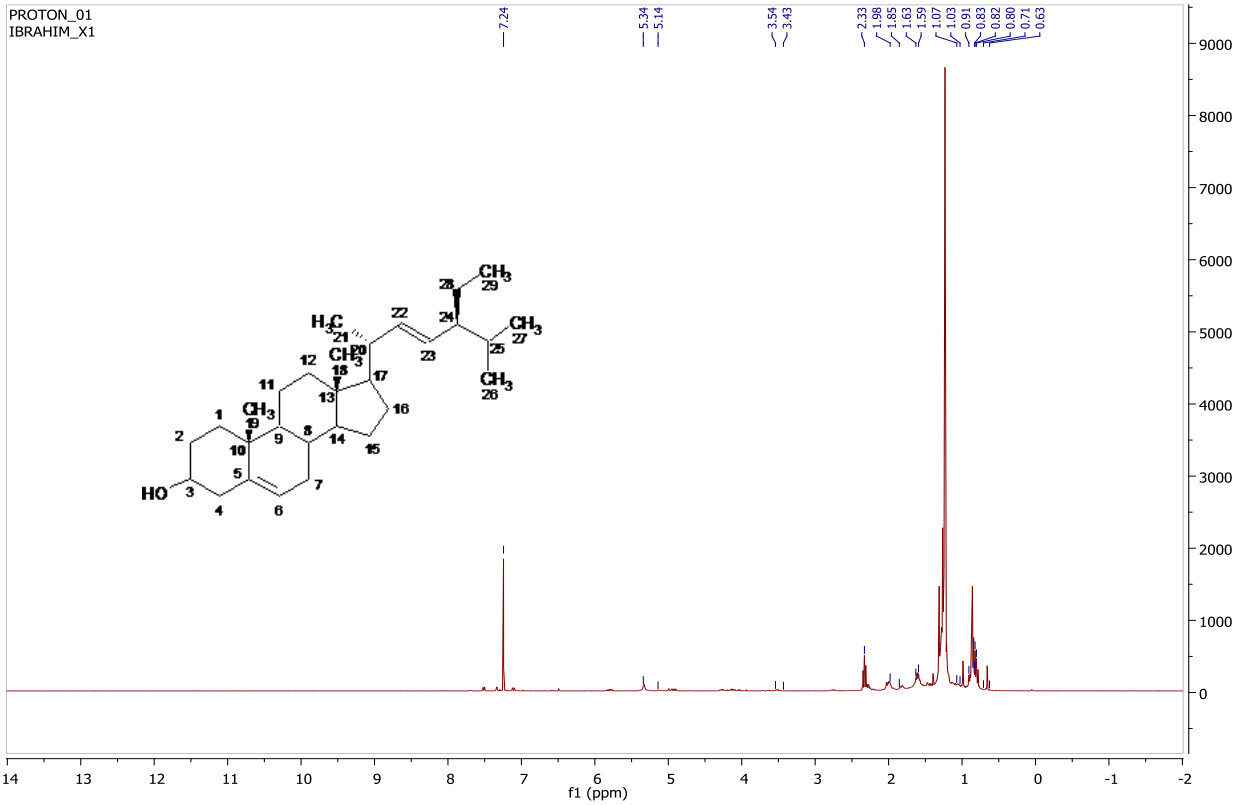


Figure 4.2: Proton NMR Spectrum of Compound X1

4.4.3 ^{13}C NMR (δppm, 400MHz, $CDCl_3$) Spectral Analysis of Compound X1

The 13C – NMR spectrum of compound X1 is shown in Figure 4.3.

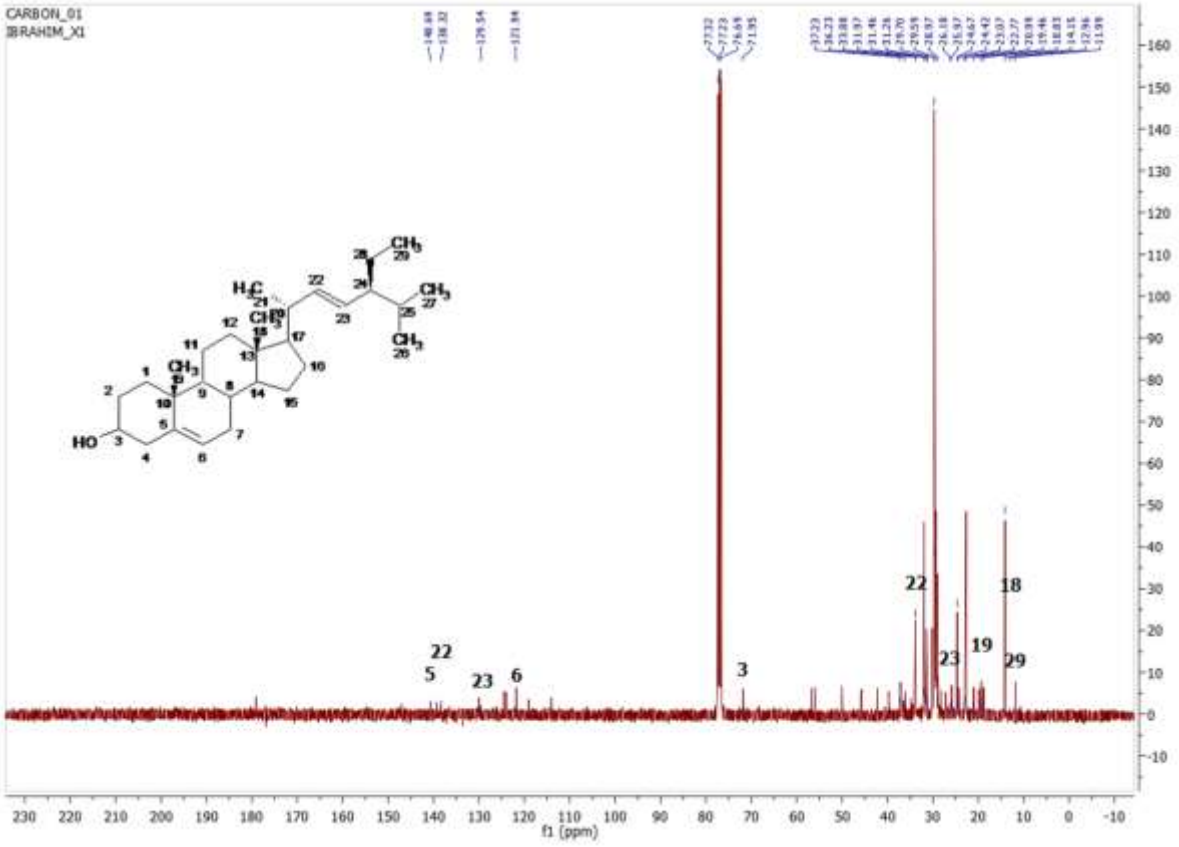


Figure 4. 3: ^{13}C - NMR Spectrum of Compound X1

Table 4. 5: **1H and 13C NMR chemical shift values for compound X1 recorded in $CDCl_3$ (400 MHz) with literature data (Luhata and Munkombwe, 2015)**

Carbon Atom	^{13}C NMR Experimental	^{13}C NMR Literature	^{1}H NMR Experimental	^{1}H NMR Literature	CHn
C-1	37.20	36.72			CH_2
C-2	29.70	29.71			CH_2
C-3	71.82	71.97	3.51 (m, 1H)	3.53 (m, 1H)	CH
C-4	42.22	42.35			CH_2
C-5	140.90	140.94			C
C-6	121.75	121.32	5.34 (s, 1H)	5.38 (s, 1H)	CH
C-7	31.85	31.71			CH_2
C-8	29.24	29.24			CH
C-9	50.05	50.03			CH
C-10	36.13	36.16			C
C-11	24.28	24.32			CH_2
C-12	39.72	39.82			CH_2
C-13	42.22	42.45			C
C-14	56.72	56.90			CH
C-15	24.67	24.32			CH_2
C-16	28.93	28.90			CH_2
C-17	55.97	56.03			CH
C-18	11.96	12.06	1.03 (d, 3H)	1.29(d, 3H)	CH_3
C-19	19.39	19.06	0.71 (d, 3H)	0.74(d, 3H)	CH_3
C-20	39.72	39.82			CH
C-21	22.99	23.12	0.91 (d, 3H)	1.20(d, 3H)	CH_3
C-22	132.5	138.40			CH
C-23	124.44	129.34	5.14 (m, 1H)	5.20(m, 1H)	CH
C-24	49.80	51.26			CH
C-25	33.88	34.01			CH
C-26	21.05	21.12	0.80 (d, 3H)	0.84(d, 3H)	CH_3
C-27	22.70	22.82	0.82 (d, 3H)	0.97(d, 3H)	CH_3
C-28	25.93	25.32			CH_2
C-29	11.84	12.06	0.83 (t, 3H)	1.04(t, 3H)	CH_3

4.5.1 Spectroscopic analysis of compound X2

4.5.1.1 FTIR Analysis of compound X2

The FTIR spectrum of Compound X2 (Figure 4.4).

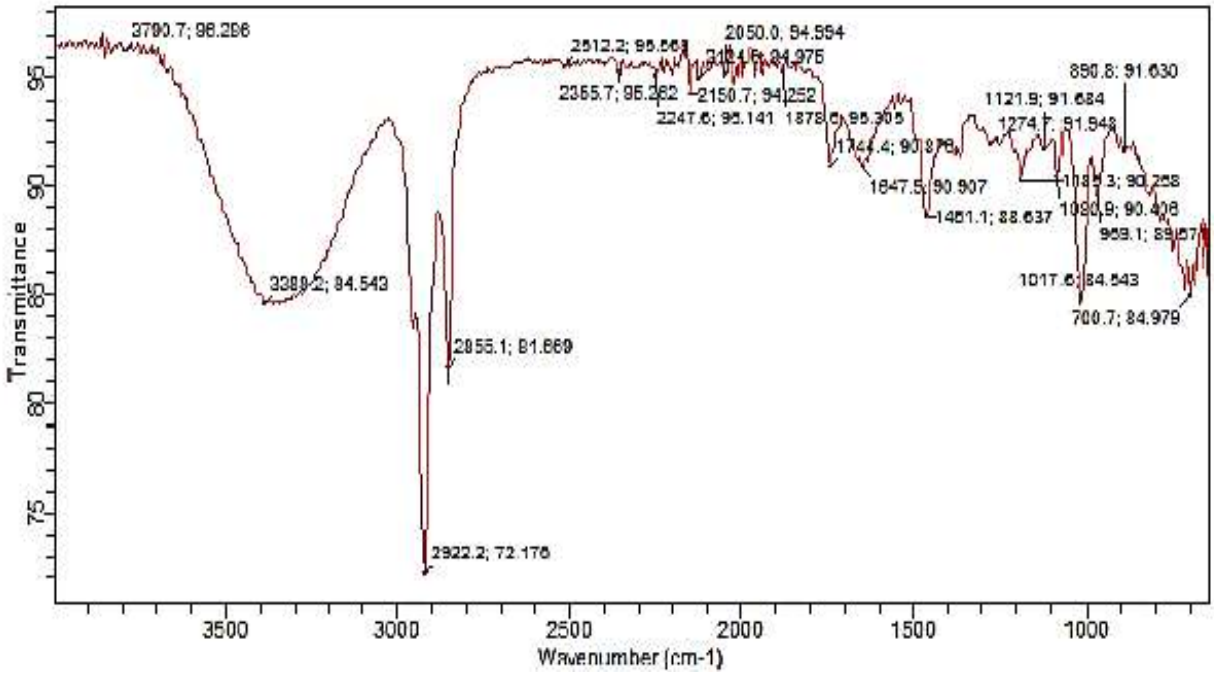


Figure 4.4: FTIR Spectrum of Compound X2

The proton NMR spectrum of compound X2 is as shown in Figure 4.5.

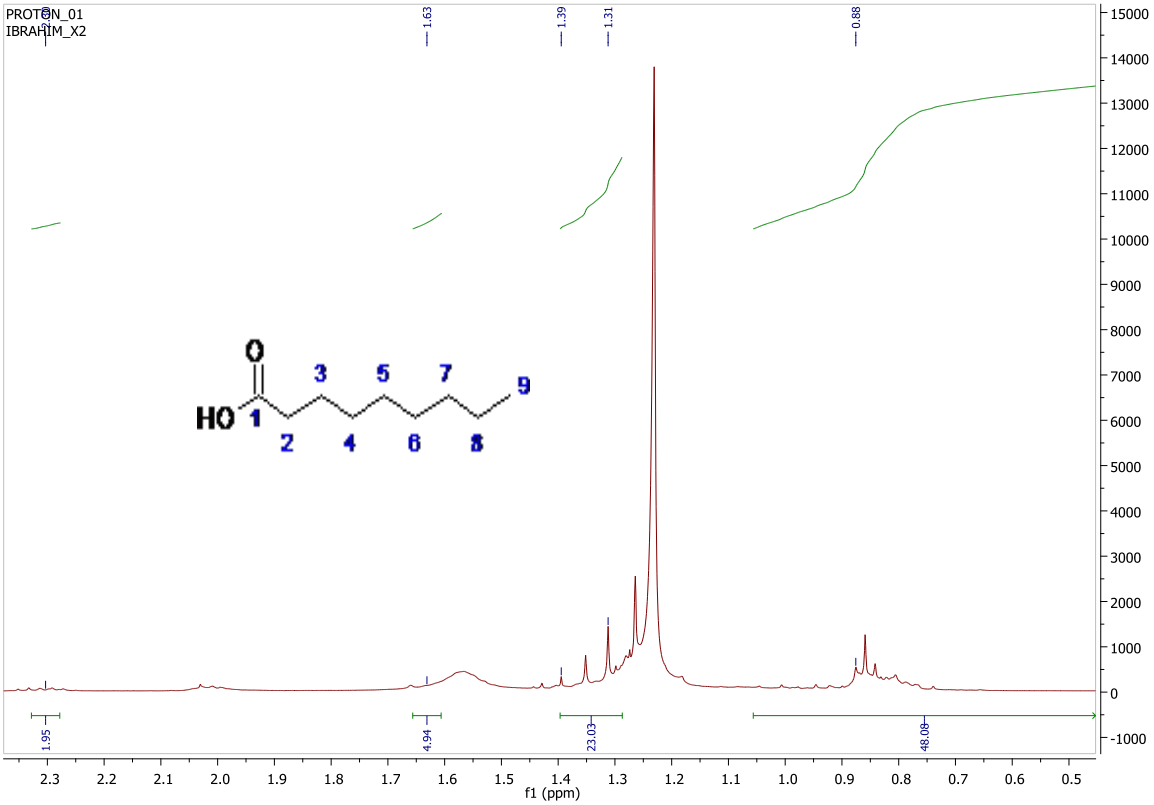


Figure 4. 5: Proton NMR spectrum of Compound X2

4.5.1.2 ^{13}C NMR (δppm, 400MHz, $CDCl_3$) is shown in Figure 4.6.

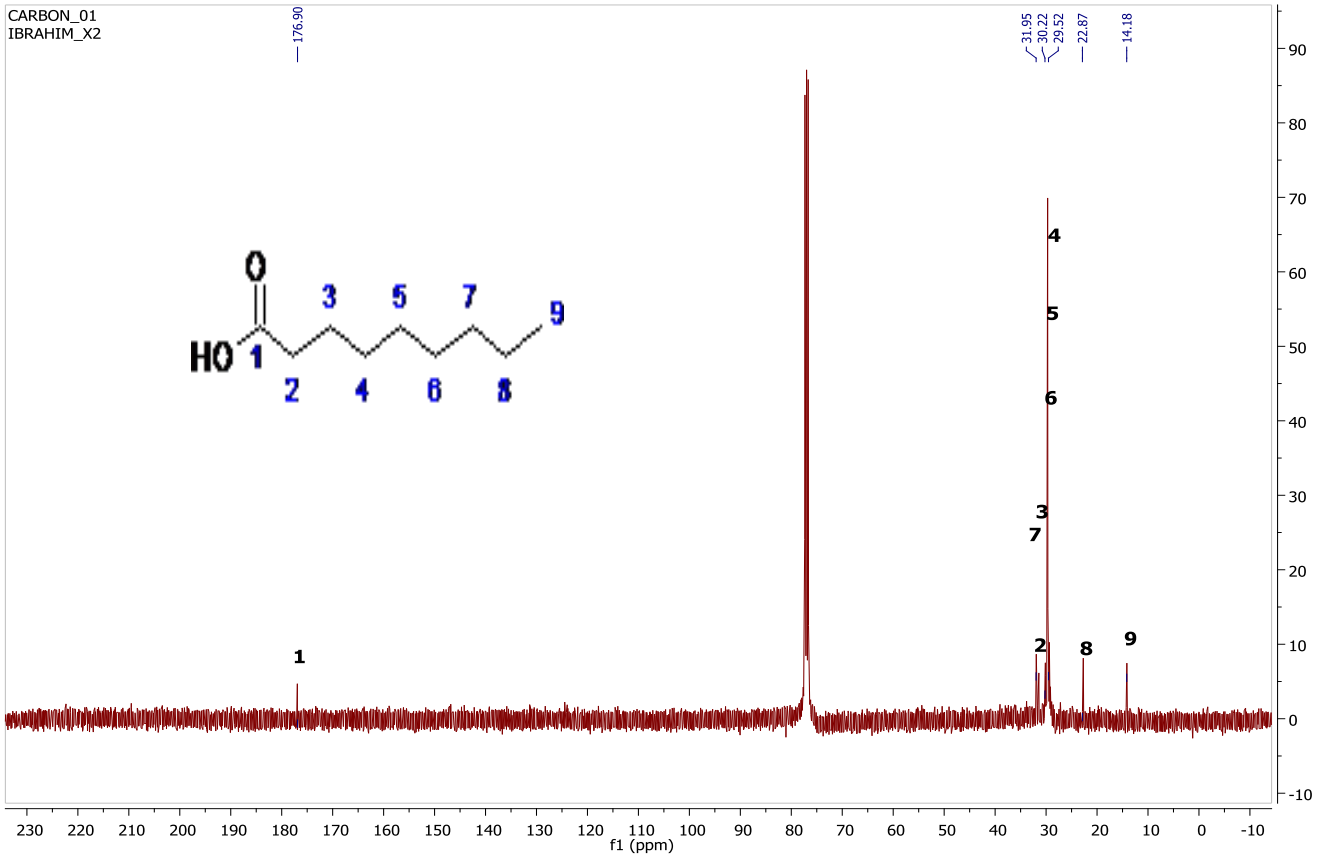


Figure 4. 6: Carbon 13 NMR spectrum of Compound X2

Table 4. 6: **1H and 13C NMR chemical shift values for compound X2 recorded in $CDCl_3$ (400 MHz) with literature data** (Yun-Woo *et al*., 2012)

Carbon Atom	**^{13}C NMR Experimental**	**^{13}C NMR Literature**	**^{1}H NMR Experimental**	**^{1}H NMR Literature**	**CHn**
C-1	176.9	179.8			C
C-2	31.4	34.0	2.30 (2H t, *J* = *9.2* Hz)	2.35 (2H t, *J* = *7.2* Hz)	CH_2
C-3	30.1	24.7	1.63 (2H m)	1.63 (2H m)	CH_2
C-4	29.7	29.1	1.31-1.23(10H m)	1.38-1.23(10H m)	CH_2
C-5	29.3	29.1			CH_2
C-6	29.1	29.1			CH_2
C-7	31.9	31.8			CH_2
C-8	22.7	22.6			CH_2
C-9	14.14	14.1	0.88 (3H t, *J* = *6.8* Hz)	0.88 (3H t, *J* = *6.6* Hz)	CH_3

4.5.2 Antimicrobial screening

Determination of inhibitory activity (sensitivity test) of *Adenodolichos paniculatus* leaf extracts on the test organism (Table 4.7) shows the zones of inhibition (mm) at varying concentration (mg/ml) of the extracts.

Table 4.7: Zone of Inhibition (mm) at Varying Concentration (mg/ml) of the Extracts

Test Organis	**MeoH Extract**				**EA Extract**				**CHC Extract**				**n-hex Extract**				**Ciprofloxacin**
	(100	50	25	12.5)	(100	50	25	12.5)	(100	50	25	12.5)	(100	50	25	12.5)	10µg
Staphylococcus aureus	16	14	12	10	19	16	14	10	-	-	-	-	14	12	10	-	35
Bacillus subtilis	-	-	-	-	17	13	10	-	-	-	-	-	-	-	-	-	41
Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	39
Salmonella typhi	15	13	12	10	18	16	13	10	-	-	-	-	-	-	-	-	38
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35
Candida albicans	18	15	13	16	19	16	14	12	-	-	-	-	-	-	-	-	38

MeoH= methanol, EA= ethyl acetate, CHC= chloroform, n-hex= n-hexane, - = No inhibition

The results of the MIC and the MBC/MFC tests of the leaves extract of *Adenodolichos paniculatus* on the test microorganisms are as shown in table 4.8.

Table 4.8: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) of the Extract Against the Test Organism.

Test Organisms	**MIC (mg/ml)**				**MBC/MFC (mg/ml)**			
	MT	EA	n-H	CL	MT	EA	n-H	CL
Staphyloc aureus	12	25	50	ND	50	25	-	ND
Bacillus subtilis	ND	50	ND	ND	-	ND	ND	ND
Escherichia coli	ND	ND	ND	ND	ND	ND	ND	ND
Salmonella typhi	50	50	ND	ND	-	-	ND	ND
Pseudomonas aeruginosa	ND	ND	ND	ND	ND	ND	ND	ND
Candida0 albicans	25	25	ND	ND	50	50	ND	ND

MT= methanol, EA= ethyl acetate, n-H= *n*-hexane, CL= chloroform, ND= not determine, - = Bacteriostatic

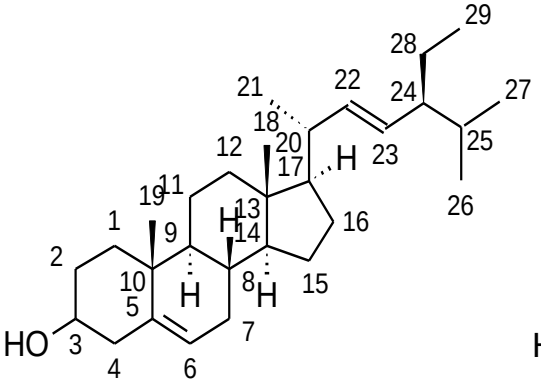
The isolated compounds showed activity against all the bacterial strains with inhibition zones of 13-18 mm at lower concentrations than were observed for the extracts except against *E. coli* and *P aeroginosa* (Table 4.9), where they show no inhibition.

Table 4.9: Result of Antimicrobial Activity of Compounds X1, X2 and Ciprofloxacin

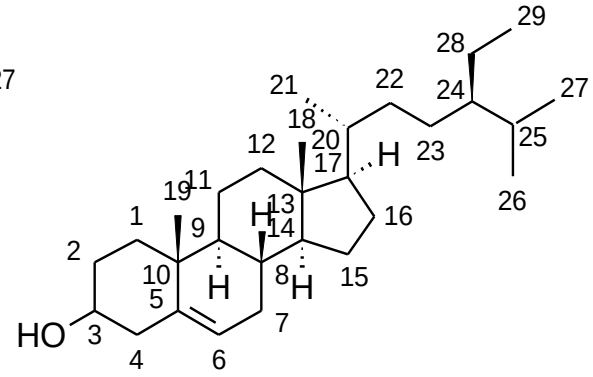
Organism	**Zone of Inhibitions (mm)**		
	C/P **(0.03mg/disc)**	**X1 (0.05 mg/ml)**	**X2 (0.05 mg/ml)**
S. aureus	35	17	18
B. subtilis	32	0	0
E. coli	32		
S. typhi	32	18	13
P. aeruginosa	25		
C. albicans	25	0	18

C/P= Ciprofloxacin

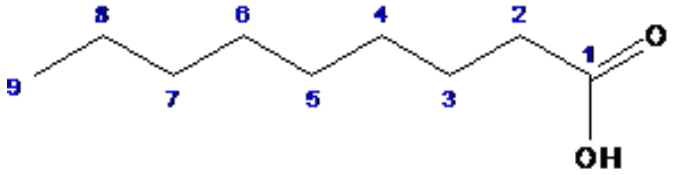
4.6 Proposed Structures of Compound X1 and X2



Stigmasterol (XVII)



β – sitosterol (XVIII)



Nonanoic acid (XIX)

CHAPTER FIVE

5.0 DISCUSSION

The result of preliminary phytochemical Screening (Table 4.2) carried out on the crude ethanol, ethyl acetate, chloroform and *n* – hexane extracts revealed the presence of flavonoids, tannins, steroids/triterpenes, saponins, phenols and alkaloids. These secondary metabolites have been associated with several pharmacological properties of plants (Cowan, 1999).

Purification of the ethyl acetate extract through repeated column chromatography over silica gel, 60 – 200 mesh size, and eluting with different solvent systems led to the isolation of two compounds coded X1 (white crystalline solid) and X2 (yellowish oil with slight odour). The melting point of X1 and X2 were determined to be 134.2-136.4℃ and 11.2-12.3°C respectively. This short range of melting point (mp) indicate a good purity, because pure compounds have sharp melting points. The positive chemical test with Liebermann-Buchard test and Salkowski's test observed with compound X1 indicated a steroidal ring (Silva *et al.,* 1998) and the appearance of transparency on filter paper observed with X2 indicated the presence of fatty acids (Sofowora, 1993).

The FTIR spectrum of Compound X1 (Fig. 4.1) showed absorptions bands (cm^{-1}) for OH at 3306.1, C – H, stretching vibration at 2944.6 and 2832.8, and also the bands observed in the finger print region are due to various C-H stretching and bending modes (Ododo *et al.*, 2016; Luhata and Munkombwe, 2015; Mofiz *et al*., 2015; Wade, 2006).

^{1}H NMR (δH, 400 MHz, $CDCl_3$, ppm) spectrum of Compound X1 (Fig.4.2) showed signals at δ 0.83 (H - 29), 0.82 (H - 27), 0.80 (H - 26), 0.91 (H - 21), 0.71 (H - 19) and 1.03 (H - 18) due to methyl protons (CH_3), signals at δ 1.25 (H - 1), 1.25 (H - 2), 1.29 (H - 28), 1.38

(H - 22), 1.49 (H - 23), 1.52 (H - 11), 1.57 (H - 12), 1.60 (H - 15), 1.60 (H - 16), 2.04 (H - 7) and 2.23 (H - 4), due to eleven methylene protons (CH_2), signals at δ 1.25 (H- 24), 1.40 (H - 25), 1.44 (H - 20), 1.45 (H - 17), 1.47 (H - 14), 1.47 (H - 9), 1.64 (H - 8), 5.34 (H - 6) and 3.51(H- 3) due to nine methine and one OH group. The singlets at δ 0.70 (H - 19) and 1.03 (H - 18) confirmed the presence of two CH_3 attached to quaternary carbons. However, lack of signal at C5, C10 and C13 shows lack of protons on these carbons, they are quaternary carbons. (Isah *et al.,* 2014; Luhata and Munkombwe, 2015; Bulama *et al.*, 2015; Mofiz *et al.,* 2015; Ododo *et al.,* 2016).

The ^{13}C – NMR spectra data of compound X1 agrees with those reported by Luhata and munkombwe, (2015) and Ododo *et al,* (2016). The ^{13}CNMR showed recognizable signals at 140.90 ppm and 121.75 ppm which were assigned to C5 and C6 double bonds respectively. The peak at 19.39 ppm corresponds to an angular carbon (C19), 132.50 ppm for C-22 and 124.44 ppm for C-23. The spectra showed twenty nine carbon signals which included six methyls, nine methylenes, eleven methine and three quaternary carbons. The alkene carbons appeared at 140.90 (C-5), 121.75 (C-6), 132.50 (C-22) and 124.44 (C-23) ppm (Prakash *et al.,* 2012; Luhata and Munkombwe, 2015). According to literature β-sitosterol and stigmasterol have the same R_f values and are always in a mixture form (Prakash *et al.,* 2012; Bulama *et al.,* 2015; Luhata and Munkombwe, 2015; Mofiz, *et al.,* 2015; Ododo *et al,* 2016). The only difference between the two compounds is the presence of a double bond between C22 and C23 in Stigmasterol and a single bond in β-sitosterol in the same position. Furthermore, literature has shown that sitosterol is difficult to be obtained in pure state (Luhata and Munkombwe, 2015). Therefore, compound (X1) is a

mixture of stigmasterol and β-sitosterol (XVII, XVIII). The mixture is a colourless solid with a melting point of 134.2-136.4℃.

The melting point of Compound X2 was determined to be 11.7 – 12.5°C. The sharp melting point displayed by the compound is an indication of purity. The positive test for fatty acids observed for Compound X2 confirmed that it is a fatty acid (Sofowora, 1993a).

The FTIR spectrum of Compound X2 (Fig.4.4) showed absorptions band (cm^{-1}) for OH at 3388.2, C – H stretching vibration at 2922.2 and 2855.1, and also the bands observed in the finger print region are due to various C-H stretching and bending modes

^{1}H NMR, spectrum (Figure 4.5) (in $CDCl_3$) of Compound X2 showed signals which were assigned to seven methylenes at δ 2.30, 1.63, 1.31, 1.28, 1.27, 1.26 and 1.23, the signal at δ 0.88, was assigned to methyl proton.

The ^{13}C NMR spectrum (Figure 4.6) (in $CDCl_3$) showed nine carbons environment. The signal at 176.9 was assigned to a carbonyl carbon (C-1). The signal assigned to the methylene carbons were; 31.4 (C-2), 30.1 (C-3), 29.7 (C-4), 29.3 (C-5), 29.1 (C-6), 31.9 (C-7), 22.7 (C-8). The signal at 14.1 was assigned to the terminal methyl carbon (C-9). These assignment base on the analysis of the spectroscopic data and with comparison with literature value (Yun-woo *et al*., 2012), the structure of X2 was proposed to be nonanoic acid (XIX).

Antimicrobial studies on the crude extracts and isolated compounds showed strong activity against *Staphylococcus aureus, Salmonella typhi,* and *Candida albicans.* The result of zone of inhibition for the methanol extracts reveals 10 – 16 mm against *Staphylococcus aureus,* 10 – 15 mm against *Salmonella typhi,* and 16 – 18 mm against *Candida albicans.* For the ethyl acetate extract 10 – 19 mm was recorded against *Staphylococcus aureus,* 10 – 14 mm

against *Bacillus subtilis*, 10 – 18 mm against *Salmonella typhi*, 12 – 19 mm against *Candida albicans*. Methanol, ethyl acetate extract have strong activity against *Staphylococcus aureus*, *Salmonella typhi*, and *Candida albicans* with MIC ranging from 12 – 50 mg/ml, and MBC ranging from 25 – 50 mg/ml which is found to be bacteriostatic against *Salmonella typhi*. The isolated compounds X1 (XVII, XVIII) and X2 (XIX) showed activity against *Staphylococcus aureus*, *Salmonella typhi*, and *Candida albicans* with inhibition zone of 13 – 18 mm. *Staphylococcus aureus* is known to play a significant role in skin diseases including superficial and deep follicular lesion (Srinivesan *et al.*, 2001). The strong activity of both the methanol and ethyl acetate extracts indicated that the plant can be effective against skin infections. The ability of the extracts to inhibit *S. typhi* with inhibition zones of 18mm showed the plant to be promising in controlling the incidences of typhoid fever, especially now that there are reports of *S. typhi* acquiring resistance to the existing antibiotics used in its control (Oluwole *et al*., 2013). It is important to note that the strong activity of both the methanol and ethyl acetate extracts against *Candida albicans* indicated that the plant can be used as a good source of anti-fungal agent, for the treatment of fungal diseases such as tinea (ringworm) and for the treatment of skin, hair and nail infections often cause by these microorganisms. It can serve as good agent for the treatment of thrush, vaginitis and other conditions such as pulmonary and generalized infections including endocarditis caused by *Candida albicans* (Aibinu *et al.*, 2007).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Preliminary phytochemical investigation of the *n* - hexane, chloroform, ethylacetate and methanol extracts of *Adenodolichos paniculatus* revealed the presence of steroids/triterpenes, flavonoids, saponins, phenols, cardiac glycoside, tannins and alkaloids.

Column chromatography of the ethyl acetate extract led to the isolation of Compound X1 mixture of stigmasterol and β-sitosterol (XVII, XVIII) and X2 nonanoic acid (XIX).

The extracts showed antimicrobial and antifungal activity against a wide range of bacterial organisms and fungi (*Candida albicans*) tested but they did not show antimicrobial activities against *Escherichia coli,* and *Pseudomonas aeruginosa*. The ethyl acetate extract from which compound X1 was isolated showed antibacterial activity against *Staphylococcus aureus* which is one of the most important economic microorganisms in the world of research today, because of it resistance to most of the available antibiotics. This further shows the importance of medicinal plants (*Adenodolichos paniculatus*) in the fight against *Staphylococcus aureus* and drug resistance.

6.2 Conclusion

This research into the validation of the use of *Adenodolichos paniculatus* in the management of infectious diseases has achieved the stated aim. Two bio-active principles (Mixture of stigmasterol and β-sitosterol X1, and nonanoic acid X2) were isolated.

To the best of our knowledge, this is the first report of isolation of Mixture of stigmasterol and β-sitosterol, and nonanoic acid from *Adenodolichos paniculatus.*

Based on the findings in this work, it can be concluded that the traditional use of *Adenodolichos paniculatus* in the treatment of diseases caused by bacterial and fungal infections has scientific basis. These research results are in agreement with some works that had been carried out before by Prakash *et al.* (2012); Luhata and Munkombwe (2015); Bulama *et al.* (2015) and Mofiz *et al.* (2015). The antimicrobial activity of the extracts can be linked to β -sitosterol and stigmasterol, the antifungal activity can be linked to nonanoic acid and other compounds that could be present in the plant.

6.3 Recommendations

1. We therefore wish to recommend that further studies aimed at isolation, characterizing and bioassay of other compounds that could be present in the plant to explore other medicinal uses of this plant and in the search for other bioactive compounds from nature.
2. Further studies need to be carried out on the isolated compounds, to establish it other applications.
3. There is also need to carry out chronic toxicity studies on the extract of this plant to found its safety upon prolonged usage.

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APPENDIX

APPENDIX I

PREPARATION OF REAGENTS USED

Spraying reagents

Sulphuric acid (10%) was used i.e. 10 ml of concentrated sulphuric acid in 90ml of methanol. This was used to spray the chromatogram and heated for 10 minutes at 100℃ in an oven. The spots were visualized under UV light.

Preparation of Culture Media

The culture media used for the analysis were;

Muller Hinton Agar (MHA) for the sensitivity test, 38g of the powder was dissolve in $1dm^3$ distilled water, the mixture was heat to dissolve then sterilized by autoclave at 121 ℃ for 15 m. then allowed to cool to about 45 ℃ then it was dispense in sterile petri dish.

Potato dextrose agar (PDA) for the sensitivity test, 39g of the powder was dissolve in $1dm^3$ distilled water, the mixture was heat to dissolve then sterilized by autoclave at 121 ℃ for 15 m. then allowed to cool to about 45 ℃ then it was dispense in sterile petri dish.

Muller Hinton Broth (MHB) for the determination of minimum inhibitory concentration (MIC) 20g of the powder was dissolve in $1dm^3$ distilled water, the mixture was heat to dissolve then sterilized by autoclave at 121 ℃ for 15 m. then allowed to cool to about 45 ℃ then it was dispense in sterile test tubes of 5 ml each.

Nutrient Agar (NA) for the determination of the minimum bactericidal and fungicidal concentration (MBC and MFC) 28g of the powder was dissolve in $1dm^3$ distilled water, the mixture was heat to dissolve then sterilized by autoclave at 121 ℃ for 15 m. then allowed to cool to about 45 ℃ then it was dispense in sterile petri dish.

APPENDIX II

Serial dilution of extract for the sensitivity test

The extract (100 mg) each of the *n* - hexane, chloroform, ethylacetate and methanol were prepared by dissolving their crude extracts in dimethylsulfoxide (DMSO) (1 ml) and sterile distilled water (9 ml) respectively to give 100 mg/ml.

5 ml was dispense out of the 100 mg/ml in a measuring cylinder and make up to 10 ml to give 50 mg/ml.

5 ml was dispense out of the 50 mg/ml in a measuring cylinder and make up to 10 ml to give 25 mg/ml.

5 ml was dispense out of the 25 mg/ml in a measuring cylinder and make up to 10 ml to give 12.5 mg/ml.

APPENDIX III

Summary of Spectral Data for Compound X1

Position	**^{13}C NMR**	**^{1}H, *J* (Hz)**	**CHn**
C-1	37.20		CH_2
C-2	29.70		CH_2
C-3	71.82	3.51 (m, 1H)	CH
C-4	42.22		CH_2
C-5	140.90		C
C-6	121.75	5.34 (s, 1H)	CH
C-7	31.85		CH_2
C-8	29.24		CH
C-9	50.05		CH
C-10	36.13		C
C-11	24.28		CH_2
C-12	39.72		CH_2
C-13	42.22		C
C-14	56.72		CH
C-15	24.67		CH_2
C-16	28.93		CH_2
C-17	55.97		CH
C-18	11.96	1.03 (d, 3H)	CH_3
C-19	19.39	0.71 (d, 3H)	CH_3
C-20	39.72		CH
C-21	22.99	0.91 (d, 3H)	CH_3
C-22	132.5		CH
C-23	124.44	5.14 (m, 1H)	CH
C-24	49.80		CH
C-25	33.88		CH
C-26	21.05	0.80 (d, 3H)	CH_3
C-27	22.70	0.82 (d, 3H)	CH_3
C-28	25.93		CH_2
C-29	11.84	0.83 (t, 3H)	CH_3

Summary of Spectral Data for Compound X2

Position	**^{13}C NMR**	**^{1}H, *J* (Hz)**	**CHn**
C-1	176.9		C
C-2	31.4	2.30 (2H t, J =9.2 Hz)	CH_2
C-3	30.1	1.63 (2H m)	CH_2
C-4	29.7	1.31-1.23 (10H m)	CH_2
C-5	29.3		CH_2
C-6	29.1		CH_2
C-7	31.9		CH_2
C-8	22.7		CH_2
C-9	14.14	0.88 (3H t, J = 6.8 Hz)	CH_3