ISOLATION AND CHARACTERISATION OF SECONDARY METABOLITES AND ESSENTIAL OILS FROM *Costus lucanusianus* J. Braun & K. Schum AND THEIR ANTIMICROBIAL ACTIVITY

BY

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CERTIFICATION

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DEDICATION

I dedicate this work to the Almighty God, for His inspiration, strength and help.

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ABSTRACT

The resistance of microorganisms to antimicrobial agents has resulted into a huge challenge in the cure of infectious diseases. Some currently used synthetic antimicrobial drugs have side effects such as skin irritation and vomiting. This necessitated a search for potent antimicrobial compounds from natural sources. *Costus lucanusianus* has been identified to be bioactive against infectious diseases from Nigeria ethnomedicine. However, the isolation of its bioactive chemical constituents to support the uses has not been reported. The study was designed to isolate and characterise bioactive compounds from *Costus lucanusianus* and investigate the *in vitro* antimicrobial activity.

Aerial parts (inflorescence, leaf, stem) and rhizome of *Costus lucanusianus* were collected in Ibadan and authenticated at Forestry Research Institute Herbarium, Ibadan. The dried and pulverised samples from the aerial and rhizome parts were cold macerated with methanol separately to obtain crude extracts which were successively partitioned with n-hexane and ethyl acetate. The leaf, stem and rhizome parts were hot extracted with n-hexane and methanol successively and methanol extracts partitioned with ethyl acetate. The extracts were subjected to phytochemical screening, column and thin layer chromatography using standard methods. Fresh samples (390 g each) were subjected to hydrodistillation and essential oils obtained were analysed using gas chromatography-mass spectrometry. The compounds obtained were characterised using infra-red (IR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and mass spectroscopy. The cold extracts, essential oils and isolated compounds were evaluated for *in vitro* antimicrobial activity against clinical strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Candida albicans* obtained from the Pharmaceutical microbiology laboratory, University of Ibadan by Agar well diffusion techniques at concentrations of 12.5-100 μg/mL. Gentamicin and Tioconazole were used as positive standards. Minimum inhibitory concentration (MIC) of the compounds was determined using Agar dilution method. Data were analysed using descriptive statistics.

The cold and hot extracts yields were 0.01-1.20% and 0.07-3.4%, respectively. Saponins, tannins, flavonoids, phlobatannins, anthraquinone, phenol, alkaloids, resin, terpenoids and reducing sugar were detected, which indicate bioactive potential of the plant. Three new (3,27-dihydroxy-1-methoxy-22-cholest-5-enone, β-sitosterol-3-O-β-D-3-deoxyxylo-4-hydroxy4,5-dimethyl-pent-2-one and 20-N-methoxyaminolucanu-1,4,6-triene-3-xylo-2-pero-4-methylpentan-2-one) compounds were isolated from the extracts. 3,27-dihydroxy-1-methoxy-22-cholest-5-enone (yield=20mg), showed two strong IR absorption bands at 3500 and 1655 cm^{-1} indicating hydroxyl and olefinic functional groups, respectively. The ^{1}H NMR spectrum showed the presence of four methyl singlets between δ 0.9 and 1.60 ppm and a singlet at δ 3.90 due to methoxy group. ^{13}C NMR and DEPT 135 revealed five methyl, ten methylene, nine methine and four quaternary carbons. The mass spectrum showed a protonated molecular ion peak at *m/z* 446.30 which corresponded to $C_{28}H_{45}O_4$. The yields of essential oils ranged from 0.05-0.18% (w/w) with 11-octadecenoic acid, methyl ester (41.0%) and heptacosane (30.2%), 3',8,8'-trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone (22.1%) and 4-(1,3,3-Trimethyl-bicyclo[4.1.0]hept-2-yl)-but-3-en-2-one (28.1%) as major components. The rhizome hexane extract and its essential oil showed the highest antimicrobial activity (10-31 mm) at 100 μg/mL. 3, 27-dihydroxy-1-methoxy-22-cholest-5-enone and β-sitosterol-3-O-β-D-3-deoxyxylo-4-hydroxy4,5-dimethyl-pent-2-one showed inhibition zones (18-28 mm) with MIC value of 25-50 μg/mL.

Three new compounds and essential oils were obtained from *Costus lucanusianus* and they all displayed antimicrobial activity.

Keywords: 3,27-dihydroxy-1-methoxy-22-cholest-5-enone, hydrodistillation, heptacosane, microbial inhibition.

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LIST OF ABBREVIATION

A. niger	*Aspergillus niger*
B. subtilis	*Bacillus subtilis*
BDE	1,2- Benzenedicarboxylic acid, (2-ethylhexyl) ester
CCS	Column Chromatography Separation
COSY	Correlation Spectroscopy
C. albicans	*Candida albicans*
DEPT	Distortionless Enhancement by Polaristion Transfer
E. coli	*Escherichia coli*
ETF	Ethyl acetate fraction
GC-MS	Gas Chromatography-Mass Spectrometry
HEF	Hexane fraction
HMBC	Heteronuclear Multiple Bond Correlation
HME	Hexadecanoic acid, methyl ester
HMQC	Heteronuclear Multiple Quantum Correlation
HSQC	Heteronuclear Single Quantum Correlation
IR	Infra-red
IZD	Inhibition Zone Diameter
K. pneumoniae	*Klebsiella pneumoniae*
MET	Methanol extract
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
OEE	Octadecane, 3-ethyl-5-(2-ethylbutyl)-
P. notatum	*Penicillum notatum*
P. aeruginosa	*Pseudomonas aeruginosa*

R. stolonifer	*Rhizopus stolonifer*
S. typhi	*Salmonella typhi*
S. aureus	*Staphylococcus aureus*
TPB	3,8,8 –trimethoxy-3-piperidyl-2,2-binaphthalene-1,1,4,4-tetrone
UTI	Urinary Tract Infection

CHAPTER ONE

INTRODUCTION

1.1 Background

Infectious diseases are known as global health threats which account for 13.4 million deaths per year (Kuete, 2017). In tropical countries, they are responsible for about one-half deaths (Iwu *et al.,* 1999) and 20% of death in the Americas (Mahady, 2005). Micro-organisms such as fungi, viruses, protozoa and bacteria are the causes of infectious diseases. They mostly need a carrier to enter into the human body system. Examples of the carriers are air, houseflies, lice, mosquitoes and animals. Infectious diseases have huge impact in emerging countries due to increasing drug-resistant infections and relative unavailability of drugs (Okeke *et al.,* 2005). The predominance of infectious diseases is mainly ascribed to socioeconomic and climatic conditions.

The resistance of microorganisms to antimicrobial agents has resulted in a great challenge in the cure of infectious diseases and this is responsible for the quest for novel sources of chemical compounds with antimicrobial properties from a natural source (Nunes *et al.,* 2006).

Africa is known to have the highest percentage of infectious disease challenges in the World. Research, infrastructure and trained infectious disease specialists in Africa are extremely inadequate (Davis and Lederberg, 2001). The World Health Organisation (WHO) recently classified 17 infectious diseases (parasitic and bacterial) as neglected tropical diseases (NTDs), which are known to be prevalent in tropical and subtropical countries. Examples of NTDs are lymphatic filariasis, trachoma and dengue (WHO, 2015). Diseases such as diarrhoea, respiratory infections, Covid-19, HIV/AIDS, NTDs and tuberculosis are the predominant origins of death and morbidity globally. Whiting *et al.* (1998) and Rweyemamu *et al.,* 2006 documented malaria, HIV/AIDS,

tuberculosis and gastroenteritis as severe infectious diseases in Africa, they account for most premature deaths among people aged 15-59 years. Among the well known bacterial infectious diseases are Urinary Tract Infections (UTIs). The UTIs are known to have challenges associated with health care and economical properties (Ejrnaes, 2011; Behzadi and Behzadi, 2016).

Herbal medicines have been reported to be well known to the people compared with orthodox or western medicine (synthetic drugs) (Nasri and Shirzad, 2013). More than 75% of the medications for infectious diseases are from natural sources such as plants (Newman *et al*., 2003). This is as a result of the safe, natural, inexpensive and accessible plant materials in the cure of bacterial infections (Nasri and Shirzad, 2013). Medicinal plants contain chemical constituents which have been useful prototypes for several potent drugs for over 100 years. Several medicinal plant species show sensitivity against the growth of an array of micro-organisms (Mahady, 2005).

Medicinal plants from families Lamiaceae, Asteraceae, Salicaceae and Fabaceae, among others are known to be effective in curing various communicable ailments (Kamatenesi-Mugisha *et al.,* 2008; Bahmani *et al*., 2015; Tolom *et al.,* 2018). *Costus lucanusianus* which belongs to the group of plants is known for its efficacy in the remedy of urethral discharge, stomach problems, venereal diseases and filariasis in ethnomedicines (Burkill, 1985; Vaghasiya and Chanda, 2007).

1.2 *Costus lucanusianus* J. Braun & K. Schum

1.2.1 Morphological description of *Costus lucanusianus*

Costus lucanusianus is classified into the family Costaceae. Its mostly well-known as the African spiral flag and Spiral ginger. It is also commonly referred to as monkey sugar cane in the South-south region of Nigeria. The Ijaw of South Nigeria recognizes *C. lucanusianus* from the related African species *C. afer*, which tend to be identical in medicinal usage as male (owei ogbodo) and *C. afer* as "female" (ere ogbodo). *Costus deisteli*, *Costus dubius*, *Costus englerianus*, *Costus Schlechteri* and *Costus Spectabilis* are other species known in West Africa to have medicinal uses. *Costus lucanusianus* is an evergreen, perennial, rhizomatous, herbaceous and aromatic plant species with a thin stem that grows nearly vertically (Figure 1). The mature height of the plant is about 6-8 feet (Burkill, 1985). The bracts on the cone structure are open. Each bract covers one flower.

The individual flowers are 1-1.5 inches across. Each has a thin, tissue-like texture. The stem grows about 7 feet tall before it throws out a terminal inflorescence. *Costus lucanusianus* reproduces by seed (sexually) and asexually (apomixis) through plantlet

Figure 1: Matured plant of *Costus lucanusianus*

Photograph was taken in June 2016 at natural habitat, Akobo area, Ibadan North Local Government of Oyo State.

formation on the inflorescence (Edeoga and Okoli, 1996). The inflorescence is globose and the corolla of the flower has yellow throat and red lips. *Costus lucanusianus* is the only *Costus* with fragrance and a vigorous grower. *Costus lucanusianus* has been reported to produce hybrids in southern Nigeria (Burkill, 1985).

A contraction of the rat uterus by *C. lucanusianus* leaf aqueous extract was documented by Owolabi *et al.,* (2010). However, Foungbe *et al.* (1987) reported the uterine relaxant property of the plant by the inhibition of the oxytocin-induced contraction in isolated rat uterus. *Costus lucanusianus* is famous for its anti-abortive properties in Southern Ivory Coast (Sawadogo, 1986) and the stem juice also demonstrated tocolytic property (Komenan, 1986; Foungbe *et al.,* 1987). At a concentration 20 mg/mL, *C. lucanusianus* leaf cold aqueous methanolic extracts also showed antimicrobial activities (Baba and Onanuga, 2011).

Costus lucanusianus leaf aqueous extract displayed activities like anti-inflammatory and antidiarrhoeal (Owolabi *et al.,* 2007, Owolabi and Nworgu, 2009). Also the hepatoprotective and renoprotective potential have been documented (Saliu and Fapounda, 2016). Ezerioha and Kagbo, (2017) stated the antimalarial property of methanolic root extract of *C. lucanusianus*. Inflorescence infusion of *C. lucanusianus* is used as a remedy for tachycardia and stomach problem. The sap from the stem is used as a remedy for venereal disease, urethral discharge and, in Gabon, as an eye drop to control filariasis. Pulped stem in water is taken as a diuretic in Southern Nigeria, the sap from the stem is used by the Ijaw for malaria and to clear urine (Burkill, 1985). The ingestion of the rhizome decoction is also useful in venereal, malaria and leprosy disease (Lambert *et al,* 1988). It is extensively used in tropical Africa as a medicinal plant. Nevertheless, there is a limited knowledge about its chemical components (Saliu and Fapounda, 2016).

1.3 Justification for the Study

The ethnomedicinal uses of *C. lucanusianus* for treating urinary tract infection, venereal disease, stomach problem and as well as cough, urethral discharge and diuretic, among others, have been reported (Burkill, 1985). However, to great cognizance, no scientific report is available to corroborate these claims and no available report on the essential oil and chemical constituents from the plant parts. This

plant was, therefore, selected with a view to isolate and characterise the secondary metabolites and essential oil constituents that may be active against micro-organisms especially uropathogens which cause urinary tract infections.

1.4 Aim of the Study

The aim of the research was to isolate and characterise chemical (non-volatile) and essential oil constituents of *C. lucanusianus* that might be active against micro-organisms especially urinary tract infection causative organisms.

1.5 Objectives of the Study

The objectives of the study were to:

- Collect *Costus lucanusianus* inflorescence, leaf, stem and rhizome parts, extract, fractionate and carry out phytochemical screening using standard phytochemical methods;
- Extract essential oils from the inflorescence, leaf, stem and rhizome parts; and
- Carry out column chromatography separation of the extracts that will lead to isolation of pure compounds;
- Characterise the pure compounds obtained by spectroscopic techniques: infra-red (IR), mass spectrometry (MS) and nuclear magnetic resonance (NMR);
- Determine the chemical composition of the essential oils using gas chromatography-mass spectrometry (GC-MS).
- Determine the antimicrobial potency of the isolated compounds, essential oils, and crude extracts.

CHAPTER TWO

LITERATURE REVIEW

2.1 Medicinal Plants

Medicinal plant products are formed by subjecting plant parts such as the leaf, stem, and root to various processes. These includes, extracting, fractionating, purifying, concentrating or other biological or physical processes. These are produced as instant intake or as a source of herbal products (WHO, 2001). Medicinal plants serve as an alternative therapy to antibiotics for many pathogenic micro-organisms (Kala *et al.,* 2006). This is because they contain some important organic compounds like alkaloids, terpenes, flavonoids, carbohydrates, steroids and tannins which, under *in vitro* conditions are responsible for anti-microbial properties (Cowan, 1999).

The use of medicinal plants has become popular because they are less expensive than synthetic drugs and most products derived from medicinal plants are classified as safe for consumption under GRAS (Generally Regarded as Safe) and seldomly with side effects (Khayyat and Saddiq, 2015). Over two-thirds of the world populace still make use of herbal medicine for taking care of their health necessities (Farnsworth *et al.,* 1985). Medicinal plants and their products usages in emerging countries, particularly in tropical Africa for curing ailments are still in practice (Okpuorl and Oloyede, 2009; Sofowora *et al.,* 2013). Requests for medicinal plants are growing rapidly in advanced and developing nations. The various effects of medicinal plants on the living system, including anti-inflammatory, antioxidant, antipyretic and antimicrobial properties are reported (Okwu and Ezenagu, 2008). Screening of organic compounds obtained from plants parts has also resulted in the finding of novel medicinal drugs which are useful in curing numerous ailments, like Alzheimer’s disease, tuberculosis, asthama and cancer. Purification process to obtain pure active components from medicinal plants may sometimes result in making it become too powerful or losing its potency.

The types of plants used for a particular ailment, the formation and application have been inherited from the ancient generation. Purification and identification of active chemical constituents in these medicinal plants resulted in drugs like Quinine, Codeine and Nicotine. The increased concern in the current information about natural cure to cardiovascular, endocrine, gastric, respiratory, neurological diseases, tumour progression and HIV/AIDS, to mention a few, has led to research for novel compounds effective against these diseases (Wright and Phillipson, 1990; Adesegun and Coker, 2001; Okwu and Ezenagu, 2008). Some examples of medicinal plants that have been documented are *Thea folium*, *Mandragora officinarum*, *Podophyllum cinnamon* stem bark, camphor and *Costus afer,* which is one of the registered plants known for its antibacterial property against *Staphylococcus aureus, Mycobacterium fortuitum*, *Candida albicans and Bacillus cereus* (Wiart, 2006; Magasomba *et al.,* 2007; Kelly, 2009). These plants are usually prepared or formulated in the form of decoctions, infusions, essential balms, tea, tablet, and among others.

Herbal medicines are renowned for their effectiveness in the remedy of acute and chronic Urinary Tract Infections (UTIs) (DiPasquale, 2008). Among these are *Vaccinium macrocarpon* (Cranberry) which possesses astringent properties that prevent microbes from bonding to the mucous membrane of the urinary system. It has also been established to produce an anti-inflammatory effect responsible for the reduction in clinical UTI episodes. Other medicinal plants useful in the remedy of UTI are *Galium aparine* (Golden seal root) which has been used for long in the cure of urinary infection (Lavigne *et al.,* 2008; Vasileiou *et al*., 2013), *Ammi visnaga* (Apiaceae) commonly known as toothpick weed or Bishop's weed, which has been traditionally used to relieve the pain of spastic urinary tract disorders and *Cucurbita pepo* (Cucurbitaceae) also commonly known as pumpkin and vegetable marrow (Khan *et al*., 2001; Babita and Vishnu, 2013). *Punica granatum* (Pomegranate) and *Cinnamomum verum* (Lauraceae) known as cinnamon tree, extracts also possess antibacterial activities against UTI-causing pathogens (Dhanalakshmi and Selvi, 2013). Species of *Costus* have been renowned to be diuretic and valuable in the remedy of urethral discharge and venereal diseases (Burkill, 1985; Sivarajan and Balachandran, 1994).

2.2 The *Costus* Genus

Costus is from the Costaceae family. The Costaceae is a family of pantropical monocotyledon. There are 7 genera in Costaceae consisting of about 143 species, making it the largest genus in the family Costaceae (Christenhusz and Byng, 2016). Plants in the Costaceae family are self-supporting, perennial, angiosperm and native to tropical climates of South America, Africa, Asia and Central America. Costaceae is also known as rhizomatous herbs with simple spirally-arranged leaves, elliptic leaf blade, fruits in the form of 2 or 3-valved capsules containing black seeds, cone-like inflorescence and bisexual. The origin of *Costus* is known to be Malay Peninsula of South-east Asia and now extensively dispersed all over the evergreen forest and the tropicals (Gupta *et al.,* 2008; Gupta, 2010).

The Genera of the family include *Monocostus* (1 Species), *Dimenocostus* (2 Species), *Tapeinchilas* (16 Species), *Paracostus* (2 Species)*, Chamaecostus* (8 Species), *Helenia* (5 Species), *Costus* (80 Species) (Specht and Dennis, 2006). The family of Costaceae in Africa comprises of two genera, namely *Costus* and *Paracostus*. The genus *Costus* is prevalent in tropical America and tropical Africa. The only *Paracostus* in Africa is *Paracostus englerianus* (Salzman *et al.,* 2015).

Costus species are mostly terrestrial, generally, 1.5-4 m tall however some are known to be epiphytic, for example*, C. lilaceus, C. lateriflorus* and *C. talbotii* (Salman *et al.,* 2015). The diverse species of *Costus* differ in bract, flowers and leaves. *Costus* species are also known to contain steroidal saponin (diosgenin), a well-known starting material for many drugs productions (Shetty *et al*., 2010; Sulakshana and Rani, 2014). It is generally grown for its ornate properties away from sun in well-drained organic soils (Whistler, 2000). It reproduces vegetatively by stem-cutting and division of culms rhizomes but has low seed germination (Merina, 2004). High yields of lipid, protein and carbohydrate are obtained from the rhizome of *Costus* genus and the aerial parts are eaten as vegetable. The rhizomes are principal origin of diosgenin, an anti-diabetic (Burkill, 1985). Sterols and steroidal saponin are abundant in the *Costus* genus (Hegnauer, 1986).

2.2.1 Medicinal uses of *Costus* genus

In homeopathic, folk and ayurvedic methods of medicine, *Costus* species have been found widely useful mostly for its carminative, tonic, stimulant, antiseptic, diuretic and

digestive activities. The extract from the rhizome is useful as a remedy for gall bladder, liver problem, abdominal and chest pain (Sivarajan and Balachandran, 1994; Joshi, 2000).

Costus species are significant medicinal plants, serving as a basis of antihyperglycemic and antimicrobial agents (Joshi, 2000; Behera *et al.,* 2016). According to Habsah *et al*. (2000), dichloromethane and methanol extracts of *Costus, Zingiber* and *Alpine* exhibit extremely high antimicrobial properties. The antibacterial property of *C. igneus* rhizome, flower and leaf, *C. pictus* and *C. speciosus* rhizome extracts was studied by Ambarish *et al*. (2011) and Sulakshana and Rani, (2014). *C. speciosus* rhizome and leaves ethyl acetate extracts have also been reported to display antifungal property (Duraipandiyan and Ignacimuthu, 2011). Malabadi, (2005) also documented both *C. speciosus* rhizomes and leaves methanol, and hexane extracts possessing antibacterial property. *Costus igneus* and *C. pictus* are recognised as insulin plants and also are useful in India folk medicine for curing diabetes (Merina, 2004; Shetty *et al.,* 2010). Rhizomes of *C. speciosus*, which are principal source of diosgenins, curcumins and curcuminoids, have been reported to exhibit various activities like CNS depressant, diuretic, cardiotonic and hydrochloretic (Srivastava *et al.,* 2011; Prabhu *et al.,* 2014). Hypotensive along with spasmolytic activities of *C. speciosus* seeds saponins have been documented (Mahmood *et al*., 1984).

Jasmin and Narasimhacharya, (2008) documented the anti-hyperlipidemic and anti-hyperglycemic activities of *C. speciosus* root extract. The hypoglycemic activity of methanol leaf extract (Momoh *et al*., 2011), antihyperglycemic property on streptozotocin-induced hyperglycemia, anti-inflammatory property (Singh *et al.,* 1992; Iwu, 1993; Anaga *et al*., 2004) and antioxidant property (Tchamgoue *et al.,* 2015) of *C. afer* have been documented. The stem aqueous extract of *C. afer* is strongly diuretic and infusion of the above ground parts is also used as a remedy for hypertension (Iwu, 1993). Omokhua, (2011) reported the antimalarial action of *C. afer* stem.

This has equally been shown to possess abortifacient property at the last three months of pregnancy, antipyretic potential (Anaga *et al.,* 2004; Odoh *et al.,* 2010) and anti-arthritic properties (Anyasor *et al.,* 2014). It has been documented that *C. afer* possesses antioxidant and anti-inflammatory activity (Soladoye and Oyesika, 2008; Anyasor *et al.,* 2010). Asolkar *et al.* (1992), Singh *et al.* (1992) and Bandara *et al*.

(1998) worked on the antifungal properties of the steroid saponins and sapogenin isolated from *C. speciosus* and antimicrobial property of the rhizome essential oils. Its antihelminthic, anticarcinogenic, anti-inflammatory, antioxidant and antifertility activities are known (Hussein *et al.,* 1992; Singh *et al*., 2008; Vijayalakshmi and Sarada, 2008). Likewise, *C. pictus* aerial parts are known for treating renal disorder and tremendous activities which include anti-inflammatory, antioxidant and anticancer (Martinez, 1996; Merina, 2004; Sathuvan *et al.,* 2012).

Anti-inflammatory properties displayed by *C. Speciosus* and *C. lucanusianus* are known (Hussain *et al.,* 1992; Owolabi *et al.,* 2009). A contraction of rat uterus by *C. lucanusianus* leaf aqueous extract has been reported by (Owolabi *et al*., 2010). Though, Foungbe *et al.* (1987) documented the uterine relaxant property of the oxytocin-induced contraction in isolated rat uterus. *C. lucanusianus* is famous in Southern Ivory Coast because of its anti-abortive properties (Sawadogo, 1986) and the stem juice is known to demonstrate tocolytic property (Komenan, 1986; Foungbe *et al*, 1987). Kagbo and Obinna, (2017) reported the antifertility effect of *C. lucanusianus* stem extract in male albino rats. Cold aqueous methanol extracts *C. lucanusianus* leaf showed antimicrobial activities at concentrations of 20 mg/mL (Baba and Onanuga, 2011). The antihyperglycemic, renoprotective, antidiarrhoeal and hepatoprotective use of the leaf aqueous extract of *C. lucanusianus* have been documented (Owolabi *et al.,* 2007; Saliu and Fapounda, 2016). An infusion of *Costus pictus* is used to treat renal disorders (Martinez, 1996). Antihelmintic, antifertility activities of *C. speciosus* (Hussain *et al*., 1992) and antioxidant property of the leaves extracts (Vijayalakshmi and Sandra, 2008) have been reported.

Inflorescence infusion of *C. lucanusianus* is applied for the treatment of tachycardia and stomach problem. Ezerioha and Kagbo, (2017) documented the antimalarial property of *C. lucanusianus* methanolic root extract in the remedy of malaria. Weesam *et al.* (2011) documented the cytotoxic property of *C. malortieeanus* root methanol extract. Quintans *et al. (*2010) have also documented *C. spicatus* to possess antinociceptive and anti-inflammatory properties. Beta-amyrin isolated from *C. igneus* leaves was documented to exhibit anti-inflammatory action (Krishnan *et al.,* 2014). The above ground parts and roots of *C. tonkinensis* contained Alpha and Beta-amyrin acetate (Frank *et al*., 1997). Muscle relaxant and antispasmodic properties of an

alkaloid obtained from *C. speciosus* rhizomes in laboratory animals were reported (Srivastava *et al.,* 2011).

2.2.2 Non-ethnomedicinal use of *Costus lucanusianus*

The stem sap with lime juice has been reported to be used to coagulate Landophia latex in the southern part of Nigeria (Burkill, 1985). In Ivory Coast, the plant is considered fetish and is believed to be able to confer protection against epidemics and to repel evil spirits. The sap is also used in Gabon as a part of benediction rituals. It has been made known in Southern America and in the United States as an ornamental plant (Burkill, 1985). Kayode *et al*. (2009) stated the usage of *C. lucanusianus* leaves in the preservation of bitter kola. The fruits, tender young shoots and rhizome of *Costus* species are eaten as vegetable. The tubers are cooked into syrup and preserved in some parts of India (Nadkarni, 2009).

2.2.3 Isolated compounds from *Costus* genus

Phytochemical reports on *Costus* genus indicated that it is rich in sapogenins, oxalates, steroidal saponins, furan and their derivatives, and starches (Oliver, 1986). Some isolated phytochemicals from *Costus* genus are shown in Figure 2.1-2.18. Steroidal sapogenin 2.1, quercetin 2.2 and diosgenin 2.3 were obtained from the rhizomes of *C. igneus* (Kalailingam *et al.,* 2011). Thambi and Shafi*,* (2015) reported the antimicrobial property of a cyclic sesquiterpenoid, zerumbone 2.4 and anti-inflammatory activity of Humulene 2.5 from essential oils isolated from the rhizome of *C. speciosus*.

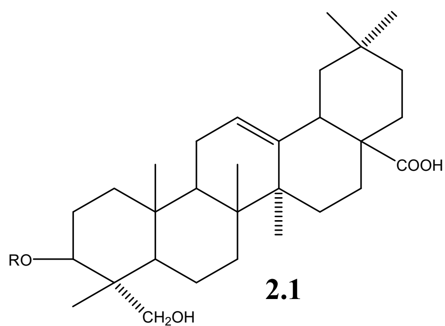
Tigogenin 2.6, dioscin 2.7, gracillin 2.8, β-sitosterol glycosides and diosgenin (2.6%) were isolated from *C. speciosus* rhizome (Gupta *et al.,* 2008) and sitosterol 2.9, D-glucose 2.10 and quinines 2.11 from the roots and tubers (Mahato *et al.,* 1980). According to Duraipandiyan *et al.* (2012), sesquiterpene lactones, costunolide 2.12 and eremanthin 2.13 obtained from *C. speciosus* rhizome hexane extract displayed antimicrobial and antioxidant properties. Likewisely, Lupeol 2.14 and Stigmasterol 2.15 were obtained from the stem of *C. igneus* (Manjula *et al.,* 2012), while from the rhizomes were quercetin 2.16 and diosgenin isolated (Kalailingam *et al.,* 2011). Frank *et al*. (1997*)* also isolated from *C. tonkinensis* α- amyrin 2.17 and β-amyrin 2.18.

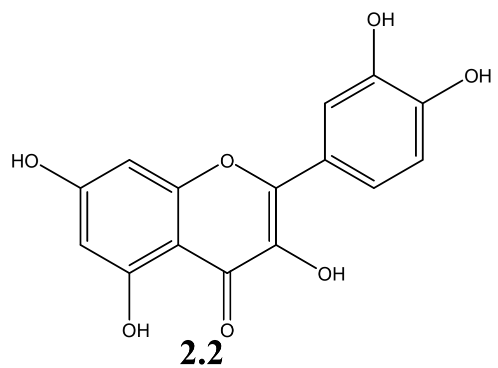
Other compounds isolated from *Costus speciosus* are α-amyrinsterate, lupeol and β-amyrin from the rhizome and palmitates from the leaves (Srivastava *et al.,* 2011).

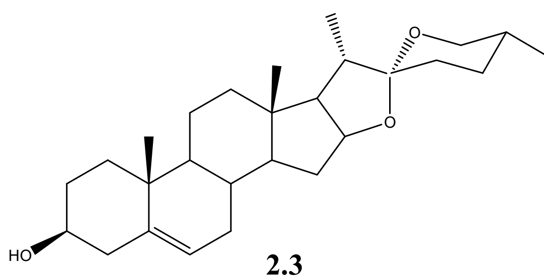
Five compounds:- 14-oxo heptacosanoic acid, tetradecyl 13-methylpentadecanoate, 15-oxo octacosanoic acid, 14-oxo tricosanoic acid and tetradecyl-11-methyl tri decanoate were also obtained from *C. speciosus* rhizomes (Gupta *et al.,* 1981). Bis-(2-ethylhexyl) phthalate (BDE) was found in the rhizome extracts of *C. speciosus* and *C. pictus,* butanol fraction of *C. afer* leaf and ether fraction of *C. pictus* leaf (Farooqui and Shukla, 1987; Rastogi and Mehrotra, 2004; George *et al.,* 2007; Anyasor *et al*., 2014). *Costus speciosus* rhizome contains aliphatic hydroxyl ketone, oxo- acids, triterpene, fatty acids, starch mucilage, corticosteroids and steroids as well as alkaloids and saponins, like tigogenin, sapogenin and diosgenin (Rajesh *et al.,* 2009; Dubey *et al*., 2010; Muniyandi *et al.,* 2013).

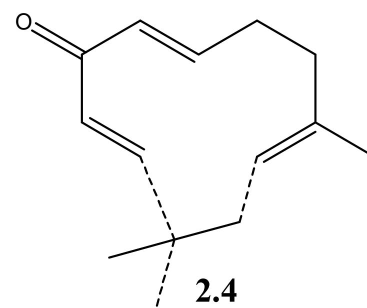
Costus speciosus seeds contained quinones, dihydro phytyl plastoquinone along with the 6-methyl derivatives and α- tocopherolquinone (Gupta *et al.,* 1981; Srivastava *et al.,* 2011), also cycloartanol, 31-norcycloartanone cycloartenol and cyclolaudenol in its roots (Rastogi and Mehrotra, 1998); and in the rhizomes, Methyl 3-(4-hydroxyphenyl)-2E propenoate (Srivastava *et al*., 2011). Oleic acid, palmitic acid, stearic acid, arachidic acids are some of the seed fatty acids. The defatted seeds have been reported to contain galactose, diosgenin, rhamnose and glucose (Rastogi and Mehrotra, 2004).

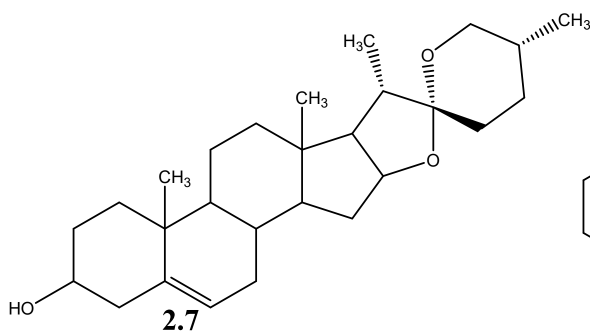
β-sitosterol-β-D-glucoside was obtained from *C. lacerus* rhizome ethanolic extract along with dioscin, prosallogenin A, and gracillin (Prawat *et al*., 1989). *Costus igneus* contains essential oils, resinoids and three alkaloids-inulin, resin and saussurine (Nagarajan *et al.,* 2011). *C. pictus* leaves which are sour in taste, have been reported to contain oxalic acid (Sathiraj and Augustin, 2011). The rhizome of a related species in Africa, *C. afer* contains many sapogenins, notably diosgenin, costugenin and stigmasterol (Iwu, 2014). Asekun and Adeniyi, (2003) identified sesquilavandulyl acetate, 3-caropyllene, and Z-, E-farnesol in *C. afer* leaves essential oils.

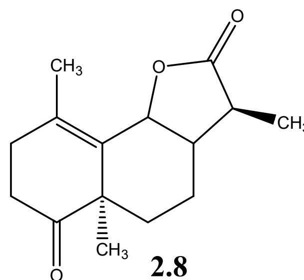




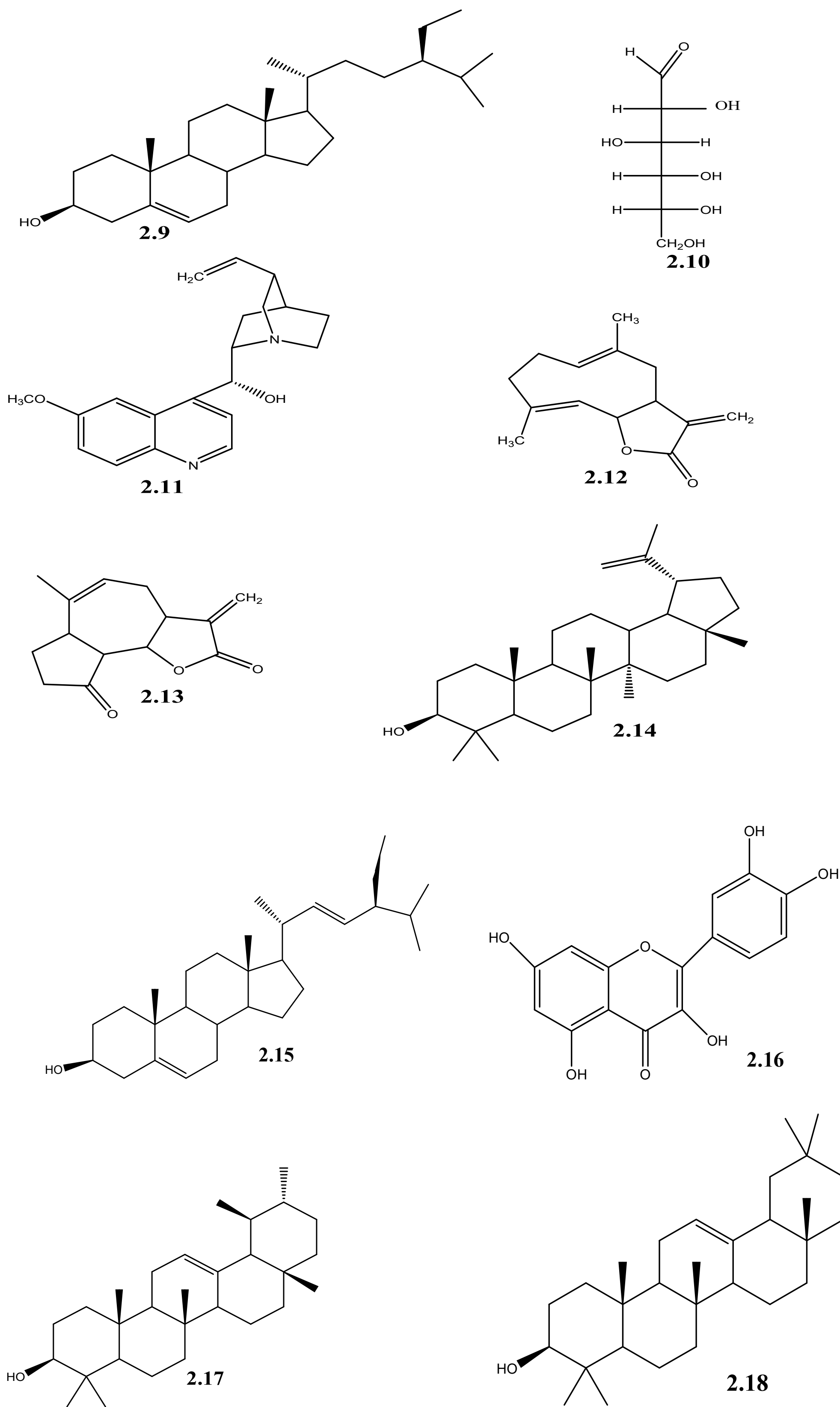








Figures 2.1-2.8: Some Isolated compounds from *Costus* genus.



Figures 2.9-2.18 Some Isolated compounds from *Costus* genus.

2.3 Plant Secondary Metabolites

These are products of secondary metabolism that takes place in plants. Secondary metabolites, like phenolic, steroids, terpenoids, and alkaloids compounds, independently or jointly, are accountable for the medicinal properties of plant materials. These are produced and deposited in the plant parts (Talib, 2011). Plants also produce a range of chemical compounds which are useful in performing important biological functions as well as to act as protector against the attack of predators, like insects, fungi and man (Lai and Roy, 2004). Secondary metabolites, depending on their biosynthetic basis, can be categorized as terpenoids and essential oils (mono-, sesqui-, di-, tri-, and tetra-), alkaloids (compounds derived from plant origin having nitrogen in the ring and phenolic (flavonoids, tannin and phenyl propanoids) (Bakkali *et al.,* 2008). The main categories of phytochemicals that possess antimicrobial properties are terpenoids, steroids, and alkaloids (Pandey and Kumar, 2013).

2.3.1 Alkaloids

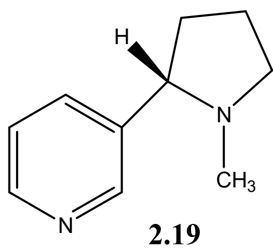
The category of organic compounds with basic nitrogen atoms is called Alkaloids, most of which are solids with a bitter taste (Liang *et al*., 2017). Generally, the position of the nitrogen atoms determines the properties of the alkaloids. Apart from nitrogen, oxygen and hydrogen, sulfur and rare elements, like phosphorus, bromine and chlorine may be present in alkaloids. Oxygen-free alkaloids (for example nicotine) are colourless and volatile oily liquids. Berberine (yellow) and sanguinarine (orange) are examples of coloured alkaloids. Limited alkaloids are present as glycoside of sugars. Examples of sugar found in solanum and veratrum groups are galactose, rhamnose and glucose. Other alkaloids, such as tropane, veratrum and aconitine groups, occur as an ester of various acids and as amides, like in piperine. Alkaloids are categorized into three major classes grounded on the nitrogen type and their biochemical origin according to Hegnauer's (1988) classification:

1. True alkaloids of amino acids origin and possess heterocyclic ring with nitrogen atoms;

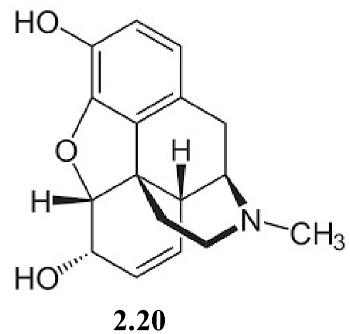
2. Pseudo alkaloids that are not of amino acids origin and possess heterocyclic ring with nitrogen atom; and

3. Proto alkaloids of amino acids origin and of which the heterocyclic ring do not possess nitrogen atom.

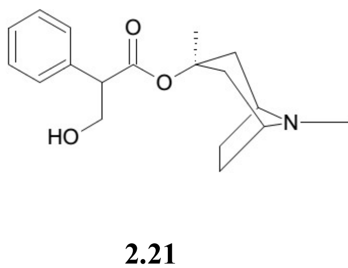
Some alkaloids-containing drugs are shown in Figure 2.19 – 2.22; nicotine 2.19, morphines 2.20, atropine 2.21 and strychnine 2.22.



2.19



2.20



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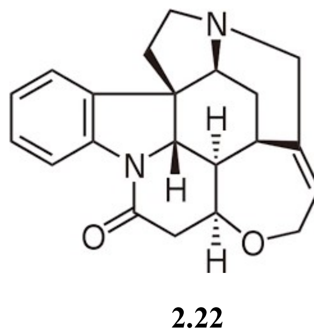


2.22

Figure 2.19-2.22. Some alkaloids containing drugs.

Isolation and identification of alkaloids

The first step in isolation of alkaloids involves the identification of alkaloids in the plant extract with various alkaloidal reagents, such as Mayers (potassium mercuric-iodide: brown precipitate), Dragendorffs (potassium bismuth iodide: reddish brown precipitate), Wangers (iodine-potassium iodide: cream precipitate), Schebler reagent (Phospho-tungstic acid) and Sonnenschein reagent (phosphomolybdic acid). The presence of alkaloids is determined by the appearance of a turbidity or precipitate observed (El-Sakka, 2010).

Solvent extraction with acids, bases or organic solvents is the general method for the isolation of total alkaloids from plant sources. Owing to the fact that alkaloids usually occur as a salt of the common plant acids, the alkaloid bases are separated from the salts by treatment with bases (calcium hydroxide, ammonium hydroxide and sodium hydroxide). After which extraction with organic solvent, such as ether, methylene chloride, methanol, ethanol or chloroform is carried out. Aqueous acid is then added to concentrated organic extract. Alkaloids are separated out as their salts in the aqueous layer.

Acid extraction involves adding of weak acids solutions, such as acetic acid in water to the plant material. Chloroform or other suitable organic solvents are added to the acidic extract remove impurities and pigments. The dried plant material is usually defatted with light petroleum prior to extraction since most alkaloids and their salts are insoluble in petroleum (El-Sakka, 2010).

Chromatography techniques are the most appropriate in the separation of individual alkaloids from complex mixtures. However, the application of ^{1}H NMR, ^{13}C NMR tandem mass spectrometry, Fourier Transform- Infrared (FT-IR), quadrupole time-of-flight tandem mass spectrometry and high-pressure liquid chromatography in the identification of alkaloids has been reported (Rousa and Cook, 1984; Bulduk and Taktak, 2013; Shangguan *et al.,* 2018).

Pharmacological relevance of alkaloids

Alkaloids are among the greatest therapeutically effective and important plant substances. Alkaloids-containing plants were not used frequently in traditional medicine except for external application because they are generally extremely toxic.

Alkaloids frequently possess pharmacological properties. They are usually used in entheogenic rites, as medication and recreation drugs (Cassiano, 2011). Alkaloids isolated from plant along with their synthesized derivatives are known to possess analgesic as well as antispasmodic effects. Chelerythrine, an antibacterial (Cushine *et al*., 2014); homoharringtonine, an anticancer; quinine, an antimalarial; ephedrine, an anti-asthma (Kittakoop *et al.,* 2014); emetine, an antiparasitic; lobeline, an expectorant; reserpine, an anti-hypertensive and piperine, an antihyperglycemic are examples of alkaloid-containing compounds (Qiu *et al.,* 2014). A tropane alkaloid called scopolamine is used in medicine as anticholinergic and pilocarpine alkaloid in the treatment of glaucoma (Cassiano, 2011).

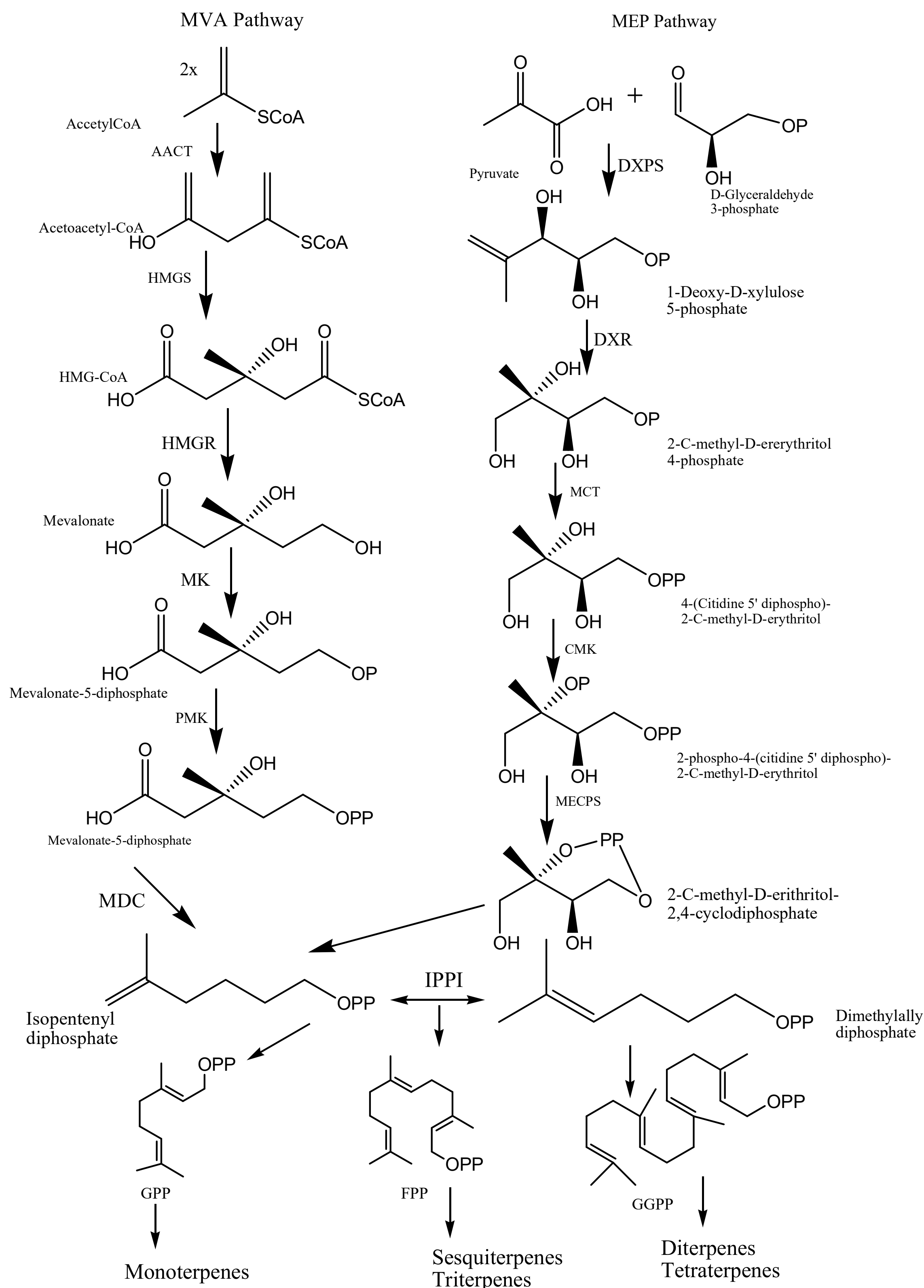
2.3.2 Terpenoids

Terpenoids are known as the major category of compounds obtained from natural source which possess several biological properties, such as activities against bacteria, malaria and cancer. Artemisinin (antimalarial drug) is a famous terpene-based drug. Terpenoids are synthetic products obtained from two 5-carbon isomers, isopentenyl-diphosphate (IPP, C5) and dimethylallyl diphosphate (DMAPP, C5). Biosynthesis pathways for the starting materials (precursors) are Mevalonate and Deoxyxylulose 5-phosphate pathways (Scheme 2.1), which also occurs in certain eubacteria, for example, *E. coli* (Markus and Croteau, 1999).

The two pathways known for the biosyntheses of these universal precursors are represented in Scheme 2.1. The classification of terpenoids into C_5-hemiterpene, C_{10}-mono, C_{15}-sesquiterpene, C_{20}- diterpene, C_{25}- sesterterpene and C_{30}- triterpene is based on the amount of 5-carbon isoprene unit (Singh and Sharma, 2015). Sesterterpenes are the least type of terpenoids from marine and fungi organisms (Dewick, 2002). Eleutherobin is a marine diterpene from *Eleutherobia* sp. (Lindel *et al.,* 1999).

The most significant groups of terpenes are saponins (triterpene glycosides) and triterpenes which have wide biological activities, like antimicrobial, antiviral,

cytotoxic and anticancer. Terpenoids vary in their structures and chemical reactions. This variation is related to the difference in the rearrangement and cyclization reactions of the isoprene unit present (Rohner, 1999).



Scheme 2.1. Biosynthesis of Terpenoids (Mahmoud and Croteau, 2002; Withers and Keasling, 2007).

The cyclization and rearrangement of geranyl pyrophosphate (GPP) result in monocyclic and bicyclic terpenes, while the large (FGPP) bring about a larger number of terpene carbon skeletons (Eisenreich *et al.,* 1998).

The triterpenoids are classified into several groups, such as acyclic, tetracyclic and pentacyclic. The pentacyclic and tetracyclic are the most abundant (Sandjo and Kuete, 2013). The tetracyclic triterpenoids consisting of Tirucallane and Dammarane, among others, are considered as methylated steroids (Gunatilaka, 1986). The basic skeleton of some types of triterpenoids are shown in Figure 2.23 – 2.30. The type of carbon skeletons is a determining factor in dividing pentacyclic triterpenoids into many sub-groups. Examples of pentacyclic triterpenes are lupeol, ursane, oleanane, friedelane, betuline and lupine (Patocka, 2003). The famous constituents of essential oils are monoterpenes (Zhang and Demain, 2007).

Extraction and identification of terpenes

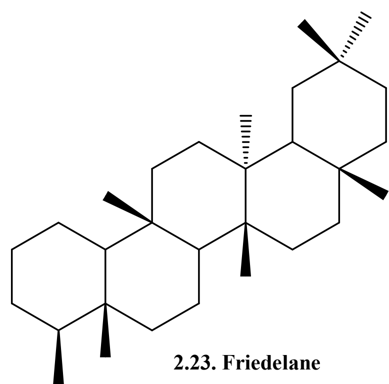
The methods of extraction of terpenes include the following steps:

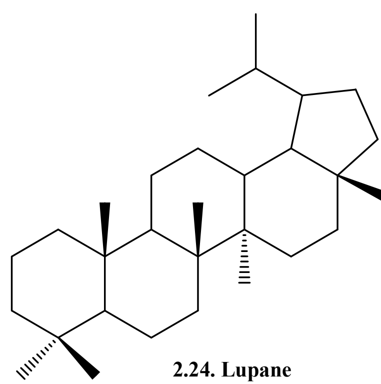
1. extraction of the sample with an appropriate solvent, or through distillation;
2. separation of the desired terpenes from other impurities; and
3. the use of an appropriate method of analysis, like Liquid Chromatography (LC) or Gas Chromatography (GC) to determine the class of terpenes (Jiang *et al.,* 2016).

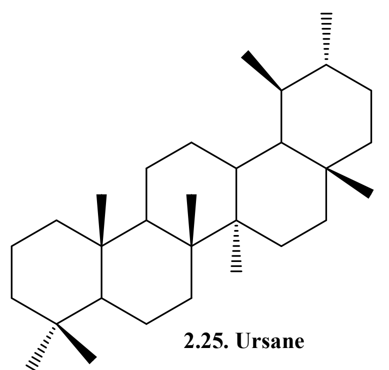
Extraction and identification of volatile terpenes, such as sesquiterpenes and monoterpenes, are done through different methods notably hydrodistillation, microwave-assisted extraction, extraction by organic solvents, or solid phase micro-extraction (Pawliszyn, 2012). The extraction of non-volatile terpenes is carried out using a non-polar organic solvent, like hexane (Jiang *et al.,* 2016).

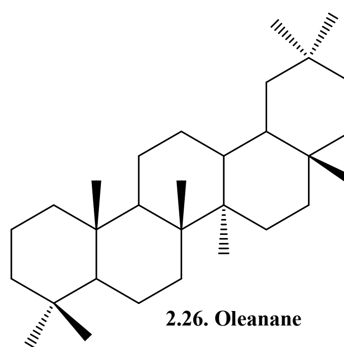
Isolation of triterpenoids

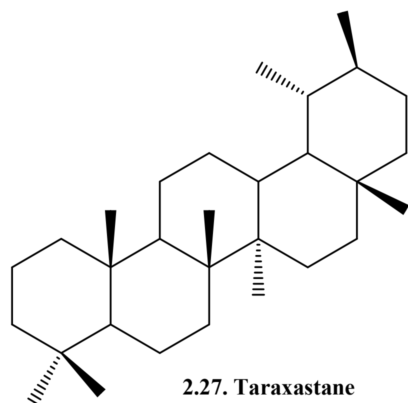
Triterpenoids occur in nature in the free state (sapogenins), as esters or as saponin (bound to glycosides). The extraction of dried plant materials with solvent (non-polar-moderate) followed by purification has been the method used to isolate these triterpenoids. Different methods that have been used for purification include column chromatography over silica gel, preparative thin layer chromatography and precipitation. Sodium hydroxide or sodium carbonate solubility has been used in the fractionation of phenolic and acidic triterpenoids (Gunatilaka, 1986).

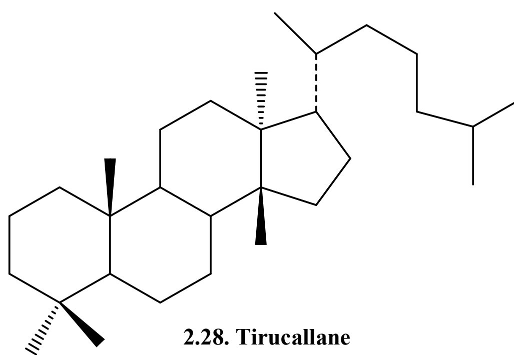


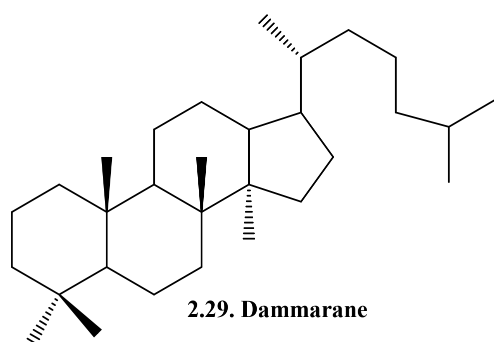












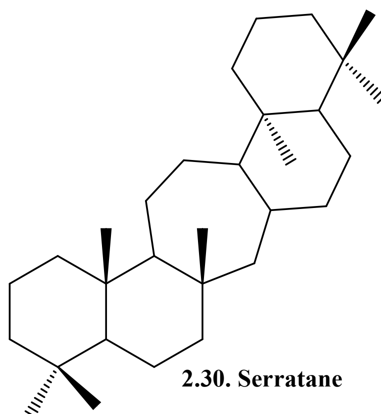


Figure 2.23-2.30 Basic skeleton of some common types of triterpenoids.

Pharmacological relevance of terpenoids

In the production of food, chemical and drug, terpenoids derived from plants have been found useful. Also, in the improvement of biofuel products, terpenoids recently have been reported to be useful (Tholl, 2015). They are a huge collection of natural compounds with various biological properties, amongst which are antimalarial, anti-inflammatory, antiparasitic, analgesic, antibacterial, antiviral and cancer preventive (Singh and Sharma, 2015). In the year 2002, about US$ 12 billion were realised from the global sales of terpene-based drugs. The artemisinin (antimalarial) and taxol, an anticancer drug, are the most popular medications with terpenes constituents (Wang *et al.,* 2005). Terpenoid oils with pungent odour repel insects and prevent fungus. Beta-caryophyllene, a sesquiterpene, is gastro-protective and anti-inflammatory.

2.3.3 Essential oils

Aromatic plants are widely known to possess medicinal, antioxidant and scent properties. Aromatic plants' essential oils possess the capability of preventing the growth of micro-organisms (Burt and Reinder, 2003). Essential oils can be obtained from the whole plant, including, branches, rhizome, leaves, flowers, twigs, heartwood, buds, bark and resin, and these oils consist of varying mixtures of terpenes, such as mono, di- and sesquiterpenes. Molecules such as acids, aliphatic hydrocarbon, aldehydes, acyclic ester or lactones and alcohols may also occur. Compounds comprising of sulphur and nitrogen seldomly occur.

Essential oils in plants function as an attraction of pollinators, dispersal agents, allelopathy, defence against insects and other animals, protection of plants, resin release and as pheromones (Bakkali *et al.,* 2008). Essential oils could be classified into three, namely Top Note, Middle Note and Base Note. Those with a fruity, sharp aroma which tends to evaporate rapidly within a few minutes are reffered to as Top Note Essential Oils. Examples are lemon grass, bay and peppermint while the Middle Note Essential Oils are oils with herbaceous, evergreen aroma which evaporates over a period of an hour.

Examples are pine and lavender. Base Note Essential Oils are oils with scent that tends to be very relaxing and evaporates over several hours. Examples are Nutmeg, Clove and Cinnamon.

Extraction of essential oils

There are different techniques utilized in obtaining plants' essential oils. The adopted methods are dependent on factors such as the plants parts used, susceptibility of the oil components to chemical reactions and stability of the oils to heat. Microwave-assisted process, steam distillation, cold pressing, hydrodistillation, solvent extraction, effleurage and hydrodiffusion are commonly utilised techniques for essential oil extraction (Hamid *et al.,* 2011).

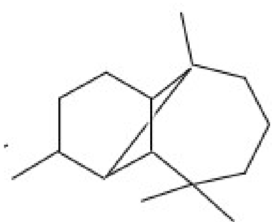
Pharmacological and medicinal relevances of essential oils

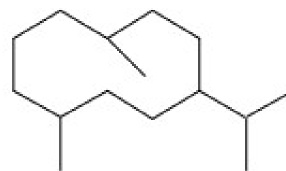
Essential oils display numerous activities among which are antidepressant, relaxant, antimicrobial and stimulant. Essential oils are used as cures for several infectious diseases (pathogenic) such as caused by fungi, bacteria and virus and non-pathogenic diseases such as diabetics and cancer (De Angelis, 2001; Abdollahi *et al.,* 2003; Jirovetz *et al.,* 2005; Ultee *et al.,* 2006). Parts of plant extracted, kind of the soil the plant is grown in, the quality of extraction from the essential oil distillers and the right temperature used during the extraction of the essential oil are the conditions that define the chemical constituents, percentage yields and aromatherapy values of essential oils. Some common examples of cyclic sesquiterpenes from plants are shown in Figure 2.31 – 2.34.

2.3.4 Saponins and sapogenins

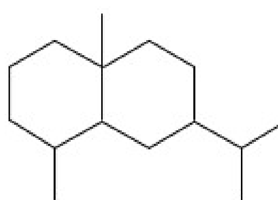
The saponins are glycosides with high molecular-weight. They contain a steroid or a triterpene aglycone joined to sugar unit (s) and known to occur naturally. Saponins are mostly known to be produced by some bacteria, plants and smaller aquatic animals. Many plant drugs and traditional medicines contain saponins (which have detergent properties) and this has resulted in a great interest in investigation and characterization of the pharmacological and biological properties of saponins (Yoshiki *et al*., 1998; Samuelsson, 2004 and Hostettmann and Marston, 2005).

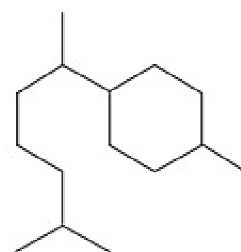
Saponins can have one to three sugar moieties (in straight or branched form) mostly of D-galactose, L-arabinose, L-rhamnose, D-glucuronic acid, D- xylose, D-fucose or D-glucose linked to a sapogenin or non-sugar with hydrophobic nature, which may also be of triterpenoids or steroids type, usually at C-3 (Vincken *et al.,* 2007). They are categorised into three main groups namely steroid alkaloid glycosides, steroid and triterpene glycosides (Figure 2.35 – 2.37) depending on the structure of the sapogenin.





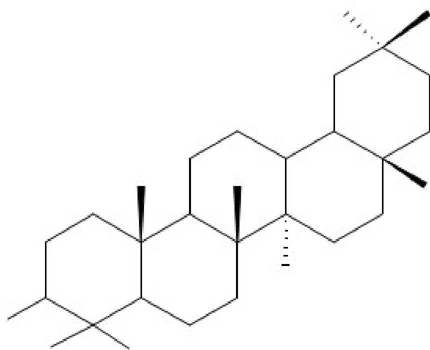
2.31. Longipinane **2.32.Germacrane**



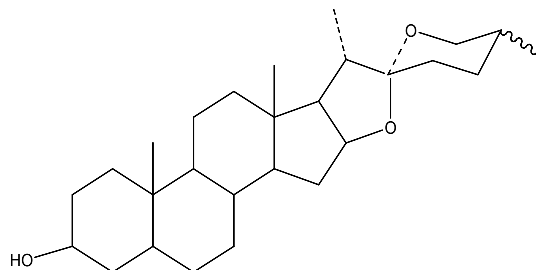


2.33. Eudesmane **2.34. Bisobalane**

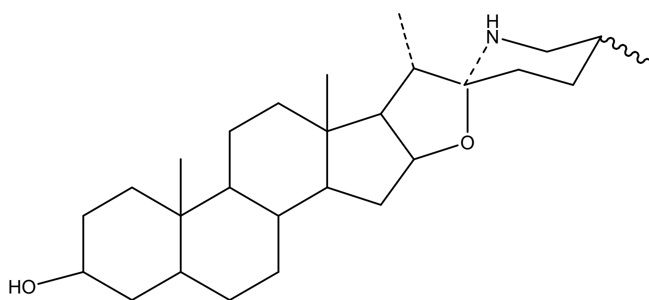
Figures 2.31-2.34: Some types of cyclic sesquiterpenes.



2.35 Tritepene Class



2.36 Steroid Class



2.37 Steroid Alkaloid Class

Figure 2.35-2.37: Different groups of sapogenins present in the three classes of saponin.

The steroid saponin can further be grouped into pseudo-spirostanol, spirostanol, furostanol and iso-spirostanol saponins (Wu and Yao, 2003). The triterpenoid saponins are also further grouped into pentacyclic triterpenoid saponins and tetracyclic triterpenoid saponins. In spirostanol saponin, L-rhamnose and D-xylose generally are found at the terminal positions.

Saponins have a connection of one or more sugar moieties attached to the sapogenin. There is commonly a linkage of one sugar moiety to C-3 position in monodesmosidic saponins while in bidesmosidic the two sugar moieties are linked to the aglycone at C-3 and C-28 positions. Side chain type, attached moieties connectivity position and variation of the aglycone structure are the factors that determine the complexity of saponin structure (Riguera, 1997; Hostettmann and Marston, 2005).

Steroids

A sterol compound is recognised by its four-membered hydrocarbon ring as shown in Figure 2.38. As shown in Figure 2.39 – 2.42, most steroids are derived from basic hydrocarbons namely gonane (2.39), pregnane (2.40), chloane (2.41) and cholestane (2.42).

The steroids of each class are characterized by the location and nature of the substituent group and the presence of unsaturation. In most cases, steroids have oxygen atom at C-3, which may be in the form of a hydroxyl group. The hydroxyl group may be masked by the formation of a derivative in some cases. These derivatives are water-soluble and very polar. A carbonyl (ketone) is another oxygen function commonly found at C-3 of steroids. Possible sites for hydroxylation and addition of carbonyl groups are C-3, C-11, C-17 and C-21. Unsaturation (double bond) is also commonly found at 4, 5- or 5, 6- positions.

Steroidal saponins

C-27 steroidal saponins are famous precursors utilised in the production of steroidal hormones. This is made up of a C-27 steroidal sapogenin attached to one or more monosaccharides sugar moiety. Steroidal saponins are grouped into two groups, namely furostanol saponins and spirostanol saponins.

The spirostanol saponins have a hexacyclic A, B, C, D, E and F ring system (Figure 2.43) while the furostanol saponins possess a skeleton with an open F-ring (Figure 2.44). Furostanol saponins are considered as precursors for spirostanol biosynthesis (Yang *et al*., 2006).

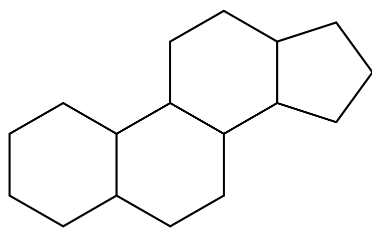
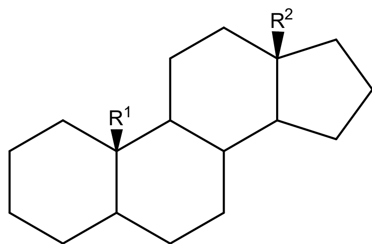


Figure 2.38 Structure of four-membered hydrocarbon ring of a sterol compound.



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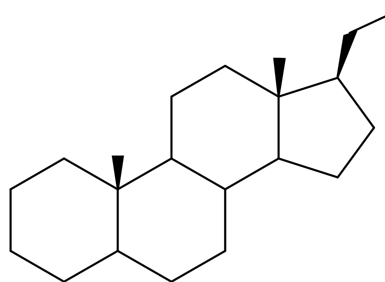

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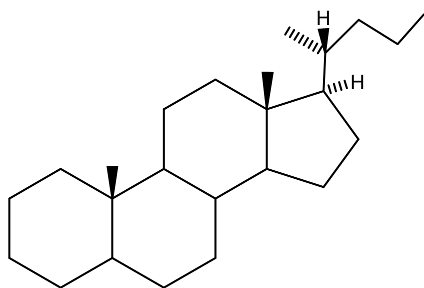

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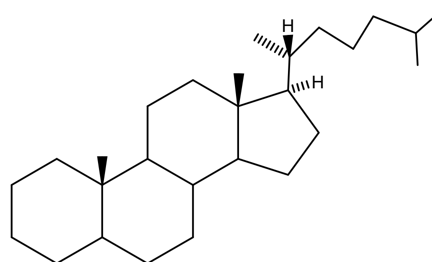
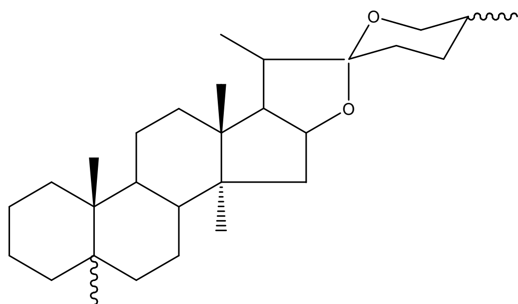
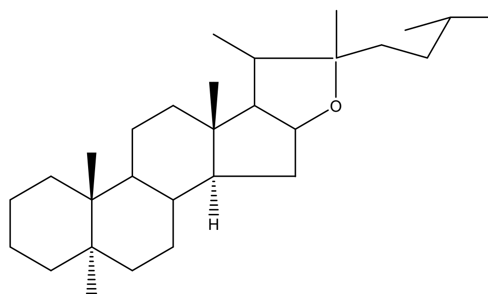


2.42

Figure 2.39-2.42: Some basic hydrocarbon skeleton of steroids





2.43 **2.44**

Figure 2.43-2.44: Basic skeletons of spirostanol and furostanol saponins

Biological properties of saponins

Saponins are known to possess numerous biological properties. The chemical structure of saponin: hydrophilic sugar and the lipophilic sapogenin, determines most of these properties. These activities are the reaction of cell membranes and saponins. Saponins exhibit diverse range of biological activities (Hostettmann and Marston, 2005). Saponins possess an extensive range of pharmacological properties, like hypoglycaemic, anti-inflammatory, antiparasitic, antifungal, expectorant, antiviral, anticancer and antimicrobial (Simoes *et al.,* 1999; Shibata, 2001; Quiroga *et al*., 2001; Kwak *et al*., 2003; Sparg *et al.,* 2004).

Isolation and identification of saponins

Reversed-phase chromatography, silica chromatography, and macro-reticular resin chromatography are the usual techniques for the isolation of saponins (Yang *et al.*, 2003). Different methods of characterisation of saponins comprise of acid hydrolysis, followed by characterisation of the sugar moieties and aglycone and use of Infra-red and ^{13}C NMR, among others. However, the hydrolysis method results in the cyclisation of furostanol to spirostanol aglycone, while the NMR method needs a purification of the samples (Agrawal *et al.,* 1995; Agrawal *et al.,* 2005).

Infra-red spectroscopy, which shows several strong bands between 875 and 1350 cm^{-1} as properties of spiroskeletal side chain, has been reported as a suitable instrument in the identification of steroid sapogenins. Electrospray ionization (ESI) is also a powerful instrument in determining saponins molecular weights. This technique, in connection with collision-induced dissociation (CID), can help to identify the site glycosidic linkage of the saponin to the backbone (Li *et al.,* 2005; Ha *et al.,* 2006).

2.3.5 Sugar derivatives

The hydroxyl groups in sugar moiety can undergo different reactions to produce different sugar derivatives. The hydroxyl group at C-1 (anomeric carbon) undergoes oxidation and glycosylation reactions, while the hydroxyl groups at other positions other than anomeric carbon undergo esterification, an amino group replacing the hydroxyl group at C-2 to form amino sugar, dehydroxylation, epoxy formation, methylation and esterification. Amino sugar, deoxy sugar, acidic sugar, sugar alcohol, glycosylamines, and sugar-phosphate are examples of sugar derivatives.

Most sugar derivatives exist naturally and possess functions and characteristics different from usual sugars.

Amino sugars

These are sugar molecules which have the C-2 hydroxyl unit replaced with an amine (NH_2). Several amino sugars have been isolated and recognised as constituents of antibiotics and examples include sialic acid, galactosamine (2-amino-2-deoxy-D-galactose) and D-glucosamine (Figure 2.45 – 2.47).

Deoxy sugars

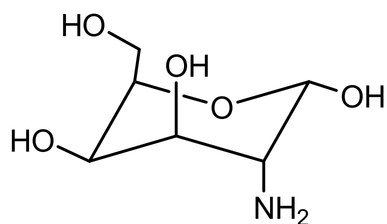
Deoxy sugars are formed by the replacement of hydroxyl group at a particular position by a hydrogen atom. Common examples are deoxyribose (2-deoxy-D-ribose), L-fucose (6-deoxy-L-galactose), deoxyxylose and L-rhamnose (6-deoxy-L-mannose) (Figure 2.48 – 2.51). In some cases, there is a replacement of two or three hydroxyl groups with hydrogen atoms forming dideoxy and trideoxy sugars respectively (Carey and Neil, 2011).

2.4 Structural Elucidation of Isolated Plant Secondary Metabolites

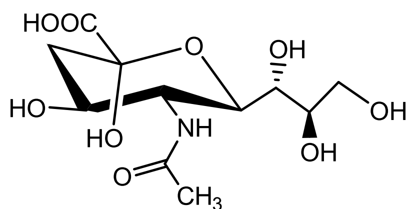
Several 1D and 2D nuclear magnetic resonance (NMR) experiments are widely utilised in the characterization of pentacyclic triterpenes. These techniques include ^{13}C-NMR and ^{1}H-NMR, Attached Proton Test (APT), Distortionless Enhancement by Polarization Transfer (DEPT), Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Correlation (HSQC), Heteronuclear Multiple Quantum Coherence (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC).

2.4.1 Nuclear Magnetic Resonance (NMR) spectroscopy

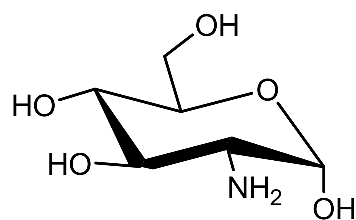
Compounds identification by the means of NMR is centred on the information obtained by the electronic, chemical, physical and structural details about the molecules. These factors are due to their Zeeman shift effect or chemical shift, or a combination of both effects on the existing nuclei resonant frequencies in the compound. The NMR is also known as a physical occurrence which involves the absorbance and re-emittance of electromagnetic radiation by nuclei in a magnetic field.



2.45. Galactosamine

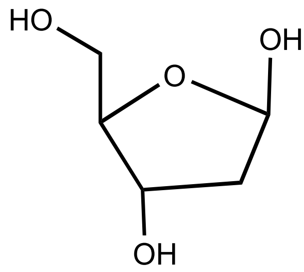


2.46. Sialic acid (Beta-N-Acetylneuraminic Acid.

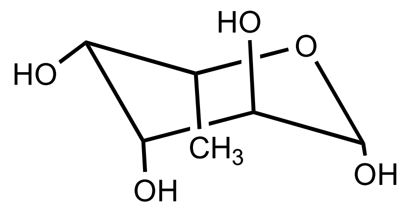


2.47. Alpha-D-glucosamine

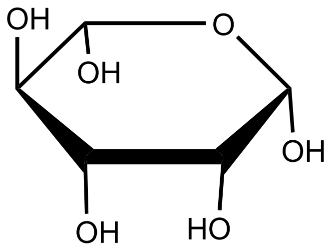
Figure 2.45-2.47: Some types of amino sugars.



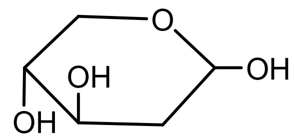
2.48. Deoxyribose



2.49. Fucose



2.50. Rhamnose



2.51. Deoxyxylose

Figure 2.48-2.51: Common deoxy sugars.

Generally, NMR principle comprises of two successive steps:

1. Aligning the nuclear magnetic spins in a continuous magnetic field applied; and
2. Disturbance of the alignment of the nuclear magnetic spins by an electromagnetic

force, such as radio frequency pulse. The nuclei type and static magnetic field determines the frequency required. Two-dimensional and three-dimensional techniques have been used to improve the effectiveness of NMR (Mahato and Kundu, 1994).

^{1}H NMR spectroscopy

This involves the use of NMR spectroscopy with reference to the ^{1}H nuclei situated within the molecule. It identifies the carbon-hydrogen framework of an organic compound. The spectra give information about the functional groups as well as atom positions in the molecule. The ^{1}H NMR spectrum supplies structural information, such as the number of signals, splitting of signals, chemical shifts and the position of signals. Solvents like CCl_4 without hydrogen may be used (Bruice, 2006).

^{13}C NMR spectroscopy

Carbon atoms of organic molecules are determined by the use of NMR-13C. The compositions of organic compounds with high molecular weights are determined by the use of ^{13}C NMR (Caytan *et al.,* 2007). ^{13}C NMR identifies solely ^{13}C isotope. The ^{13}C NMR and ^{1}H NMR are used for molecular structure elucidation (Silverstein *et al*., 1991).

Correlation Spectroscopy (COSY)

This is a 2D experiment useful in the identification of nuclei that share a scalar (J) coupling. This is also usually used to analyze coupling connections between protons. This may also be useful in the correlation of high-abundance homonuclear spin (Mahato and Kundu, 1994).

Heteronuclear Single Quantum Correlation (HSQC)

This is another 2D proton-detected experiment. It is similarly related to heteronuclear multiple quantum correlation (HMQC) by providing the same information on one bond H-X correlations. HSQC experiment is majorly used for the enhancement of resolution

that is acquired in the X-dimension. The advantage of HSQC over HMQC experiment is in the absence of broadened resonances obtained by homonuclear proton coupling in HSQC.

Distortionless Enhancement by Polarisation Transfer (DEPT)

This pseudo-2D NMR experiment has chemical shifts present on one axis and on the other is the self-diffusion coefficient of the solutes. The DEPT experiment distinguishes among CH_3, CH_2 and CH groups by changing the selected angle parameter. Signals from carbons without attached protons or quarternary carbons are always not present (Caytan *et al.,* 2007).

Heteronuclear Multiple Bond Correlation (HMBC)

HMBC provides information about the chemical shifts of carbons that are about 2-3 bonds away from the proton to which they correlates. Hence they are useful in locating the quaternary carbons and in determining long-range ^{1}H-^{13}C connectivities.

Heteronuclear Multiple Quantum Correlation (HMQC)

In HMQC, only protons directly bonded to ^{13}C nuclei produce cross peaks. This also allows the pairing of NH or CH resonances.

2.4.2 Mass Spectroscopy (MS)

The afore-mentioned methods of analysis are non-destructive techniques. The principle involved is centred on absorption of electromagnetic radiation, transformation of molecules from low state energy to an excited state energy level and finally back to the ground state level. However, the concept in mass spectroscopy involves the formation and use of ions rather than molecules. The conversion of ions to molecules is impossible. Therefore, it is generally referred to as a destructive method of analysis (Jones, 2000).

Basic principles of mass spectroscopy

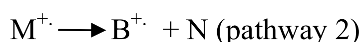
The mass spectroscopy elementary principle involves the production of ions from molecules or atoms by any appropiate techniques, such as thermally, or by the impact of energetic electrons, ions or by an electric field. The ions produced may be molecules, single ionized atoms, clusters and/ their fragments. The separation of the

ions is centred on the mass-to-charge ratios (m/z) of the ions and finally the ions are detected quantitatively or qualitatively by their relative m/z abundance. The separation of ions is effected by magnetic fields, static or dynamic electric field. It requires very small quantities and a very low concentration of compounds for identification (Price, 1991).

Ionization and fragmentation

The molecular ions ($M^{+\cdot}$) derived fragment into new products, namely a neutral particle and a fragmentation ion through two fragmentation pathways.

$M^{+\cdot} \longrightarrow A^{+} + N^{\cdot}$ (Pathway 1) Fragmentation to an even-electron-fragment cation and an odd –electron neutral species



In the two mechanisms, the positive charge and odd electron status must be conserved.

Techniques in mass spectroscopy

Several techniques have been used in mass spectroscopy. These include Electron Impact (EI), Field Desorption (FD), Chemical Ionization (CI), Field Ionization (FI), Matrix-Assisted Laser Desorption Ionization (MALDI) and Fast-Atom-Bombardment (FAB) (Barber *et al*., 1981; Munson, 2000).

Quadrupole-Time-of- Flight (TOF) Mass Spectrometry (QqToF)

This technique is widely recognised by the analyticals as a strong instrument with exceptional properties of combining the great efficiency of time-of-flight analysis in tandem MS (MS/MS) and Mass Spectrometry (MS) modes with the ionisation techniques of Atmospheric Pressure Chemical Ionization (APCI) and Electrospray Ionization (ESI). The precursor and product ions high mass accurateness and high sensitivity have led to their wide application in nanospray analysis of biological samples, analysis of peptides and liquid chromatography of drugs (Morris *et al.,* 1996).

The QqToF comprises of three quadrupoles namely Q0, Q1, and Q2 and a reflecting ToF mass analyzer coupled with orthogonal injection of ions. The direction of the collided and cooled ions into the instrument is achieved by Q0 quadrupole (ion guide).

Ions produced from APCI, ESI or a high-pressure electrospray are passed through quadrupole ion guide Q0 into Q1. Either hexapole or quadrupole is useful as a collision cell Q2 and ion guide Q0. Quadrupoles supply both axial and radial collisional restraining of ions motions. Neutral gas molecules (usually nitrogen and argon) are used to thermalize the ions by colliding with them.

In determining the molecular weight of saponins, Electrospray Ionisation (ESI) which is a powerful technique is utilised due to the low levels of sample needed, high sensitivity and rapid analyses time (Ha *et al*., 2006). The combination of Collision-Induced Dissociation (CID) with the ionisation technique helps in the identification of glycosidic linkage sites of saponins (Li *et al*., 2005).

The CID is a process that involves the transfer of energy to an ion by colliding them with a neutral gas, like argon, nitrogen, and helium. The energy transfer results in bond breakages and rearrangement of the chosen ion. Fragmentations of stable gaseous ions are obtained (Gross, 2011).

2.4.3 Gas Chromatography- Mass Spectrometry (GC-MS)

In analysing and quantifying semi-organic and organic volatile compounds, this recognised analytical technique (GC-MS) has been found useful. The separation of mixtures into individual constituents by gas chromatography is achieved by the use of a capillary column that is temperature-controlled, while mass spectroscopy is useful in the identification of different chemical components using their mass spectra. In the determination of the structural elucidation of compounds in relation to their molecular weight, mass spectrometry has been found very valuable (Sparkman, 2000). The peculiar mass spectrum of each compound is compared with a mass spectral database for identification.

There are two methods of ionization:

1. Electron Ionization (EI) and
2. Chemical Ionization (CI)

Electrons at a fixed voltage (70 eV) are projected on the compounds separated as they go through the detector in electron ionization process. The fixed voltage (70 eV) enables for a reproducible fragmentation pattern for the interested analyte and hence

the comparison of data. Fixed voltage also allows the generation of a massive database of compounds.

2.4.4 Infrared spectroscopy (IR)

This involves a variety of methods centred on absorption spectroscopy. It also deals with light that defers from visible light in longer wavelength and lesser frequency. For liquid, solid or gaseous samples, the techniques of infrared spectroscopy involve the use of an instrument called spectrophotometer. This produces an infrared spectrum where on the horizontal axis is the wavelength or frequency and on the vertical axis is the infrared light absorbance. Unit of frequency is reciprocal centimetre (cm^{-1}). Infrared radiation causes the excitation of the bending and stretching mode vibration of covalent bonds in the molecules.

IR spectrum is divided into two parts, 4000-1000 cm^{-1} (functional group regions) and less than 1000 cm^{-1} (fingerprint region). The most useful information obtained from the IR spectrum is the type of existing functional groups within the molecules.

2.5 Urinary Tract Infection (UTI)

This is an invasion of urinary tract tissues:- bladder, kidney and urethra or ureter by microbes. It is widely known as one of the best clinical cases presented for anti-microbial cure in both the primary and secondary health care centers (Obiogbolu *et al.,* 2009). This is also the highest frequently experienced bacterial infection in humans (Nicolle, 2002). Each year, there are over 8.6 million cases and over 1 million admissions in the United States. A prevalence of 39.69% of UTI was reported in the rural community (Okada) in Edo State, Nigeria (Oladeinde *et al*., 2011). In Nigeria, prevalences of 40%-60% have also been reported between the year 2007 and 2018 at different states (Ojo *et al.,* 2007; Otajevwo and Amedu, 2015; Oluwafemi *et al.,* 2018) among others.

Women get it four times as often as men and up to 50% of female experience one occurrence by 32 years of age (Patton *et al.,* 1991; Foxman and Brown, 2003). The increasing resistance of microorganisms to antibiotics is the major factor responsible for the progressive difficulty encountered in Urinary Tract Infection teatment (Foxman *et al*., 2000). In clinical practice, it is among the most frequently encountered infectious diseases that have been tremendously studied (Dulawa, 2004). In women,

UTI is extremely common, as shown by international studies; that one out of five women in her life time experience UTI (Stamm and Norrby, 2001; Behzadi and Behzadi, 2008). A total of 85% of UTIs, are caused by bacteria from human's own vagina or intestine. Uncomplicated UTI means the attack of an anatomically normal urinary tract by a non-resident infectious microbe.

2.5.1 Types of UTI

Urinary tract infections are classified anatomically into two:

1. lower UTI- urethritis and cystitis, and
2. An upper UTI- pyelonephritis and perinephric abscess.

Cystitis: It is an irritation of the lower urinary tract mucosa and it is not invasive. Symptoms are localized in nature and include painful urination, increased rate of urination and leukocytes present in urine (Orenstein and Wong, 1999; Wilson and Gaido, 2004).

Urethritis: This is the inflammation and infection of the urethra only. Transmission is usually through sex.

Pyelonephritis: This infection is as a result of bacteria from the lower urinary tract ascending into the kidney or decending from the lungs to the kidney in patients with pneumonia. This is an invasive disease. Complication includes sepsis and death in some cases (Orenstein and Wong 1999; Wilson and Gaido 2004).

2.5.2 Causes and epidemiology

Urinary Tract Infection-causing pathogens are *Enterococcus aerogenes, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Proteus vulgaris* and *Escherichia coli* (Nicolle, 2008). In complicated cases of UTI, the most common causes of pathogens are *E. coli*, *K. pneumoniae* and *P. aeruginosa* (Wilson and Gaido, 2004). *E. coli* is the greatest infecting organism in patients having uncomplicated UTI (Johnson, 1991). Oladeinde *et al*. (2011) reported *S. aureus* as the main organism responsible for urinary tract infection in males and *E. coli* has been reported to be caused in both sexes.

Uropathogenic bacteria are known to attack and spread within the urinary tract via two important routes, namely the ascending and hematogenous pathways. Infections of the kidney with gram-negative Bacilli via the haematogenous pathway rarely occur. Rate of bacteria present in the urine among males above 75 years are a range of 7 to 10% (Woodford and George, 2011), 2 to 10% in pregnant women (Smail and Vazquez, 2007), 2 to 7% in women of childbearing age and 50% in elderly women in care homes (Dielubanza and Schaeffer, 2011). The microbial attack of the urinary tract tissues could be liable for the Urinary Tract Infections picked up by the community (Da Silva *et al*., 2007).

2.5.3 Symptoms and treatment

Women are more susceptible to cystitis. This is most likely due to the fact that the urethral opening in women is near the bacteria from the vagina and anus. An increasing risk of cystitis has been attributed to the use of diaphragm by women and sexual intercourse (Ronald, 1996). Post-menopausal women are exposed to greater risk of infection due to a decrease of oestrogen and bladder prolapse.

Males experience a rapid increase in UTI at about the age of 50. Cystitis in men is uncommon. It is associated with urinary conditions, such as prostatitis, infected stones and chronic urinary retention (Foxman, 2003).

The most common symptoms of lower UTI are

1. burning with frequent urination and cloudy urine.
2. painful urination (Dysuria)
3. difficulty initiating urination (Hesitancy)
4. pains above the pubic bone or lower back
5. fever, nausea, and vomiting which may be experienced in addition to classic symptoms
6. feeling of incomplete voiding.
7. On rare occasions, the urine may be bloody (Jacqueline and Bettima, 2010).

Fever may be the only symptom of UTI in children (Nicolle, 2008). Untreated UTI can cause a kidney infection and even deadly blood poisoning. Conditions that may be

mistaken for a UTI are vaginitis and sexually transmitted diseases (STDs) that cause vaginal discharge or inflammation.

Urinary Tract Infections that occur due to fungi are typically asymptomatic and the majority of patients do not have common symptoms like dysuria or fever. UTIs due to *Candida albicans* are commonly catheter-related (Richards *et al.,* 2000).

Risk factors of getting UTI: Some of these factors are long use of catheters placed in the bladder of patients, urinary tract abnormality, sexual intercourse for sexually active women, use of diaphragm and diabetes (Wilson and Gaido, 2004). Antibiotics are the usual treatment for UTI but the resistance of extended-spectrum beta-lactamase (ESBL) which is a strain of *E. coli* to many drugs have been reported.The ESBL *Esch. coli* are the causes of more UTI (Sanchez *et al.,* 2012). The resistance of uropathogens to antibacterial agents has contributed to the significant increase of UTI financial problem (Stamm and Norrby, 2001). Some of the factors influencing the selection of anti-microbial agents for the treatment of UTI include medical history, drug allergy, cost and spectrum of activity and result of gram staining (the type of causative organism) (Juan and Steven, 2013).

Some orthodox drugs used in the treatment of UTI are fosfomycin, trometamol, nitrofurantoin monohydrate macrocrystals, ciprofloxacin, levofloxacin, amoxicillin-clavulanate and cefpodoxine (Chamberlain, 2009).

2.5.4 Prevention

1. Avoidance of the use of spermicides containing contraceptives
2. Taking of oral or vaginal oestrogen by post-menopausal women
3. Increasing water intake (Patton *et al*., 1991; Foxman *et al*., 2003).

2.6 Uropathogen Bacteria and Fungi

2.6.1 *Pseudomonas aeruginosa* (*P. aeruginosa*)

This is a gram-negative bacteria also identified as an opportunistic pathogen, and is the commonly known pathogen isolated from patients after one week of hospitalisation. These pathogens are common in water, animals, plants and man. *Pseudomonas aeruginosa* is rarely pathogenic in a healthy person. *P. aeruginosa* infections are complicated, can affect any part of the body and can also lead to death. For example,

ear (otitis), central nervous system (CNS) (meningitis) and gastrointestinal tract, (urinary tract, diarrhoea). The general cause of nosocomial infections, like UTI, pneumoniae and bacteremia is *Psedomonas aeruginosa.*

Pseudomonas aeruginosa infection pathogenesis involves three stages, namely:

1. Colonization after bacterial infection ss
2. Infection locally, and
3. Spreading in the bloodstream and finally general ailment (Pollack, 2000).

2.6.2 *Klebsiella pneumoniae* (*K. pneumoniae*)

This is also another type of gram-negative bacteria which commonly occurs among sick patients. They are found in the human stool and in the human intestine, where they do not cause disease and are responsible for healthcare-associated infections, like meningitis, bloodstream infection (Wilson and Gaido, 2004).

2.6.3 *Staphylococcus aureus* (*S. aureus*)

This is one of the gram-positive bacteria and it is non-moving, small, round-shaped and capable of surviving at various levels of oxygen. It is the most important *Staphylococcus* bacteria in the human, having the capability of causing various skin infections and infections on other organs in the body. *Staphylococcus aureus* invades through broken skin or mucous membrane. *Staphylococcus aureus* causes cystitis and ascending pyelonephritis, which is very uncommon among gram-positive organisms. *Staphylococcus aureus* is transmitted through air droplets (coughing and sneezing), also through direct contact with contaminated objects or bites of infected animals. This has also become resistant to many common antibiotics, like erythromycin and penicillin. About 500,000 patients in American hospitals are affected annually (Chamberlain, 2009). Common medications used to treat UTI caused by *S. aureus* are Cipro oral, Augmentin oral, Bacterium oral.

2.6.4 *Escherichia coli* (*E. coli*)

Escherichia coli live in digestive tracts of Man and animals. Some *E. coli* can cause bloody diarrhoea and other gastrointestinal infections. *E. coli* (0157: H7) may cause anaemia or kidney failure. It causes around 85% of community-acquired infection and approximately 50% of nosocomial infection (Sobel, 2009). *E. coli* has been reported to

possess severe public health implications in several large disease epidermics (Quilliam *et al.,* 2011). Humans are infested with *E. coli* by consuming contaminated water or food. Meningitis in newborn babies and neonates is caused by *E. coli*. Other bacteria responsible for meningitis are *Pseudomonas, Klebsiella* and *Salmonella.*

The greatest common uropathogenic gram-negative bacteria are *E. coli*, also known as Enterobacteriaceae, and *K. pneumoniae* (Behzadi and Behzadi, 2008; Obiogbolu *et al.,* 2009). *Escherichia coli* causes 70-95% upper and lowers UTIs (Stamm and Norrby, 2001). Researchers found that between the years 2000 and 2010, *E. coli* is accountable for 3% to 17% increase in the resistance of drug ciprofloxacin to UTI from all cases and from 17.9% to 24.2% in the case of trimethoprim-sulfamethoxazole (Sanchez *et al*., 2012). The cost of getting the drugs to combat the bacteria is relatively high for people, especially in the third World countries. This resulted in the use and administration of orthodox medication for curing the ailment (Sanchez *et al*., 2012).

2.6.5 *Candida albicans* (*C. albicans*)

The fungi UTI caused by *Candida* genus have tremendously increased in the last few years (Brito *et al.,* 2006; Da Silva *et al.,* 2007). These are very recurrent in women. Urinary Tract Infection which is caused by bacterial pathogens accounts for a total of 93.2% while 6.8% is due to *Candida albicans* (Behzadi *et al*., 2010). The best common cause of fungi infections is *Candida* species (Richard *et al.,* 2000) and the best common form of yeast infection found in the intestinal tract, vagina, mouth and sometimes on the skin and mucous membrane is called *Candida albicans*. The majority of the fungal infection in the urinary tract is due to *Candida albicans* (yeast). With the exception of *candida*, UTIs are rarely due to fungal contagions.

Candida albicans is part of the microbials that act as fungal uropathogens in UTI and exists in the vagina, but in small numbers which are harmless. However, when the number grows too large, a woman may suffer a yeast infection called candidiasis. The most frequent forms of genitourinary candidiasis include balanitis in men (an inflammation of the glans penis, which is generally sexually acquired), vulvovaginal candidiasis (VVC) in women and candiduria in both sexes (Edwards, 1996). Candiduria occurs typically in hospitalized, elderly, immunocompromised patients and neonates. Yeast infections are also common and about 75% of females experience a yeast infection in a lifetime. Yeast infection may be transmitted during sexual

intercourse. However, it is not acknowledged to be a sexually transmitted infection, as it can also affect women who are not sexually active.

Rare fungal diseases, such as *Aspergillosis*, can affect the urinary tract but usually occur as part of disseminated disease in immunocompromised patients, like those with HIV (Foxman, 2003). Lower urinary tract infections caused by fungi occur less regularly in men than in women (Cantillep *et al.,* 1995). The most important uropathogenic-causing candiduria, also known as urinary tract candidiasis in human, is *Candida albicans* (Lundstrum and Sobel, 2001; Abelson *et al.,* 2005; Binelli *et al*., 2006). Candiduria can be asymptomatic or symptomatic (Calltillep *et al.*, 1995). In the last few years, the fungal UTIs produced by *Candida* yeast have increased greatly (Da Silva *et al.*, 2007). Anti-fungal agents such as Clotrimazole, Miconazole, Fluconazole, and Nystatin are used to treat fungal infections.

CHAPTER THREE

3.1 Materials

3.1.1 Reagents and chemicals

The reagents and chemicals utilised were :- ethyl acetate, hexane, ethanoyl chloride, ethanol, chloroform, acetone, olive oil, 20% sodium hydroxide, Dragendorffs reagent, potassium hydroxide, methanol, hydrochloric acid, glacial acetic acid, ferric chloride, Fehling solutions A and B, copper acetate, concentrated tetraoxosulphate VI acid, aqueous silver nitrate, ammonia solution, iodine crystals, potassium permanganate, silica gel F_{254} (pre-coated aluminum sheets, 0.25 mm thick) and silica gel (60-230 mesh, Merk).

3.1.2 Equipment and apparatus

Soxhlet apparatus, water-bath, weighing balance (OAUS, UK), condenser, electric pump, iodine tank, capillary tubes, filter papers, litmus paper, Fourier Transform Infra Red (FT-IR) spectrophotometer (Bruker Platinum ATR Tensor 27), NMR Spectrometer (Bruker Avance III 500 MHz), Bruker Topsin 400 and 600 MHz Spectrometer, Ultraviolet-visible spectrophotometer double beam (UVD 3200), Buchner funnel, rotary evaporator (ROVATOR 1100), separating funnel, melting point apparatus (Gallenkamp, UK), GC-MS (Agilent technologies 5975 series MSD), UV lamp for visualising TLC plates, 254/365 nm and Quadrupole-time-of Flight (ToF) mass spectrometer.

3.1.3 Plant collection and Authentication

C. lucanusianus plant samples were obtained fresh from Akobo in Ibadan, Oyo state, Nigeria on 14th October 2015. The plant was identified at Forestry Research Institute of Nigeria (FRIN) herbarium, Ibadan with voucher number FH110048 and specimen left at the herbarium. The collected inflorescence, leaf, stem and rhizome were dried in

the air for two weeks separately under shade. These were ground separately and kept in airtight containers until use.

3.2 Extraction Procedures

Each of coarse inflorescence, leaf, stem and rhizome (50 g) was cold macerated for 72 hours with 200 mL of 70% aqueous methanol and distilled water separately. Extracts filterations were with Whatman filter paper using Buchner funnel. Concentration of each filterate obtained was subsequently carried out by the use of rotatory evaporator at $40^{o}C$ under reduced pressure (Cowan, 1999). Thereafter, the concentrated methanol extracts were reconstituted by adding distilled water in the ratio 1:2. Subsequently, the non-polar fraction was removed with n-hexane. Utilising rotary evaporator at $40^{o}C$, the defatted fractions were concentrated and further partitioning of the methanol extracts were carried out with ethyl acetate. Rotary evaporator was also used to evaporate solvents from the fractions. The extracts and fractions were put in a desicator over anhydrous sodium sulphate until use. The weights and percentage yields (w/w) of the extracts were determined (Baba and Onanuga, 2011).

More extracts were gotten by hot extraction method using soxhlet apparatus. The extraction of ground inflorescence (830 g), leaves (1.3 kg), stem (4.150 kg) and rhizome (2.70 kg) in a soxhlet apparatus with hexane for 22 hours was carried out. Further extractions of the marcs obtained with methanol for 30 hours were done until the solvent in the thimble became clear. Using separating funnel, the resulting methanol portion was partitioned with ethyl acetate by solvent fractionation method. With the aid of a steam bath, solvents were evaporated from the extracts until almost dryness. The extracts were kept in a desicator over anhydrous sodium sulphate. The weights and percentage yields (w/w) of the extracts were determined.

Comparative Study of the Cold and Hot extracts.

Extracts solution in their individual solvents of extraction/fractionation were obtained and co-spoted on a pre-coated thin layer chromatography (TLC) plates. Different solvent mixtures such as Hexane: ethyl acetate 3: 2, 2: 1 and 1:3; Methanol: ethyl acetate 3:5 and 2:5 were utilised. The UV lamp and iodine vapour were utilised for the visualisation of the spots.

3.3 Qualitative Phytochemical Screening

In preparing the stock solution of each cold extracts; 100 mg of each extract was added to 10 mL of its mother solvents. They were then phytochemically screened in order to determine the presence of secondary metabolites like tannins, alkaloids, flavonoid, saponin, triterpenes, resin, glycosides, reducing sugar, anthraquinone, phlobatannin and phenols using standard methods (Trease and Evans, 1989; Sofowora, 1993).

3.3.1 Test for alkaloids

Some drops of Dragendorff's reagent were added to (0.5 mL) of the extract solution already added into a test-tube. Alkaloid presence was indicated by a color change to orange.

3.3.2 Test for flavonoids

The addition of extract solution (1 mL) to 3 mL of 20% NaOH yielded a yellow solution which turned colorless on adding dilute HCL. Flavonoid was indicated by color change.

3.3.3 Test for saponins

Distilled water was added to the extract solution (0.5 mL) and shaken. Frothing which occured persisted after heating. This provided first evidence of saponins presence. Thereafter, little drops of olive oil were introduced into the solution and shaken very well. A soluble emulsion formed thereby signified the existence of saponins.

3.3.4 Test for tannins

A mixture of few drops of 5% ferric chloride solution and extract solution (1 mL) resulted in colour change to blue-black, indicating the existence of hydrolysable tannins. A further test to confirm the presence of tannin was carried out: 1mL of 10% potassium hydroxide (freshly prepared) was introduced into 0.5 mL of extract solution. A dirty/ash precipitate proved the existence of tannins.

3.3.5 Test for glycosides

Extract solution (0.5 mL) was poured into a test tube, then a mixture of glacial acetic acid (1 mL) and some drops of ferric chloride were added. Thereafter, concentrated sulphuric acid (1 mL) was introduced meticulously along sides of the test tube. The

presence of glycoside was confirmed by the formation of a reddish brown ring below the boundary and greenish upper layer.

3.3.6 Test for anthraquinones (Borntrager's test)

Extract (2 mg) solution in 1 mL of ether-chloroform mixture was prepared. Into the solution were added little drops of 10% hydrochloric acid. Following filteration and addition of 10% NaOH (1 mL) into 1 mL of the filtrate, a red coloration was formed. This established the presence of anthraquinones.

3.3.7 Test for triterpenes (Liebermann Burchard Test)

Concentrated sulphuric acid, chloroform and glacial acetic acid of 0.2 mL each was added into previously prepared extract solution (0.5mL). A purple-pink color specified the existence of triterpenes.

3.3.8 Test for phlobatannin

A solution of 1 mg of the extract in aqueous 1% hydrochloric acid (2 mL) was heated. A red precipitate specifying the existence of phlobatannin was detected.

3.3.9 Test for reducing sugar

Fehling solutions A and B of equal volume (2 mL) were added to extract solution (2 mL). Subsequently, a brick-red precipitate noticed after heating the mixture confirmed that reducing sugar was present.

3.3.10 Test for resin

Mixture of copper acetate solution (1 mL) and the extract solution (1 mL) was shaken vigourously. A green coloration formed specified the presence of resin.

3.3.11 Test for phenol

Ferric chloride (10%) was added to 0.5 mL of extract solution in drops. Blue-black or brown coloration specified the existence of phenol.

3.4 Extraction of Essential Oils from the Inflorescence, Leaf, Stem and Rhizome of *C. lucanusianus*

Each of fresh leaf (325.45 g), inflorescence (416.77 g), stem (596.25 g) and rhizome (265.0 g) of *C. lucanusianus* were chopped and separately subjected to extraction

using hydrodistillation method with Clevenger apparatus for three hours following British Pharmacopoeiae specifications, 1988 with modification. (Jose and Reddy, 2010; Thambi and Shafi, 2015).

Into a 3 L round-bottomed flask containing 850-1500 mL distilled water were the samples added and heated. There was an evaporation of the essential oil along with water vapour which was then collected using diethyl ether in a condenser. The separation of the lower phase from the upper phase containing the essential oils in diethyl ether (DEE) was done. The DEE was evaporated from the oils, and anhydrous sodium sulphate was used for drying the oils isolated. Extracted oils were conserved in a sealed sample tube at 4 ^{0}C until analyses (British Pharmacopoeiea, 1988; Jose and Reddy, 2010). The percentage yields (w/w) were determined.

3.5 Antibacterial and Antifungal Assay of Essential oils and Plant Extracts using Agar Difussion Method

3.5.1 Test microorganism

Clinical strains were supplied by the Pharmaceutical Microbiology Laboratory, University of Ibadan. Six bacteria strains used were*, Salmonellae typhi (S. typhi), Pseudomonas aeruginosa (P. aeruginosa), Staphylococcus aureus (S. aureus), Escherichia coli (E. coli)*, *Bacillus subtilis (B. subtilis)* and *Klebsiella pneumonia (K. pneumoniae).* While *Penicillium notatum (P. notatum), Candida albicans (C. albicans), Aspergillus niger* (*A. niger*) and *Rhizopus stolonifer* (*R. Stolonifer*) were the four fungi strains used.

3.5.2 Sample preparation of graded concentration of the extracts and essential oils

Extracts (1.0 mg each) was added into 10 mL of methanol for proper dilution. From the solution, 5 mL was taken and added to another 5 mL of the solvent. This process was repeated up to the 4th test tube while the 5th and 6th test tubes were negative and positive controls respectively. The positive control for bacteria was gentamicin 10 µg/mL while that for the fungi was tioconazole 70%. The same procedure was used to prepare different concentrations of essential oils. Four concentrations of the essential oils and crude extracts (12.5 µg/mL-100 µg/mL) were utilised for the experiment.

Positive control used for the essential oils was the same as in the extracts while Dimethylsulphoxide (DMSO) was the negative control used.

3.5.3 Agar Well Diffusion Assay

Preparation of inoculums

The strains were preserved as stock strains in 50% glycerol and sustained at 33°C until resuscitation. The resuscitation of the organisms was carried out by obtaining a loop full of each glycerol stock-culture and inoculating into a tube of 5 mL each sterile Mueller-Hinton Broth (MHB) and vortexed carefully well, following this was incubation at 37°C for 24 hours.

Standardisation of the bacterial suspension (inoculum) was performed with sterile distilled water to 10^8 CFU/mL (turbidity= McFarland barium sulphate 0.5). The preparation of an initial 1:100 dilution of the organisms was achieved by adding 0.1 mL of the overnight culture to sterile distilled water (9.9 mL). Thereafter, 0.2 mL of the solution of the organism was taken and added into sterile nutrient agar (20 mL) which was at 45°C.

Pour Plate Method (Bacteria)

The mixture of sterile nutrient agar and the organisms were carefully transferred into sterile petri dishes (60 x 15 mm) and solidification for approximately 45 to 60 minutes took place. Thereafter, into the solidified nutrient Agar (8 mm diameter) wells were made by making use of a sterile cork borer in accordance with the number of different concentrations (12.5 µg/mL-100 µg/mL) of the samples and controls. Different graded concentrations of the sample were poured into each well ensuring that no spillage occurred. This experiment was done in triplicates and the plates were set aside on the table for 2 hours to permit pre-diffusion. Subsequently, at 37°C the plates were incubated for 24 hours. By means of a metre ruler, the observed clear Inhibition Zone Diameter (IZD) were measured in millimetres (mm) (Perez *et al.,* 1990; Duraipandiyan *et al*., 2012).

Surface Plate Method (Fungi)

The sterile Sabouraud Dextrose Agar (SDA) (62 g/L) prepared was allowed to solidify for 45 minutes in sterile plates in duplicate. All the surface of the agar was covered

with 0.2 mL of the 10^{-2} dilution of the organism using a sterile spreader. Wells were made inside the set plates using 8 mm diameter sterile cork borer.

The extracts of different concentrations and the control (tioconazole 70%) were poured into the wells and there was a perfect diffusion of the extract into the agar for 120 minutes. Thereafter, the incubation of the plates uprightly for 48 hours at 28°C in the incubator took place. The experiment was done in triplicate. The Inhibition Zone Diameters (IZDs) in mm were measured.

3.6 Column Chromatography Seperation of *Costus lucanusianus* Plant Extracts

Monitoring of the fractions obtained with TLC on pre-coated plates was with the aid of iodine, spraying the plates with potassium permaganate ($KMnO_4$) followed by heating in the oven at 100°C and ultra-violet light (254 and 365 nm).

3.6.1 Column Chromatography Separation of the Leaf Ethyl Acetate Fraction

Silica gel (Merk 60-200 mesh, 300 g, 32.8 mm x 60 mm) was utilised for purifying ethyl acetate fraction (10 g) by making use of a stepwise increase of ethyl acetate in hexane solvent system of (5%, 10%, 20%, 30%, 50%, 70% and 100%) and finally with 5% methanol in ethyl acetate. An aggregate of 187 fractions containing 100 mL each were obtained from the column. A pre-coated silica gel plate and different concentrations of ethyl acetate in hexane (5:1, 4:1, 3:2, 3:1, 2:3 and 1:5) were utilised for the TLC analyses. Fractions having identical R_f values in TLC pattern (which were monitored using iodine vapour, and ultraviolet light 254 nm and 365 nm), were pooled together to get 25 fractions. Sub-fraction (6) of hexane: ethyl acetate (7:3) (yield 45 mg) was purified using silica gel column by a stepwise increase of ethyl acetate in hexane. A white crystalline solid labelled compound C was obtained at hexane: ethyl acetate (9:1) (Duraipandiyan *et al.*, 2012). R_f values of compound C were determined in a mixture of hexane: ethyl acetate (3:1 and 3:2) and dichloromethane: hexane 3:1. The melting point and yield of compound C was also determined.

Chemical test carried out on compound C

Test for hydroxyl group (-OH).

Ethanoyl chloride (1 mL) was added to 1 mg of isolated compound C. The white fume evolved was tested with blue litmus paper and aqueous silver nitrate.

Another 9 g of ethyl acetate fraction was purified on silica gel (60-200 mesh, 270 g, 32.8 mm x 60 mm) using a solvent gradient of a mixture of ethyl acetate in hexane (10%, 30%, 40%, 60%, 80% and 100%).

An aggregate of 178 fractions containing 200 mL each were obtained and these were pooled together into 17 sub-fractions following the TLC pattern. The purification of sub-fraction 17 (yield=80 mg) obtained from fractions (168-178) of 100% ethyl acetate by a second column chromatography utilising the same solvent gradient method gave a solid (55 mg) at 80% ethyl acetate in hexane. Further purification of the solid obtained was achieved by making use of a mixture of ethyl acetate 5: methanol 1 in isocratic column separation followed by recrystallization from hot methanol to obtain an ash colored solid which was labeled compound 1. The melting point and yield of compound 1 were determined. The R_f values were also determined using ethyl acetate: methanol 5:1 and 7:1; diethyl ether: methanol 4:1.

Carbon and Proton NMR data of compound C were reported with Bruker AVANCE III at 125.75 and 500.13 MHz respectively. Spectra were recorded in deuterated chloroform and in 5 mm NMR tubes. Coupling constants quoted are given in Hertz. The chemical shift values for all spectra obtained were in parts per million. References were made against the internal standard tetramethylsilane (TMS), which occurs at zero parts per million or the solvent peaks. The NMR spectra of isolated compound 1 was determined using Bruker Topsin 400 and 100 MHz spectrometer for ^{1}H and ^{13}C respectively, and reported in relation to tetramethylsilane (δ 0.00 ppm). Bruker Platinum ATR Tensor 27 FT-IR Spectrophotometer was used to acquire Fourier Transform Infra-red (FT-IR) spectra.

3.6.2 Column Chromatography Separation of the Leaf Hexane Extract

The purification of (38.55 g) hexane extract by solvent gradient method using silica gel (Merk 60-200 mesh, 1200 g, 45.5mm x 90 mm) was done by utilising a mixture of ethyl acetate in hexane with an initial solvent 100% n-hexane, (2%, 5%, 10%, 30%, 50%, 80% and 100%). Totally, 190 fractions containing 200 mL each were collected and the rate of flow was 200 mL/14 min. Similar fractions were pooled together to form 18 sub-fractions following the TLC patterns as visualised in iodine, UV lamp and $KMnO_4$ spray (heated in oven at 100°C for 5 minutes). Fractions (86-101) from 5-10% ethyl acetate in hexane (1.90 g) which was a mixture of need-like crystals and

powdered solid was purified by another column chromatography utilising different solvent mixtures of 1%, 2%, 3%, 4% and 5% ethyl acetate in hexane.

An aggregate of 124 fractions containing 50 mL each obtained were pooled into 14 sub-fractions. Different solvent mixtures were used for the TLC (hexane: ethyl acetate 8:1 and 3:1 and diethyl ether: Hexane 3:1) and hexane 8: ethyl acetate 1 which gave the best separation was used in an isocratic method for further purification of the solid (0.64 g) obtained from the sub-fraction 14. An aggregate of 138 fractions of 5 mL each were collected. Fractions (16-32) afforded a colorless amorphous powder (20 mg) labeled Xp. The melting point and R_f values of Xp in hexane: ethyl acetate 3:1 and 3:2 and hexane: diethyl ether 3:1 were determined.

3.6.3 Column Chromatography Separation of the Rhizome Hexane Extract

The separation of hexane extract (6.80 g) with silica gel (60-200 mesh, 205 g, 32.5 mm x 66 mm) was carried out by making use of mixtures of ethyl acetate in hexane (5%,10%, 30%, 50%, 80% and 100%). An aggregate of 125 fractions containing 200 mL each obtained were pooled together according to the TLC patterns as visualised with iodine vapour, UV lamp and $KMnO_4$ spray (which was heated for 5 minutes in an oven at 100°C) to give 38 sub-fractions. Fractions (64-73) obtained from 10% ethyl acetate in hexane (1.03 g) was purified with repeated column chromatography (CC) to give a solid (compound Z) which was visualised as a single spot on TLC plates using three different solvent mixtures, ethyl acetate: hexane 7:3, 6:1 and chloroform 19:1. Melting point and yield of compound Z was determined.

3.6.4 Column Chromatography Separation of the Stem Ethyl acetate Fraction

Silica gel (60-200 mesh, 220 g, 32.8 mm x 60 mm) was utilised in the purification of the stem ethyl acetate fraction (7.24 g). An aggregate of 160 fractions containing 200 mL each were collected with n-hexane and mixtures of different proportion of ethyl acetate in hexane of (5%, 10%, 20%, 30%, 50%, 70%, 80% and 100%). These were further pooled into 17 fractions following the TLC pattern and visualised with iodine vapour, UV lamp (256-365 nm) and spraying with $KMnO_4$ followed by heating in an oven at 100°C. Fractions 124-139 (30 mg) from 70% ethyl acetate in hexane gave two spots on TLC plates using different mixtures of solvents.

The different solvent systems tested were dichloromethane: methanol 10:1, ethyl acetate: methanol 5:1 and 7:1. Ethyl acetate: methanol 5:1 provided the utmost separation and minimal tailing out of the solvent systems utilised. Therefore, these compounds labeled A and B were separated with a mixture of ethyl acetate 5: methanol1 by isocratic elution method and 5 mL fractions were collected each time.

3.6.5 Column Chromatography Separation of the Stem Hexane Extract

Purification of hexane extract (8.1 g) on a silica gel column (60-200 mesh, 240 g, 32.8 mm x 60 mm) by making use of mixtures of ethyl acetate in hexane of (5%, 10%, 20%, 30%, 40%, 50%, 60%, 80% and 100%) was carried out. An aggregate of 130 fractions of 150 mL each were obtained, these fractions pooled together gave 29 fractions following the TLC pattern and visualization under UV lamp 256 and 365 nm, iodine vapour and $KMnO_4$ solution spray on the plates, thereafter heating in an oven to 100^oC for 5 minutes.

Fractions (113-115) of 0.19 g obtained from 60% ethyl acetate in hexane was purified using different mixtures of ethyl acetate in hexane (10%, 12%, 15%, 20%, 25%, 30%, 40% and 50%). An aggregate of 211 fractions each containing 50 mL using a rate of flow 10 mL/min was collected. These were pooled together into 10 sub-fractions following TLC patterns. No solid was obtained at 50% ethyl acetate in hexane.

Crystallisation of a white solid obtained and labelled C_{2018} which was obtained from fractions (179-188) of 40% ethyl acetate in hexane was carried out with hot methanol.

Melting point, yield and R_f values of compound C_{2018} in ethyl acetate: hexane 3:2 and 5:1 dichloromethane: hexane 3:1 were determined.

Chemical Test on Compound C_{2018}

Test for Alkaloids

A solution of compound C_{2018} (1 mg) in chloroform (2 mL) was prepared. 1 mL of the solution and little drop of dragendorffs reagent were mixed together. There was an appearance of an orange color.

Figure 3.1 shows the summary of isolation and purification procedure.

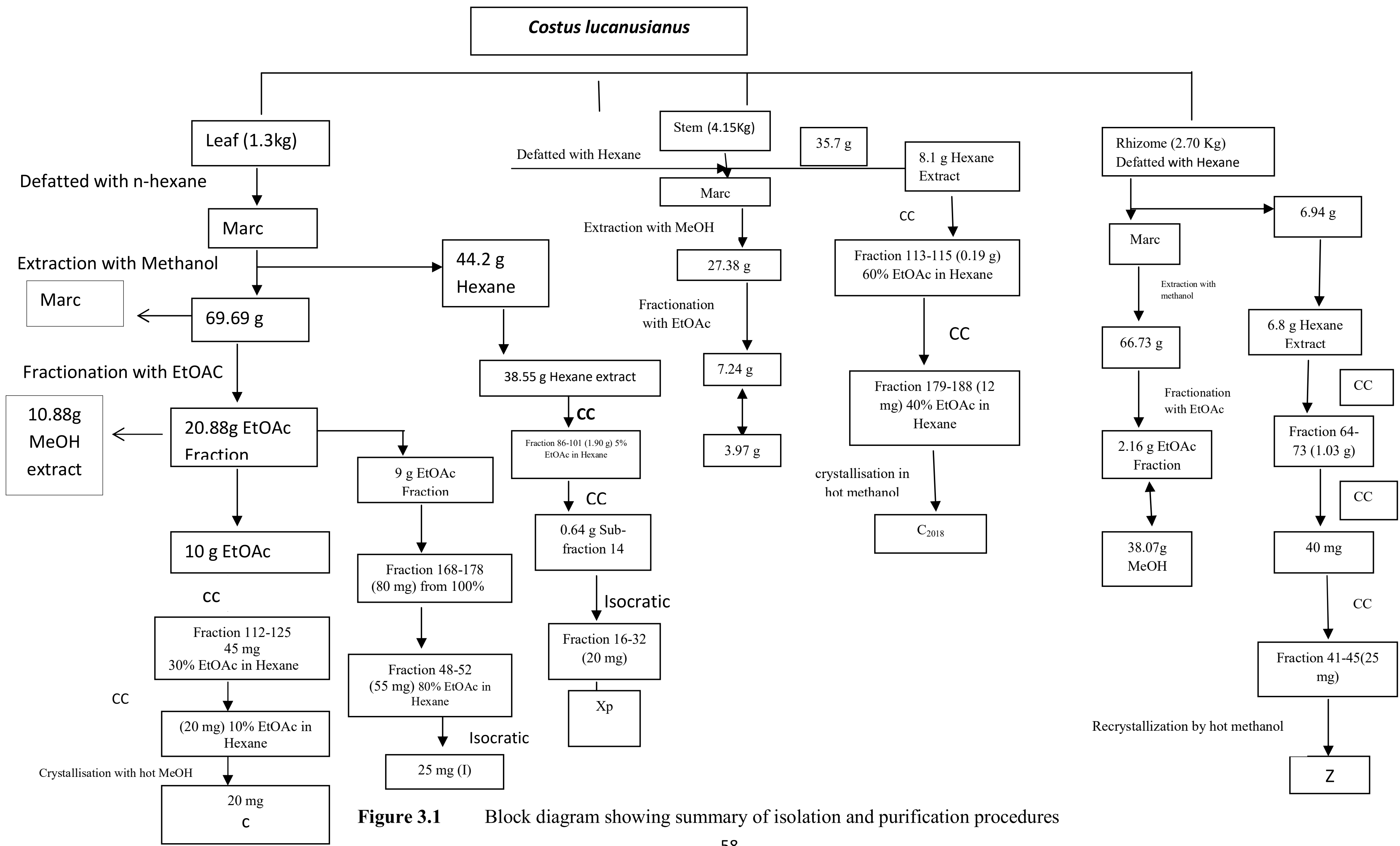


Figure 3.1 Block diagram showing summary of isolation and purification procedures

QqTOF-MS/MS analysis

In preparing solutions of isolated compounds, 0.1% formic acid (0.2 μg/mL) was used to acidify 50% acetonitrile-water. The acidified mixture was introduced into the compounds and analysed with electrospray ionisation (ESI), attached proton chemical ionisation (APCI) with the positive mode of collision-induced dissociation (CID), using ESI-QqToF-MS/MS equipment (Waters GynaptG_2). Nitrogen gas of high purity was utilised as a curtain gas and the APCI interface conditions were as follows: Cone gas rate of flow at 50L/Hr, source gas rate of flow at 0 ml/min, desolvation gas rate of flow at 650 L/Hr, probe temperature 550 ^oC, source temperature 120 ^oC, corona current 12 μA on a Waters BEH C_{18} 2.1 x 100 mm x 1.7 μm. Harvad syringe pump was utilised in introducing the samples into the mass spectrometer.

3.7 Antifungal and Antibacterial Assay of Isolated Compounds

The same procedure earlier explained was utilised in carrying out the antimicrobial assay of the isolated compounds.

Minimum Inhibitory Concentration (MIC)

Various concentrations of the obtained compounds were made by dissolving them in dimethylsulphoxide. Agar dilution method (micro-dilution technique) was utilised for determining the MIC. Prepared sterilized agar was allowed to cool to 45^oC, and 18 mL each was thoroughly mixed with different concentrations (2 mL each) of the compounds. The mixture was permitted to uniformly spread in a 96-well microplate which was kept in an oven for 24 hours at 37^oC, after which the micro-organisms were applied on the surface. The experiment was done in triplicate and the MIC of the compounds in millimetre was determined.

3.8 Gas Chromatography- Mass Spectrometry Analysis of the Essential Oils

GC-MS analyses of the essential oils were performed using Agilent Technologies 7890B coupled to a 5975 VLMSD mass spectrometer and an injector 7890B series device was utilised for the analyses of the obtained oils. Essential oils (3 μL) were introduced into a gas chromatograph GC system An Agilent (9091)-413:325^oC HP-5 column (30mm x 320 μm id, film thickness 0.50 μm) was used. Setting of the oven temperature was at 70^oC -240^oC and at 50^oC/min.

The electron ionization was set at 70eV and ion source fixed at 240°C. The carrier gas, helium was set at a flow rate of 1.4mL/min with 50:1 split ratio and 70.615 mL/min split flow. The scanning range was from 30-500 amu. After being diluted with hexane, oil samples (3.0 μL) were injected into the system.

Identification of compounds

Essential oils constituents were mostly identified by comparing their Retention Indices (RI) with the reported values in the literature. Identification of others was achieved by comparison of their mass spectra with those of standards obtained on a nonpolar HP-5MS column, Wiley Library Mass Spectra database of the GC/MS system (Adams, 2001; Babushok *et al.,* 2011).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Solvent Extractions

Hot and cold extracts yields (%) are presented on Table 4.1. Amongst the extracts, the highest yields of 3.4% and 1.6% were obtained for the hot hexane extract and ethyl acetate fractions of the *Costus lucanusianus* leaf respectively. The leaf hot hexane extract gave a greenish jelly-like semi solid. A colourless solid of 3.2 g crystallized out of the hot methanol extract of the stem. Amongst the cold extracts, the rhizome methanol extract gave the highest yield of 1.20%.

The Thin Layer Chromatography (TLC) comparative analysis of the cold and hot extracts showed that there was no difference between phytochemicals present. However, cold extracts gave lower yields than the hot extracts. Duraipandiyan *et al.* (2012) also documented low yields (0.95-1.4%) for *C. speciosus* rhizome cold extracts. So also, Owolabi and Nworgu, (2009) documented a low yield (5.53%) for *C. lucanusianus* leaf aqueous extract. Therefore, column chromatography separation of the hot extracts was carried out in this research.

4.2 Phytochemical Composition of Crude Extracts

Phytochemical compositions are beneficial for detecting the presence of bioactive components and also in the qualitative separation of compounds that are chemically active (Millikharjuna *et al.,* 2007). The phytochemical composition of *C. lucanusianus* solvent extracts are presented in Table 4.2

Several secondary metabolites like saponins, phenols, alkaloids e.t.c which are found to be present in medicinal plants are accountable for their healing characteristics. Extracts obtained from the inflorescence, stems, rhizomes and leaves of *C. lucanusianus* contained saponins, alkaloids, flavonoids, phlobatannins, anthraquinones

and tannin among others which may have led to their usage in ethnomedicine. The nature of constituents in plant extracts determines its pharmacological action (Mukherjee, 2006).

Table 4.1: Percentage yields of hot and cold extracts from *Costus lucanusianus*

Plant parts	Hexane Cold	Hexane hot	Ethyl acetate cold	Ethyl acetate Hot	Methanol Cold	Methanol Hot
Inflorescence	0.01	0.24	0.04	0.07	0.40	0.67
Leaf	0.07	3.40	1.12	1.60	0.51	0.70
Stem	0.04	0.85	0.08	0.17	0.08	0.10
Rhizome	0.05	0.26	0.01	0.08	1.20	1.41

Table 4.2: Qualitative phytochemical composition of *Costus lucanusianus* plant extracts

	Inflorescence			Leaf			Stem			Rhizome		
Bioactive components	Hexane	Ethyl acetate	Methanol	Hexane	Ethyl acetate	Methanol	Hexane	Ethyl acetate	Methanol	Hexane	Ethyl acetate	Methanol
Saponin	-	+	+	+	+	+	-	+	+	+	-	+
Tannin	+	-	+	-	-	+	-	+	+	-	+	+
Flavonoids	-	+	-	-	+	-	-	+	+	-	-	+
Phlobatannins	-	-	+	-	-	-	-	-	-	-	-	-
Reducing sugar	+	+	+	+	+	+	+	+	+	+	+	+
Anthraquinones	-	+	-	-	-	-	-	-	-	-	-	-
Phenol	-	-	+	-	-	+	-	-	-	-	-	-
Alkaloids	+	-	-	-	+	+	+	-	-	+	-	+
Resin	+	+	+	-	+	+	-	+	+	+	-	+
Glycosides	+	-	+	+	+	+	+	+	+	-	+	+
Terpenoids	-	-	+	-	+	+	-	+	+	+	+	-

+= Detected, - = Below detectable limit

The phytochemical screening of bioactive components of the inflorescence and rhizome of *C. lucanusianus* are reported for the first time.

Eleven phytochemicals were tested to determine their presence or absence in the extracts. The highest number of phytochemicals (8) was detected in the infloresence and leaf methanol extracts while the least (3) was present in the leaf and stem hexane fractions based on TLC results. The hexane fractions (HEF) of the rhizome and inflorescence contained more phytochemicals than leaf and stem HEF. Nevertheles, the rhizome HEF contained terpenoids which were not detected in the other HEF. The rhizome ethyl acetate fraction (ETF) contained the least phytochemical constituents among other ETF. The inflorescence methanol extract (MET) contained phlobatannin while anthraquinone was detected in its ETF. It was observed that the entire extracts obtained from the plant parts contained reducing sugar.

The phytochemical screening result of the cold aqueous methanol of the leaf agreed with previous works of (Baba and Onanuga, 2011). In this present study, we hereby report the existence of saponin and absence of alkaloid in the stem methanol extract. However, Traore *et al*. (2017) recorded the existence of alkaloid and absence of saponin. Climatic conditions, physiological state and geographical localisation of plants are part of the reasons that might be responsible for the varying chemical compositions (Dinan *et al*., 2001). Alkaloids are recognised to possess anti-inflammatory and antimicrobial properties (Sofowora, 1993) and also known to interfere with nucleic acid and DNA targeting RNA polymerase (Cowan, 1999). Anthraquinones have been reported to have a laxative effect (Dave and Ladawani, 2012). Antibacterial, antiviral, anti-parasitic (Akiyarma *et al*., 2001; Shrivastava *et al.,* 2012) and the healing property of tannin (Okwu and Okwu, 2004) have been reported. Also, plants with secondary metabolites of the phenolic structure backbone are reported to possess high activities against microorganisms (Osman *et al.,* 2003). Terpenoids are recognised for their antimicrobial activities (Mahizan *et al.,* 2019).

4.3 Essential Oils

The chemical compounds recognised in the oils by GC-MS are shown in Table 4.3.

Hydrodistillation of the inflorescence, leaf, stem and rhizome of *C. lucanusianus* yielded colorless essential oils with percentage yields (w/w) of 0.11%, 0.05%, 0.026% and 0.18% respectively. Long chain saturated and unsaturated alkanes, oxygenated terpenoids and nitrogen, fatty acids and its derivatives, chlorine and silicon-containing compounds were found to be abundant in the oils. The amount of essential oils extracted from plants is dependent on numerous causes such as harvest period, geographical and climatic conditions, and stages of plant growth and extraction technique.

Table 4.3: Chemical Constituents of the Essential Oils from *Costus lucanusianus* plant*

S/N	Compound	% composition				RRI	Class of compound
		INF	LF	STM	RHIZ		
1	Methyl Salicylate	-	2.91	-	-	1187	Non-Terpenoid
2	Chromone, 3-methyl-7-nitro-	-	-	3.67	-	-	Non-Terpenoid
3	Butylated hydroxytoluene	-	2.84	-	-	1533	Non-Terpenoid
4	4-(1,3,3-Trimethyl-bicyclo[4.1.0]hept-2-yl)-but-3-en-2-one	5.09	-	28.10	-	-	Oxygenated bicyclic Sesquiterpene
5	Oleic acid	-	-	4.56	1.24	-	Monounsaturated fatty acid
6	Tetradecanal	5.09	-	-	-	1611	Long chain aldehyde
7	Oleic acid, eicosyl ester	-	-	5.91	0.93	3922	Fatty acid derivative
8	Docosahexaenoic acid	-	-	4.21	-	2520	Omega-3-poly unsaturated fatty acid
9	Octadecanoic acid, methyl ester	-	2.35	-	-	2130	Fatty acid derivative
10	1,2- Benzenedicarboxylic acid mono (2-ethylhexyl) ester	13.80			25.58	-	Other (plasticizer)
11	5,9,13-pentadecatrien-2-one,6,10,14-trimethyl-	-	0.85	-	-	1921	Unsaturated fatty acid derivative
12	Hexadecanoic acid, methyl ester	-	10.19	-	-	1928	Fatty acid derivative
13	n-hexadecanoic acid	-	2.24	-	-	1964	Fatty acid
14	1- Heptadecene	-	2.08	-	-	1694	Unsaturated long chain alkane

15	11-octadecenoic acid, methyl ester	-	41.00	-	-	2089	fatty acid ester
16	Hexadecane, 8-hexyl-8-pentyl-	-	-	-	8.24	-	Long chain alkane
17	Tricosane	-	2.40	-	-	2300	Long chain alkane
18	3',8,8'-trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone	-	-	22.06	-	-	Other (Aromatic compound)
19	Tritetracontane	4.68	-	-	-	3401	Long chain alkane
20	Tetrapentacontane, 1,54-dibromo-	-	0.87	-	-	-	Long chain alkane derivative
21	9-Octadecenoic acid (E)	-	-	2.99	0.44	2141	Fatty acid
22	n-Propyl 11-octadecenoate	-	-	-	3.24	-	Fatty acid ester
23	trans-13-octadecenoic acid	-	3.33	-	0.64	2163	Fatty acid
24	Caryophyllene	-	-	8.66	-	-	Bicyclic sesquiterpene
25	Eicosane	6.70	-	-	-	2000	Acyclic alkane
26	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	-	-	-	18.23	-	Alkane
27	1-Octadecene	-	-	-	4.51	1795	Long chain unsaturated alkane
28	6-Octadecenoic acid	3.15	-	-	-	2073	Fatty acid
29	Heptacosane	30.23	-	-	-	429.45	Long chain alkane
30	9-tricosene, (z)-	10.27	1.20	-	-	2298	Long chain unsaturated alkane

31	Heneicosane	-	-	-	3.46	-	Long chain saturated aliphatic hydrocarbon
32	2-tetradecanol	-	-	-	7.10	-	Long chain fatty alcohol
33	Octadec-9-enoic acid	-	-	2.28		-	Fatty acid
34	Squalene	-	16.40	-	-	2847	Triterpene
35	Carbonic acid, octadecyl 2,2,2-tri chloroethyl ester	-	-	4.28	-	-	Fatty acid derivative
36	cis-vaccenic acid	-	0.39	4.00	-	-	Fatty acid
37	Phytol	17.41	-	-	-	2122	Diterpene alcohol.
38	Carbonic acid, hexadecyl 2,2,2-tri chloroethyl ester	8.62	-	-	-	-	Fatty acid derivative
Yield (% w/w)		0.11	0.05	0.026	0.18		
Sesquiterpene hydrocarbon		-	-	8.66	-		
Triterpene		-	16.40	-	-		
Oxygenated sesquiterpene		5.09	-	28.10	-		
Oxygenated diterpene		17.41	-	-	-		
Long chain saturated and unsaturated		51.88	6.55	-	34.44		
Fatty acid and its derivatives		11.77	60.35	28.23	6.49		
Others		13.80	5.75	25.73	32.68		
Total(%)		99.95	89.05	90.72	73.61		

INF=Inflorescence, LF= Leaf, STM= Stem, RHZ=Rhizome. RRI=Retention relative index to C_9-C_{24} n-alkanes on HP-5 column.

The essential oils from *C. lucanusianus* plant parts are recounted for the first time. An aggregate of 9 compounds constituting 99.95% in the inflorescence oil, 14 compounds (89.05%) in the leaf oil, 11 compounds (90.72%) in the stem oil and 11 compounds constituting 73.64% in the rhizome oil were realised in this study.

The inflorescence oil was dominated by 30.23% of long chain alkane (heptacosane). Other constituents were phytol, a diterpene alcohol (17.41%), 1, 2-Benzenedicarboxylic acid (2-ethylhexyl) ester (BDE) (13.80%), 9-Tricosene, (z) - (10.27%) and 6-octadecenoic acid (3.15%). The principal constituents of the leaf oil were 11-octadecenoic acid, methyl ester (41%), squalene (16.40%), hexadecanoic acid, methyl ester (HME) (10.19%), methyl salicylate (2.91%) and n-hexadecanoic acid (2.24%). In the stem oil, 4-(1,3,3-trimethyl-bicyclo[4.1.0] hept-2-yl)-but-3-en-2-one (BHE), an oxygenated sesquiterpene (28.10%) and 3',8,8'-trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone (TPB) (22.06%), caryophyllene (8.66%) and cis-vaccenic acid (4.00%) were detected as the main constituents.

The rhizome oil was dominated by octadecane, 3-ethyl-5-(2-ethylbutyl)- (OEE) (18.23%) and BDE (25.58%). Other constituents are 2-tetradecanol (7.10%), heneicosane (3.46%) and trans-13-octadecenoic acid (0.64%).

The acidic nature of the essential oils was revealed by their analyses, similar to some other species in the genus *Costus.* Oils from all the four parts contained fatty acid and its derivatives in different proportions. Reports have implicated fatty acids as the major constituents of essential oils obtained from the *Costus* species (Jose and Reddy, 2010; Srivastava *et al*., 2011; Srinivasan *et al*., 2016). All parts of *C. lucanusianus* have been reported to have an acidulous taste (Burkill, 1985).

In this present study, BDE which was isolated from the inflorescence and rhizome had reportedly been detected from the rhizome extract of *C. speciosus*, ether fraction of *C. pictus* leaf and butanol fraction of *C. afer* leaf (Farooqui and Shukla, 1987; George *et al.,* 2007; Thambi and Shafi, 2015).

The *C. lucanusianus* essential oils were characterised by the presence of fatty acids and its derivatives (6.49-60.35%). The leaf and stem oils were with higher amount of fatty acids and its derivatives (60.35% and 28.23%) respectively. However, the inflorescence and rhizome oils were with higher amount of saturated and unsaturated

alkanes (51.88% and 34.44%) respectively. The long chain saturated and unsaturated alkanes were absent in the stem oil.

Oleic acid and stearic acid are known to induce the crystallization of rubber chains which justifies the use of *C. lucanusianus* stem for the coagulation of rubber (Crafts *et al.,* 1990). HME, vaccenic acid, eicosane, 9, 12-octadecanoic acid methyl ester, phytol, 2-methoxy-4-vinyl phenol and n-hexadecanoic acid were found to be the principal components of *C. afer* leaf butanol fraction (Anyasor *et al.,* 2014). Sesquiterpenoids were also reported by Asekun and Adeniyi, 2003 as the most abundant class of volatile components of *C. afer* essential oils. Also, an extensive range of triterpenoids are present in many *Costus* species. The seed oil fatty acid composition of *C. speciosus* were mainly of palmitic (55.97%), oleic (22.75%), stearic (8.3%) and linoleic acid (6.8%) (Shruti *et al.,* 2011). *Stevia rebaudiana* (bertoni) leaves also contain tritetracontane among other constituents (Hossain *et al.,* 2010).

The principal constituents of *C. lucanusianus* essential oils which are yet to be reported in the essential oils of other *Costus* genus are octadecane, OEE, BHE, squalene, TPB and BDE (Asekun and Adeniyi, 2003; Jose and Reddy, 2010; Thambi and Shafi, 2015; Srinivasan *et al*., 2016). The medicinal advantages of *C. lucanusianus* essential oils were attributed to the existence of squalene (16.40%), phytol (17.41%) and caryophyllene (8.66%), which are known to possess medicinal values in the inflorescence, leaf and stem oils respectively.

4.4 Antimicrobial Assay

4.4.1 Antibacterial assay of *C. lucanusianus* essential oils

Table 4.4 contains the results of the essential oils antibacterial activity. The antimicrobial activity of *C. lucanucianus* oils at (12.5 -100 µg/mL) was evaluated against six bacterial strains namely *S. aureus, B. subtilis, E. coli, P. aeruginosa, S. typhi* and *K. pneumoniae* by pour plate method.

The antibacterial screening results of the essential oils revealed that the oils inhibited bacteria with varying sensitivity. The inflorescence oil at 12.5 µg/mL showed inhibition zone diameter (IZD) against *S. aureus* (14 mm), *E. coli* (12 mm), *B. subtilis* (12 mm)*, P. aeruginosa* (14 mm), *K. pneumoniae* (10 mm) and *S. typhi* (10 mm). At 25 µg/mL, the leaf oil inhibited *S. aureus* (10 mm), *E. coli* (10 mm), *K. pneumoniae*

(10 mm) and *S. typhi* (14 mm) and at 50 µg/mL, *P. aeruginosa* and *B. subtilis* (10 mm). Stem oil showed IZD against *S. aureus*, *B. subtilis* (10 mm) at 25 µg/mL, *E. coli*, *S. typhi* and *K. pneumonia* (10 mm) at 50 µg/mL. Stem oil displayed no activity against *P. aeruginosa*. Rhizome oil showed IZD against *S. aureus* (21 mm), *P. aeruginosa* (21 mm) *E. coli* (23 mm), *K. pneumoniae* (22 mm), *B. subtilis* (19 mm) and *S. typhi* (24 mm) at 12.5 µg /mL.

The diversity of the chemical compostion of the oils is responsible for the different antimicrobial activity displayed. Like natural extracts, essential oils antimicrobial property is dependent on the quantity/percentage of the each constituent present as well as their chemical constituents. The antibacterial activity demonstrated by most essential oils against gram-positive bacteria is higher than gram-negative bacteria (Trombetta *et al.,* 2005).

However, in this present study, *C. lucanusianus* rhizome oils exhibited highest antibacterial activity among oils from other plant parts. Nikaido, (1994) reported the relative inhibitory effect of gram-negative bacteria to hydrophobic antibiotics and toxic drugs. The major chemical components detected in the rhizome oil were BDE and OEE. The antibacterial activity of *C. speciosus* and *C. pictus* plant aerial, rhizome and root has also been reported (Thambi and Shafi, 2015; Srinivasan *et al.,* 2016).

The rhizome oil of *C. speciosus* at a concentration of 1 mg/mL showed antibacterial activity against tested bacterial with IZD between 10 mm-16 mm while the rhizome oil of *C. pictus* displayed antibacterial activity with IZD between 6 mm-15 mm.

4.4.2 Antifungal assay of *C. lucanusianus* essential oils

In determining the antifungal property of the oils, four fungal species used were namely *Candida albicans, Aspergillus niger*, *Rhizopus stolonifer* and *Penicillium notatum* were employed.

As shown in Table 4.5, different concentrations of the oils repressed all the fungal species with different degrees of sensitivity except for the stem oil which was inactive against every tested fungal species. The inflorescence oil at 12.5 µg/mL displayed IZD against *C. albicans* (14 mm), *A. niger* (12 mm), and at 25 µg/mL against *P. notatum* (10 mm) and *R. stolonifer* (10 mm). The leaf oil at 25 µg/mL repressed *C. albicans* and *A. niger* (10 mm), *R. stolonifer* (10 mm) at 100 µg/mL and *P. notatum* (10 mm) at 50

µg/mL. Rhizome oil showed activity against *C. albicans, P. notatum* and *R. stolonifer* (14 mm) and *A. niger* (16 mm) at concentration 12.5 µg/mL. The inflorescence and rhizome oils similarly inhibited *C. albican*s.

Of all the tested oils, the rhizome oil exhibited the most IZD against the fungi tested. Furthermore, the highest antifungal activity was against *A. niger* (16 mm). In contrast, only the root and leaf essential oils of *C. pictus* are known to possess antifungal property (Srinivasan *et al.,* 2016).

C. lucanusianus stem oil which was inactive against the fungal strains contained the highest amount of oxygenated sesquiterpenes (28.10%) which possess high antimicrobial property (Bakkali *et al*. (2008); Lang and Buchbauer, (2012); Zengin and Baysal, 2014; Thambi and Shafi, 2015). Similarly, *C. afer* (a related species) which contained oxygenated sesquiterpene, sesquilavandulyl acetate (17.0%) as the highest abundant constituent in its leaf essential oils have been reported to display antimicrobial inactivity (Asekun and Adeniyi, 2003). The rhizome oil among the tested oils, exhibited the maximum inhibition against the entire fungi tested and the highest activity was against *A. niger* (16 mm).

The antibacterial activity displayed by stem oil was ascribed to the existence of fatty acids and its derivative which are recognised to exhibit antimicrobial activity (Debois and Smith, 2010). Antifungal inactivity of the stem oil may be because of the absence of long chain saturated and unsaturated alkanes which were present in other oils but of a lowest percentage in the leaf essential oils (5.68%). The antibacterial and antimicrobial properties of hexadecane which is a long-chain alkane, eicosane, octadecanoic acid, and hexadecanoic acid are documented (Kalpana *et al.,* 2012; Chandrasekar *et al.,* 2015). *Costus pictus* root oil which displayed antifungal activity was reported to contain fatty acid and long chain hydrocarbon as the main constituents (Srinivasan *et al.,* 2016).

Table 4.4: Antibacterial Activity results of *Costus lucanusianus* Essential Oils*

Part of plant/ Concentration	*S. aureus*				*E. coli*				*P. aeruginosa*				*K. pneumonia*				*B. subtilis*				*S. typhi*			
µg/mL	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5
Inflorescence	20	18	16	14	18	16	14	12	20	18	16	14	16	14	12	10	18	16	14	12	16	14	12	10
Leaf	14	12	10	-	14	12	10	-	12	10	-	-	14	12	10	-	12	10	-	-	18	16	14	12
Stem	14	12	10	-	12	10	-	-	-	-	-	-	12	10	-	-	14	12	10	-	12	10	-	-
Rhizome	31	26	24	21	32	29	26	23	30	27	24	21	29	27	25	22	28	25	22	19	31	29	27	24
- ve standard DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
+ve standard Gentamicin (10 µg/mL)	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38	40	40	40	40

- = Beyond detectable limit, DMSO= dimethylsulphoxide

**S. aureus*= *Staphyloccocus aureus*, *E. coli* = *Escherichia coli*, *P. aeruginosa* = *Pseudomonas aeruginosa, K. pneumoniae* = *Klebsillae pneumoniae, B. subtilis* = *Bacillus subtilis, S. typhi* = *Salmonellae typhi*

Table 4.5: Antifungal Activity results of *Costus lucanusianus* Essential Oils*

Part of plant/ Concentration	*C. albicans*				*A. niger*				*P. notatum*				*R. stolonifer*			
(µg/mL)	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5
Inflorescence	20	18	16	14	18	16	14	12	14	12	10	-	14	12	10	-
Leaf	14	12	10	-	14	12	10	-	12	10	-	-	10	-	-	-
Stem	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhizome	20	18	16	14	21	19	18	16	20	18	16	14	20	18	16	14
- ve standard DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
+ ve standard Tioconazole 70%	28	28	28	28	28	28	28	28	26	26	26	26	28	28	28	28

- = Beyond detectable limit, DMSO= Dimethylsulphoxide

* *C. albicans = Candida albicans, A. niger = Aspergillus niger, P. notatum = Penillinum notatum, R. stolonifer = Rhiziopus stolonifer*

Perez *et al*. (1994), Oluwatosin and Bewaji (2011) also reported n-hexadecanoic acid, octadecanoic acid, 1-eicosene and 17-pentatriacontene as the active principles used in the treatment of trypanosomiasis. Octadecanoic acid esters also exhibit antibacterial, antiviral and antioxidant properties (Oladimeji *et al.,* 2013). The antifungal and antibacterial activities of carboxylic acids with long hydrocarbon chains (fatty acids) have also been reported (Agoramoorthy *et al.,* 2007).

Plant components with phenolic structures demonstrate great activity against microorganism (Osman *et al.,* 2003). For example, the antioxidative and antimicrobial properties of phytol, a fatty alcohol is known (Kumar *et al.,* 2010; Hema *et al*., 2011).

The antimicrobial activity results of *C. lucanusianus* revealed that the rhizome and inflorescence oils amongst others possessed broad-spectrum activity against *E. coli* and *C. albicans*. Existence of BDE in the inflorescence and rhizome oils only was attributed to their antibacterial property similarity. The existence of fatty acid and its derivatives and a considerable amount of the long chain saturated and unsaturated alkanes with different polarity in both the inflorescence and rhizome oils characteristically act synergetically to enhance the antimicrobial activities of the rhizome and inflorescence oils (Bassole and Juliani, 2012).

Finally, the antimicrobial activity displayed by the rhizome oil could also be attributed to the presence of 2-tetradecanol (7.10%), a long chain fatty alcohol which was present only in the rhizome oil. Long chain fatty alcohol have been reported to have antibacterial properties which were attributed to the type of unsaturated bonds and their positions, and the quantity of carbon atoms present (Tanaka *et al.,* 2002; Togashi *et al*., 2007).

4.4.3 Antibacterial Activity of *Costus lucanucianus* Part Extracts

Six bacterial strains namely *S. aureus, E. coli, K. pneumoniae, B. subtilis, S. typhi* and *P. aeruginosa* were used in determining the antibacterial activity of *C. lucanusianus* extracts. The summary of the results is shown in Table 4.6.

Table 4.6: Antibacterial activity results of *Costus lucanusianus* cold extracts*

Parts of plant used	Solvent extracts/ Concentration	*S. aureus*				*E. coli*				*P. aeruginosa*				*K. pneumonia*				*B. subtilis*				*S. typhi*			
	(µg/mL)	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5
	Aqueous	14	12	10	-	14	12	10	-	-	-	-	-	12	10	-	-	-	-	-	-	12	10	-	-
	Hexane	-	-	-	-	16	14	12	10	16	14	12	10	14	12	10	-	-	-	-	-	16	14	12	10
Inflorescence	Ethyl acetate	14	12	10	-	20	18	14	12	14	12	10	-	14	12	10	-	14	12	10	-	16	14	12	10
	Methanol	14	12	10	-	14	12	10	-	16	14	12	10	16	14	12	10	14	12	10	-	16	14	10	-
	Aqueous	16	14	12	10	16	14	12	10	16	14	12	10	14	12	10	-	18	16	14	10	14	12	10	-
	Hexane	-	-	-	-	16	14	10	-	16	14	12	10	14	12	10	-	-	-	-	-	14	12	10	-
Leaf	Ethyl acetate	14	10	-	-	20	18	16	14	14	12	10	-	14	12	10	-	16	14	10	-	16	14	10	-
	Methanol	18	16	14	12	14	12	10	-	18	16	14	12	18	16	14	12	16	14	12	10	20	18	16	14
	Aqueous	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	Hexane	14	12	10	-	16	14	12	10	18	16	14	12	18	16	14	12	12	10	-	-	16	14	12	10
Stem	Ethyl acetate	16	14	12	10	24	20	18	16	18	16	14	12	18	16	14	12	16	14	12	10	20	18	16	14
	Methanol	14	12	10	-	16	14	12	10	16	14	12	10	18	16	14	12	12	10	-	-	16	14	12	10
	Aqueous	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hexane	28	24	20	18	28	26	24	20	28	26	22	20	27	26	24	20	25	24	20	18	30	27	25	23
Rhizome	Ethyl acetate	16	14	12	10	18	16	14	12	18	16	14	12	18	16	14	10	20	18	16	14	16	14	12	10
	Methanol	18	16	14	12	22	18	16	14	23	21	18	16	22	18	16	14	24	21	15	13	21	18	16	14
	+ve standard Gentamicin			40					38				38				38			38					38
	-ve standard Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* *S. aureus= Staphyloccocus aureus, E. coli = Escherichia coli, P. aeruginosa = Pseudomonas aeruginosa, K. pneumoniae = Klebsillae pneumoniae, B. subtilis = Bacillus subtilis, S. typhi = Salmonellae typhi*

Infloresecence

The inflorescence aqueous extract at 25 µg/mL displayed IZD against *S. aureus*, *E. coli* (10 mm) and at 50 µg/mL, *S. typhi, K. pneumoniae* (10 mm) and no IZD was observed for *B. subtilis* and *P. aeruginosa* at all concentrations. Its HEF inhibited *P. aeruginosa, S. typhi* and *E. coli* (10 mm) at 12.5 µg/mL*, K. pneumonia* (10 mm) at 25 µg/mL but displayed no IZD against *S. aureus* and *B. subtilis*. The ETF at 25.5 µg/mL showed IZD against *S. aureus, K. pneumoniae, B. subtilis* and *P. aeruginosa* (10 mm), and at 12.5 µg/mL, *E. coli* (12 mm) and *S. typhi* (10 mm) while at 25.5 µg/mL the MET inhibited *S. aureus, S. typhi, E. coli, B. subtilis* (10 mm), *P. aeruginosa* and *K. pneumonia* (10 mm) at 12.5 µg/mL. The inflorescence aqueous and methanol extracts inhibited *Esch. coli* and *Stap. aureus* (10 mm) at 25 µg/mL. The ETF which inhibited *E. coli* (12 mm) at 12.5 µg/mL showed the highest activity. The antibacterial activity of the inflorescence is reported for the first time.

Leaf

Leaf aqueous extract at 12.5 µg/mL exhibited IZD against *E. coli, B. subtilis, P. aeruginosa, S. aureus* (10 mm), and at 25 µg/mL, *K. pneumoniae* and *S. typhi* (10 mm). HEF at 25 µg/mL displayed IZD against *E. coli, K. pneumoniae, S. typhi* (10 mm), and *P. aeruginosa* (10 mm) at 12.5 µg/mL. However, *S. aureus* and *B. subtilis* did not exhibit any IZD.The ETF at 25 µg/mL, inhibited *P. aeruginosa, K. pneumoniae, B. subtilis, S. typhi* (10 mm), and at 12.5 µg/mL, *E. coli* (14 mm) while the MET at concentration 12.5 µg/mL, inhibited *S. aureus, P. aeruginosa, K. pneumonia* (12 mm), *B. subtilis* (10 mm) and *S. typhi* (14 mm).

In this present study, the leaf aqueous methanol extract repressed *B. subtilis* (12 mm), *E. coli* (10 mm), *K. pneumoniae*, *P. aeruginosa, S. aureus* (14 mm) and *S. typhi* (16 mm) at 25 µg/mL as against standard gentamicin.

In a study, Baba and Onanuga, (2001) reported that cold aqueous methanol extract of the leaf of *C. lucanusianus* at 20 mg/mL inhibited *E. coli* (9 mm), *S. aureus* (9 mm), *P. aeruginosa* (14 mm) and no IZD for *K. pneumoniae* as against standard ciprofloxacin. The differences observed could have been as a result of the fractionation of the methanol extract to remove the non-polar and moderately polar fractions. The antagonist effects of these fractions could have been responsible for a lower

antimicrobial activity recorded by (Baba and Onanuga, 2001), even at a higher concentration. In this present work, *C. lucanusianus* leaf aqueous methanolic extract displayed highest IZD against *S. typhi* (20.00 mm) at 100 µg/mL. This could be attributed to the presence of more phytochemical constituents especially saponin which is known to possess antibacterial activity.

High antibacterial properties of *C. speciosus* leaf methanol extract at 100 mg/mL against *S. typhi* (IZD=24.00 mm) was also reported by Vasantharaj *et al.* (2013); Arunprasath and Gomathinayagam, (2014). *Costus igneus* methanol leaf extract at 2.5 µg/mL has also shown activity against *E. coli, B. subtilis, S.typhi, A. niger* and *C. albicans* with IZD ranging from (1.0-1.2 cm) with the highest against *B. subtilis* (1.2 cm). The *C. pictus* methanol leaf extract at 150 µg/mL displayed the highest IZD (18.11 mm) against *K. pneumonia* (Vasantharaj *et al.,* 2013; Arunprasath and Gomathinayagam, 2014).

The inflorescence and leaf HEF showed similarity in their inactivity against *B. subtilis* and *S. aureus*, which are gram-positive bacteria at all concentrations. The inflorescence and leaf ETFs at 12.5 µg/mL displayed the greatest IZD against *E. coli* (12 mm) and (14 mm) respectively.

Stem

The aqueous extract displayed no IZD against all tested bacterial strains at all concentrations. However, the HEF revealed activity against *K. pneumonia, P. aeruginosa* (12 mm), *S. typhi, E. coli* (10 mm) at concentration 12.5 µg/mL. It also inhibited *S. aureus* (10 mm) and *B. subtilis* (10 mm) at concentration 25 and 50 µg/mL respectively. The ETF at 12.5 µg/mL inhibited *S. aureus, B. subtilis* (10 mm), *P. aeruginosa, K. pneumoniae* (12 mm), *S. typhi* (14 mm) and *E. coli* (16 mm) and MET at 12.5 µg/mL inhibited *E. coli, P. aeruginosa, S. typhi* (10 mm), *K. pneumoniae* (12 mm), and *B. subtilis* and *S. aureus* (10 mm) at 50 µg/mL and 25 µg/mL respectively.

However, Traore *et al*. (2017) documented that *C. lucanusianus* stem methanol and aqueous extracts showed no antibacterial activity against *E. coli, K. pneumoniae, S. aureus* and *P. aeruginosa* at 50 mg/mL-200 mg/mL.

On the other hand, the aqueous leaf and stem extracts of *C. pictus* have been reported to show antibacterial activity against *K. pneumonia, B. subtilis* and *E. coli* (Arunprasath and Gomathinayagam, 2014).

Rhizome

No antibacterial activity was displayed by the rhizome aqueous extract. However, at 12.5 µg/mL the rhizome HEF repressed *P. aeruginosa, E. coli,* and *K. pneumoniae* (20 mm), *B. subtilis, S. aureus* (18 mm) and *S. typhi* (23 mm). The ETF at 12.5 µg/mL also repressed the growth of *S. typhi, S. aureus, K. pneumoniae* (10 mm) and *E. coli, P. aeruginosa* (12 mm), and *B. subtilis* (14 mm) whereas the MET revealed IZD against *S. typhi, E. coli, K. pneumoniae* (14 mm), *S. aureus* (12 mm), *P. aeruginosa* (16 mm) and *B. subtilis* (13 mm) at 12.5 µg/mL.

The rhizome aqueous extract displayed no antibacterial activity, unlike *C. speciosus* rhizome aqueous extract which showed antibacterial activity against *S. aureus* (Saraf, 2010). The *C. lucanusianus* rhizome HEF, ETF and MET showed significant antibacterial activity against all the tested bacterial at all concentrations with HEF showing the highest IZD (30.00 mm) against *S. typhi* followed by IZD (28.00 mm) against *S. aureus* and *K. pneumonia* at 100 µg/mL. On the other hand, Duraipandiayn *et al*. (2012) found out that the *C. speciosus* rhizome MET extract showed no activity against any bacterial. The presence of terpenoids in the rhizome HEF which were absent in the other hexane fractions could therefore be responsible for its best antibacterial activity.

Amongst the aqueous extracts, only the leaf extract at all concentrations displayed activity against the entire tested bacterial strains.The *C. lucanusianus* extracts at different concentrations (12.5-100 µg/mL) inhibited the growth of *B. subtilis* at varying inhibition zones except for HEF of leaf and inflorescence which showed no activity at all concentrations used. Information on the antimicrobial properties of crude bioactive compounds against *B. subtilis* is limited (Faridha *et al.*, 2016). *C. lucanusianus* rhizome methanol extract and its fractions showed activity against *B. subtilis* even at the least concentration 12.5 µg/mL. The organic solvent extracts displayed more activity against bacterial strains. Mostly, gram-negative bacteria are expected to be more resistant to plant extracts than gram-positive.

In this study, the inflorescence and leaf HEFs were more effective against gram-negative bacteria than gram-positive. It was also eminent that the efficacy of *C. lucanusianus* extracts and fractions used in this study was less active than that of standard gentamicin. This could have been due to varied concentration used and also the chemical compositions of the extracts.

4.4.4 Antifungal Activity of *Costus lucanucianus* Parts Extracts

The antifungal activity was carried out with four fungi species namely; *C. albicans, P. notatum, A. niger* and *R. stolonifer* (Table 4.7).

The inflorescence aqueous extract displayed no IZD against the entire tested fungal, however the HEF at 25 µg/mL displayed IZD against *C. albicans* and *A. niger* (10 mm). The ETF exhibited inhibitory property against *P. notatum, C. albicans, R. stolonifer* (10 mm) at concentration 25 µg/mL and *A. niger* (10 mm) at 12.5 µg/mL. The MET inhibited *C. albicans, A. niger* (10 mm) at 50 µg/mL and *P. notatum, R. stolonifer* (10 mm) at 25.5 µg/mL.

The aqueous leaf extract inhibited *C. albicans* and *A. niger* at (12 mm) and (10 mm) respectively at concentration 12.5 µg/mL. However, no activity was displayed against *P. notatum* and *R. stolonifer* at all concentrations. There was no IZD demonstrated by HEF against all tested fungi however, the ETF inhibited *C. albicans, A. niger* (10 mm) at 12.5 µg/mL, and *P. notatum, R. stolonifer* (10 mm) at 25 µg/mL, while the MET inhibited *A. niger*, *R. stolonifer* and *C. albicans* (10 mm) at 12.5 µg/mL and *P. notatum* (10 mm) at 25 µg/mL.

There was no antifungal activity displayed by the stem aqueous extract. Although, the stem MET and its HEF at 25 µg/mL repressed *A. niger* and *C. albicans* (10 mm).

Table 4.7: Antifungal activity results of *Costus lucanusianus* cold extracts*

Parts of plant used	Solvent extracts	*C. albicans*				*A. niger*				*P. notatum*				*R. stolonifer*			
	Concentration (µg/mL)	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5
	Aqueous	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hexane	14	12	10	-	14	12	10	-	-	-	-	-	-	-	-	-
Inflorescence	Ethyl acetate	14	12	10	-	16	14	12	10	14	12	10	-	14	12	10	-
	Methanol	12	10	-	-	12	10	-	-	14	12	10	-	14	12	10	-
	Aqueous	18	16	14	12	16	14	12	10	-	-	-	-	-	-	-	-
	Hexane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leaf	Ethyl acetate	16	14	12	10	16	14	12	10	14	12	10	-	14	12	10	-
	Methanol	16	14	12	10	16	14	12	10	14	12	10	-	16	14	12	10
	Aqueous	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	Hexane	14	12	10	-	14	12	10	-	-	-	-	-	-	-	-	-
Stem	Ethyl acetate	20	18	16	14	18	16	14	12	18	16	14	12	20	18	16	14
	Methanol	14	12	10	-	14	12	10	-	-	-	-	-	-	-	-	-
	Aqueous	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hexane	18	16	15	14	19	17	15	13	19	17	15	13	18	17	15	12
Rhizome	Ethyl acetate	16	14	12	10	16	14	12	10	18	16	14	12	16	14	12	10
	Methanol	18	16	14	12	16	14	12	10	16	14	12	10	18	16	14	12
	+ve Standard Tioconazole (70%)	28				26				28				28			
	-ve Standard Methanol	-				-				-				-			

**C. albicans= Candida albicans, A. niger=Aspergillus niger, P. notatum=Penicillium notatum, R. stolonifer= Rhizophus stolonifer.*

The ETF at 12.5 µg/mL displayed IZD against *C. albicans, R. stolonifer* (14 mm), *P. notatum* and *A. niger* (12 mm).

The antimicrobial results revealed that at 12.5 µg/mL the ETFs of the leaf and stem showed the best antimicrobial property against *E. coli* and *C. albicans*.

There was also no antifungal activity displayed by the rhizome aqueous extract although the ETF revealed inhibition against *R. stolonifer*, *C. albicans* and *A. niger* (10 mm), and *P. notatum* (12 mm) at 12.5 µg/mL. The HEF of the rhizome revealed inhibition against *C. albicans, A. niger* (14 mm) at concentration 12.5 µg/mL and the MET inhibited *A. niger*, *P. notatum* (10 mm) and *C. albicans, R. stolonifer* (12 mm) at concentration 12.5 µg/mL.

There was no antimicrobial activity displayed by the stem and rhizome aqueous extracts of *C. lucanusianus* against the tested organisms. Traore *et al*. (2017) documented the antibacterial inactivity of *C. lucanusianus* stem aqueous and methanol extracts against *K. pneumoniae*, *P. aeruginosa, S. aureus* and *E. coli* at concentrations 50-200 mg/mL, so also the antimicrobial inactivity of *C. speciosus* rhizome methanol and aqueous extracts against *E. coli, S. aureus, K. pneumoniae* and *P. aeruginosa* was documented by Duraipandiyan *et al*. (2012). The inactivity displayed by the inflorescence, rhizome and stem aqueous extracts against all tested fungi may be because the active components are present in an insufficient amount in the concentrations of the extracts used to display activity (Taylor *et al.,* 2001).

In this present study, *C. lucanusianus* rhizome hexane extract amongst other extracts displayed the best broad-spectrum activity against tested microorganisms. However, according to Duraipandiyan *et al*. (2012), *C. speciosus* rhizome hexane extract among others tested at 12.5 mg/mL displayed the highest antimicrobial property only against *B. subtilis* (12 mm) and *S. aureus* (15 mm). The antimicrobial activity displayed by the rhizome HEF of *C. lucanusianus* could have been due to the presence of terpenoids which were absent in the other hexane fractions. The antimicrobial activity of terpenoids has been reported (Mahizan *et al.,* 2019). The inactivity of the inflorescence HEF against *P. notatum* and *R. stolonifer* might be due to the absence of saponin as revealed by the phytochemical screening (Killen *et al.,* 1998; Quiroga *et al*., 2001). Although, saponin was absent in both stem hexane extract and the rhizome ethyl acetate fraction, the antimicrobial activities observed in these extracts would have been

due to other bioactive constituents present. *C. lucanusianus* inflorescence, leaf, stem and rhizome ETFs displayed antifungal activity. The highest activity was displayed by the stem ETF Duraipandiyan and Ignacimuthu, (2011) reported the promising antifungal property of *C. speciosus* rhizome ETF against *A. niger* and *C. albicans.*

The inflorescence, leaf and stem hexane fractions and the stem methanol extract of *C. lucanusianus* are resistant to *R. stolonifer* and *P. notatum*. The inactivity exhibited by the inflorescence, rhizome and stem aqueous extracts against all tested fungi may be because the active components are present in an inadequate amount in the concentrations of the extracts used for it to display activity (Taylor *et al*., 2001). Activity against other fungi and bacterial species not utilised in this present work may be shown by the extracts (Shale *et al.,* 1999).

In conclusion, in this study, the cold aqueous extracts of the stem and rhizome displayed no antimicrobial activity against all tested microorganisms. The cold aqueous extract of *C. specious* rhizome was also detected to be inactive against the tested bacterial and fungal strains. In contrast, the hot aqueous extract displayed only antibacterial activity, and this was against *P. aeruginosa* and *S. aureus*. The high temperature might be required to activate the constituents present in these extracts. The traditional way of making use of plant materials for treating bacterial infections is by boiling the plant in water or obtaining a decoction of the plant (Tiwari *et al*., 2011).

Antimicrobial property of the aerial and rhizome solvent extracts of *C. lucanusianus* plant showed promising activities against uropathogenic organisms and could, therefore, be employed in the treatment of UTI, while the other extracts could also be useful in the cure of some other infectious diseases.

4.5 Characterisation of Isolated Compounds

4.5.1 Characterisation of isolated compound C

Compound C was a white crystalline powder (25 mg) with a melting point of 131°C-133°C. It gave R_f values of 0.55, 0.66 and 0.35 as a singular spot in hexane: ethyl acetate (3:1 and 3:2), and dichloromethane: hexane (3:1) respectively.

The IR (KBr) cm^{-1} spectrum (Appendix 1) showed significant absorptions as listed in Table 4.8.

The data obtained from IR spectrum suggested the existence of a combination of two compounds with similar functional groups. The absorption peaks at 3786 cm^{-1} and 3393 cm^{-1} indicated the existence of two hydroxyl groups.

The proton NMR spectra of compound C (Appendices 2-3) ranged from δ_H 0.63- δ_H 5.3.The proton spectra displayed high intensity peaks between δ_H 0.63 and δ_H 1.59 for methyl groups. Other signals between δ_H 1.59 and δ_H 2.2 were assigned to the methylene protons. The δ_H 2.2 was assigned to (2H, CH_2-4).The multiplet (triplet of doublet) at 3.52 ppm was assigned to the H-3 of the sterol moiety and doublet at δ_H 5.3 was assigned to C-6 (presence of a proton attached to C=C). δ_H 5.14 (dd, J = 15.0, 8.6 Hz) and δ_H 5.03 (dd, J =15.0, 8.6) were given to C-22 and C-23 respectively.

^{13}C spectra (Appendices 4-5) revealed 44 carbon atoms which were resolved with DEPT 135 (Table 4.9). Out of these carbon atoms, 29 were similar to those documented for stigmasterol (Syafrinal *et al.,* 2015; Edilu *et al.,* 2015). The remaining carbon atoms δ_C [121.6 (C-6), 50.0 (C-9), 36.1 (C-10), 42.1 (C-13), 56.8 (C-14), 24.8 (C-15), 28.2 (C-16), 56.0 (C-17), 18.7 (C-21), 33.9 (C-22), 26.0 (C-23), 45.7 (C-24), 18.9 (C-27) and 11.9 (C-29)] were compared with the signals of β-sitosterol as reported by (Chaturvedula and Prakash, 2012; Sen *et al*., 2012; Edilu *et al*., 2015). The overlapping of the carbon signals observed could have been due to the similarity in the chemical structure of these two compounds. Signals with high intensity represented the overlappings in the 600MHz. The three quaternary carbon atoms in both compounds were observed at δ_C 140.7, 36.4 and 42.2). The only difference is the C-22/C23 double bond which is present in stigmasterol but absent in sitosterol. It has been reported that obtaining sitosterol in the pure state is a difficult task. Sitosterol/ stigmasterol mixture usually contain a greater percentage of stigmasterol (Pateh *et al.,* 2008). The ^{13}C NMR spectrum and data obtained in this research were comparable to those documented for a mixture of sitosterol and stigmasterol by (Luhata and Munkombwe, 2015). The protons attached to the heteroatoms which do not always exhibit coupling with neighboring protons were also noticed as singlets at δ 4.71 and δ 4.66 ppm. In a delayed COSY experiment (Appendix 7) emphasizing long-range coupling, the singlet at δ 4.77 which corresponds to the -OH functional group exhibited cross-peaks with the proton-doublet occurring at δ 5.35 and δ 5.36. A signal observed at δ C 71 in the DEPT 135 experiment specified the existence of hydroxyl moiety at C-3 position. This

was further established through HMBC experiment (Appendix 8) by the observed correlation between δ H 5.3 (d) and the carbon at δ C 121. Other correlations noticed in the HMBC experiment were between δ_C 129 and δ_H 4.9, 5.0; δ_C 138 and δ_H 5.0, 5.1 and 5.2.

The structure of compound C was further established through the correlations observed in the HMBC (Appendix 8) and NOESY (Appendix 9) experiments. Compound C was a mixture of sitosterol and stigmasterol.

Table 4.8: The significant IR absorption peaks of Compound C from *Costus lucanusianus*

Absorption (cm^{-1})	Group assignment
3786	O-H stretching vibration
3434	O-H stretching vibration
2938	C-H stretching in CH_2
2873	C-H stretching in CH_3 group
1639	Weak C=C stretching
1464-1383	Gem dimethyl vibration
1050-1022	C-O bond
968	Out of plane C-H bending of terminal alkane

Table 4.9: ^{13}C NMR data of a mixture of sitosterol and stigmasterol from *Costus lucanusianus*

Carbon Position	Compound**1***		Compound **2***	
	Experimental	Literature	Experimental	Literature
1	37.23	37.50	37.23	37.60
2	31.86	31.90	31.62	32.1
3	71.79	72.00	71.79	72.10
4	42.27	42.50	42.18	42.40
5	140.73	140.90	140.73	141.10
6	121.68	121.90	121.68	121.80
7	31.86	32.10	31.62	31.80
8	31.86	32.10	31.62	31.80
9	50.11	50.30	50.09	50.20
10	36.49	36.70	36.13	36.60
11	21.02	21.30	21.05	21.50
12	39.74	39.90	39.65	39.90
13	42.27	42.60	42.18	42.40
14	56.84	56.90	56.74	56.80
15	25.40	26.30	24.35	24.40
16	29.10	28.50	28.92	29.30
17	56.84	56.03	56.74	56.20
18	11.96	11.98	11.84	12.06
19	19.82	19.06	19.39	19.80
20	40.50	39.82	40.50	39.82
21	18.97	18.78	18.76	18.96
22	39.91	33.93	138.32	138.40
23	26.04	26.04	129.24	129.34
24	51.22	51.30	51.20	51.13
25	29.10	29.13	28.92	29.60
26	21.20	21.12	21.06	21.20
27	19.82	19.41	19.39	19.80
28	26.00	25.32	25.41	25.40
29	12.24	12.27	12.03	12.20

^{13}C Spectrum (cdl_3) recorded on Bruker Avance III 150.0 MHz. 1*, 2* (Chaturvedula and Prakash, 2012; Sen *et al*., 2012; Edilu *et al*., 2015; Pateh *et al.,* 2008; Luhata and Munkombwe, 2015).

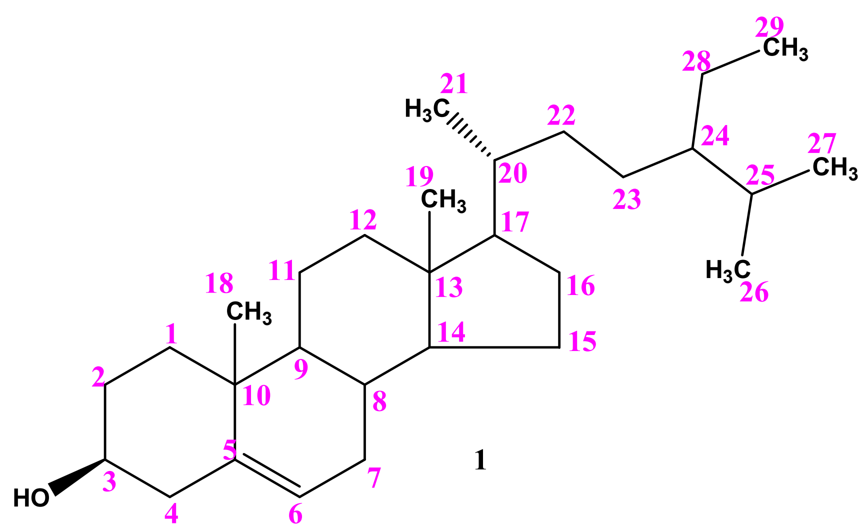


Figure 4.1: Chemical structure of β-sitosterol from *Costus lucanusianus*

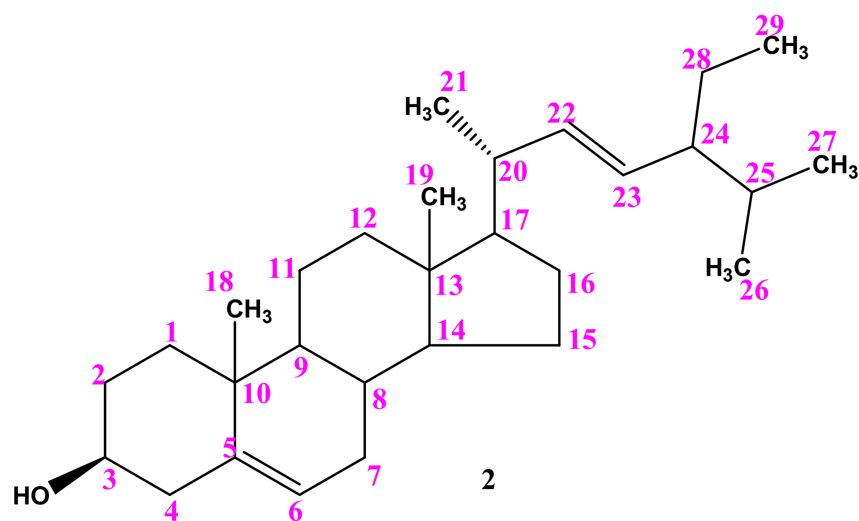


Figure 4.2: Chemical structure of stigmasterol from *Costus lucanusianus*

4.5.2 Characterisation of Isolated Compound Xp

The ^{13}C and ^{1}H NMR of compound Xp was determined using a Bruker Topsin 100 MHz and 400 MHz spectrometer respectively and reported in relation to tetramethylsilane. Fourier Transform Infra-Red (FT-IR) spectrum was acquired by bruker platinum ATR Tensor 27 FT-IR Spectrophotometer.

Compound Xp appeared as an amorphous white powder (20 mg) with R_f values of 0.6, 0.8 and 0.6 in diethyl ether: hexane 3:1, hexane: ethyl acetate 3:2 and 3:1 respectively and a melting point of 129°C. The IR spectrum (Appendix 10) displayed two strong absorption bands at 3500 cm^{-1} and 1655 cm^{-1}. These bands were attributed to hydroxyl and olefinic functional groups respectively. Also, an etheric bond (C-O) at 1021 cm^{-1} was observed. Many strong bands between 875 and 1350 cm^{-1} were detected. These are typical of the spiroketal side chain.

In the ^{1}H NMR spectrum (Appendix 11), few signals were recognisable as a result of extensive inter-proton coupling. The proton NMR chemical shifts (Table 4.10) for the signals (>3ppm) such as oxy substituted methylene and methine resonances, olefinic and the angular and secondary methyl signals are characteristic signals in the ^{1}H NMR of steroidal sapogenin because CH_3 and CH_2 resonances were observed as multiplets (overlapping of signals) (Agrawal *et al*., 1995).

Four methyl singlets were observed between δ 0.9 and δ 1.60 in the proton spectrum and the most downfield methyl singlet at δ 3.90 was as a result of methoxy moiety which showed resonance in the ^{13}C spectrum at δ C 56.96. H-1, 3 and 26 appeared as a multiplet within the range of δ 3.42-3.49.The absorption at δ 5.2 ppm (dd) was allotted to the H-6. Various connectivities in Xp were determined through HMBC (Appendix 14) experiment.

The broadband ^{13}C NMR spectrum (Appendix 12) gave information about the skeletal type of compound Xp (spirostanol type). The DEPT 135 experiment (Appendix 13) was used to resolve the broadband signals noticed in the ^{13}C NMR spectrum into four quaternary, five methyl, nine methine and ten methylene carbon atoms (Table 4.11). Compound Xp parent skeleton was established with C-22 multiplicity and chemical shift values. C-22 of compound Xp was of keto group with δ_C 206.97. The 22-deoxy-spiroidal sapogenin of 16-hydroxy cholestane skeleton type has chemical shift values δ 34.8-35.4 (Agrawal *et al.,* 1995). The presence of four methyl resonances is the

common features of all steroidal sapogenin. However, five methyl resonances may occur occasionally (Agrawal *et al.,* 1995) as in compound Xp (two tertiary methyls and three secondary methyls).

The carbonyl absorption at δC 206.97 was allotted to the keto group at C-22, while the hydroxylated methine at C-3 was δ 69.73 and oxy-substituted C-27 was at δ 67.23. The most commonly encountered unsaturation is the double bond at C-5 which resulted into the display of C-5 and C-6 at δ 141.0 (C) and δ 121.9 (CH) respectively. This can be substantiated by the absorption observed at 5.28 ppm (dd) for the H-6 signal. The unsaturation was also further established by the cross-peak observed between δ_H 5.28 and δ_C 121 in the HMQC Sp., and the correlation detected between δ_C 141 and δ_H 0.9 in the HMBC spectrum. Correlations were also noticed in the HMBC Sp between δ C 207 and δ H 2.1; δ C 31 and δ H 1.9, 2.1 and 2.3 while cross-peaks were also noticed in the HMQC spectrum between δ C 31 and δ H 2.1; δ H 0.9 and δ C 18.

All carbon and proton assignments were finally made with COSY, HMBC, HMQC and NOESY experiments (Appendices 14-17).

Table 4.10: The Proton data of compound Xp from *Costus lucanusianus*

δ value (ppm)	Multiplicity
5.20	dd
3.90	s
3.45	m
1.94	s
1.20	s
0.90	s

Proton spectrum ($CDCl_3$) recorded on Bruker Topspin 400 MHz spectrometer. d = doublet, s = singlet, m = multiplet, dd = double doublet

Table 4.11: The ^{13}C NMR data of methyl, methylene, methine and quaternary carbon of compound Xp from *Costus lucanusianus**

Carbon No.	δ value	Assignment	Carbon No	δ value	Assignment
1	72.50	CH	15	24.12	CH_2
2	34.88	CH_2	16	26.54	CH_2
3	69.73	CH	17	55.00	CH
4	38.00	CH_2	18	19.00	CH_3
5	141.00	Cq	19	12.07	CH_3
6	121.70	CH	20	31.00	CH
7	32.00	CH_2	21	17.06	CH_3
8	31.05	CH	22	206.97	C=O
9	48.98	CH	23	33.00	CH_2
10	36.40	Cq	24	30.27	CH_2
11	22.05	CH_2	25	34.44	CH
12	39.37	CH_2	26	13.85	CH_3
13	43.83	Cq	27	67.23	CH_2
14	56.50	CH	28	56.96	CH_3

^{13}C spectrum recorded on Bruker Topsin III 400 at 100.0 MHz in $CDCl_3$. Cq= quaternary carbon

The fragmentation mechanisms of compound Xp, its exact mass and structural information were acquired by Quadrupole-time-of-flight MS/MS with attached proton chemical ionisation in the positive mode as shown in Table 4.12. The mass fragmentation pattern of Xp observed in the APCI-ToF-MS/MS (Appendix 18) clearly indicated the presence of spirostanol type of skeleton (Agrawal *et al.,* 1995). Comparison of the proton and carbon data with reported data for spirostanol further established this.

The APCI-ToF-MS/MS Sp of compound Xp revealed a base peak ion at m/z 397.3070 corresponding to $C_{27}H_{41}O_2$ with a double bond equivalent 5.5 and a protonated molecular ion at m/z 446.2996. A fragment loss of m/z=31 at m/z 415.3174 proposed the existence of a methoxy group. An absorption band at 1020 cm^{-1}in the infra-red spectrum further established the existence of C-O-C bond.

In APCI-ToF/MS/MS, the main fragment ions were derived from the loss of CH_3OH (m/z=31) followed by the protonation of the oxygen at C-16 to produce m/z 416. This protonation led to the formation of positive charge on the oxygen. Subsequently, there was transfer of electron pair from C-16 to oxygen which led to the breaking up of C-16-O bond. Carbonyl at C-22 was produced by the tautomerisation due to the presence of an enol. The dissociation of the intermediate took place in three pathways (Figure 4.3).

In pathway A (main), there was the breaking up of C-17-C-20 bond. This resulted into the production of m/z 271.20.

In pathway B (minor), there was transference of hydrogen from C-23 to C-20. The breaking up of C-20-C-22 bond followed thereafter. Lastly, the formation of m/z 283.24 was an outcome of the H_2O molecule removed.

In pathway C, there was a sequential removal of two molecules of water to generate ions at m/z 397.31 and 379.29.

From the above, the molecular weight of Xp was found to be 445 with molecular formula $C_{28}H_{45}O_4$ and was named 3,27-dihydroxy-1-methoxy-22-cholest-5-enone (Figure 4.4).

Table 4.12: Data of fragment ions in the positive mode derived from spirostanol aglycone compound Xp from *Costus lucanusianus*

Fragment Ions	Accurate Mass
$C_{27}H_{43}O_3^+$	415.3174
$C_{27}H_{41}O_2^+$	397.3070
$C_{27}H_{39}O^+$	379.2967
$C_{21}H_{31}^+$	283.2400
$C_{19}H_{27}O^+$	271.2044
$C_{19}H_{25}^+$	253.1951

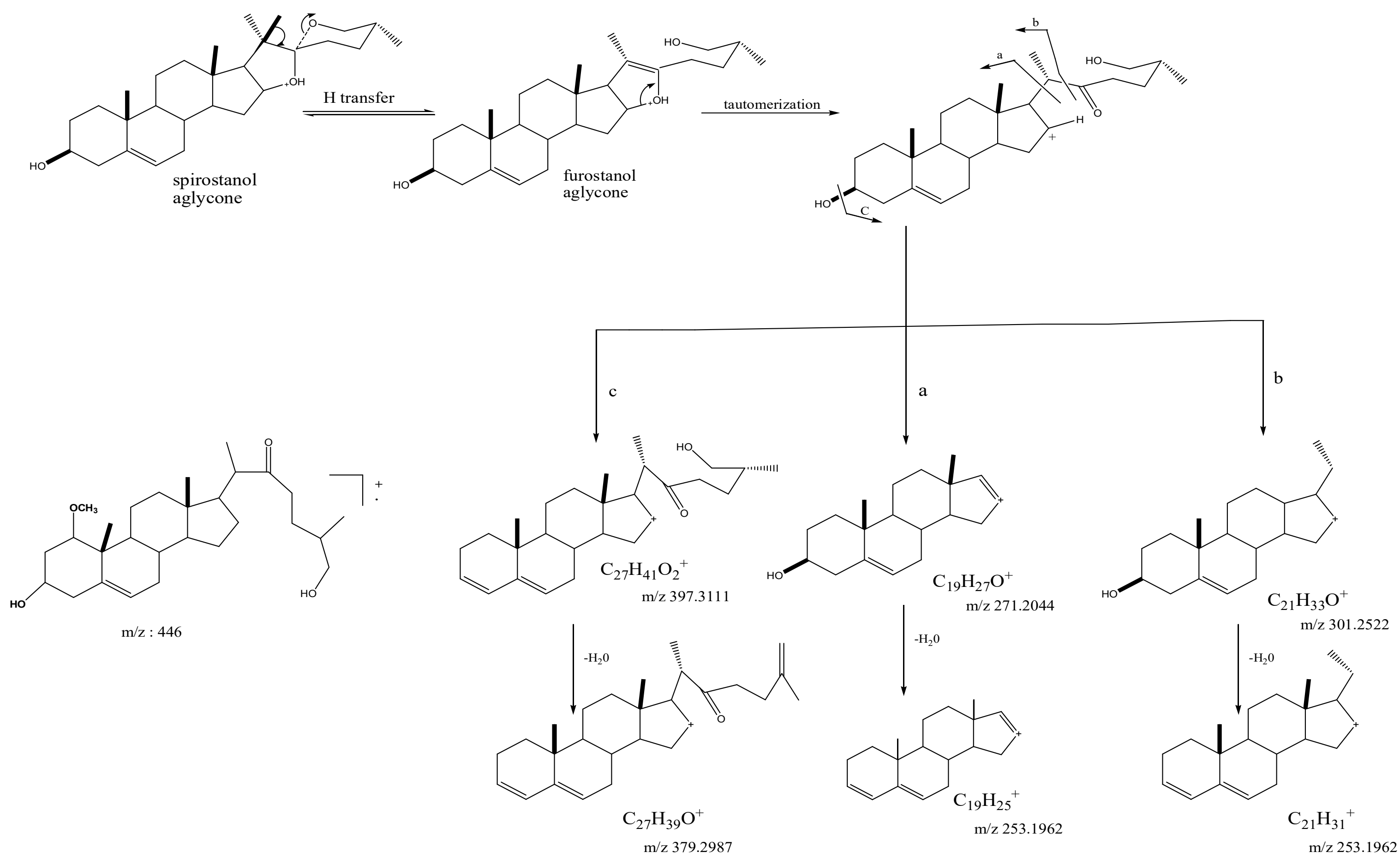


Figure 4.3: Fragmentation pathways of spirostanol aglycone (Xp) using APCI-QqTOF-MS/MS

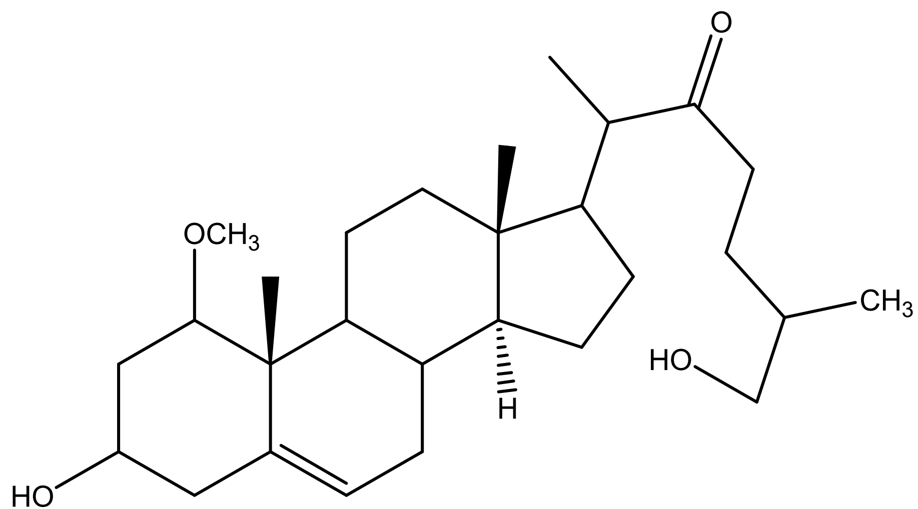


Figure 4.4 Structure of 3,27-dihydroxy-1-methoxy-22-cholest-5-enone from *Costus lucanusianus*

4.5.3 Characterisation of Isolated Compound 1

Compound 1 was an amorphous ash-colored solid (25 mg) of a melting point 235°C with R_f values of 0.81, 0.63 and 0.75 in ethyl acetate : methanol (5:1), ethyl acetate : methanol (7:1) and diethyl ether : methanol (4:1) respectively.

IR μ_{max} (KBr) cm^{-1} spectrum (Appendix 19) of compound 1 displayed broadband in the area 3420 cm^{-1} specifying the existence of hydroxyl group. Also bands observed at 2910 and 2850 cm^{-1} corresponded to asymmetric aliphatic –CH stretching of CH_3 and CH_2 groups respectively. An etheric bond (C-O) at 1021cm^{-1} and C=C stretching absorption band at 1640 cm^{-1} were noticed. The steroidal nature of compound 1 was supported by a peak at 920 cm^{-1}.

^{1}H NMR (DMSO-*d6*, 400MHz) of compound 1 (Appendix 20) displayed several signals (-CH_2, -CH_3 protons) in the aliphatic area of the spectrum (δ 0.6-1.1 ppm) as shown in (Table 4.13). Proton equivalent to the H-3 of a sterol moiety usually appears at *δ* 3.4 ppm as a multiplet (Ahmad and Alam, 1995; Kiriakidis *et al.,* 1997) while that of H-1 proton of sugar usually appear as a multiplet at *δ* 4.1 (Rawat *et al.,* 2011). However, this appeared as a broad singlet. The proton NMR also displayed a signal at *δ* 2.1 (s) which was suggested to be for the methyl protons attached to a carbonyl group. Signal at δ 3.6 (s) was due to the hydrogen of the heteroatom –OH.

The broadband (BB) spectrum ^{13}C NMR (Appendix 21) displayed altogether six signals at *δ* C 207.0, 79.0, 56.5, 49.0, 31.1 and 19.0. These signals were further resolved via distortionless enhancement by polarisation transfer (DEPT 135) (Appendix 22) to contain 38 carbon atoms (Table 4.14) out of which 29 carbon atoms were similar to those reported values for β-sitosterol (Rahman *et al*., 2009, Chaturvedula and Prakash, 2012, Njinga *et al*., 2016, Peshin and Kar, 2017). Other prominent peaks at *δ* C 102 and 71.0 which indicated carbon linked to oxygen atoms were observed in the DEPT 135. The oxygenated carbon C-1 of the xylose appeared at δ 102 ppm. The carbon signals of the xylose moiety were noticed at *δ* C 77.0, 102.0, 65.8, 49.0 and 71.00 ppm.

A carbon chemical shift at δ 102 and an anomeric proton at δ 4.1 revealed the existence of sugar unit. To the C-3 of the aglycone was assigned the absorption noticed

Table: 4.13: Proton data of compound 1from *Costus lucanusianus*

δ value (ppm)	multiplicity
1.1	m
2.1	s
3.0	m
3.4	m
3.6	s
4.1	s (Broad)
4.4	m

Proton spectrum (DMSO-*d6*) was recorded on Bruker Avance III at 400.0 MHz. s = singlet, m = multiplet

Table 4.14: Carbon data of Compound 1from *Costus lucanusianus*

Carbon no	δ value	Assignment	Carbon no	δ value	Assignment
1	36.60	CH_2	16	28.64	CH_2
2	29.21	CH_2	17	56.00	CH
3	79.00	CH	18	11.95	CH_3
4	41.60	CH_2	19	19.00	CH_3
5	140.00	C_q	20	33.28	CH
6	121.00	CH	21	18.40	CH_3
7	31.00	CH_2	22	33.00	CH_2
8	31.08	CH	23	25.38	CH_2
9	50.01	CH	24	45.61	CH
10	36.12	C_q	25	28.64	CH
11	20.50	CH_2	26	18.73	CH_3
12	38.20	CH_2	27	18.58	CH_3
13	41.60	C_q	28	22.06	CH_2
14	55.80	CH	29	12.20	CH_3
15	23.00	CH_2		Substituents	
C-1[1]	102.00	CH		207.00	C=O
C-2	77.00	CH		56.4	CH_2
C-3[1]	49.00	CH		31.00	CH_3
C-4[1]	71.00	CH		78.00	Cq
C-5[1]	65.80	CH_2		23.1	CH_3
Substituent	34.0	CH		12.0	CH_3

^{13}C Spectrum (DMSO) recorded on Bruker Avance III 400 at 100.0 MHz. Cq=quartenary carbon.

at δ 79.0.

A signal observed at δ 2.1 (singlet and intense) was assigned to the CH_3 of methyl keto. The δ_C 19 and δ_C 11.95 were allotted to the angular methyl carbons at positions 19 and 18 of sitosterol respectively (Luhata and Munkombwe, 2015). There was a methylene carbon signal also noticed at δ 56 assigned to the methylene next to the keto group. Correlation noticed between carbon at δ 140 and proton at δ 0.8 in the HMBC spectrum (Appendix 23) substantiated the unsaturation assigned between C-5 and C-6. Although an olefinic absorption was absent in the proton spectrum, the existence of an olefinic bond was sustantiated by IR band at 1640 cm^{-1}. The full structural elucidation was established by comparison with the literature and the data obtained from COSY, HMBC, HMQC, NOESY and mass spectroscopy experiments (Appendices 23-27).

All the proton signals were correlated to their respective carbons with the help of the HMQC experiment. As shown in Figure 4.5, attachment of a methyl group to the quaternary carbon was established by the correlation noticed in the HMBC Sp between protons at δ 2.1 (CH_3) and a quaternary carbon at δ 207 and also the cross peak noticed between δ_C 31 and δ_H 2.1 in the HMQC spectrum (Appendix 25). There was a broad intense singlet (which indicated the existence of a β-xyloside unit) of anomeric proton at δ H 4.1 which exhibited a long range coupling with a methine at δ 49 in the HMBC Sp, which likewise also displayed an interaction with protons at δ 3.0 and δ 3.2. The protons of sugar moiety usually appear at about δ 3.2 (Ali *et al.,* 2002). The methylene of sugar unit which usually appear at about δ 3.20 resonated as a multiplet at δ 4.4. The downfield chemical shifts of the C-2 and C-5 proton could have been due to the existence of the acetyl group in the sugar unit. In the HMQC, where the carbon at δ 49 revealed a cross-peak with proton at δ 3.2 of the sugar moiety, this suggested the point of linkage of the substituent to the sugar (Njinga *et al.,* 2016) and also the presence of 3-deoxy xylose.

The C-C linkage at δ_H 49 was established by the correlation observed in the HMBC between δ_C 49 and δ_H 4.1 and δ 3.0, δ 3.2. The absence of IR peak at 1685-1700 cm^{-1} and chemical shift value at δ C 170 $-^{+}$1.5 which indicated the absence of conjugated ketone or ester further confirmed the assignment of the keto functional group.

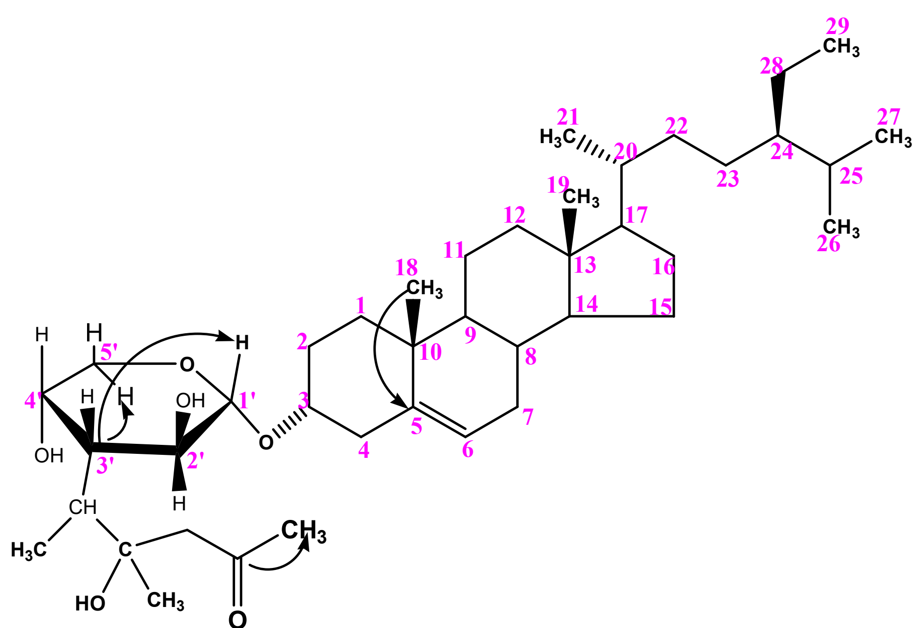


Figure 4.5: HMBC connectivities of Compound 1

The sugar unit point of attachment to the aglycone was established as 1-3 with the help of downfield methine carbon signal of sugar at δ 102 and the C-3 downfield chemical shift of (δ 79) as opposed to the δ 71 for C-3 in β-sitosterol (Luhata and Munkombwe, 2015) and connectivities noticed in the HMBC spectrum.

The ESI-ToF/MS/MS in the positive mode (Appendix 27) displayed the utmost strong peak at m/z 397 which is equivalent to $[C_{29}H_{49}]^+$ having five degree of unsaturation (DBE=5); four rings and an olefinic bond which is suggestive of the aglycone part to be sitosterol. Additional ions found were at *m/z* 124, 125, 299 and 468. Protonated ions *m/z* at 398 and 399 were also observed. A molecular ion was detected at *m/z* 658 in the spectrum. There was also a precursor ion at *m/z* 606 which fragmented to produce daughter ions. Some of the observed ions were similar to some of the fragments observed in 3-β-D-glycopyranosyl-β-sitosterol (Chaturvedula and Prakash, 2012). The peaks observed at m/z 396 and 397 are distinctive of β-sitosterol (Kiriakidis *et al.,* 1997; Aziz and Habib-ur-Rehman, 2008). Likewise, Kalo *et al*. 2006 reported m/z 299 as a specific fragment for sterol with saturated side chain and other fragments at m/z 273 and 175 as fragments from sterols, this further supported the fact that the aglycone was not stigmasteol. Few fragment ions detected could have been due to D-xylose which is renown for its existence in pyranosic form in aqueous solution. The proposed pathways for the formation of *m/z* 468 was fragmentation of the xylose (between C-3' and C-4') atom of the sugar unit to give a molecule (-486), then loss of water molecule (-18) to give *m/z* 468 (Figure 4.6).

The most characteristic peak of steroidal sapogenin is at m/z 139. The fragmentation of steroids, the loss of the methyl group is the first point of interest. The loss CH_3 (m/z 15) from sitosterol (m/z = 414) gave m/z 399. Molecular formula for compound 1 was established to be $C_{41}H_{70}O_6$. Acylated sterol glycosides are known to occur naturally in plant tissues and the acylation usually assumed to be at C-6. However C-4 acylation was also reported by Jin *et al.* (2007). Herein, we reported the C-3 acylation of deoxy-xylose.

Sitosterols have been reported to undergo oxidation processes to form sitosterol oxidated products. Different derivatives of sitosterol that have been isolated include β-sitosterol diglucosyl caprate, β-sitosterol-3-O-butyl, 7-keto sitosterol, β-sitosterol glucoside-3-O-hexacosanoicate, β-sitosterol-3-O-β-D-xylopyranoside and 7-O-β-D-

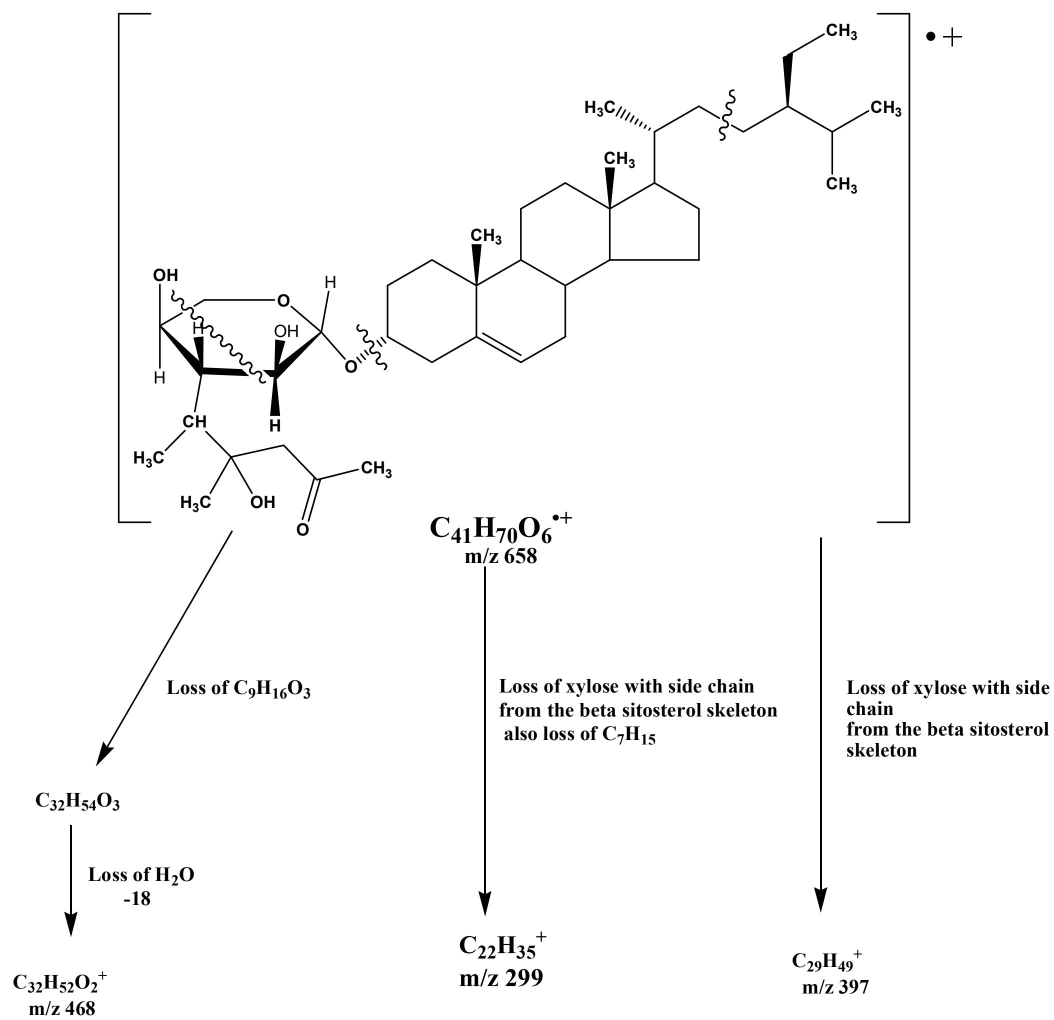


Figure 4.6: Fragmentation pathway of β-sitosterol-3-O-β-D-3-deoxyxylo-4-hydroxy4,5-dimethyl-pent-2-one

fucosyl-3-oxo-4-en clerosterol (Ali *et al.,* 2002; Saxena and Albert, 2005; Jin *et al*., 2007; Jaju *et al.,* 2010 and Osman *et al.,* 2016). However, a new addition to the family of steroids having acylated 3-deoxy-xylose is hereby reported. Compound 1 was trivally named β-sitosterol-3-O-β-D-3-deoxyxylo-4-hydroxy-4,5-dimethyl-pent-2-one (SDHP) (Figure 4.7).

4.5.4 Characterisation of Compounds Isolated from the Stem

The purification of ethyl acetate fraction of the stem afforded compounds A and B but could not be characterised due to the problem encountered with the solvent of dissolution. R_f values of compounds A and B in dichloromethane; methanol 10:1, ethyl acetate: methanol 7:1 and 5:1 were 0.53 and 0.64; 0.34 and 0.46; 0.50 and 0.66 respectively. The compounds were spotted immediately after the collection of fractions from the column. However, the compounds became insoluble after crystallisation from solution. Compound A was a white solid (yield=2.5 mg) while compound B was jelly-like colorless solid (yield=7.7 mg).

4.5.5 Characterisation of compound C_{2018}

Compound C was a white powder (yield=12 mg) of melting point 160°C and positively reacted to Dragendorff reagent giving an orange color, suggesting the presence of alkaloidal compound.

FT-IR (cm^{-1}) spectrum (Appendix 28) revealed absorptions at 3400, 2980, 2810, 1500, 1020, 1640, 850 and 1200 cm^{-1} for hydroxyl, sp^2 of CH_3 and CH_2, N-O, etheric bond (C-O-C), olefinic bond, O-O and C-N respectively. 1D, 2D NMR and QToF/MS/MS in the ESI positive mode were used in determining the elucidation of the whole structure of compound C_{2018}.

Proton and Carbon (DMSO) data were obtained by Bruker Topsin spectrometer 400 and 100 MHz respectively. In the proton NMR (Appendices 29-30), there were overlapping of signals observed between δ 0.8 and δ 1.5 (Table 4.15). These overlapping signals were assigned to the methyl and methylene signals which are characteristics of steroidal sapogenin. In the proton spectrum, a signal displayed at δ 4.1 (s) broad and intense was allotted to the anomeric proton of the sugar molecule while the δ 3.3 (m) was allotted to the C-3 proton of the aglycone.

The attachment of the observed –OCH_3 to the nitrogen atom was substantiated with IR band at 1500 cm^{-1} (N-O). Signal at δ H 2.1 (singlet and intense) suggested the presence of methyl keto group.

The signals at δ_H 4.04-4.11(m) and δ_H 4.31-4.44 (m) were assigned for the sugar protons. The signals at δ H 7.9-8.6 (Table 4.16) were also assigned to the ring unsaturated protons: δ 8.65 (dd, J=4.00, 6.83 Hz, 1H), 8.44 (d, J=6.83 Hz, 1 H) 8.29 (dd, J=4.0, 8.0 Hz, 1H), 8.19 (d, J = 6.83 Hz, 1H) and 7.93 (d, J = 7.94 Hz, 1H).

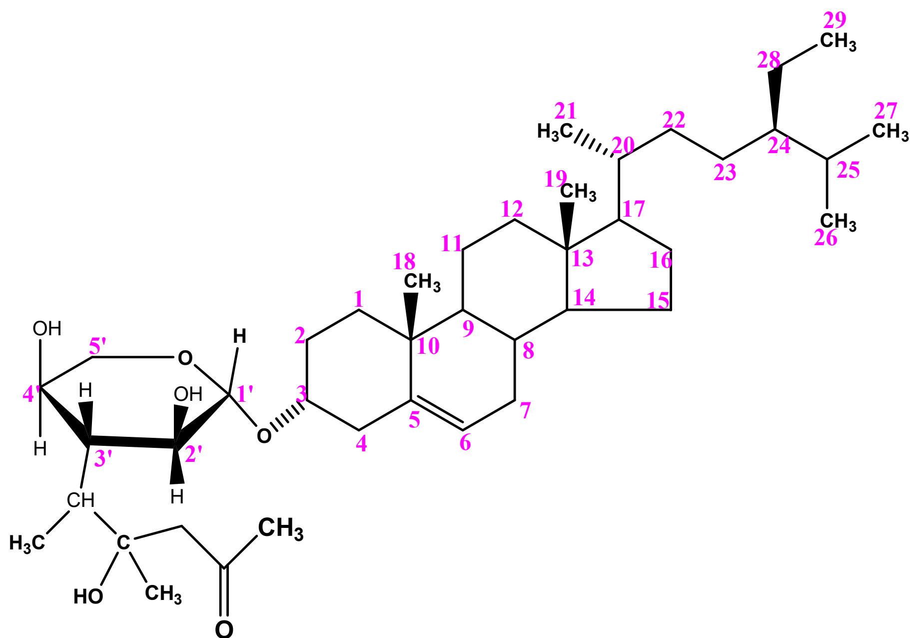


Figure 4.7: Structure of β-sitosterol-3-O-β-D-3-deoxyxylo-4-hydroxy4,5-dimethyl-pent-2-one from *Costus lucanusianus*

Table 4.15: Proton data of Compound C_{2018} from *Costus lucanusianus*

δ value (ppm)	multiplicity
8.65	dd
8.44	d
8.29	dd
8.19	d
7.93	d
4.4	m
4.1	s
3.3	m
2.3	s
2.1	s
1.9	s
1.5	m
0.9	d

Proton spectrum (DMSO-*d6*) recorded on Bruker Topsin 111 at 400 MHz. d = doublet, m = multiplet, s = singlet.

Table 4.16: Expanded proton NMR of compound C_{2018} from *Costus lucanusianus*

Name	Shift	H's	Integral	*J*'s (Hz)
A (d)	8.19	1	0.62	6.83
B (dd)	8.29	1	1.00	4.0, 8.0
C (d)	7.93	1	0.88	7.94
D (d)	8.44	1	0.92	6.83
E (dd)	8.65	1	0.87	4.0, 6.80

Proton spectrum (DMSO-d_6) recorded on Bruker Topsin 111 at 400 MHz

Table 4.17: Carbon data of compound C_{2018} from *Costus lucanusianus*

Carbon no	δ value	Assignment	Carbon no	δ value	Assignment
1	132.00	CH	17	51.20	CH
2	129.00	CH	18	16.00	CH_3
3	79.00	CH	19	23.00	CH_3
4	124.00	CH	20	52.00	CH
5	141.00	Cq	21	12.00	CH_3
6	126.00	CH	C-1	109.00	CH
7	138.00	CH	C-2	88.00	CH
8	40.10	CH	C-3	84.00	CH
9	49.00	CH	C-4	86.00	CH
10	41.50	Cq	C-5	69.50	CH_2
11	23.00	CH_2		207.00	Cq
12	39.00	CH_2		31.00	CH_3
13	41.40	Cq		42.00	CH_2
14	55.00	CH		24.00	CH
15	29.00	CH_2		14.00	CH_3
16	26.00	CH_2		77.00	CH_2
				65.20	OCH_3

Carbon spectrum (DMSO-*d6*) recorded on Bruker Topspin 111 400 at 100 MHz. Cq=quartenary carbon.

Three signals at δ 207, 40 (broadband) and 31 were observed on ^{13}C NMR spectrum (Appendix 31). These were further resolved into 33 carbon atoms of 6 methyls, 7 methylenes, 16 methines and 4 quaternary carbons in the DEPT 135 experiment (Appendix 32). Four methines and one methylene were allotted to the sugar moiety (xylose). The signals at δ 124-δ 141 were assigned to the unsaturated carbon atoms of the rings (A&B), while the δ 109 was assigned to the anomeric carbon atom. The other methine carbon atoms of the sugar moiety resonanted at δ 84-δ 86 where as the methylene carbon resonated at δ 69.5. However, the methylene carbons of pentose sugar have been reported to resonate between δ 64-δ 67 (Agrawal, 1992). The downshift of the sugar carbon atoms chemical shift values could have been due to the attached substituents. A methylene observed at δ 77.00 was finally allotted to the carbon atom attached to the peroxide (O-O).

The keto signal at δ 207 was confirmed to be for ketone functional group since no signal was observed at δ 178 in the ^{13}C NMR and no absorption at 1700-1730 cm^{-1} in the IR spectrum (Appendix 28) (suggesting the existence of carboxylic acid carbonyl). Observed correlations were between δ_C 207 and δ_H 2.1; δ_C 31 and δ_H 1.9, 2.1, 2.2; δ_C 24, 42 and δ_H 0.9 in the HMBC (Appendix 33 and Figure 4.8).

There were correlations observed also between protons at δ 0.9 and 1.5 ppm in the COSY experiment spectrum (Appendix 34). The cross-peaks observed in HMQC experiment (Appendix 35) between carbon at δ 24 and proton at δ 0.9; carbon at δ 16 and proton at δ 0.8 together with NOESY experiment (Appendix 36) were further used to establish the structure of compound C_{2018}.

The mass accuracy results from the mass spectrometry of compound C_{2018} with the aid of ESI ion source in the positive mode are summarised in Table 4.18.

The ESI-positive ion mode mass spectroscopy of compound C_{2018} (Appendix 37) displayed molecular ion and utmost intense peaks at *m/z* 590 and 552 respectively, formula $C_{33}H_{52}NO_8$, a double bond equivalent (DBE) 8.5. Seven out of the 8.5 (DBE) were assigned to a tetracyclic pregnane type of alkaloid with three double bonds.

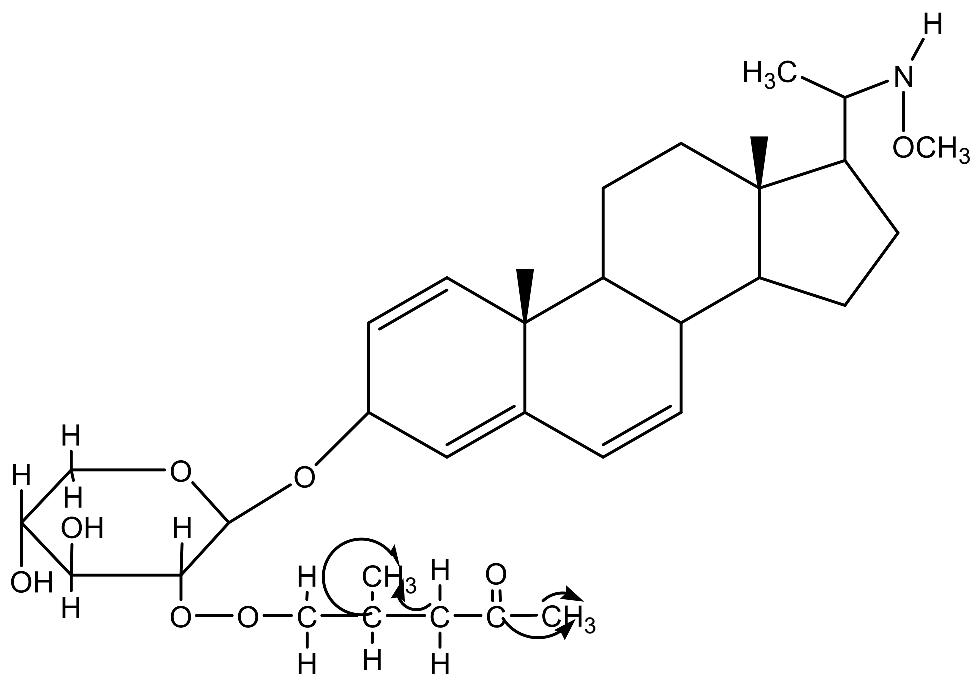


Figure 4.8: HMBC connectivities of compound C_{2018} from *Costus lucanusianus*

Table 4.18: Accurate mass data of fragment ions in ESI positive mode

Molecular ion [M^+]	Fragment ions [M^+]$^{.+}$
590	86.0973
	199.1816
	227.1761
	281.2232
	326.2448
	439.3288
	524.4182
	552.4130
	574.3948
	591.3716
	546.399

Protonated molecular ion (591.3716) was also observed. Substituents were successively lost from $[M+H]^+$ to give rise to many typical peaks detected. The major peaks in the spectrum of high mass resolution of the compound at m/z (552.4130, 574.3948 and 590.0) were selected by first stage of the analysis. These were thereafter activated by collision–induced dissociation (CID) utilising nitrogen as collision gas and then analysed by MS/MS. Figure 4.8 shows the two pathways suggested for the production of ion at *m/z* 326.

Pathway 1: This involves the loss of m/z 150 (which established xylose as the sugar moiety) and re-arrangement resulting into a peak at m/z 439. Fragment ion (m/z 325) was brought about by the loss of m/z 115 (side chain of sugar unit) from ion at m/z 439.

Pathway 2: There was a protonation of oxygen atom at C-3 of the aglycone, which was followed by electron pair transfer resulting into the loss of m/z 265 (sugar moiety and side chain) to produce a peak at m/z 326 which agreed to steroid alkaloid of pregnane skeleton. A peak noticed at $[M+H^+-46]$ = m/z 546 resulted from loss of $–NHOCH_3$ from C-20. Ion m/z 46 is generally known as a typical ion in all steroidal alkaloid (Musharraf *et al.,* 2012). The absence of characteristic peaks corresponding to m/z 84 and 71 or its losses, which are for C-3 and C-20 dimethylamino group of amino pregnane skeleton respectively further confirmed the linkage of the $–OCH_3$ to -N and assignment to C-20 (Neem *et al.,* 2005). Fragment ion (m/z 281) which resulted from the m/z 326 losing m/z 46 confirmed the aglycone unit type.

Fragment ion m/z 285 $[C_{21}H_{33}]^+$ a product of the eradication of C-3 amide moieties and/C-20 amine have been reported to be an analytical fragment utilised in the characterisation of pregnane-type steroidal alkaloids without unsaturation within the main skeleton and which also possess two substituents only at C-3 and C-20. Ion m/z 281 in compound C_{2018} resulted from the unsaturation in the rings A and B. Fragment ion m/z 281 have been reported to be a product of inductive bond cleavage as shown in (Figure 4.8) which takes place in steroidal alkaloids that possess in ring A two unsaturation (Musharraf *et al*., 2012). It was proposed that the re-arrangement of the side chain of the sugar moiety (xylose) might have led to the dissociation of the bond between the sugar moiety and the carbonyl carbon which therefore resulted into the loss of m/z 86.

Different chromatographic techniques such as silica gel and sephadex LH-20 column chromatography, preparative TLC and HPLC on C-8-, C18- silica gel have been found useful in the isolation of steroidal glycoalkaloids (Shibata *et al.,* 1982; Arnaldo and Georgina, 1982). Comparison of the fragment ions with the characteristic fragment

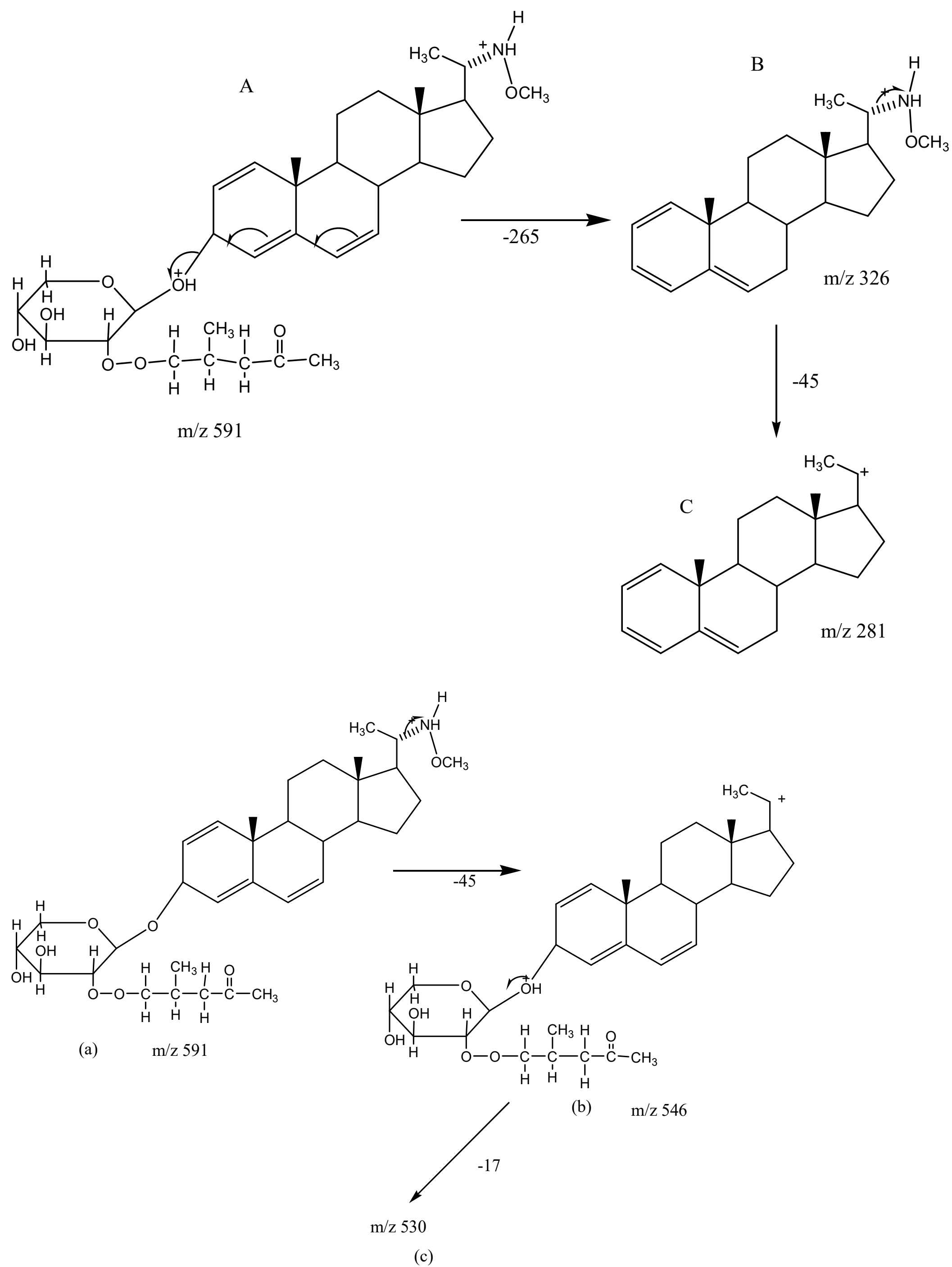


Figure 4.9: Fragmentation Pathways for fragments at m/z 326, 281, 546 and 530.

ions of Protoverine type alkaloids (m/z 436, 418), Germine type (m/z 438, 420, 402) and Zygadenin type (m/z 440, 422) in ESI positive mode, further supported the fact that compound C_{2018} was not of these types (Li *et al.,* 2007). The UV-visible absorption spectrum (Appendix 38) of compound C_{2018} in dichloromethane at λ_{max} 239 nm and an absorption of 1.46 further confirmed the structure of compound C_{2018}. Compound C_{2018} is a new addition to natural products. Compound C_{2018} was therefore named 20-N-methoxy amino lucanu-1, 4, 6-triene-2-xylo-pero-4-methylpentan-2-one (Figure 4.10). In the family Costaceae, acetylated steroidal glycoalkaloids was herein documented for the first time.

4.5.6 Characterisation of isolated compound Z

Compound Z was an amorphous colorless powder (yield=12 mg) with melting point 196-198°C. It gave R_f values of 0.41, 0.21 and 0.38 in ethyl acetate: hexane (7:3, 6:1) and chloroform:methanol (19:1) respectively.

IR µmax(KBr) cm^{-1} : 3450, 3420 (OH), 2925, 2825 (CH), 1640; 1440; 1040,1020; 920. ^{1}H NMR (600 MHz, $CDCl_3$) (Appendices 39-40) signals ranged between δ_H 0.79 and δ_H 5.36. The very close values of the proton signals (Table 4.19) suggested the presence of two similar compounds. There was comparison of proton signals with the literature (Faini *et al.,* 1984; Puri *et al*., 1993; Abdel-Aziz *et al* 1990). The carbon-13 spectrum revealed a total of 52 carbon signals of very close chemical shift values. Carbon atoms (44) were accounted for in the assignment of carbon signals. Overlapping of 10 carbon signals were noticed at postions C-1 (37.38), C-2 (31.78), C-3 (71.89), C-4 (42.43), C-5 (140.9), C-6 (121.5), C-10 (36.80), C-12 (39.95), C-18 (16.44) and C-19 (19.57). The related chemical shift values also suggested the presence of two compounds of similar chemical structures. Compound Z was a mixture of steroidal sapogenin which are greatly related. The carbon signals were compared with those reported for (25*R*)-Isonuatigenin (1) and Diosgenin (2) as stated in Table 4.20.

The methyl group at C-18, 19 and 21 displayed chemical shift values very close to that reported for Isonuatigenin and C-27 proton at δ_H 1.20 indicated the presence of 25R configuration (Kutney, 1963; Faini *et al*., 1984, Agrawal *et al*., 1995, Saracoglu *et al*., 2002). Signals at H-27 and H-26 were also used as diagnostic values for compound (1) as reported by Faini *et al*., 1984.

Proton and carbon-13 NMR data (Appendix41) of compound Z showed that Z was a mixture of (25*R*)-Isonuatigenin and Diosgenin. Mixture of steroids with almost similar chemical structures was isolated from *Porcelia macrocarpa* ethanol extract (Chaves *et al.,* 2004).

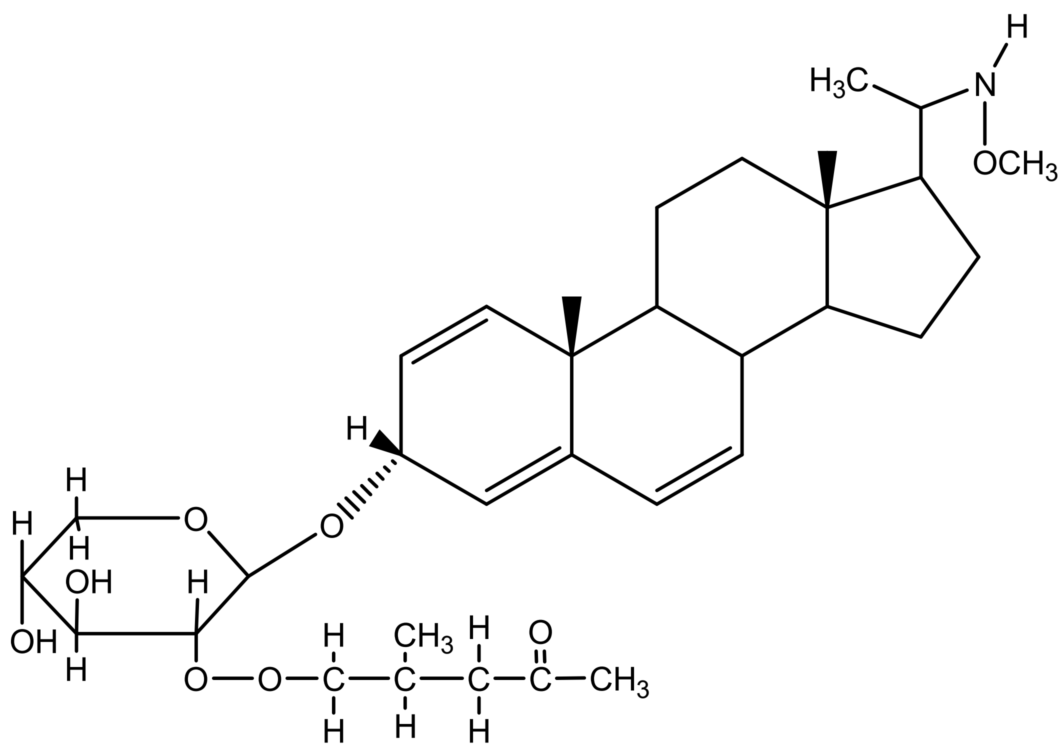


Figure 4.10: 20-N-methoxy amino lucanu- 1, 4, 6-triene-3-xylo-2-pero-4-methylpentan-2-one from *Costus lucanusianus*

Table 4.19: Proton spectra data of compounds 1 and 2 from *Costus lucanusianus*

Compd.	H-3	H-6	H-18	H-19	H-21	H-26	H-27
1	3.35-3.37, m	5.36,d,1.9Hz	0.79,s,3H	1.02,s,3H	0.96, d, 7.0 Hz	3.28,dd, 10.3,2.4, 3.6 Hz	1.20,s,3H
2	3.45-3.47, m	5.73,d,1.9Hz	0.80,s,3H	1.02,s,3H	0.98,d,6.7 Hz	3.34, d,11.0, 3.44, d,11	0.82,s,3H

600 MHz, $CDCl_3$, TMS as internal standard.

Table 4.20: ^{13}C NMR data of compounds 1 and 2 from *Costus lucanusianus*

Carbon Position	Compound 1[*1,*2]		Compound 2[*3,*4]	
	Experimental	Literature	Experimental	Literature
1	37.38	37.1o	37.38	37.43
2	31.78	31.60	31.78	31.79
3	71.89	71.30	71.89	71.89
4	42.43	42.30	42.43	42.46
5	140.95	141.5	140.95	141.02
6	121.59	121.5	121.59	121.60
7	32.96	32.90	32.29	32.25
8	31.87	31.80	31.60	31.64
9	50.89	50.60	50.22	50.25
10	36.80	37.00	36.80	36.84
11	20.98	21.20	21.03	21.07
12	39.95	40.10	39.95	39.99
13	40.52	40.60	40.42	40.46
14	56.68	56.90	55.82	56.72
15	32.01	31.90	32.21	32.04
16	80.77	81.60	80.97	81.03
17	62.20	62.40	62.26	62.28
18	16.40	16.40	16.44	16.49
19	19.50	19.50	19.57	19.63
20	41.77	41.40	41.88	41.80
21	14.63	14.30	14.67	14.73
22	109.42	109.5	109.44	109.50
23	31.52	29.90	31.55	31.59
24	34.11	34.60	28.96	28.99
25	80.66	81.60	30.46	30.49
26	67.00	69.20	67.02	67.04
27	30.43	23.80	17.56	17.35

^{13}C Spectrum (CDL_3) recorded on Bruker Avance III 600 at 150.0 MHz

*1= Faini *et al.,* 1984; *2= Agrawal *et al.,* 1995; *3= Puri *et al.,* 1993; *4= Abdel-Aziz *et al.,* 1990.

(25*R*)-Isonuatigenin (1) and diosgenin (2) are structurally similar. Rings A-E showed similar chemical shift values. The difference lies in the replacement of hydroxyl group (-OH) in 1 with H in 2 in the (ring F) spiroketal moiety. The assignment of –OH group at C-25 position resulted into a higher chemical shift of ring F carbon atoms as compared to those of diosgenin. Dept 135 (Appendices 42-43), COSY (Appendix 44), HSQC (Appendix 45) aided in the elucidation of the structures.

Angular methyl proton correlation in the HMBC experiment aided with the assignment of the quaternary carbons. There were correlations between H-18 and quartenary carbon C-5 (δ_C 140.95) and carbon C-10 (δ_C 36.80). Also, C-22 (δ_C 109.42) correlated with H-21 (δ_C 0.92) and C-13 (δ_C 40.42) with H-19 (δ1.20). The HSQC showed correlations between δ_C 121.6 and δ_H 5.3, 5.7; δ_C 80.7 and δ_H 4.4; δ_C 71.89 and δ_H 3.5; δ_C 42 and δ_H 1.8.

Faini *et al*. 1984 reported a mixture of (25*R*)-Isonuatigenin and nuatigenin. Diosgenin are present in *Costus* species. However, we reported for the first time (25*R*)-isonuatigenin from the family Costaceae. (25*R*)-Isonuatigenin has been isolated from many solanum species.

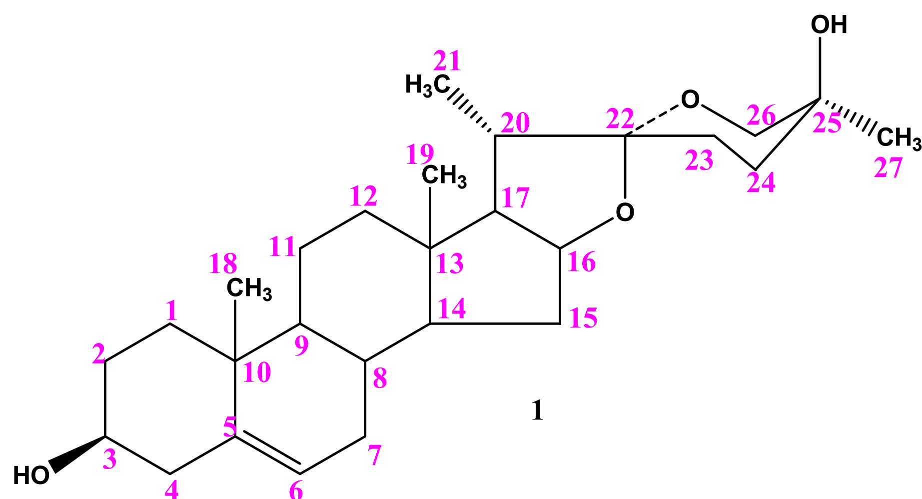


Figure 4.11: (25*R*)-Isonuatigenin from *Costus lucanusianus*

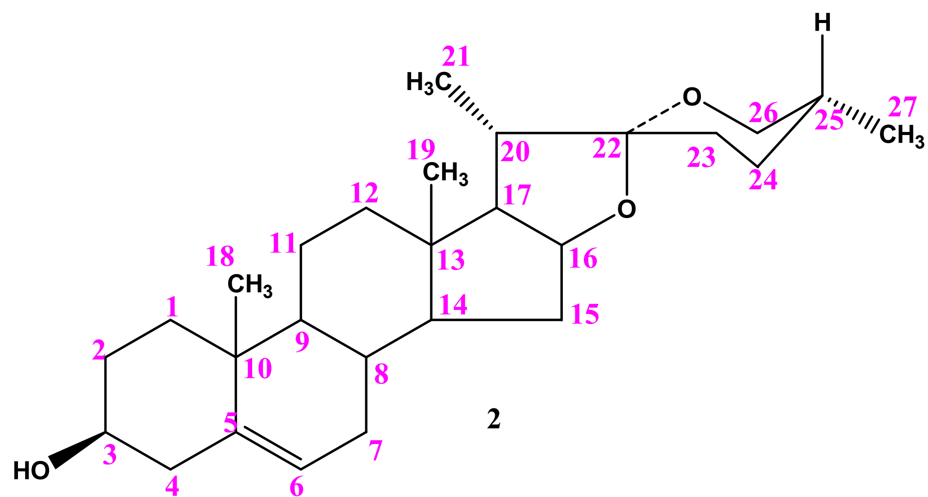


Figure 4.12: Diosgenin from *Costus lucanusianus*

4.6 Antibacterial and Antifungal Assay of Isolated Compounds

4.6.1 Antibacterial Assay of Isolated compounds

Compound X_P was obtained from the leaf hexane extract while compounds C and I were from the leaf ethyl acetate fraction

Compound C at 12.5 µg mL showed IZD against *S. aureus, K. pneumonia, E. coli, P. aeruginosa, S. typhi* (14 mm) and *B. subtilis* (12 mm). Likewise, compound 1 displayed IZD at 12.5 µg mL against *S. aureus* (18 mm), *E. coli*, *B. subtilis, S. typhi* (14 mm), *P. aeruginosa* (16 mm) and *K. pneumonia* (12 mm). Compound Xp repressed the growth of *K. pneumonia* (14 mm), *S. typhi* (12 mm*), P. aeruginosa* (14 mm), *E. coli* (12 mm), *B. subtilis, S. aureus* (17 mm) at 12.5 µg/mL. The hexane crude extract at 12.5 µg/mL inhibited only *P. aeruginosa* (10 mm) amongst other bacterial strains used.

The antibacterial properties of the isolated compounds were lower than the reference drug (Gentamycin 10 µg/mL) against all tested bacteria strains.

Compound 1 and C displayed similar activity against *S. typhi* (14 mm) and *E. coli (*14 mm) at 12.5 µg/mL. Compound Xp inhibited *E. coli* (12 mm) while compounds C and 1 (14 mm) at 12.5 µg/mL. Oh *et al.* (2006) reported the significance of the modification of β-sitosterol for it to exert its antibacterial effect. Voukeng *et al.* (2017) documented that β-sitosterol-3-O-β-D-xylopyranoside possesses no antibacterial property against *E.coli, K. pneumonia, P.aeruginosa* and *S. aureus,* and MIC of a greater value than 256 µg/mL.

The antibacterial property displayed by compound 1 in this present study could have been as a result of the sugar derivative present. The antibacterial activity shown by compound C which was a mixture of stigmasterol and sitosterol could have been due to the synergetic effect of these constituents. Stigmasterol and β-sitosterol have been known to demonstrate low to moderate antibacterial activities against different bacterial species at different concentrations (Mokbel and Hashinaga, 2005; Tamokou *et al.*, 2011; Sen *et al.,* 2012; Joy *et al*., 2012). Gomez *et al*. 1999 similarly reported the anti-inflammatory property of a mixture of stigmasterol and sitosterol.

4.6.2 Antifungal Assay of Isolated Compounds

Compounds C and 1 repressed the growth of all tested fungi used at 12.5-100 µg/mL. Compounds C and 1 inhibited *C. albicans* (12 mm) and (14 mm) respectively at 12.5 µg/mL. On the contrary, the crude ethyl acetate fraction at 12.5 µg/mL repressed *A. niger* and *C. albicans* (10 mm) and showed no area IZD against *P. notatum* and *R. stolonifer*. Compound Xp showed activity against all tested fungi, *C. albicans* (14 mm*), R. stolonifer* (10 mm), *A. niger, P. notatum* (12 mm) at 12.5 µg/mL. The crude hexane extract revealed no antifungal property against all tested fungi.

Yang *et al.* 2006 documented that the C-27 steroidal saponins antifungal property was connected with the number of monosaccharides units in their sugar moiety and the type of aglycone moieties (steroidal sapogenin) which are dependent on the number and location of the –OH group, and the presence of unsaturation and ketone functional group. The presence of antifungal activity observed in compound Xp could have been due to the presence of two –OH and an olefinic bond since no sugar moiety was present. Compounds C and 1 showed higher antimicrobial activity than the crude extract with the exception of *E. coli* which showed the same area of inhibition (14 mm) in both the crude and pure compounds at a concentration 12.5 µg/mL. Compound 1 exhibited the maximum antimicrobial properties among the pure compounds. The antimicrobial activity of the isolated compounds is reported on Table 4.21.

The lowest MIC values for compound C were obtained against all tested bacteria at 6.25 µg/ mL (Table 4.22). The lowest MIC values obtained against all tested fungi were at 12.5 µg/mL. Compound Xp MIC result as shown on (Table 4.23) revealed that it repressed the growth of all tested microorganisms at 25.0 µg/mL with the exception of *P. notatum* and *R. stolonifer* which showed no prominent growth. Compound Xp displayed IZD against *S. aureus* at 6.2-50 µg/mL suggesting the high sensitivity of *S. aureus* to compound Xp. Compound 1 was not sufficient to determine its MIC values.

Overall, the MIC results showed that compound C had a higher inhibitory property than compound Xp

To our greatest awareness, the antimicrobial components of *C. lucanusianus* are documented for the first time.

Table 4.21: Antimicrobial Activity of Isolated Compounds from *Costus lucanusianus*

Concentration (μg/mL)	*S. aureus*	*E. coli*	*B. subtilis*	*P. aeruginosa*	*S. typhi*	*K. pneumonia*	*C. albicans*	*A. niger*	*P. notatum*	*R. stolonifer*
COMPOUND C										
100	24	20	18	20	20	22	18	18	18	20
50	20	18	16	18	18	20	16	16	16	18
25	18	16	14	16	16	18	14	14	14	16
12.5	14	14	12	14	14	14	12	12	12	14
COMPOUND 1										
100	28	26	24	24	20	18	20	18	18	18
50	24	22	20	22	18	16	18	16	16	16
25	20	18	16	20	16	14	16	14	14	14
12.5	18	14	14	16	14	12	14	12	12	12
COMPOUND Xp										
100	28	18	26	24	18	20	20	18	18	18
50	24	16	22	21	16	18	18	16	16	14
25	21	14	18	18	14	16	16	14	14	12
12.5	17	12	14	16	12	14	14	12	12	10
-ve Methanol	-	-	-	-	-	-	-	-	-	-
+ve Gentamicin (10 μg/mL)	38	38	38	38	38					
Tioconazole (70%)							28	26	28	28

Table 4.22: Minimum Inhibitory Concentration result of Compound C from *Costus lucanusianus*

Concentration (μg/mL)	*S. aureus*	*E. coli*	*B. subtilis*	*P. aeruginosa*	*S. typhi*	*K. pneumonia*	*C. albicans*	*A. niger*	*P. notatum*	*R. stolonifer*
12.5	-	-	-	-	-	-	-	-	-	-
6.25	-	-	±	-	-	-	+	+	+	±
3.125	±	±	+	+	+	+	+	+	+	+
1.562	+	+	+	+	+	+	+	+	+	+

+ = No inhibition, - = Inhibition, + = Growth not prominent

Table 4.23: Minimum Inhibitory Concentration result of Compound Xp from *Costus lucanusianus*

Concentration (μg/mL)	*S. aureus*	*E. coli*	*B. subtilis*	*P. aeruginosa*	*S. typhi*	*K. pneumonia*	*C. albicans*	*A. niger*	*P. notatum*	*R. stolonifer*
50	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	±	±
12.5	-	+	±	±	±	+	+	+	+	+
6.25	-	+	+	+	+	+	+	+	+	+
3.125	+	+	+	+	+	+	+	+	+	+

+ = No inhibition, - = Inhibition, ± = Growth not prominent

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATION

5.1 Summary

Methanol extracts, the hexane and ethyl acetate fractions of inflorescence, leaf, stem and rhizome of *C. lucanusianus* constitute an array of bioactive constituents namely alkaloids, flavonoids, phlobatannin, phenol, saponins, tannin, glycosides, resin, anthraquinones, reducing sugars and triterpenes.

All the parts of the plant yielded essential oils with the rhizome oil having the highest percentage yield of 0.18. The essential oils obtained from the aerial and rhizomes of *C. lucanusianus* were rich in long chain aliphatic hydrocarbon, fatty acid and its derivatives and oxygenated triterpene. Seven pure compounds were isolated from the leaf, stem and rhizome of *C. lucanusianus* for the first time, three out of which were new additions to the natural products. These compounds were of the classes: steroids, steroid alkaloid glycoside and steroidal sapogenin. These are sitosterol, stigmasterol, (25*R*)-Isonuatigenin, Diosgenin, 3, 27-dihydroxy-1-methoxy-22-cholest-5-enone, β-sitosterol-3-O-β-D-3-deoxyxylo-4-hydroxy4, 5-dimethyl-pent-2-one (SDHP), 20-N-methoxy amino lucanu-1, 4, 6-triene-3-xylo-2-pero-4-methylpentan-2-one.

The extracts, essential oils and the isolated compounds obtained from *C. lucanusianus* showed moderate to high antimicrobial activity against tested micro-organisms especially uropathogens like *C. albicans* and *E. coli* which are the main causative organisms of Urinary Tract Infections (UTI). The rhizome hexane extract and its essential oil showed the highest antimicrobial activity. SDHP displayed the maximum antimicrobial activity amongst the tested compounds.

5.2 Conclusion

The antimicrobial activities displayed by the essential oils, isolated compounds and crude extracts of *C. lucanusianus* justify its use in curing urinary tract infection and other infectious diseases such as cough and venereal diseases in ethnomedicine. This present study may be helpful in discovering a mass production of new chemical groups of antimicrobial compounds that could be useful as an agent against infectious diseases.

5.3 Recommendation

1. Isolation and characterisation of more pure compounds from *Costus lucanusianus.*

2. Synthesis of 20-N-methoxy amino lucanu-1, 4, 6-triene-3-xylo-2-pero-4-methylpentan-2-one (a novel acetylated steroid glycoalkaloid) and determination of its biological activities.

5.4 Contributions to Knowledge

Below are listed the contributions to knowledge by the research study

1. The phytochemical screening of alkaloids, flavonoids, phlobatannin, phenol, saponins, tannin, glycosides, resin, anthraquinones, reducing sugars and triterpenes, and antimicrobial activity of *C. lucanusianus* inflorescence and rhizome extracts against *Salmonellae typhi, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Klebsiella pneumoniae, Penicillium notatum, Candida albicans, Aspergillus niger* and *Rhizopus stolonifer* were determined for the first time.
2. Essential oils were isolated for the first time from *C. lucanusianus*
3. Seven compounds were isolated and characterised for the first time from *C. lucanusianus*. 3,27-dihydroxy-1-methoxy-22-cholest-5-enone, β-sitosterol-3-O-β-D-3-deoxyxylo-4-hydroxy4,5-dimethyl-pent-2-one (SDHP), 20-N-methoxy amino lucanu-1, 4, 6-triene-3-xylo-2-pero-4-methylpentan-2-one are new compounds reported for the first time and a significant addition to knowledge in natural product chemistry.
4. An unusual steroidal sapogenin ((25*R*)- Isonuatigenin was for the first time isolated from the family Costaceae.

5. The broad spectrum antimicrobial activities displayed by the crude extracts, essential oils and isolated compounds against tested microorganisms established the antimicrobial potency of the plant.
6. The potential of *C. lucanusianus* as a new source of potent antimicrobial agent and justification of its use in ethnomedicine were established in this research.

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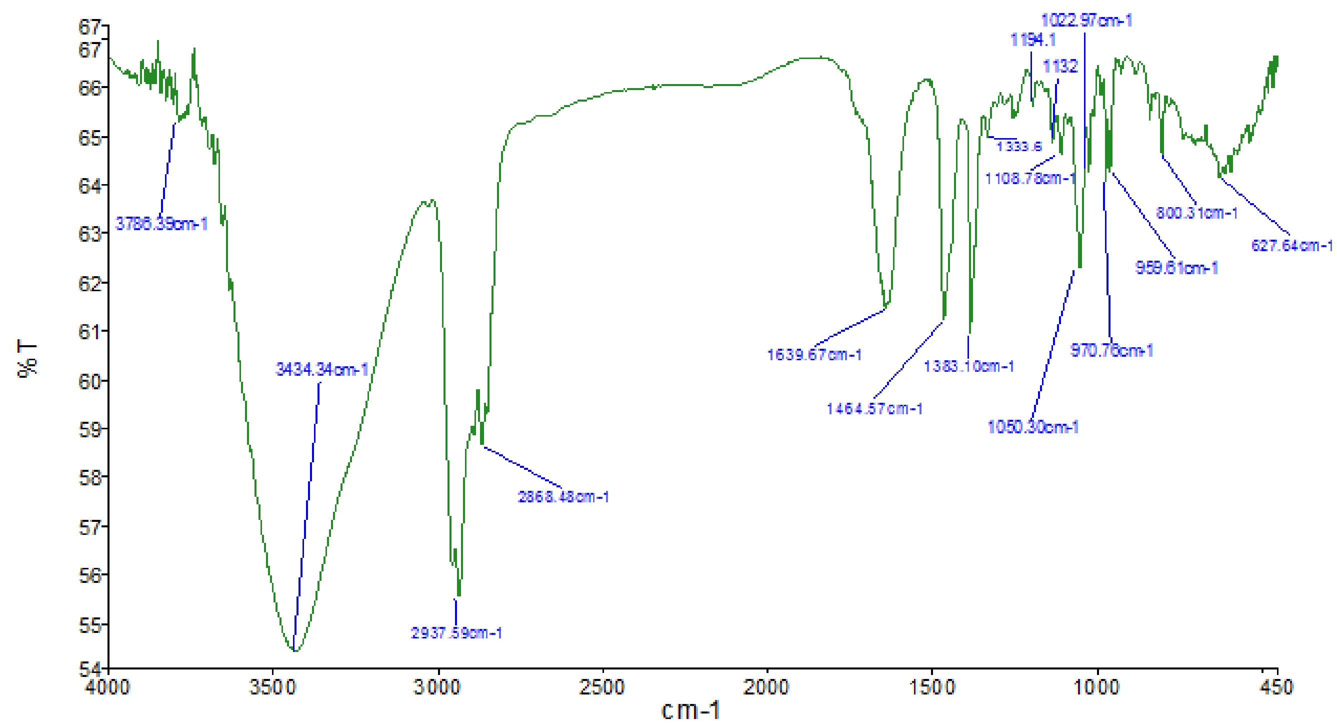
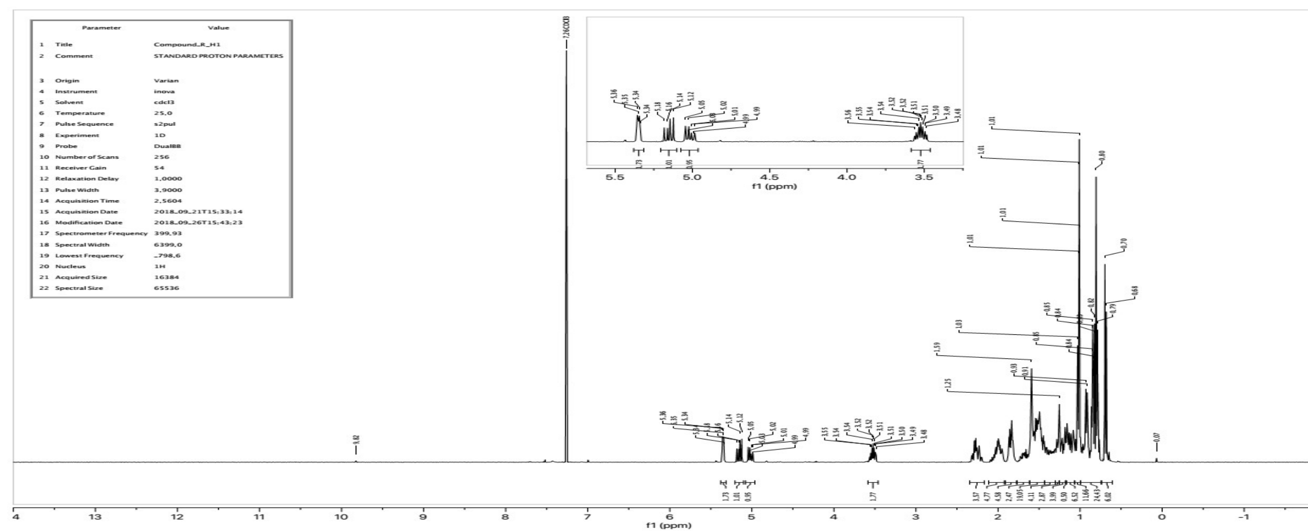
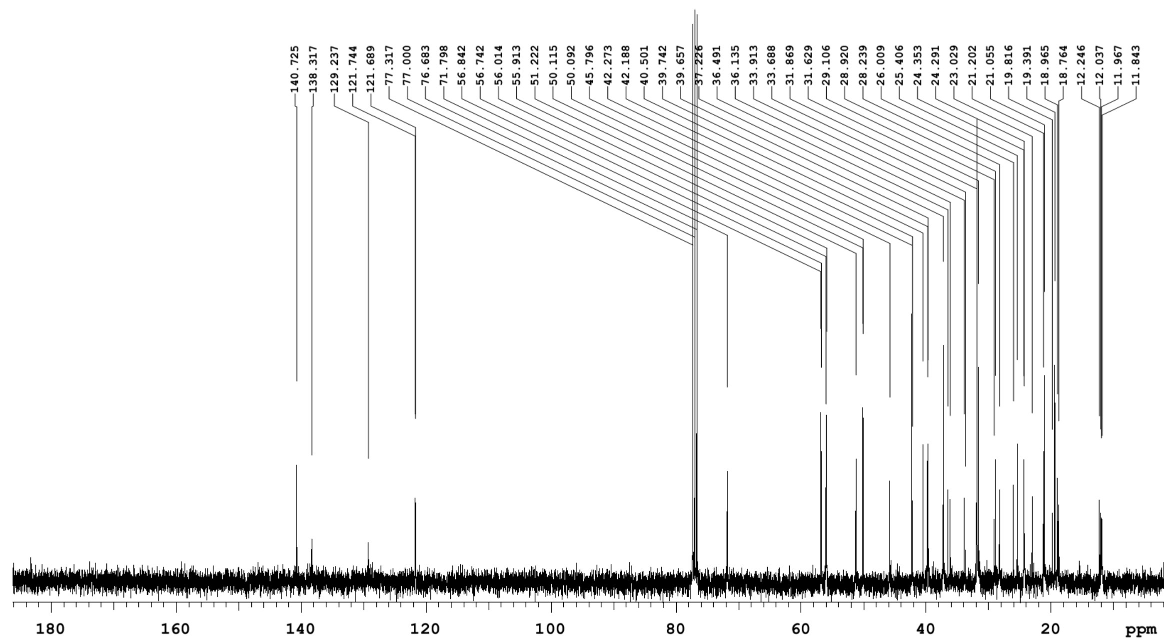


Appendix 1: Compound C IR spectrum

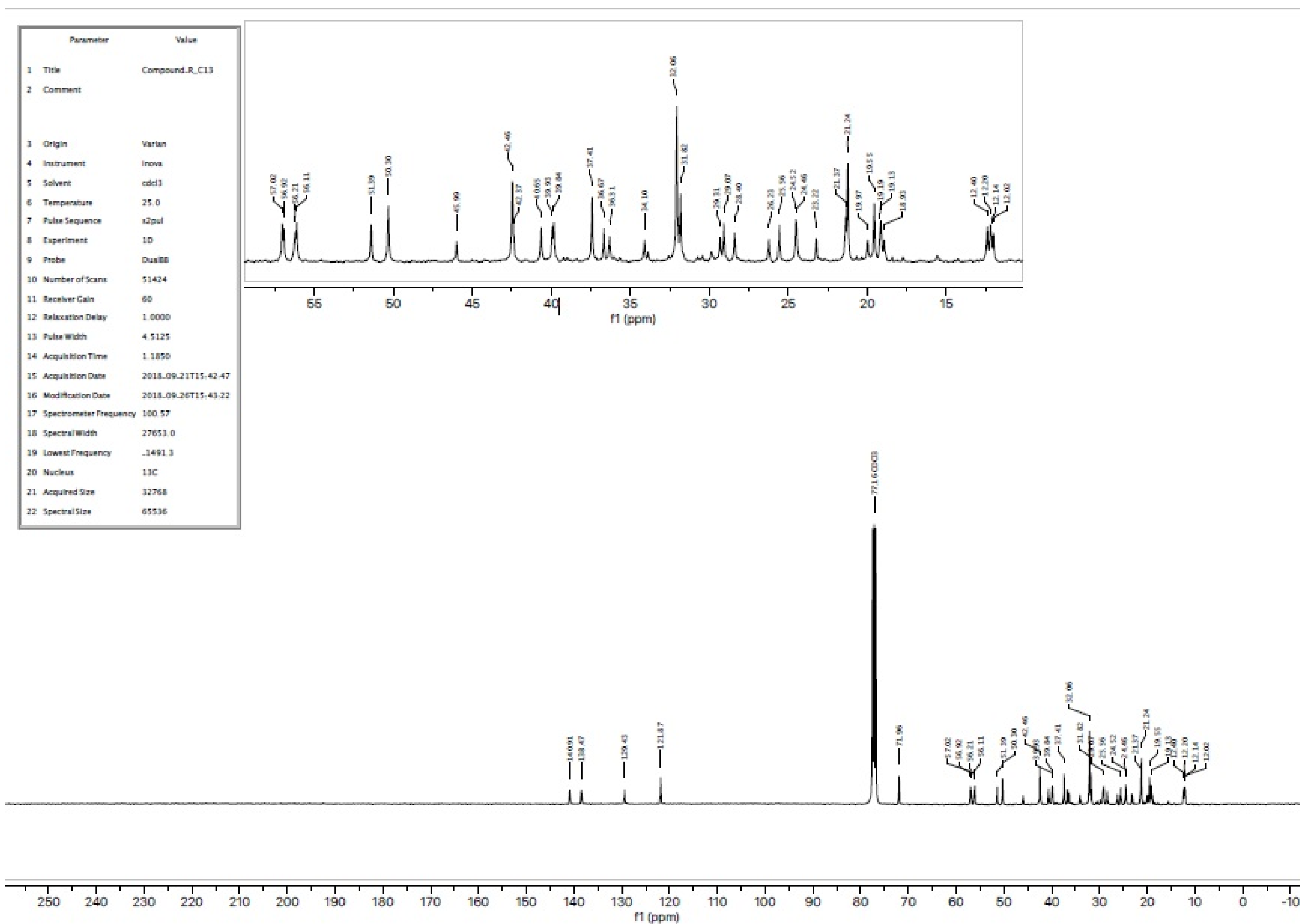
Appendix 2: Compound C proton spectrum (400 MHz)



` Appendix 3: Compound C proton spectrum (600 MHz)

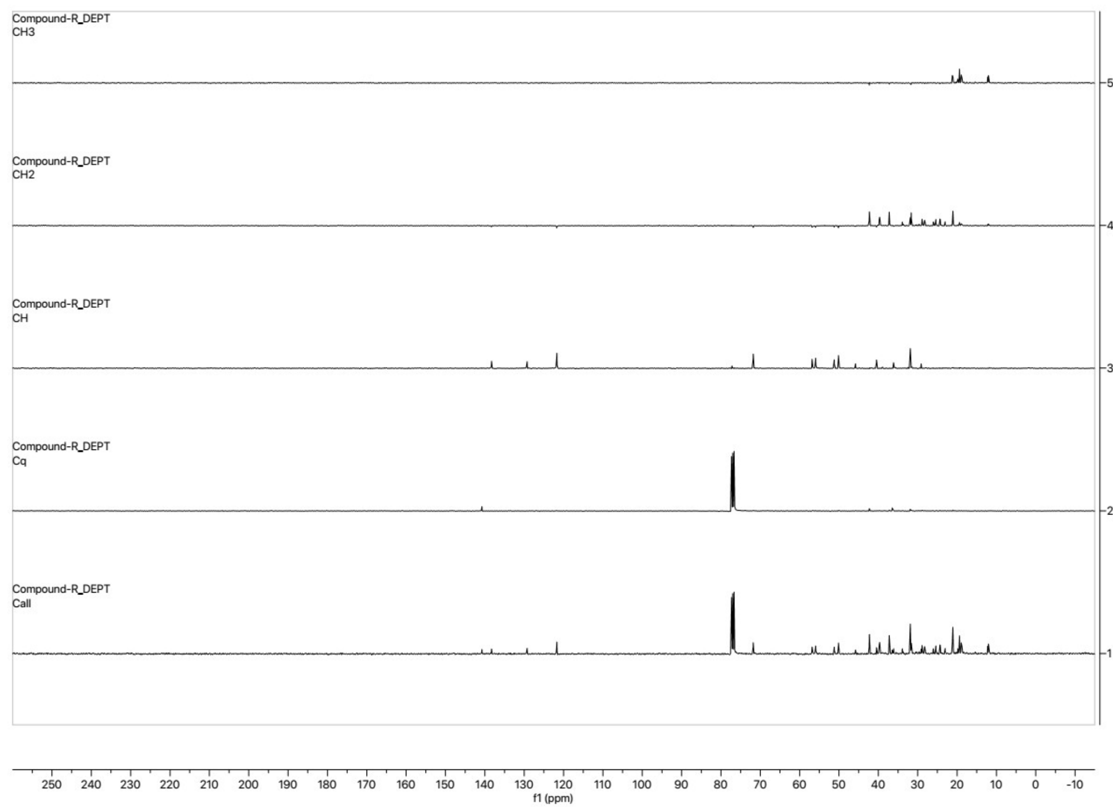


Appendix 4: Compound C carbon spectrum (400 MHz)

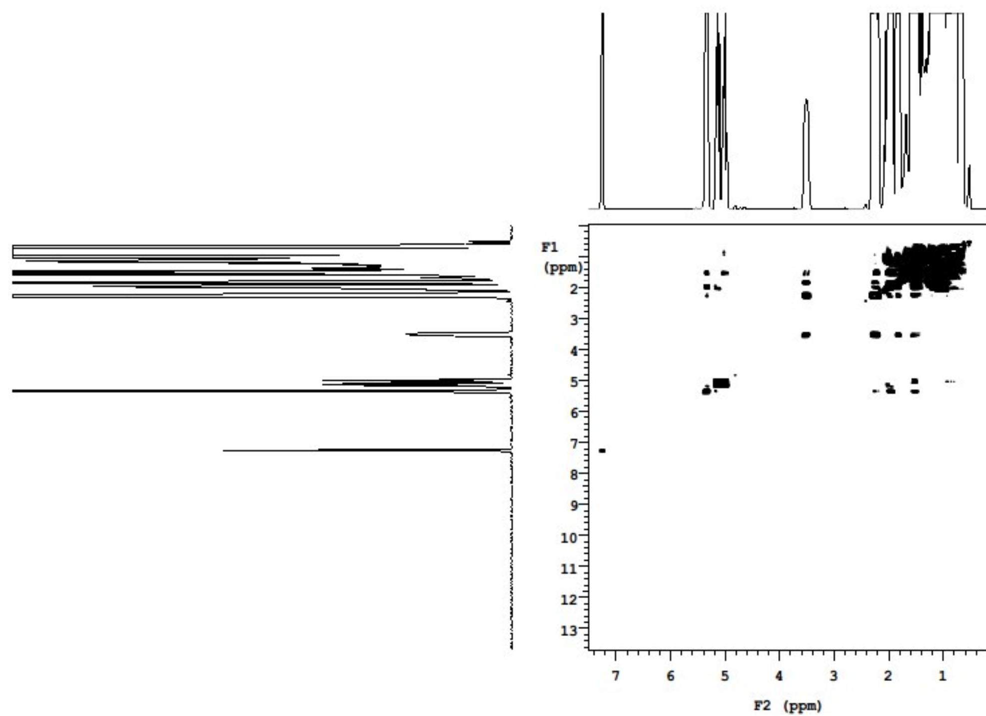


	Parameter	Value
1	Title	Compound.R_C13
2	Comment	
3	Origin	Varian
4	Instrument	inova
5	Solvent	cdcl3
6	Temperature	25.0
7	Pulse Sequence	s2pul
8	Experiment	1D
9	Probe	DualBB
10	Number of Scans	51424
11	Receiver Gain	60
12	Relaxation Delay	1.0000
13	Pulse Width	4.5125
14	Acquisition Time	1.1850
15	Acquisition Date	2018-09-21T15:42:47
16	Modification Date	2018-09-26T15:43:22
17	Spectrometer Frequency	100.57
18	Spectral Width	27651.0
19	Lowest Frequency	-1491.3
20	Nucleus	13C
21	Acquired Size	32768
22	Spectral Size	65536

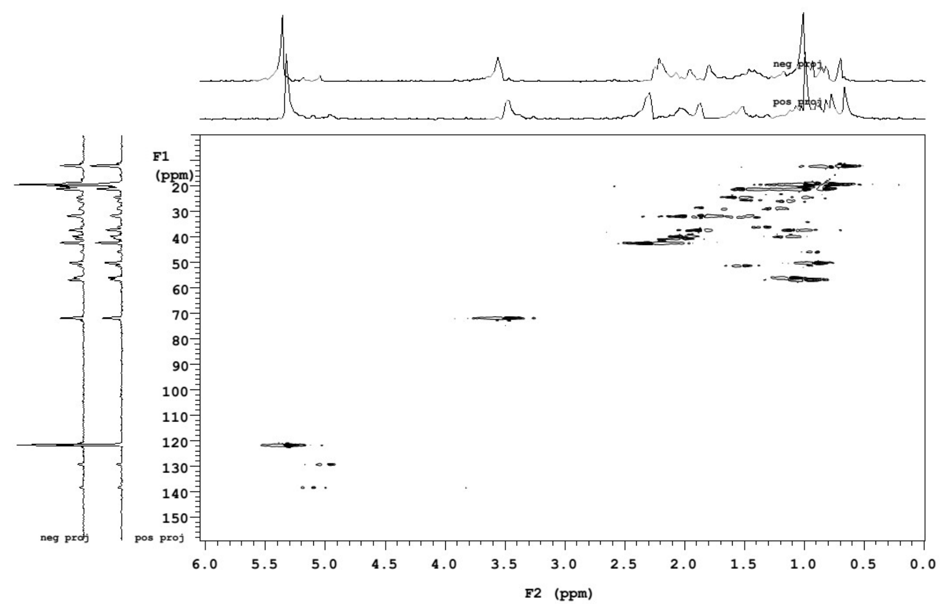
Appendix 5: Compound C carbon spectrum (600 MHz)



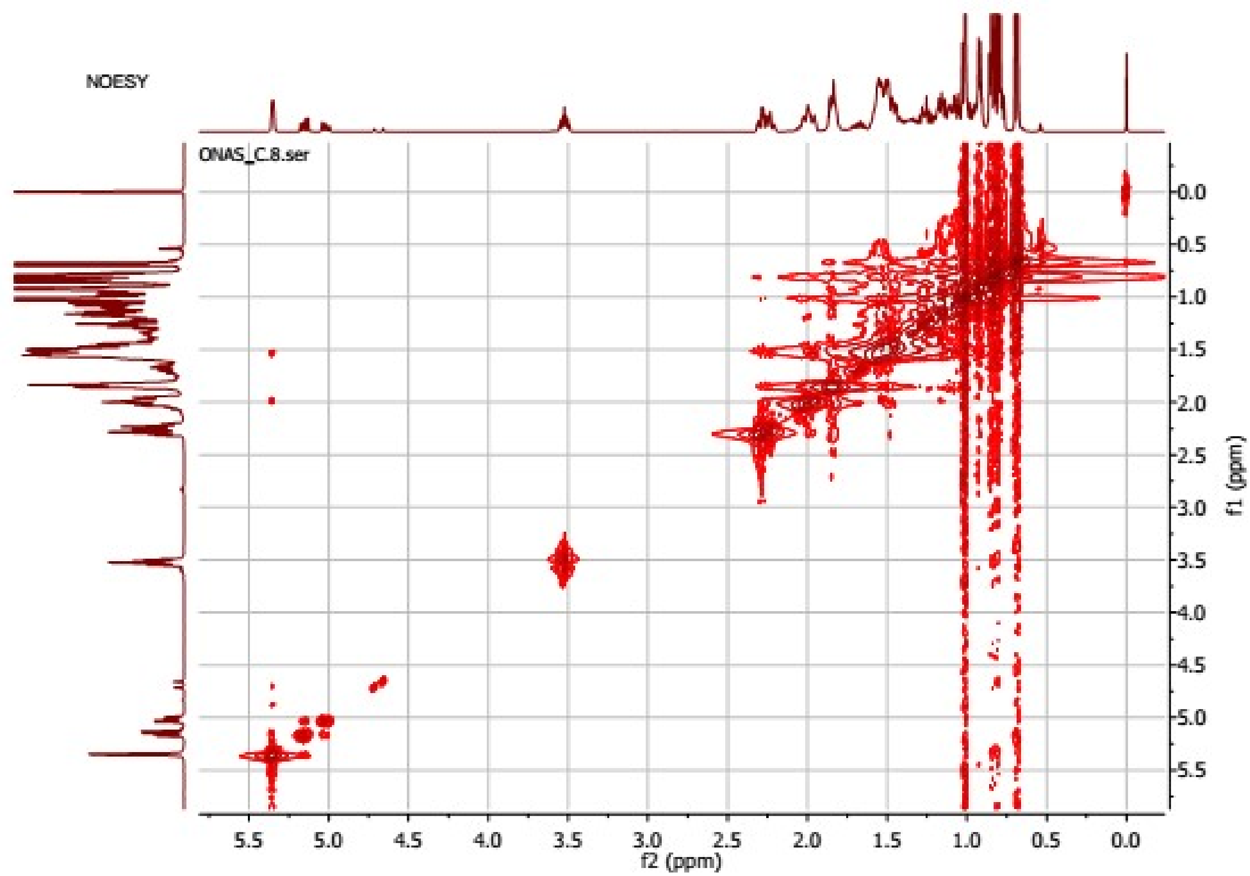
Appendix 6: Compound C DEPT 135 spectrum.



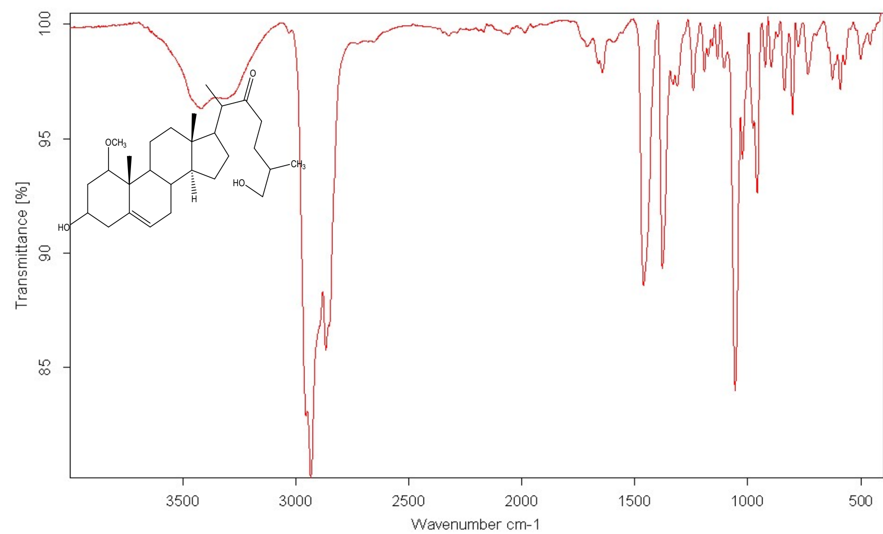
Appendix 7: Compound C COSY spectrum



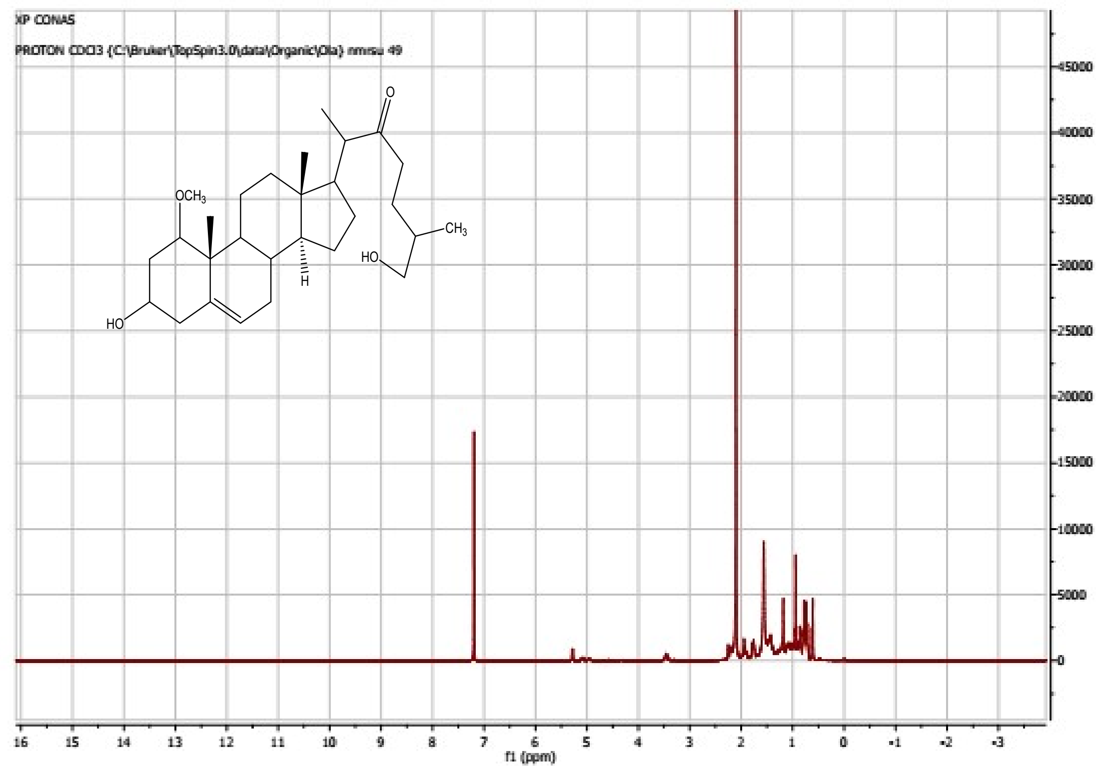
Appendix 8: Compound C HMBC spectrum



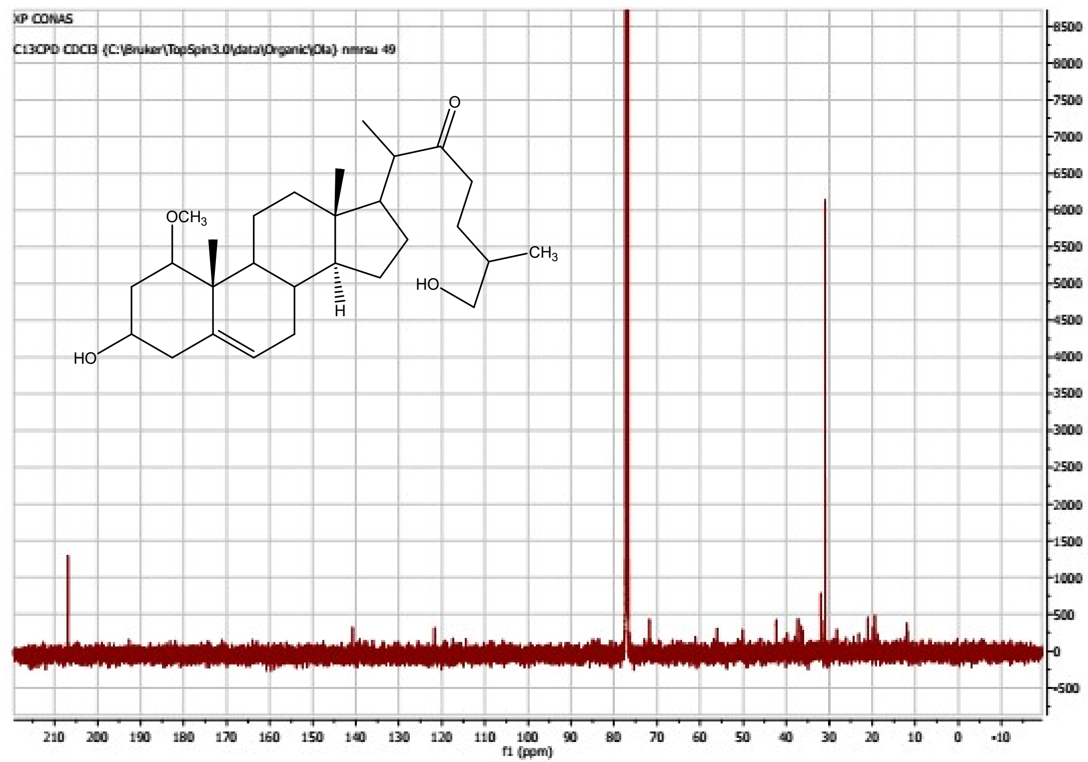
Appendix 9: Compound C NOESY spectrum



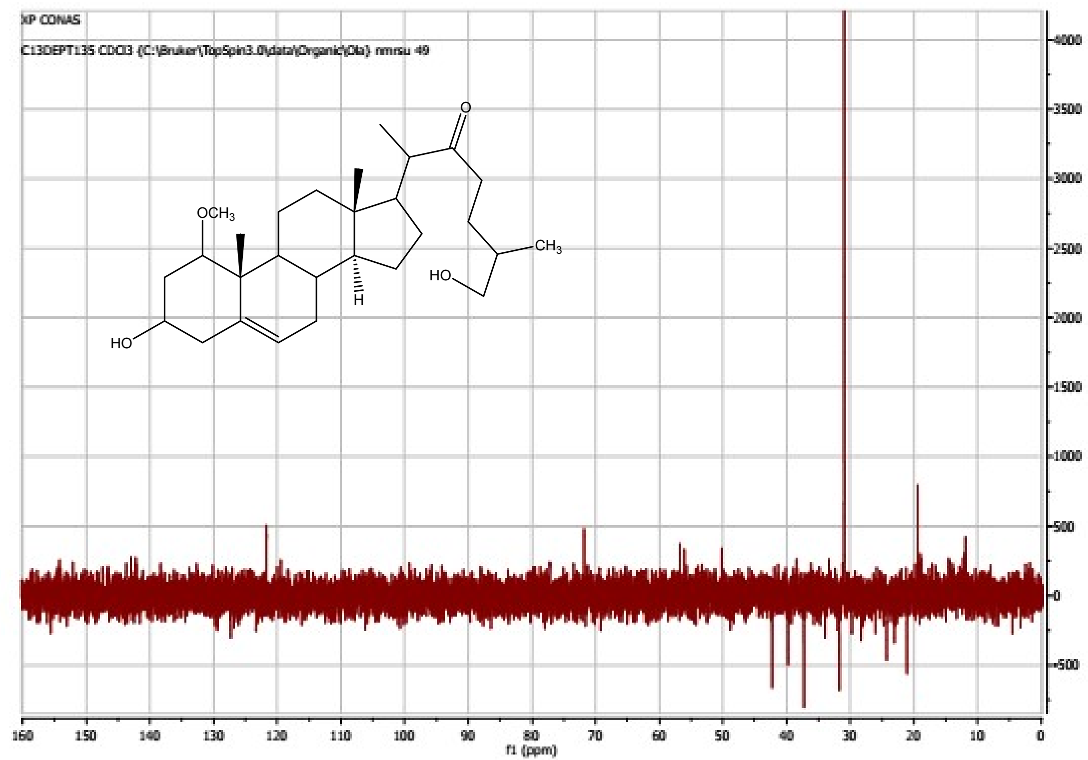
Appendix 10: Compound Xp IR spectrum



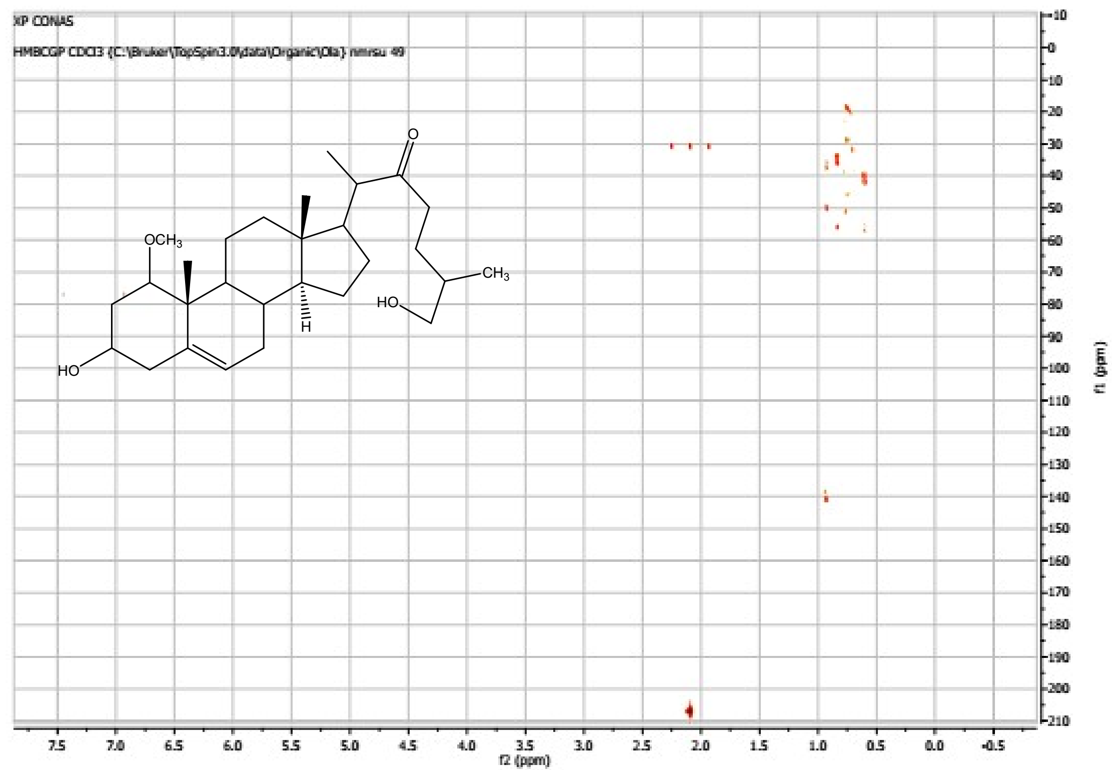
Appendix 11: Compound Xp proton spectrum



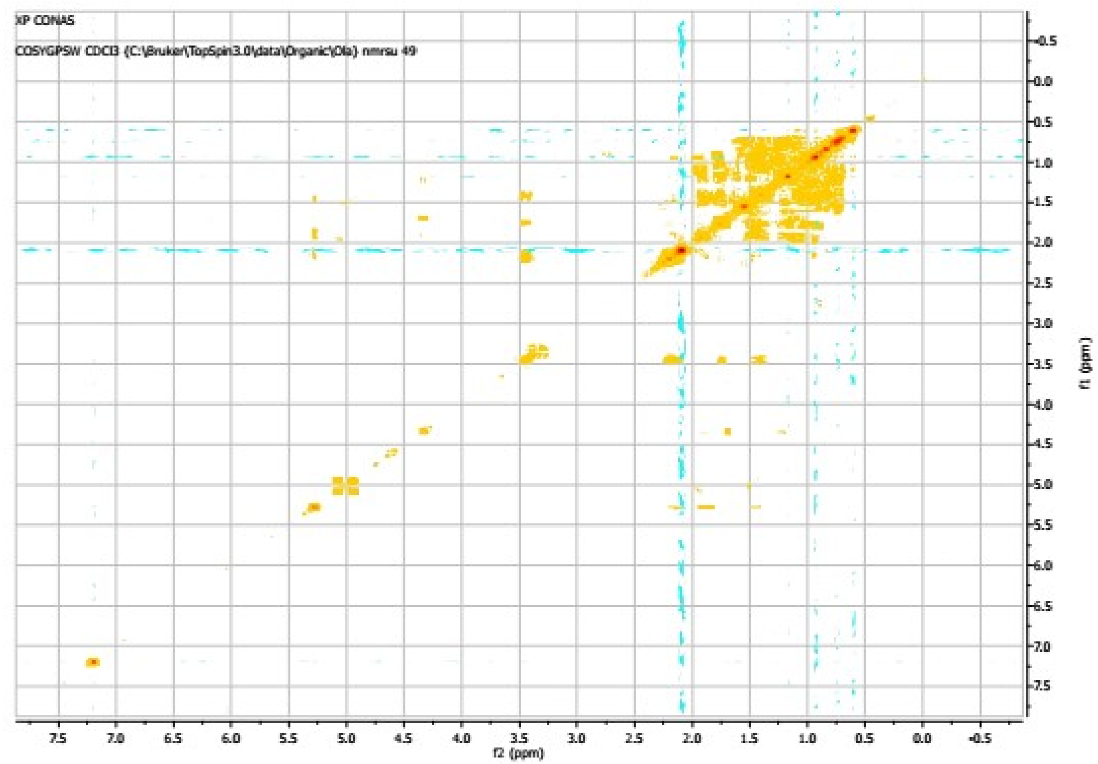
Appendix 12: Compound Xp carbon spectrum



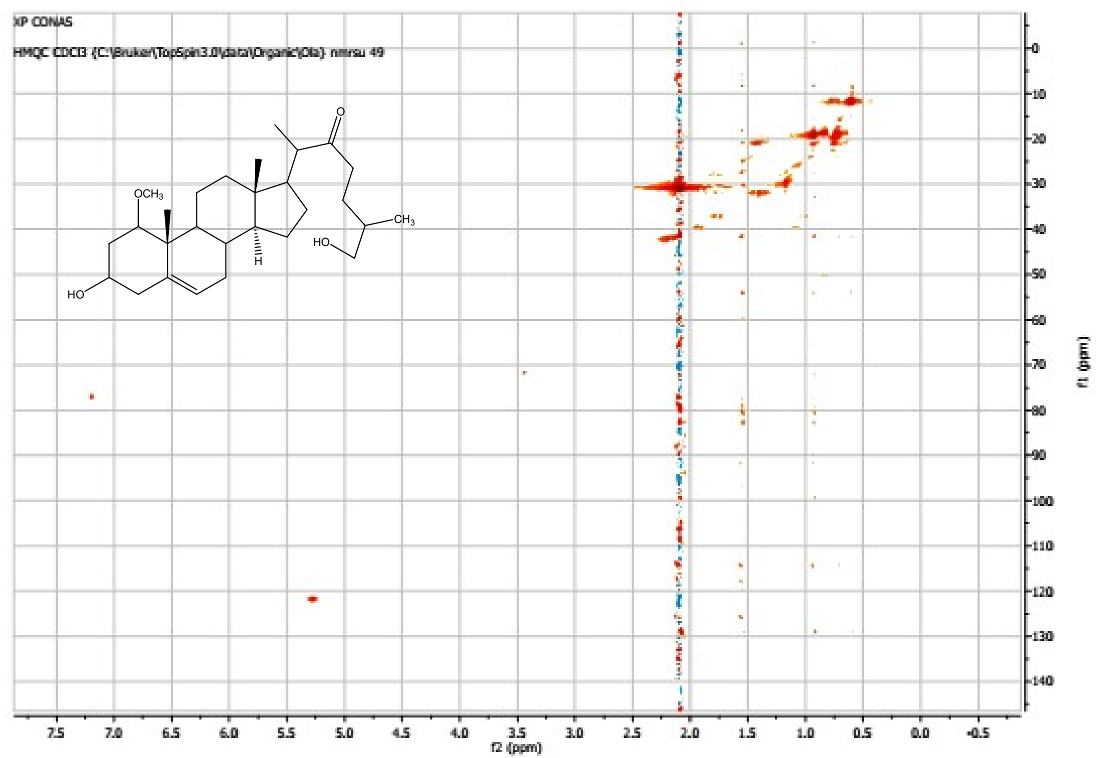
Appendix 13: Compound Xp DEPT 135 spectrum



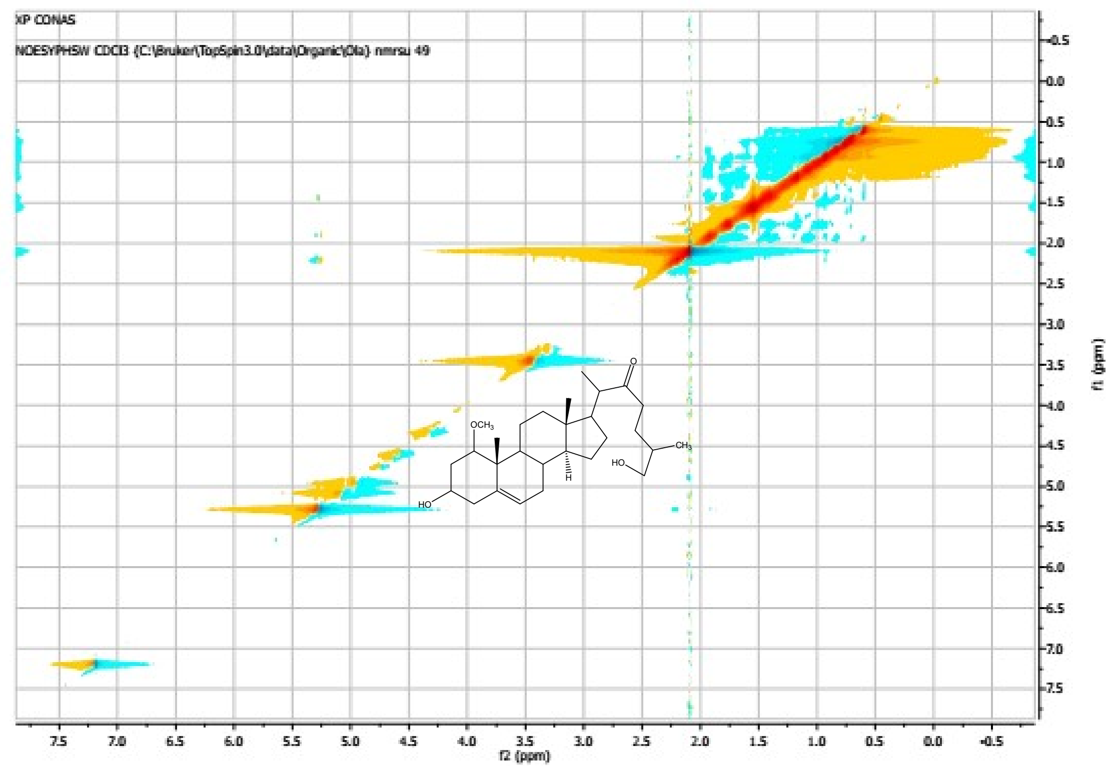
Appendix 14: Compound Xp HMBC spectrum



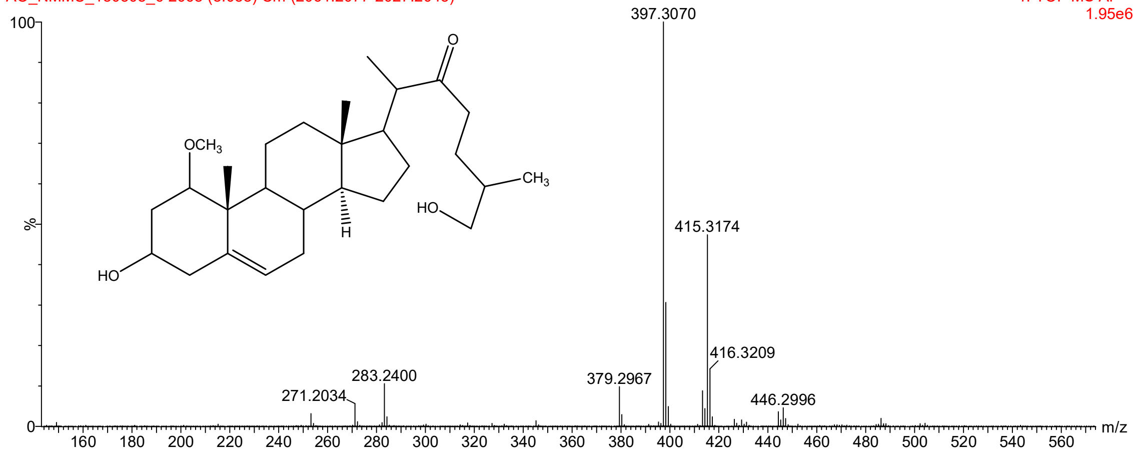
Appendix 15: Compound Xp COSY spectrum



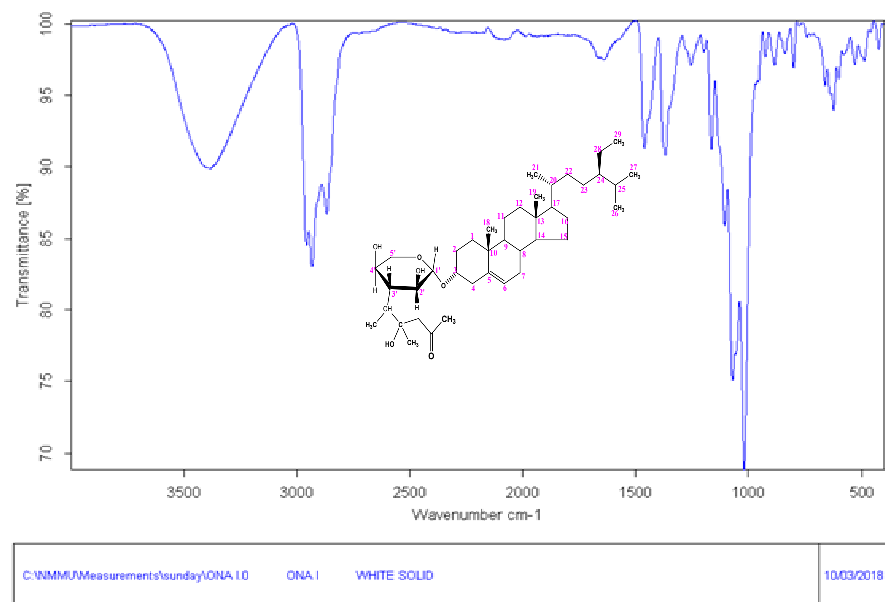
Appendix 16: Compound Xp HMQC spectrum



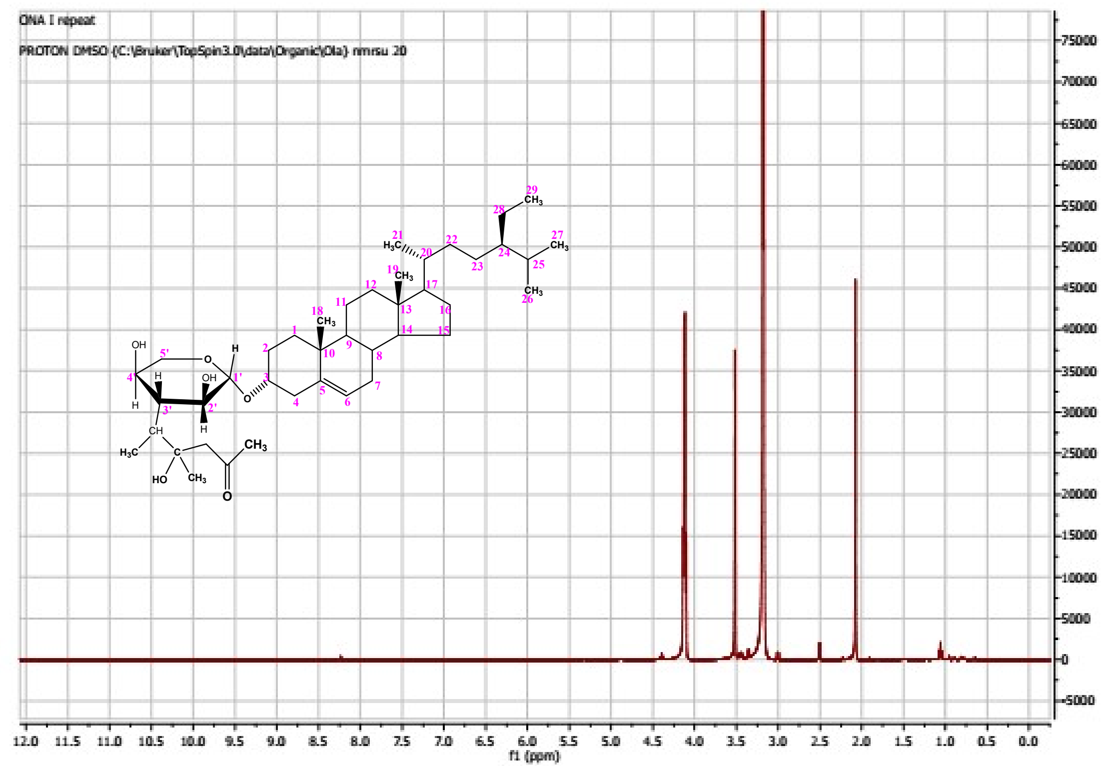
Appendix 17: Compound Xp NOESY spectrum



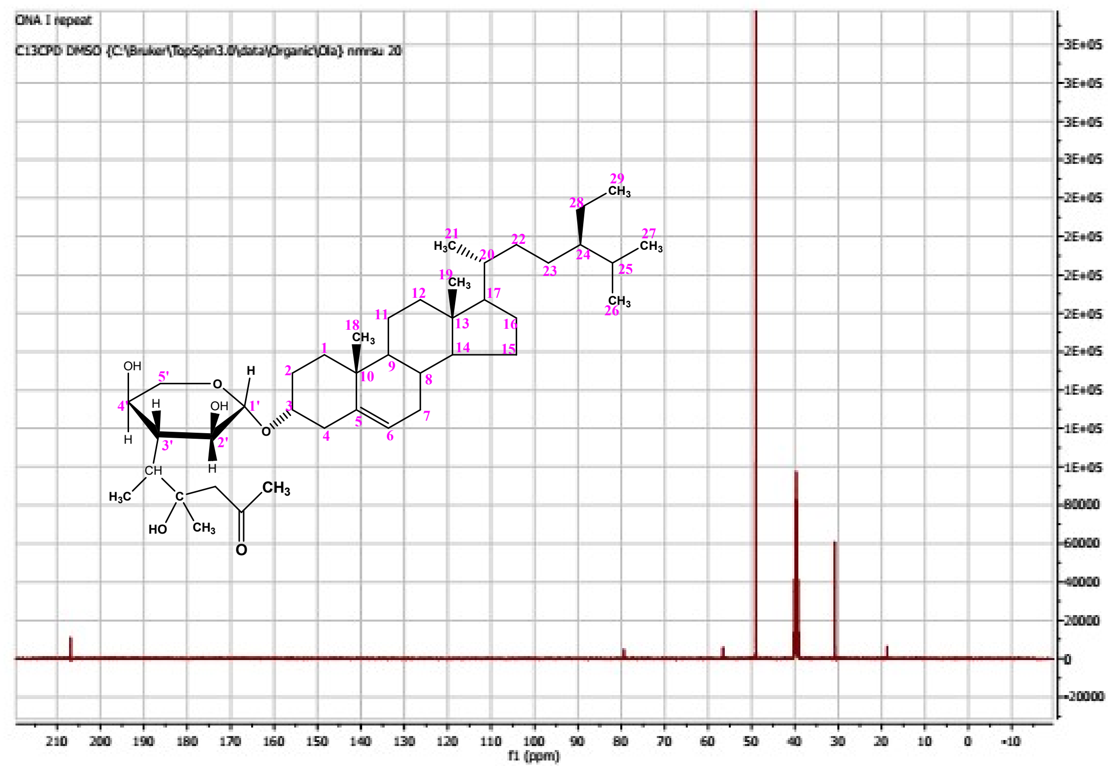
Appendix 18: APCI-ToF/MS/MS of compound Xp



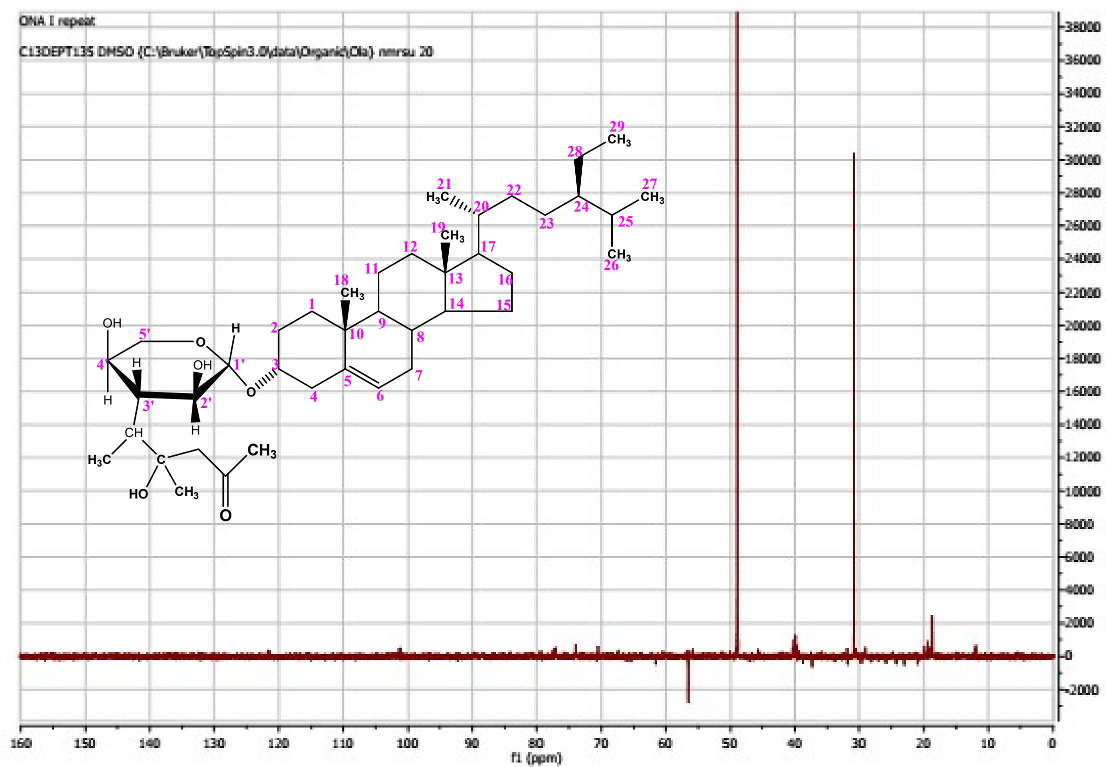
Appendix 19: Compound 1 IR spectrum



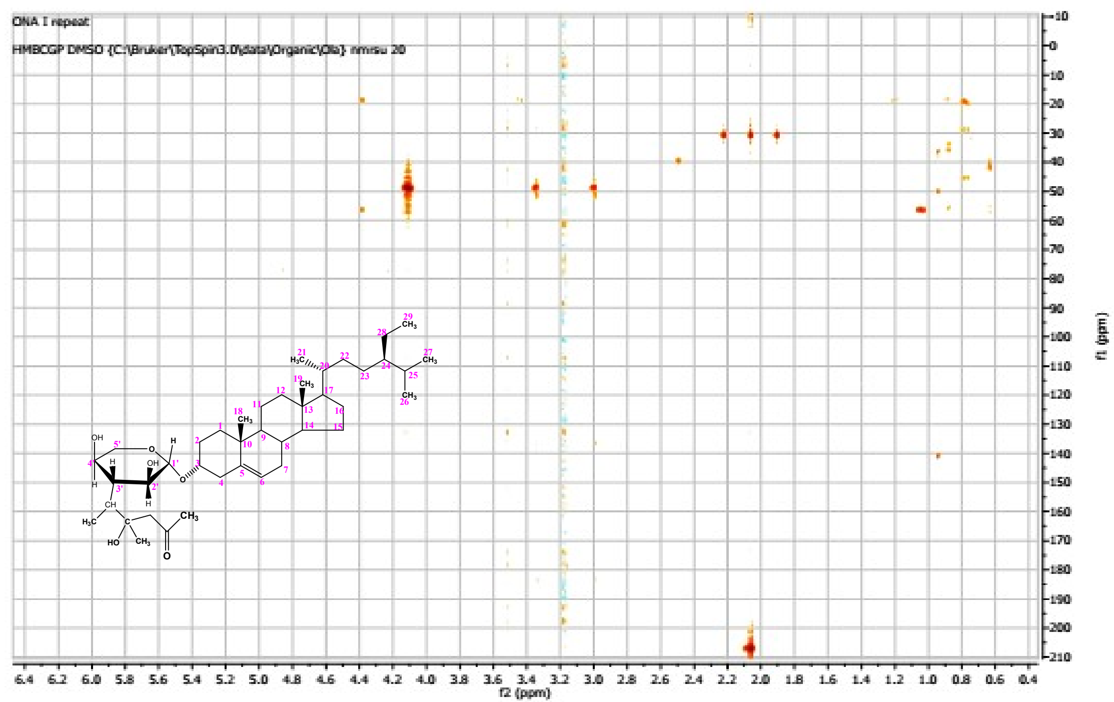
Appendix 20: Compound I proton spectrum



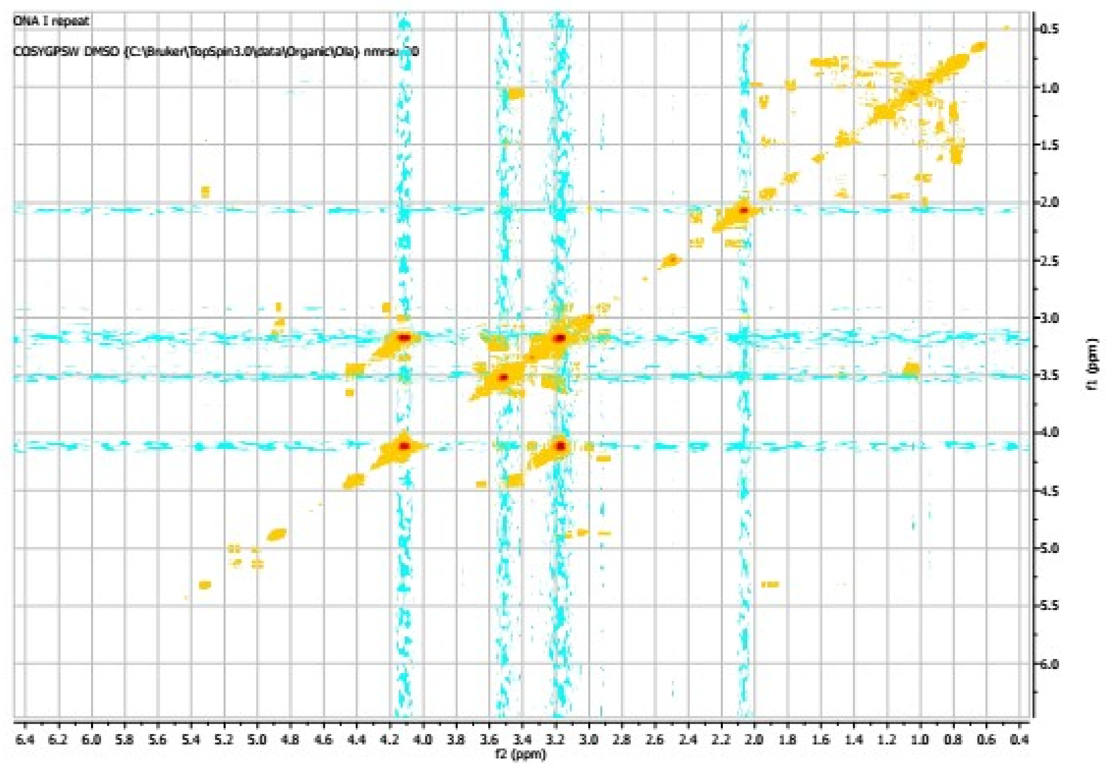
Appendix 21:Compound I carbon spectrum



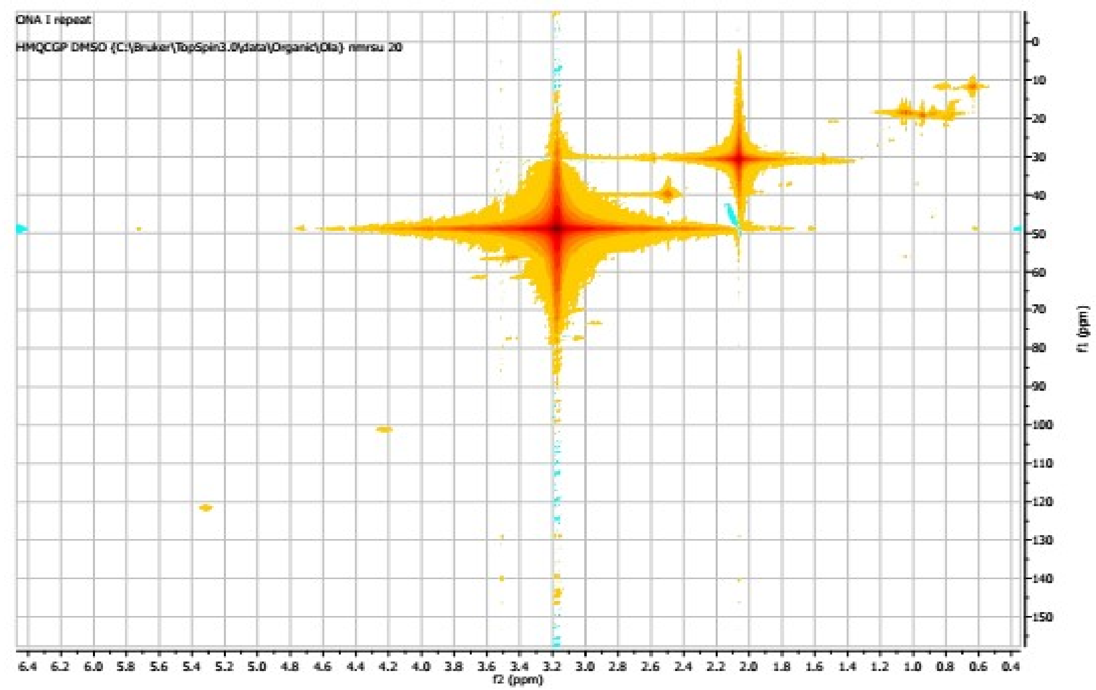
Appendix 22: Compound1 DEPT 135 spectrum



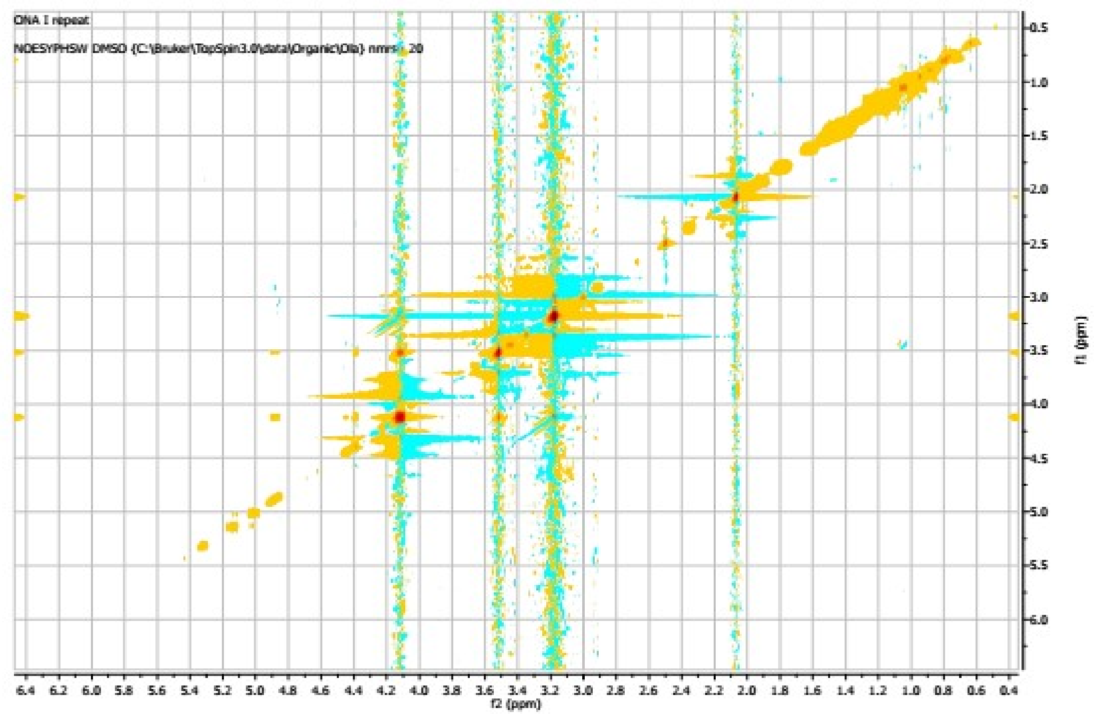
Appendix 23: Compound I HMBC spectrum



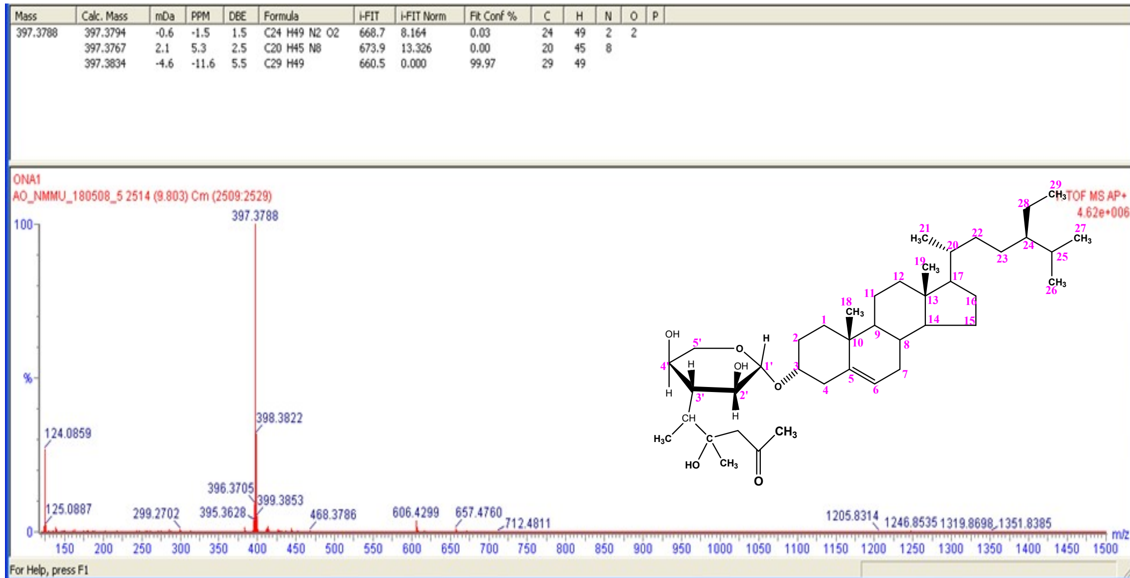
Appendix 24: Compound I COSY spectrum



Appendix 25: Compound I HMQC spectrum

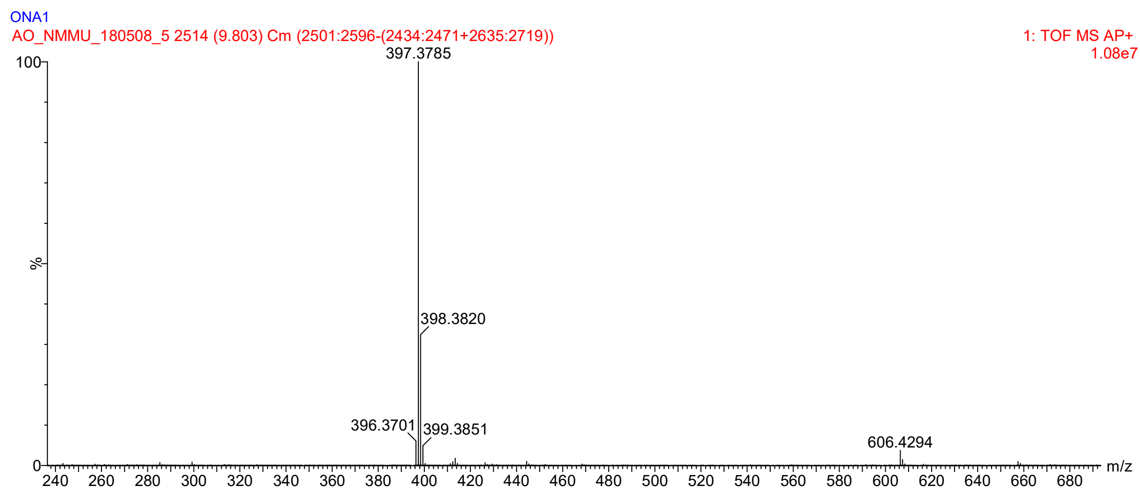


Appendix 26: Compound I NOESY spectrum

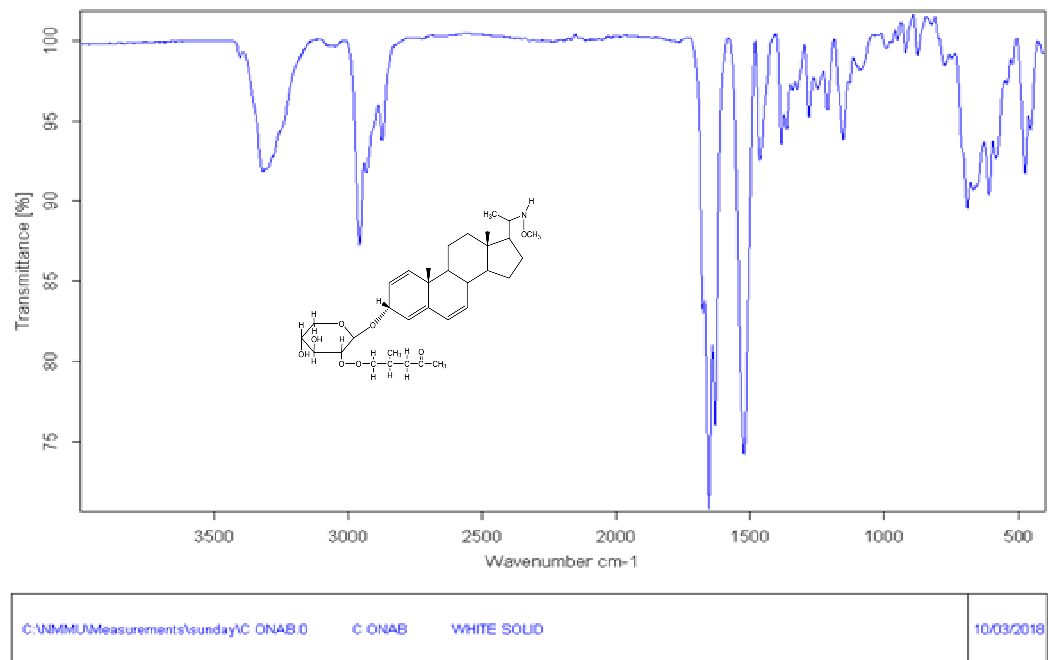


Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	P
397.3788	397.3794	-0.6	-1.5	1.5	C24 H49 N2 O2	668.7	8.164	0.03	24	49	2	2	
	397.3767	2.1	5.3	2.5	C20 H45 N8	673.9	13.326	0.00	20	45	8		
	397.3834	-4.6	-11.6	5.5	C29 H49	660.5	0.000	99.97	29	49			

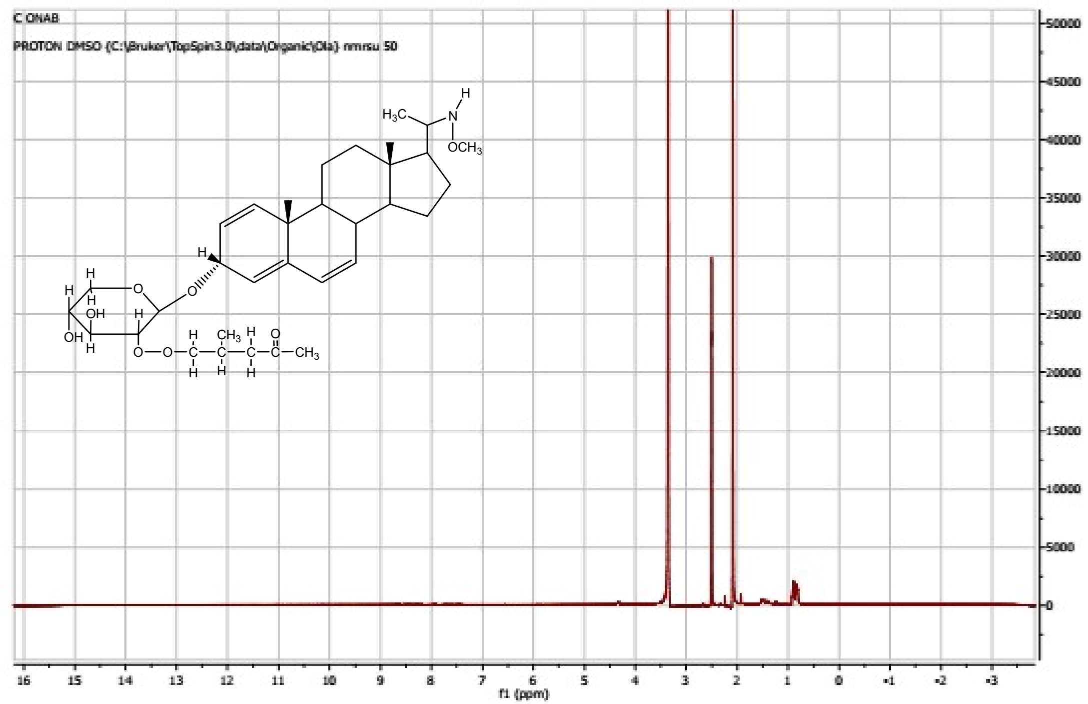
Appendix 27: APCI- TOF/MS/MS of compound I



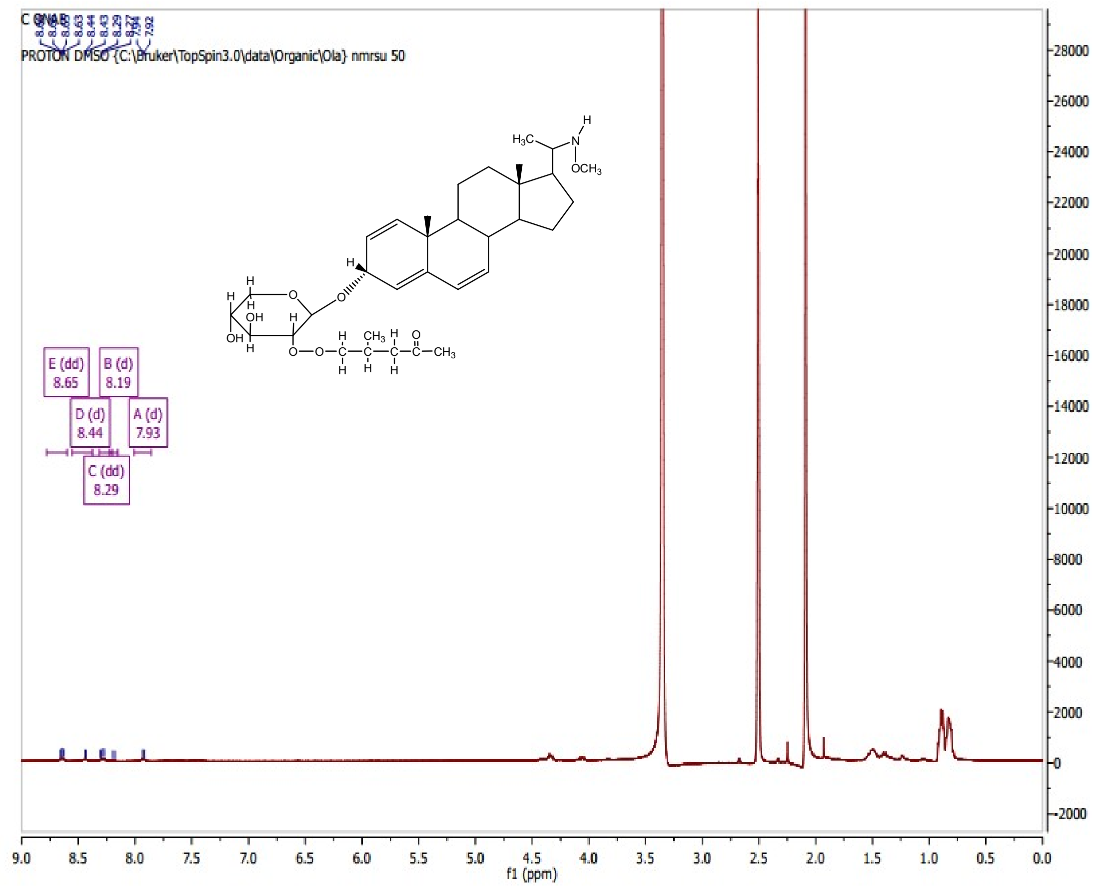
Appendix 27: ESI TOF/MS/MS of compound I



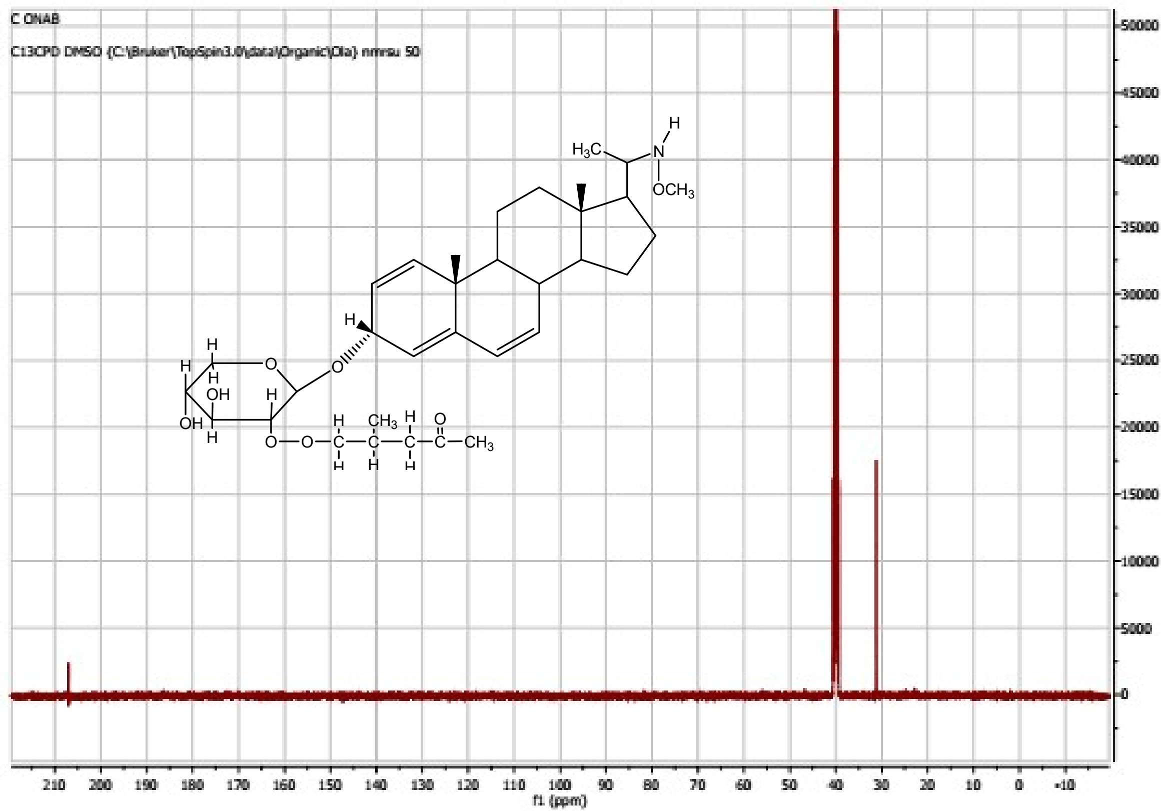
Appendix 28: Compound C_{2018} IR spectrum



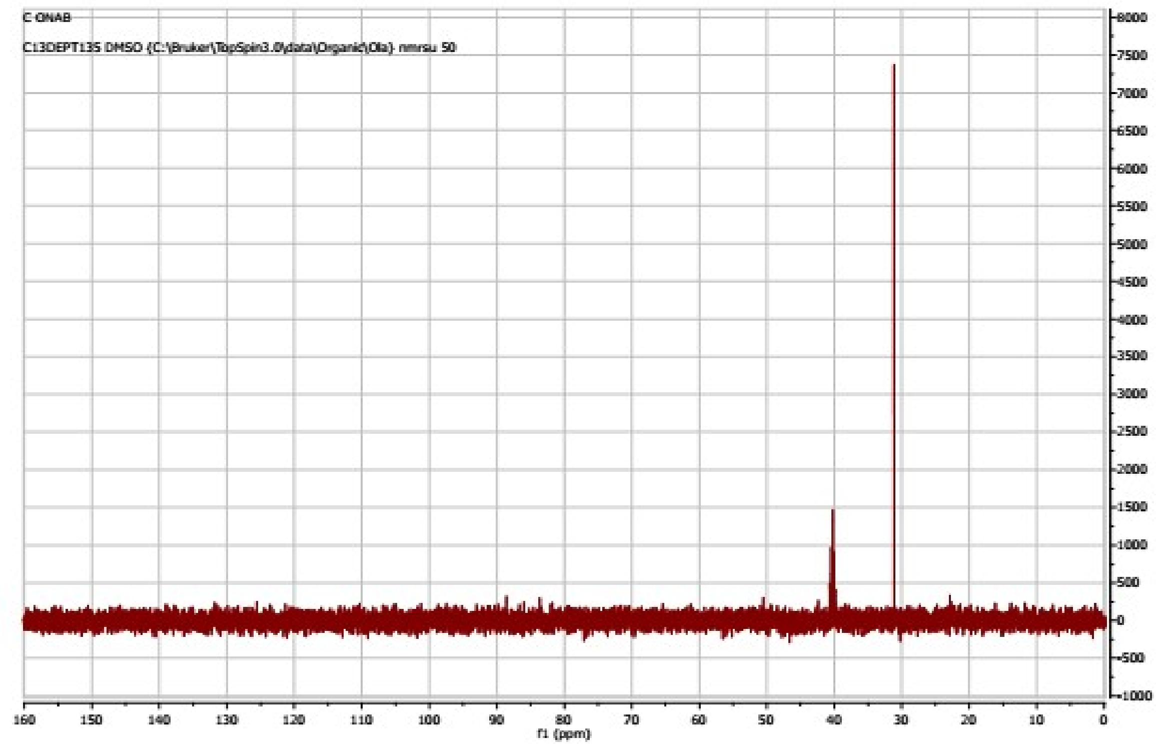
Appendix 29: Compound C_{2018} proton spectrum



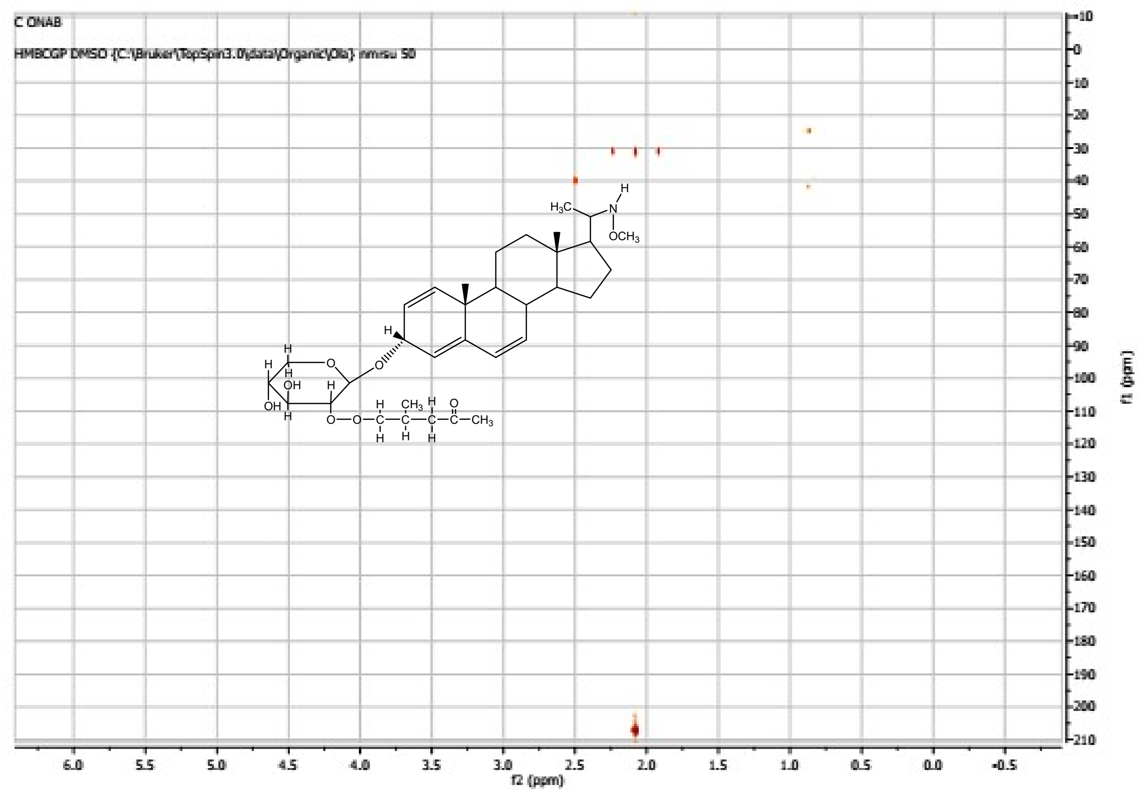
Appendix 30:Compound C_{2018} expanded proton spectrum



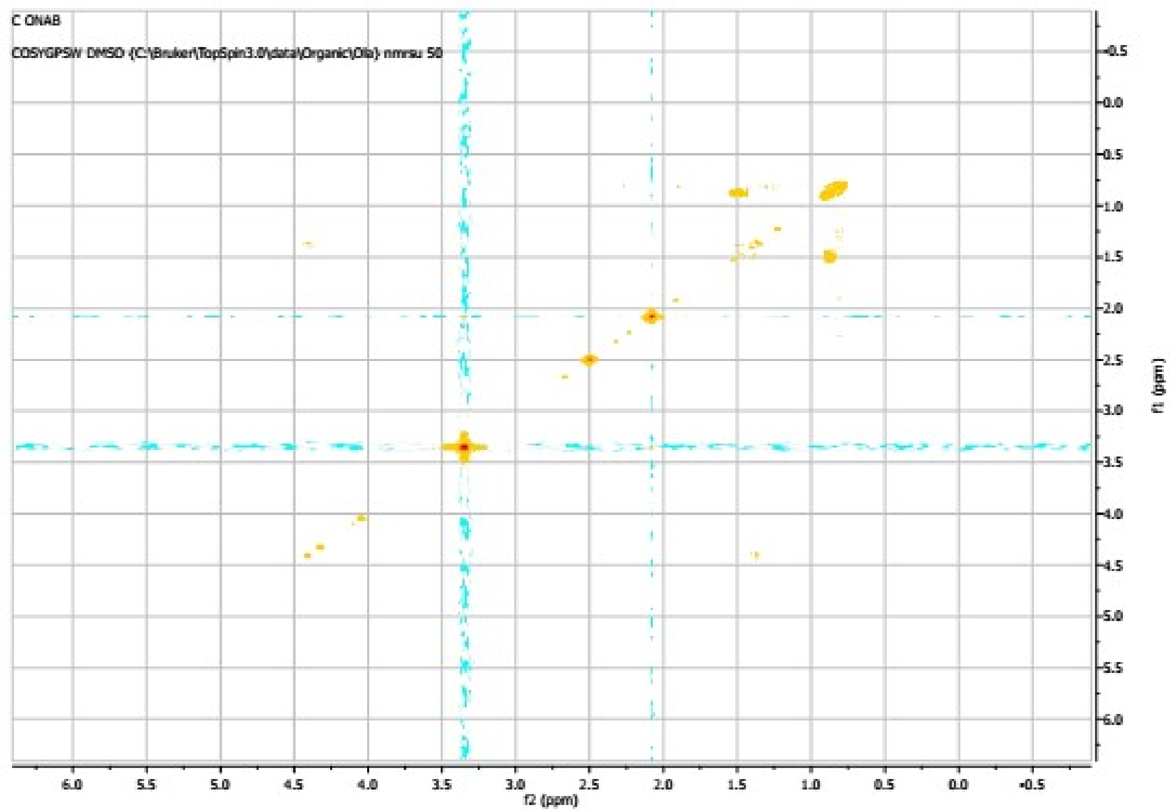
Appendix 31:Compound C_{2018} carbon spectrum



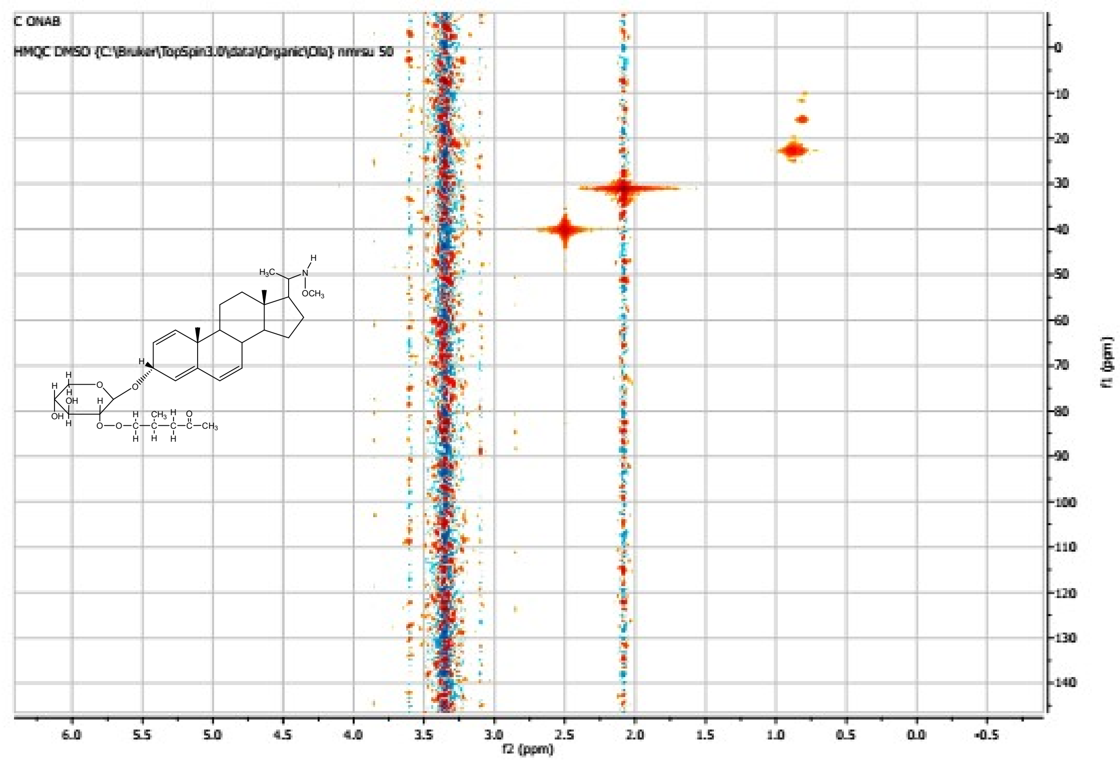
Appendix 32:Compound C_{2018} DEPT 135 spectrum



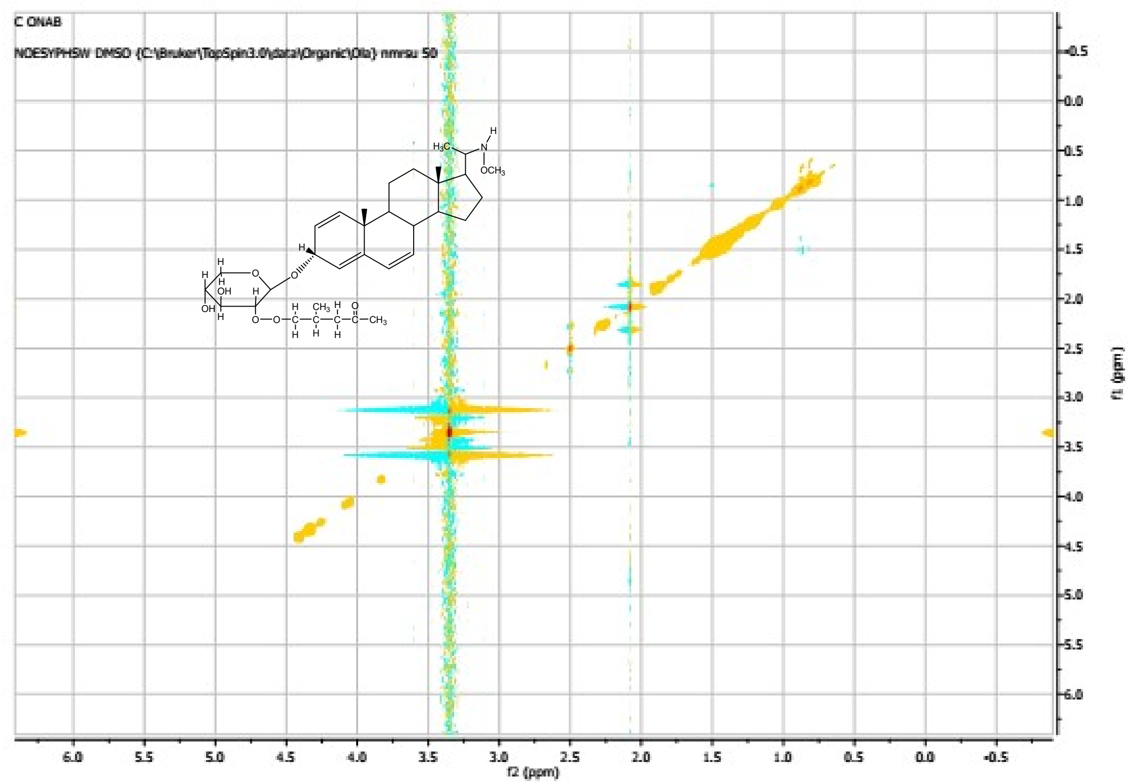
Appendix 33: Compound C_{2018} HMBC spectrum



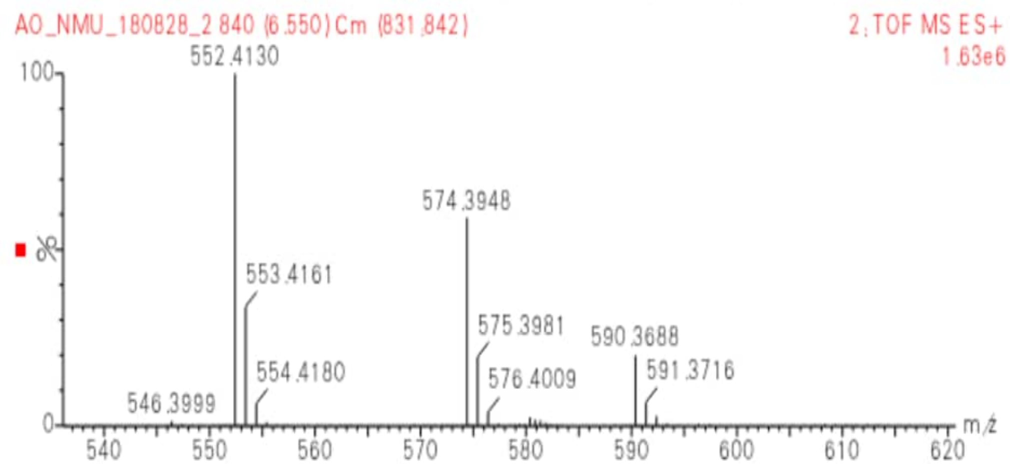
Appendix 34: Compound C_{2018} COSY spectrum

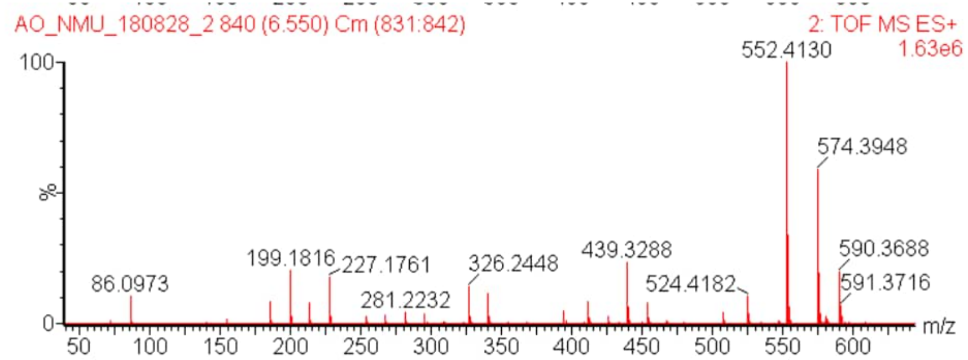


Appendix 35: Compound C_{2018} HMQC spectrum

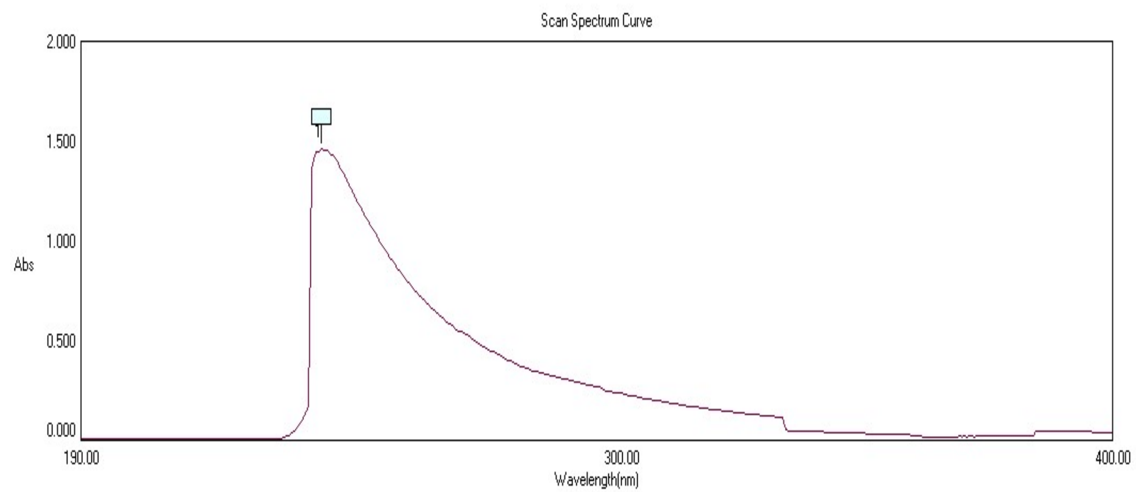


Appendix 36: Compound C_{2018} NOESY spectrum

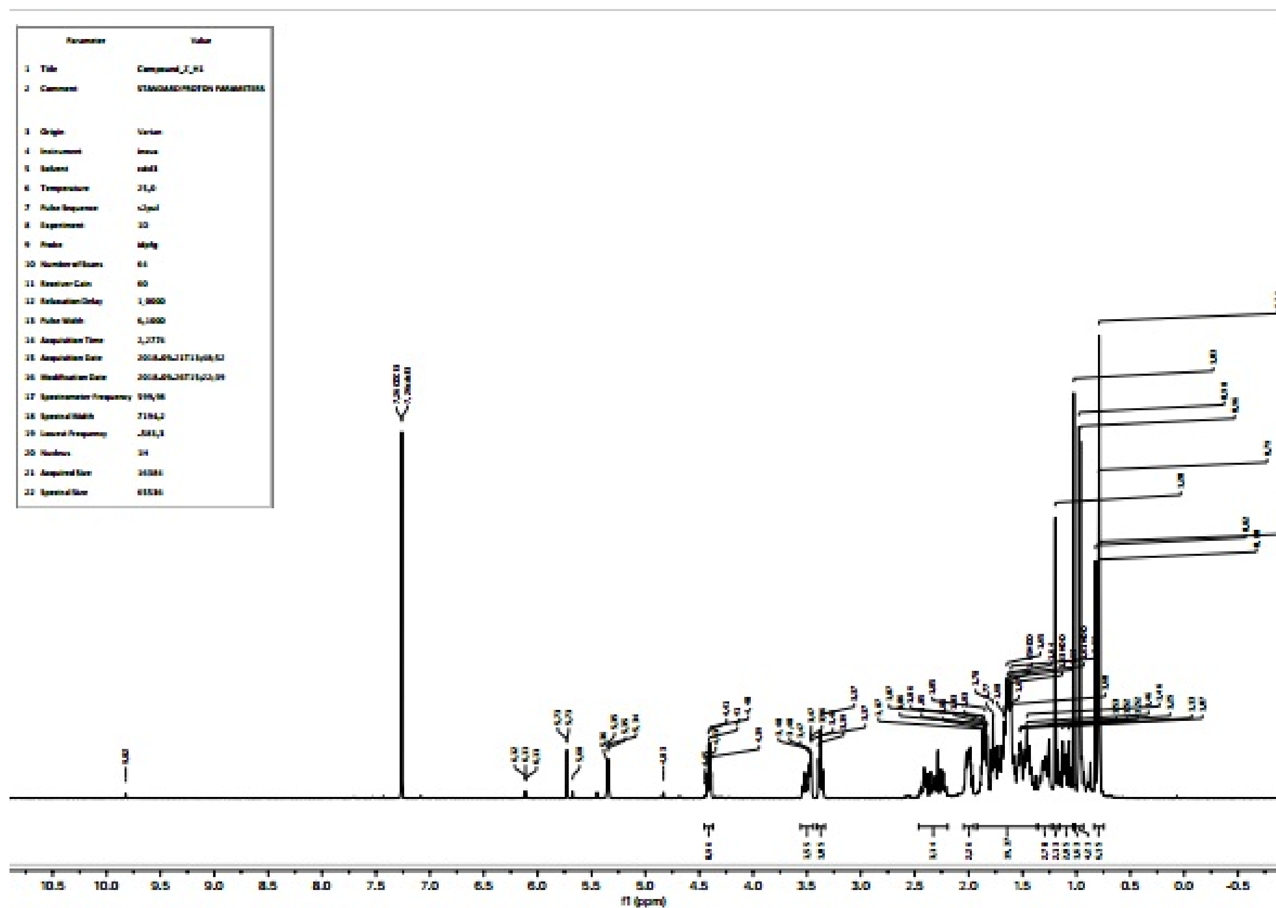




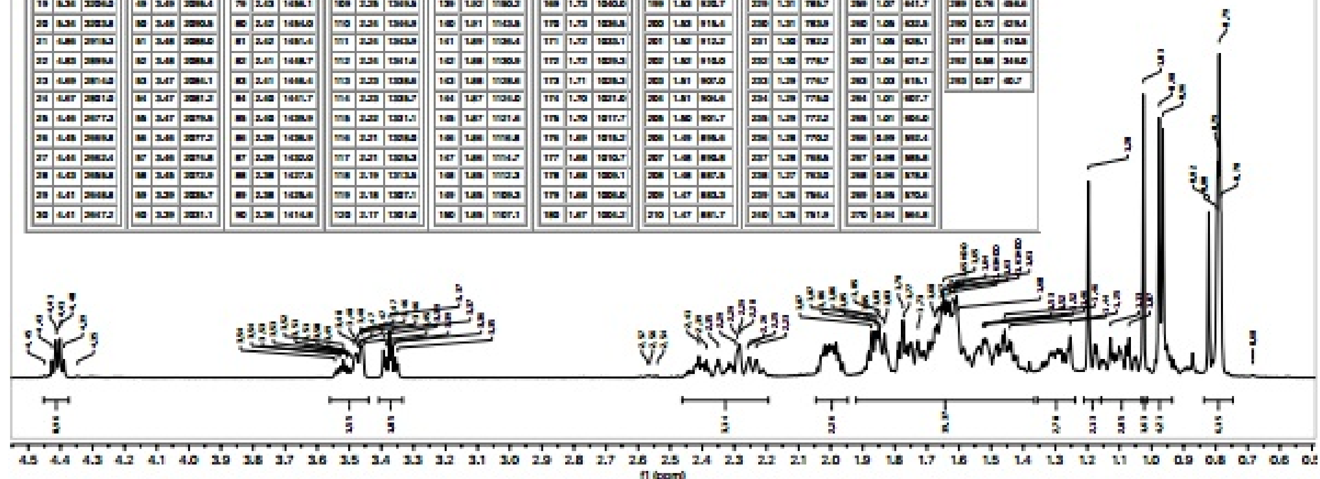
Appendix 37: ESI-ToF/MS/MS of compound C_{2018}



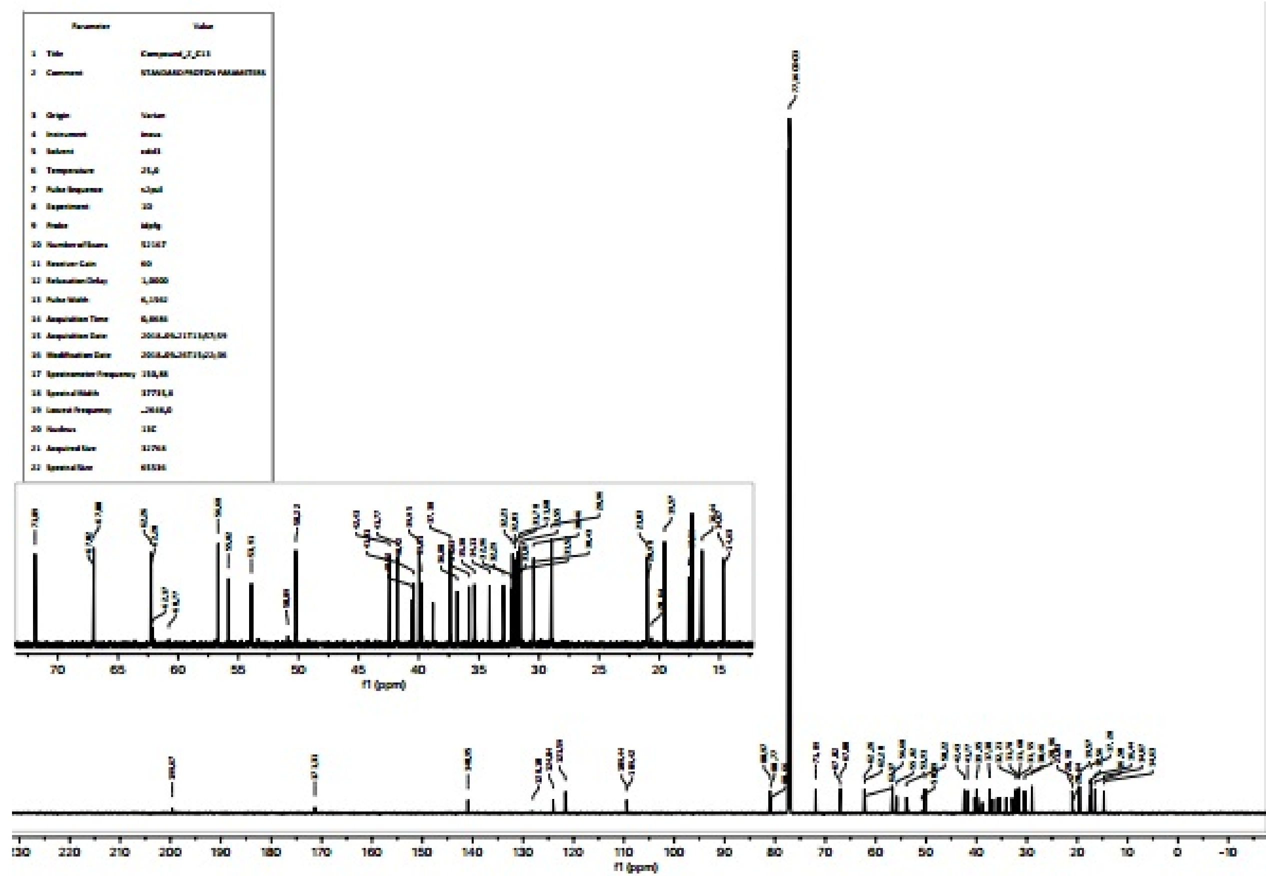
Appendix 38: Compound C_{2018} UV spectrum



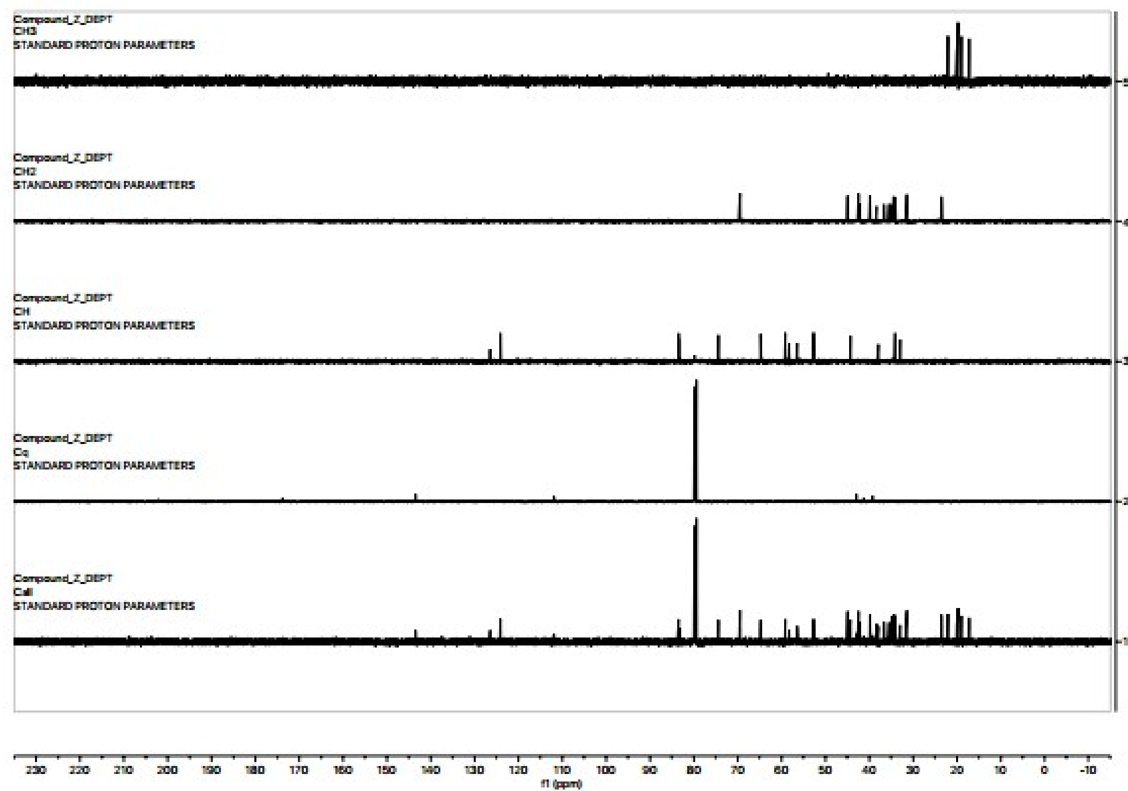
Appendix 39:Proton NMR spectrum of Compound Z



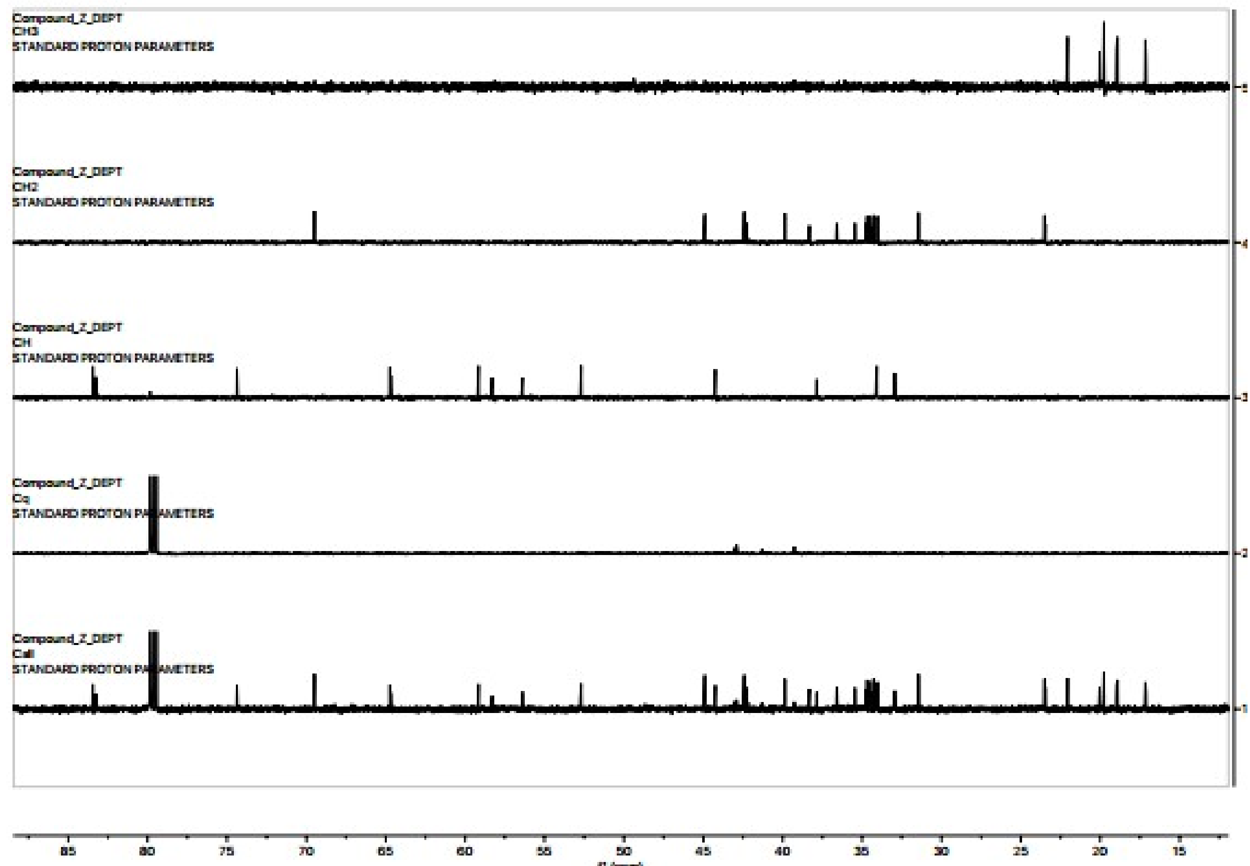
Appendix 40:Proton NMR spectrum of Compound Z



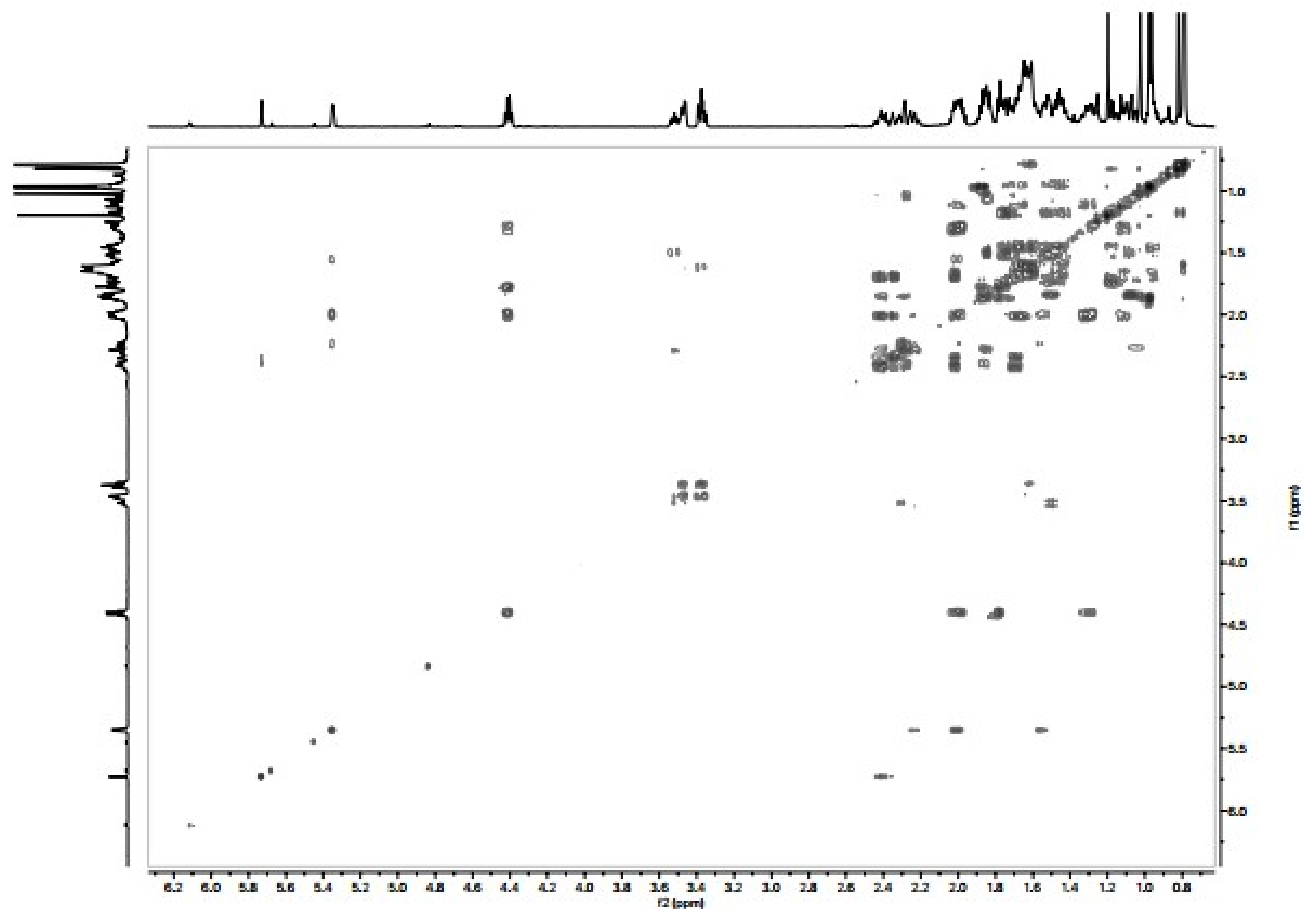
Appendix 41: Compound Z carbon spectrum



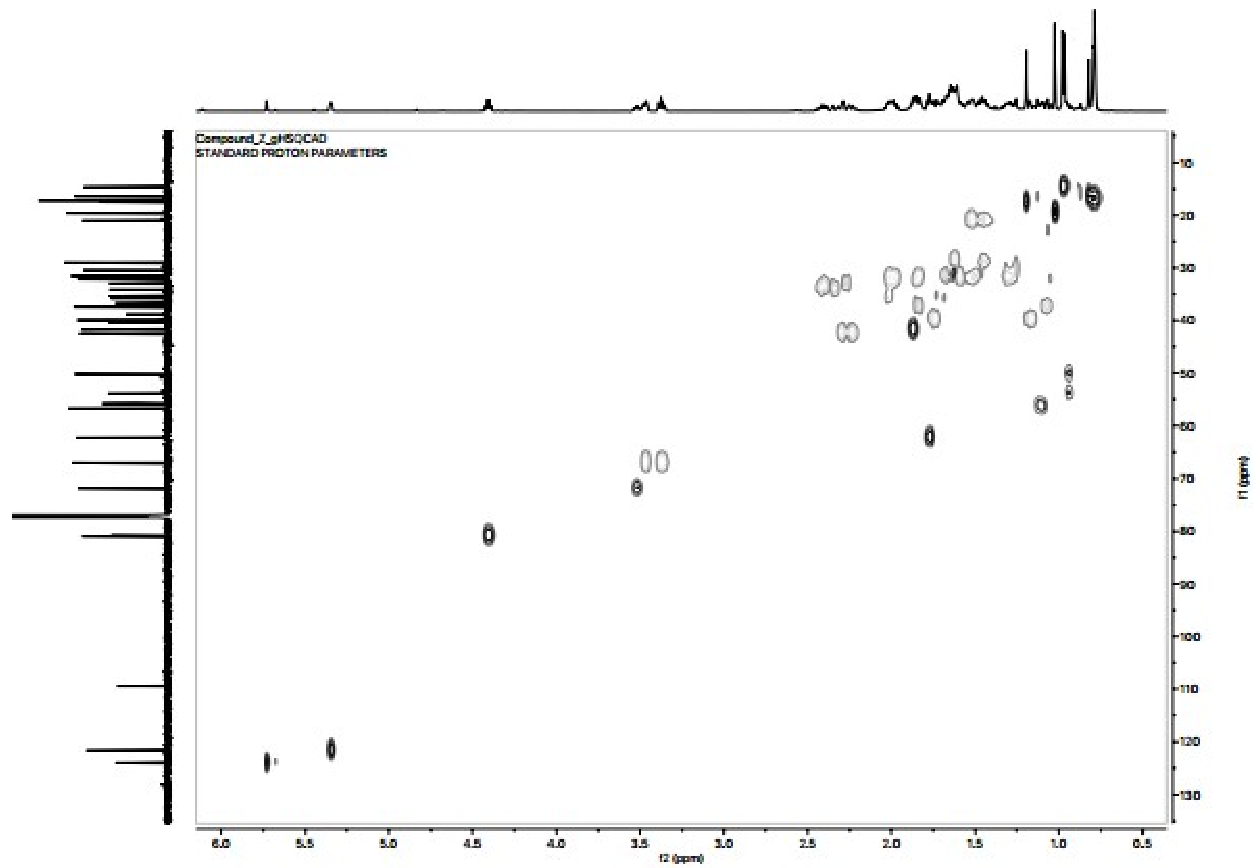
Appendix 42: DEPT 135 spectrum



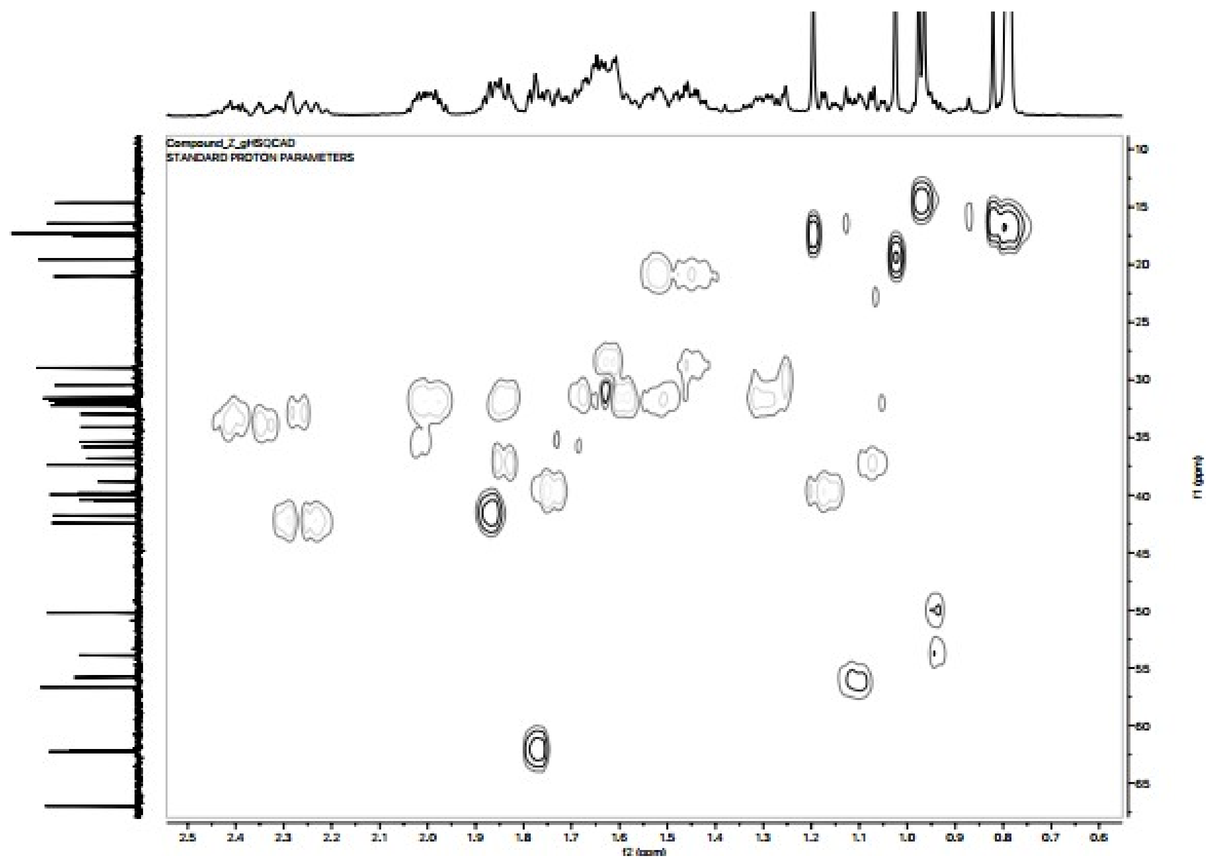
Appendix 43: DEPT 135 spectrum



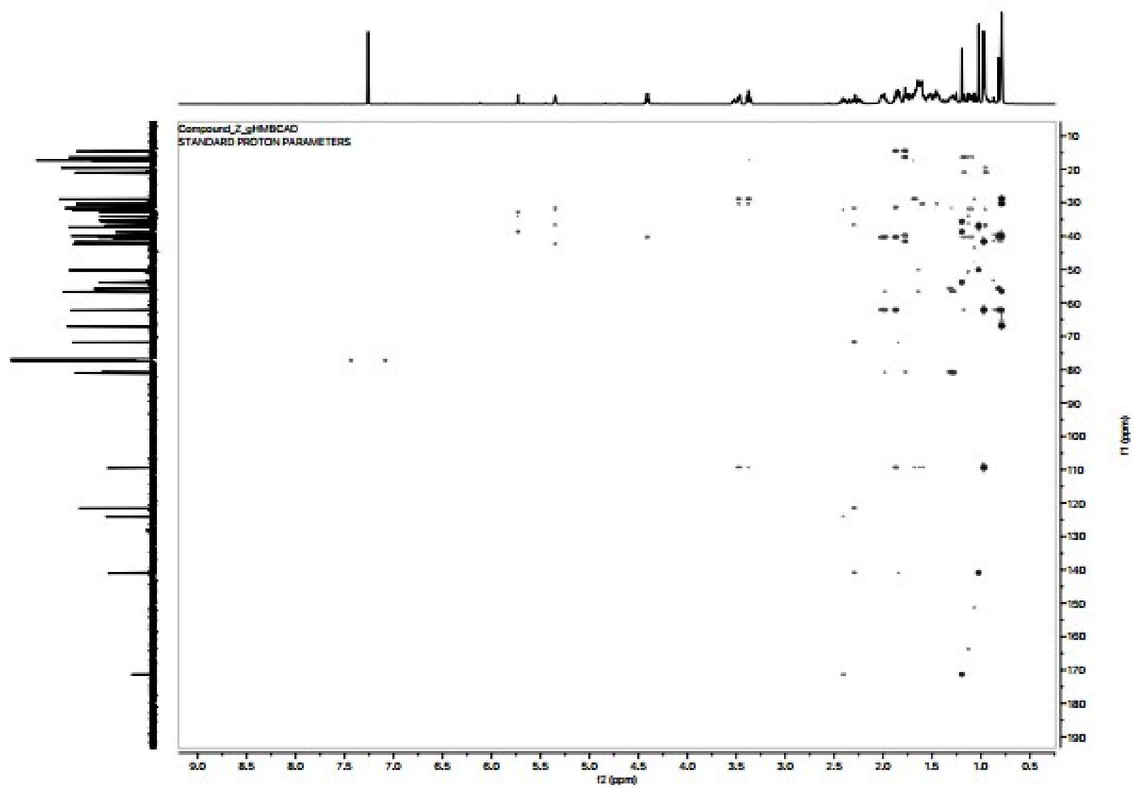
Appendix 44: COSY spectrum



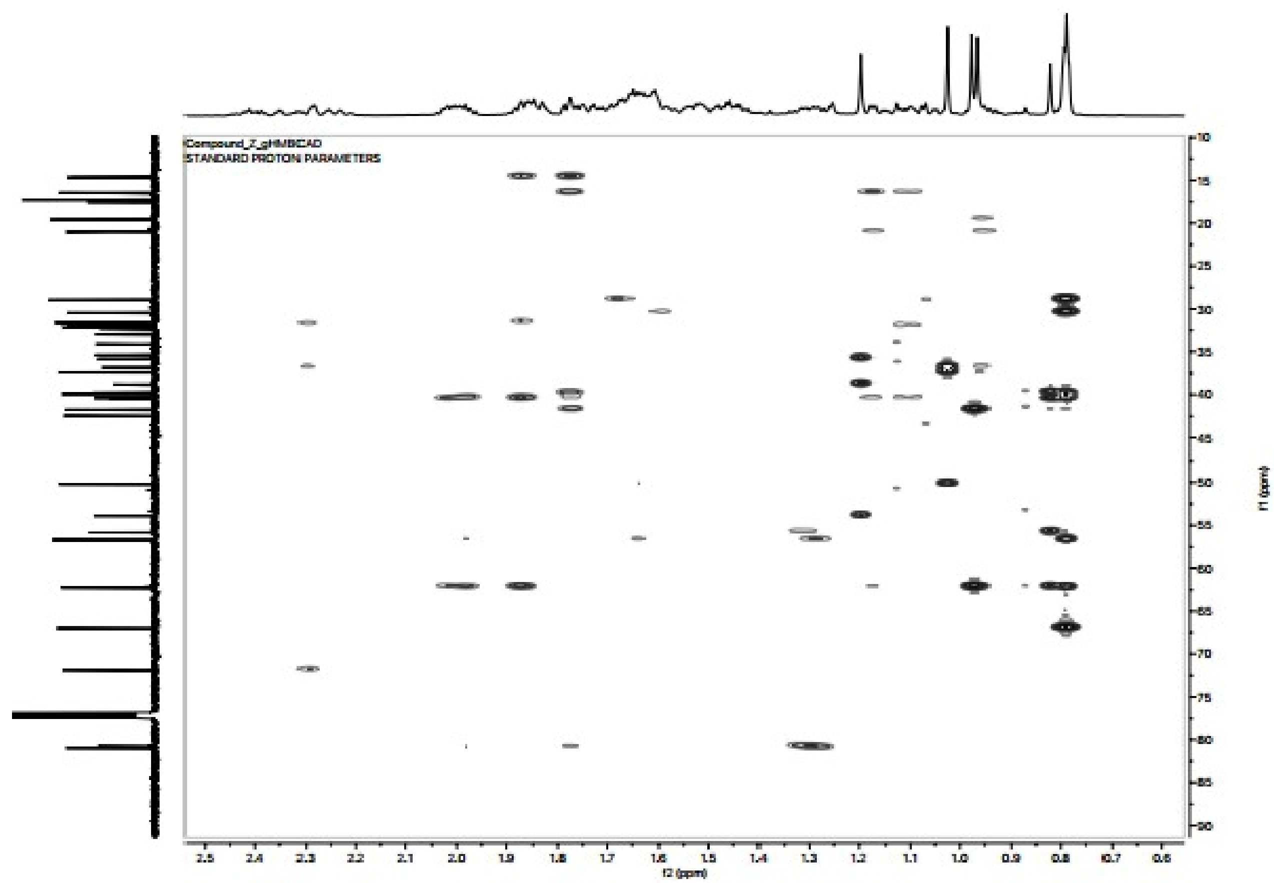
Appendix 45: HSQC spectrum



Appendix 46: HSQC spectrum



Appendix 47: HMBC spectrum



Appendix 48: HMBC spectrum