ISOLATION AND CHARACTERIZATION OF ANTIBACTERIAL SECONDARY METABOLITES FROM *Polyscias fulva* AND ITS ENDOPHYTIC FUNGI

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A thesis submitted to the Graduate School in partial fulfillment for requirements of the Master of Science Degree in Chemistry of Egerton University

EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted or presented for examination in any institution

Signature…… Date……..

Winnie Cherotich Maritim

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RECOMMENDATION

This thesis has been submitted for examination with our approval as university supervisors

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DEDICATION

This work is dedicated to my Dad Mr. Richard Maritim, my Late Mum Mrs Catherine Maritim and my husband Mr. Weldon Kirui for their moral and financial support.

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ABSTRACT

Infectious diseases remain to be a global health burden due to the development of antibiotic resistance by pathogenic microorganisms. Antibiotic resistance has led to increased number of deaths among children and adults. Medicinal plants have been used over the years in the treatment and remedy of various infections affecting human beings. The use of secondary metabolites from medicinal plants and fungal endophytes can be an alternative to disease management without the negative impact of synthetic antibiotics. Therefore, this study sought to isolate and characterize antibacterial secondary metabolites from the leaves of *Polyscias fulva* and its endophytic fungi. The leaves were collected from Kakamega rain forest; some of the leaves were dried under shade and ground into fine powder for extraction whereas the remaining fresh leaves were used for fungal endophyte isolation. The fungal endophytes were isolated from the internal tissues of *P. fulva* and cultured in Potato Dextrose Agar (PDA) media amended with streptomycin sulphate, followed by sub-culturing to obtain pure cultures. The pure cultures were then identified by DNA sequencing using ITS1F and ITS4 primers as *Fusarium* species (PF1 and PF2). The fungal endophytes were screened for bioactivity by carrying out antagonistic assay test against *Staphylococcus aureus* ATCC25922 and *Klebsiella pneumonia*e ATCC13883. *Fusarium* species (PF1 and PF2) were subjected to solid fermentation on rice media for 21 days; followed by ultra-sonication in methanol and subsequent liquid partitioning between hexane and ethyl acetate. The ethyl acetate extract was fractionated using column chromatography on sephadex LH 20. Extraction of the dried leaves' powder was carried out using methanol. The bioactivity of the pure compounds was determined using disc diffusion method against *K. pneumoniae* and *S. aureus*. Structure elucidation of the compounds isolated was successfully identified through analysis of their 1D and 2D NMR spectra and mass spectrometry data as well as comparison with literature data. Compounds **15**, **16** and **17** were isolated from the dried leaves of *P. fulva* while compound **18** was obtained from fungus *Fusarium* species (PF1). The methanol crude extract and isolated fractions showed antibacterial activities. Compound **18** was found to be the most active against *K. pneumoniae* (11.66±2.51 mm) and *S. aureus* (12.00±3.60 mm). Compound **17** was the least active against both *K. pneumonia*e (7.33±0.57 mm) and *S. aureus* (7.00±1.00 mm). These results suggest that these compounds could be lead compounds for drug development.

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ABBREVIATIONS AND ACRONYMS

COSY	Correlation spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
DNA	Deoxyribonucleic acid
ESI	Electron Spray Ionization
GPR	General Purpose reagent
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Power Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
MIC	Minimum Inhibitory Concentration.
PDA	Potato Dextrose Agar
PTLC	Preparative thin layer chromatography
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV	Ultra violet

CHAPTER ONE
INTRODUCTION

1.1 Background information

Bacteria cause serious infections in humans and have been the major cause of mortality and morbidity throughout history. *Staphylococcus aureus*, for example, causes superficial skin lesion, localized abscesses and food poisoning (Lotifpour *et al*., 2008) while *Klebsiella pneumoniae* cause nosocomial infection, urinary tract infections, infections of the respiratory tract, septicemial and pneumonia (Carsten and Karen, 2004). The rapid spread of bacteria expressing multidrug resistance has necessitated the discovery of new antibacterials and resistance-modifying agents (Starvi *et al*., 2007). Natural products medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms and terrestrial vertebrates and invertebrates (Raja *et al*., 2010). The use of indigenous knowledge of traditional medical practitioners as leads provides a useful route employed in the search for novel drugs. This is rewarding, and since most indigenous plants used in traditional medicine have not been explored in detail, the potential for discovery of more novel therapeutic compounds through bioprospecting of the flora is tremendous (Amusan *et al*., 2007). Numerous investigations have proved that medicinal plants contain diverse classes of bioactive compounds such as tannins, alkaloids and flavonoids, which exhibit various pharmacological properties (Emam, 2010). However, in the absence of any scientific proof of their effectiveness, the validity of these remedies remains questionable and their use by local communities remains restricted (Kaur and Arora, 2009).

The interest in plants with antibacterial properties has been revived because of current problems associated with the use of antibiotics (Jain *et al*., 2010). The development of resistance in infectious microorganisms towards drugs is a real problem of concern. Medicinal plants constitute a prolific source of antibacterial substances. According to World Health Organization, medicinal plants would be the best source of a variety of drugs (Njateng *et al*., 2013). About 80% of the total population in developing countries relies on traditional medicine based on plant products (Nascimento *et al*., 2000). This explains why numerous studies have been conducted on various medicinal plants extracts with the hope of discovering new and more antibacterial compounds. Among these plants is *P. fulva*, a medium size tree which grows in the tropical forests of west and central Africa (Focho *et al*., 2009). It is used in traditional medicine to fight against venereal infections and against

dermatoses (Bedir *et al*., 2001). Based on traditional uses of this plant, the extracts of the plant *P. fulva* could possess antibacterial properties. To give a scientific basis to the use of this plant, this study was carried out to assess the antibacterial potential against some selected bacteria.

1.2 Statement of the problem

According to the World Health Organization (WHO) reports, infectious diseases have time been among the leading cause of deaths in human population affecting both children and adults. This is attributed to the rise in antibiotic resistance by disease-causing microorganisms. The cause of antibiotic resistance has been linked to inappropriate misuse and overuse of antibiotics by a greater proportion of the entire human population. The developments of multi-drug resistant bacterial strains have considerably increased in the recent years. Therefore, the absence of effective agents for the control and treatment of bacterial infections has affected the economy negatively this is due to the consequences arising from illness and resultant deaths. The global health threat has greatly attracted research attention in an attempt to come up with practical measures that will help to solve the problem at hand. There is therefore need to use secondary metabolites from *P. fulva* and its endophytic fungi that can offer an alternative in managing diseases.

1.3 Objectives

1.3.1 General objective

To isolate and characterize antibacterial secondary metabolites from the leaves of *P. fulva* and its endophytic fungi

1.3.2 Specific objective

i. To isolate secondary metabolites from the leaves of *P. fulva* and its endophytic fungi.
ii. To determine the structures of secondary metabolites from the leaves of *P. fulva* and its endophytic fungi.
iii. To determine the antibacterial activity of the extracts and pure compounds from the leaves of *P. fulva* and its endophytic fungi.

1.4 Hypotheses

i. Secondary metabolites from the leaves of *P. fulva* and its endophytic fungi have similar physical and chemical characteristics.

ii. Secondary metabolites from fungal endophytes and leaves of *P. fulva* have similar structures.
iii. Extracts and pure compounds from the leaves of *P. fulva* and its endophytic fungi are inactive against human pathogens.

1.5 Justification

Antibiotic resistance among disease-causing pathogens has resulted in emergence of multi-drug resistant pathogens and the increasingly immune-suppressed population now poses a new global health challenge. Synthetic drugs are associated with negative effects that include non-biodegradability and high toxicity in organisms. Because of such problems associated with the use of synthetic antibiotics, the antibacterial properties from plants are of much interest. Therefore, there is need for alternative sources of antibiotics that are readily available, non-biodegradable and environmentally friendly. Plants have been traditionally used for treatment of various ailments. Secondary metabolites from plants and fungal endophytes used for the prevention of infectious diseases offer an alternative to discovery of antibiotics with no reported resistance. The most prevalent way of achieving this is to explore bioactive potential of medicinal plants such as *P. fulva* and its endophytic fungi with an intention of overcoming drug resistance menace.

CHAPTER TWO
LITERATURE REVIEW

2.1 The plant family Araliaceae

Araliaceae (the ginseng family) are a family of flowering plants, also known as the aralia family or ivy family. The family comprises of 41 genera and some 1350–1400 species, most of which are found in the tropical Southern Hemisphere, with centers of species richness in west and central Africa, New Caledonia, Australasia, Madagascar, Southeast Asia, and South America (Wen *et al*., 2001; Plunkett *et al*., 2004). The family includes a number of important medicinal plants, such as *Panax* (ginseng) and *Eleutherococcus* (Siberian ginseng), and several well-known ornamentals, including *Hedera* (English ivy), *Schefflera* (the umbrella trees) and *Polyscias* (Judd *et al*., 1994).

2.2 Genus *Polyscias*

The genus *Polyscias* was described by Forster & Forster (1775) based on a single collection they made in the South Pacific while serving on board Captain James Cook's second voyage. The Forsters distinguished their new genus from other Araliaceae recognized at the time (primarily the Linnaean genera *Aralia* l., *Hedera* l. and *Panax l*.) by several characters including the presence of 6 to 8 petals and as many stamens, 3 or 4 styles and a globose fleshy fruit containing 4 seeds. Over the next 90 years no additional species had been assigned to *Polyscias,* although during the same period over 60 taxa were described, mostly in the genus *Panax,* that are currently included in *Polyscias* either as accepted species or synonyms (Lowry *et al*., 2010).

2.3 The Plant *Polyscias fulva*

The plant *P. fulva* (Figure 1) is a fast growing deciduous tree of the tropical forests of sub Saharan Africa which is found at an altitude range of 1,180-2,500m, with an annual rainfall of 1,500-2,000 mm. It grows up to 30 m tall, often with a straight, slender trunk to about 9 m before developing branches, like the spokes of an umbrella. The bark is grey and smooth and the leaf scars are prominent. The flowers are green-yellow, honey scented. The fruit is small, black, and more or less oval and often ribbed (Bedir *et al*., 2001). It is usually found in wetter highland areas like Kakamega Forest in Kenya, often occurring in tea growing districts (Orwa *et al*., 2009).

Figure 1: The plant *Polyscias fulva*

Source: Lemmens (2009)

2.3.1 Traditional uses of the *Polyscias fulva*

Polyscias fulva, also known as Mwanzu in luhya communities and aounet in kalenjin communities, is a medium size tree which grows in the tropical forests of west and central Africa, is traditionally taken orally against venereal infections and other various infections. (Focho *et al*., 2009). In Kenya, a decoction of the leaves is taken to treat intestinal complaints, its stem barks is used as decoction for obesity while the pounded leaves are applied externally to treat fractures (Maundu & Tengnas, 2005). In Burundi, *P. fulva* stem barks, branch and trunk powder is used for sniffing against pulmonary tuberculosis while its powdered bark is taken orally to facilitate delivery (Baerts & Lehmann, 1989). In the Democratic Republic of Congo, the *P. fulva* bark infusion is used against fevers. In Cameroon, decoction of *P. fulva* bark is orally administered to cure venereal infections (Focho *et al*., 2009). It is also used in traditional medicine to fight against dermatoses (Bedir *et al*., 2001; Njateng *et al*., 2013).

2.3.2 Geographical distribution of *Polyscias fulva*

Polyscias fulva is distributed in the highland forests into the bamboo zone. It grows in afro-montane and undifferentiated afro-montane forests (broad- leaved forests) often in clearings and regrowth (Lemmens, 2009). It also occurs in rainforests, lowland forests and

mountain grasslands. In Uganda it grows in woodland, semi-humid and humid highland forests. In Kenya the species grows around Elburgon, North of Mt. Elgon, West of Mt. Kenya and North of the Nandi forests (Figure 2). It is usually found in wetter highland areas like Kakamega Forest in Kenya, often occurring in tea growing districts. A few remnant trees can be found in the Nairobi area. It grows as far South as South Africa (Orwa *et al*., 2009).

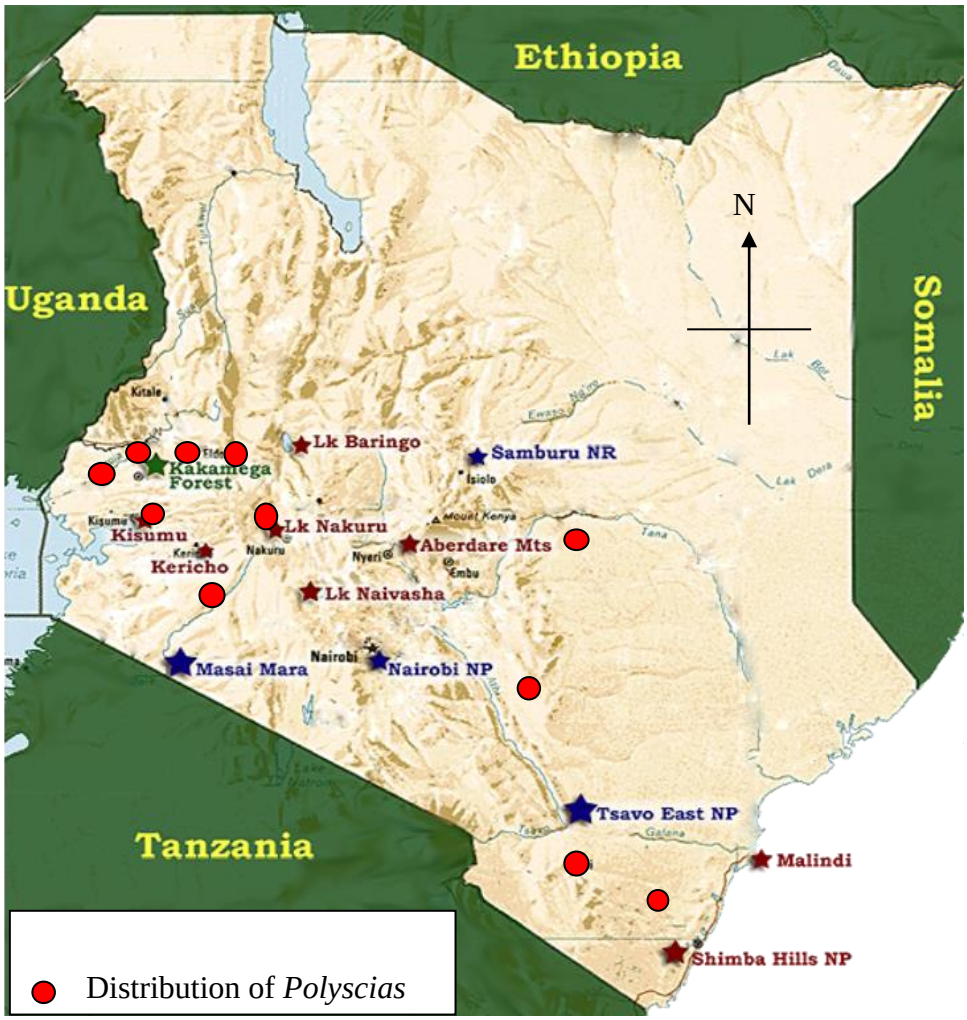


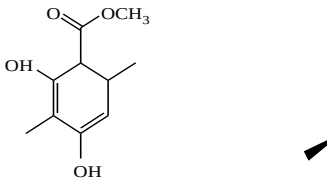
Figure 2: Distribution of *Polyscias fulva* in Kenya

Source: (Maundu and Tengnas, 2005)

2.4 Secondary metabolites from Araliaceae family

Secondary metabolites are organic compounds produced by bacteria, fungi or plants which are not directly involved in the normal growth, development, or reproduction of the organism. They often play an important role in plant defense against herbivory and other interspecies defenses. Humans use secondary metabolites as medicines, flavourings, pigments, and recreational drugs (Pichersky & gang, 2000). Secondary metabolites that have

been isolated from various polyscias species within the family Araliaceae include: methyl 2,4-dihydroxy-3,6-dimethylbenzoate (Methyl tartarate) (**1**) (Mutai *et al*., 2004), β-sitosterol (**2**) (Castola *et al*., 2002), pinoresinol (**3**) (Cespedes *et al*., 2006), oleanolic acid (**4**) (Zhong *et al*., 2001), 3-O-[α-L-rhamnopyranosyl (1-2)-α-L-arabinopyranosyl]-oleanolic acid (**5**) (Zhong *et al*., 2001), (Z, 3-O-[α-L-rhamnopyranosyl (1-2)-α-L-arabinopyranosyl]-echinocystic acid (**6**) (Grishkovets *et al*., 1996).



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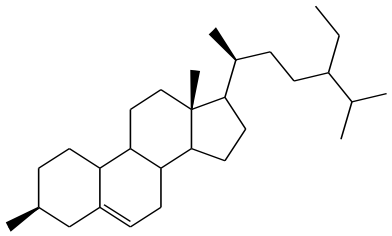

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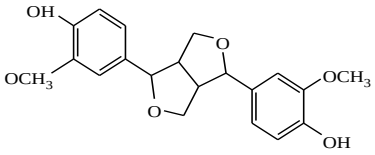

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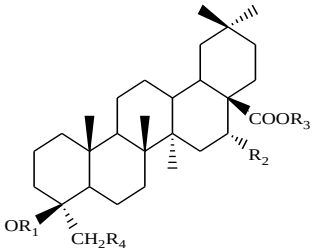
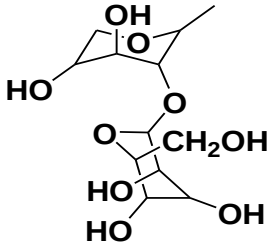
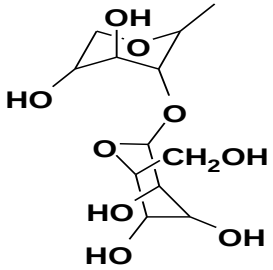


(4) Basic skeleton of Compounds 4-6

Table 1: The substituents R_1, R_2, R_3 and R_4 for compound **4**

	R_1	R_2	R_3	R_4
4	H	H	H	H
5		H	H	H
6		OH	H	H

2.5 Plant based antibiotics

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. Their role is twofold in the development of new drugs: they may become the base for the development of medicine or a phytomedicine to be used for the treatment of diseases. There are numerous illustrations of plant derived antibiotics. The isoquinoline alkaloid emetine obtained from the roots of *Cephaelis ipecacuanha*, and related species, has been used for many years as an amoebicidal drug as well as for the treatment of abscesses due to the spread of *Escherichia histolytica* infections. Another important antibiotic of plant origin with a long history of use is quinine. This alkaloid occurs naturally in the bark of Cinchona tree. Apart from its continued usefulness in the treatment of malaria, it can also be used to relieve nocturnal leg cramps (Iwu *et al*., 1999). Similarly, higher plants have made important contributions in the areas beyond anti-infective, such as in cancer therapies. Cancer therapeutic agents include taxol and several derivatives of campthotein. For example, a well-known benzylisoquinoline alkaloid, papaverine, has been shown to have a potent inhibitory effect on the replication of several viruses including measles and HIV (Turano *et al*., 1989).

2.6 Endophytes

An endophyte is a bacterial or fungal microorganism, which spends the whole or part of its life cycle colonizing inter-cellularly inside the healthy tissues of the host plant without causing any immediate, overt negative effects (Tan & Zou, 2001). The most frequently isolated endophytes are fungi (Strobel & Daisy, 2003). Examination of plant material can lead to the discovery of endophytic fungi and bacteria. The fungi isolated are often host specific (Bacon & De battista, 1991), so it is possible that of the nearly 300,000 plant species that exist on the earth, each individual plant could be host to one or more endophytes (Tan & Zou, 2001). The number of endophytic species potentially associated with plants can reach several hundred (Sanchez *et al*., 2008). Furthermore, it has been reported that endophytes are also found in marine algae, mosses and ferns (Petrini, 1992; Raviraja *et al*., 1996). They reside inside the tissues of nearly all healthy plants. The relationship that they establish with the plant varies from symbiotic to bordering on pathogenic (Strobel & Daisy, 2003). They are synergistic to their host and at least some of them are thought to be useful to the plant by producing special substances, such as secondary metabolites, that prevent the host from being attacked successfully by fungi and pests (Thongchai *et al*., 2005). Endophytes may also

produce a plethora of substances of potential use to modern medicine, agriculture, and pharmaceutical industry. The potential prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans, plants, and animals are great (Strobel & Daisy, 2003).

2.6.1 Endophytic fungi

Endophytic fungi are defined as a group of organisms capable of living in host plants tissue without causing any symptoms (Petrini, 1992). To date, endophytic fungi have been separated into four classes namely; the clavicipitaceous endophytes, the nonclavicipitaceous endophytes that colonize the whole plant, the nonclavicipitaceous endophytes that colonize shoots and the nonclavicipitaceous endophytes that colonize roots. These classes are based on host range, type of tissue(s) colonized, colonization in planta, and diversity in planta, transmission and fitness benefits (Cannon & Simmons, 2002; Arnold *et al*., 2007). Endophytic fungi are extremely ubiquitous; it is thought that the vast majority of plant species in natural ecosystems (if not all of them) harbor fungal endophytes (Rodriguez *et al*., 2009). Endophytic fungi are estimated to be represented by at least one million species residing in plants (Dreyfurs & Chapela, 1994).

2.6.2 Endophytic fungi as sources of bioactive products

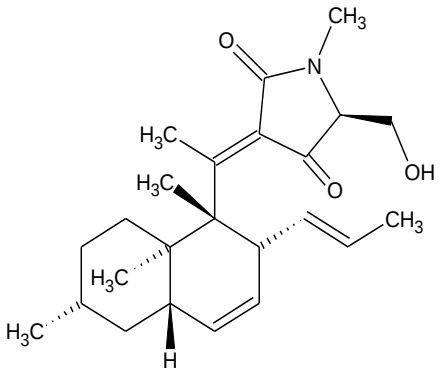
A fungal secondary metabolite is a chemical compound produced by a limited number of species in a genus. A satisfactory production of secondary metabolites depends on the medium the fungi are cultured on. The more complex a medium is the more chemical diversity the fungi will display (Frisvad *et al.*, 2008). Endophytes are ubiquitous and show a rich biodiversity; they are capable of synthesizing a variety of bioactive compounds. Hundreds of natural products have been reported from endophytes, including alkaloids (El Amrani *et al*., 2012), steroids (Zhang *et al*., 2009), terpenoids (De Souza *et al*., 2011) and flavonoids (Qiu *et al*., 2010). Most of the natural products from endophyte are antibiotics, anticancer agents or biological control agents.

The most famous example of successful drugs derived from endophytic fungi is the one-billion-dollar drug paclitaxel (Taxol®). This highly functionalized diterpenoid was first isolated from the inner bark of *Taxus brevifolia* in 1969 and is used as a cytostatic agent against breast cancer and ovarian carcinoma (Wani *et al*., 1971; Strobel *et al*., 2004). Endophytic fungi gained more interest when Strobel *et al*. (1993) reported that *Taxomyces andreanae*, an endophytic fungus of *Taxus brevifolia*, also produces paclitaxel. Screening of

the diverse group of fungi that may produce valuable medicinal plant products is a promising approach for producing drugs on a commercial scale using microbes (Strobel & Daisy, 2003).

2.6.3 Secondary metabolites from *Fusarium* species

Fusarium is a large genus of filamentous fungi which belong to a class Soidariomycetes which are distributed in plants mostly symptomless. The genus Fusarium comprises inhabitants of the soil and of organic substrata and is widely distributed throughout the world (Burgess, 1981). This genus is endowed with several means of survival, amongst which is its quick capacity for change, both morphological and physiological, when faced with environmental changes. The non-pathogenic forms of *Fusarium* can colonize the plants without causing symptoms of diseases (Appel & Gordon, 1994). These species has been reported as endophytes from several plants with diverse biological activity (Shiono *et al*., 2007; Deng *et al.*, 2009). Some secondary metabolites from *Fusarium* species have been shown to possess an antibacterial effect, including Equisetin **7** (Ratnaweera *et al*., 2015), antibiotic Y **8** (Golinski *et al*., 1986), Rohitukine **9** (Son *et al*., 2008) and Taxol **10** (Tayong *et al*., 2011).



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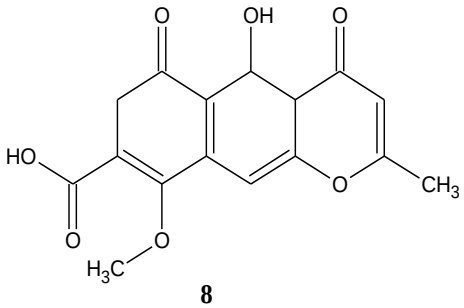

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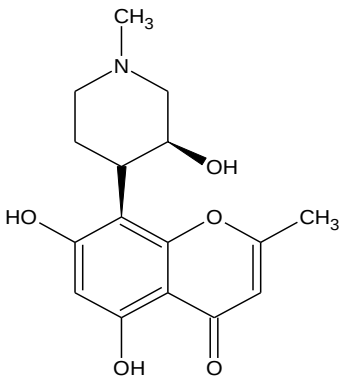

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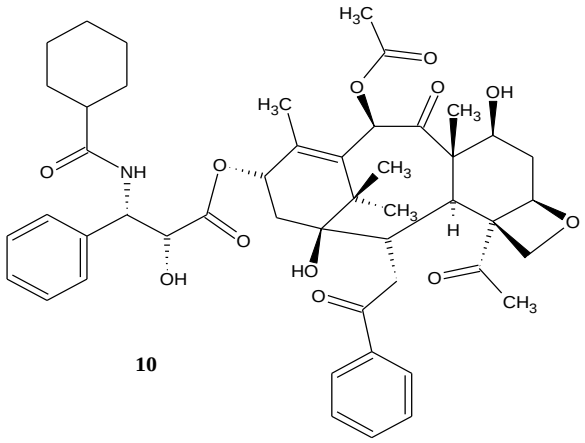

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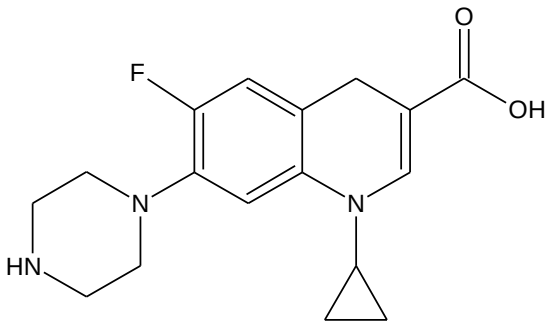
2.7 Endophytes as a source of antibiotics

Endophytes are chemical synthesizers in inside the plants; they are capable of synthesizing bioactive compounds that can be used by plants for defense against human pathogens (Owen & Hudley, 2004). Currently, endophytes are viewed as an outstanding source of bioactive natural products (Strobel & Daisy, 2003). Furthermore, fungal endophytes have been recognized as repository of novel secondary metabolites for potential therapeutic use (Tan & Zou, 2001). According to a study by Guo *et al*. (2006), most of the natural products from endophytes are antibiotics. According to Demain (1981), antibiotic compound is a low molecular weight organic natural product made by microorganisms that are active at low concentration against other microorganisms and are the most bioactive natural products isolated from endophytes. The future of modern medicine depends upon effective antibiotics (So *et al*., 2010). Antibiotics consists of different classes, these include quinolones, tetracyclines, aminoglycosides and beta lactams.

2.7.1 Quinolones

Quinolones and fluoroquinolones are broad-spectrum antibiotics and are widely used for urinary tract infections, as well as other hospital-acquired infections. These drugs act by inhibiting the bacterial DNA-gyrase. Nearly all quinolones antibiotics in use contain a fluorine atom in their chemical structure and are effective against both Gram-negative and Gram-positive bacteria (Gigosos *et al.*, 2000). The general structure consists of a 1-substituted-1, 4-dihydro-4-oxopyridine-3-carboxylic moiety, combined with an aromatic or heteroaromatic ring (**11**) (Jackson *et al*., 1998).



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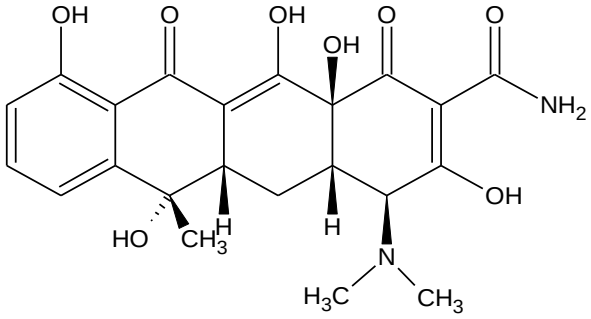
2.7.2 Tetracyclines

Tetracyclines (**12**) are a family of broad-spectrum antibiotics that includes tetracycline, oxytetracycline (OTC), chlortetracycline, doxycycline, and minocycline (chopra

& Roberts, 2001). These antibiotics inhibit protein synthesis in Gram-positive and Gram-negative bacteria by preventing the binding of aminoacyl-tRNA molecules to the 30S ribosomal subunit (Geigenmuller & Nierhaus, 1986; Ross *et al*., 1998). Owing to their broad-spectrum activity and low toxicity, tetracyclines are used in the treatment of a number of human skin and dental diseases (Chopra & Roberts, 2001).



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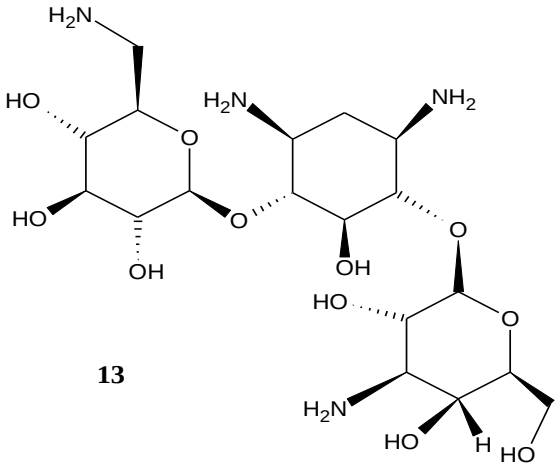
2.7.3 Aminoglycosides

Aminoglycosides (**13**) are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of life-threatening infections (Gilbert *et al*., 2005). Aminoglycosides is used in the treatment of serious infections caused by aerobic gram-negative bacilli (Kumana & Yuen, 1994; Gilbert *et al*., 2005).



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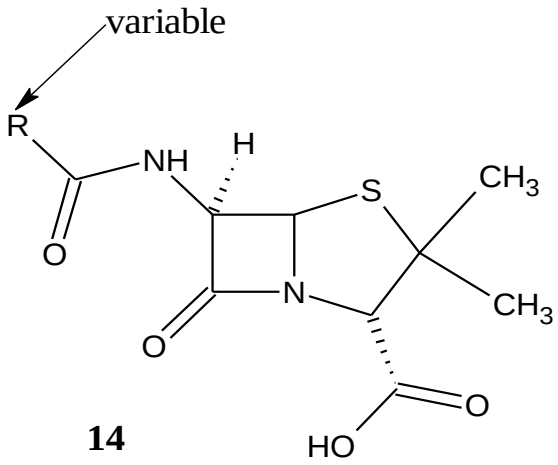
2.7.4 Beta lactams

Beta-Lactam antibiotics (penicillins and cephalosporins) are bactericidal, and they inhibit the synthesis of the bacterial cell wall. They generally act well against both gram-positive and gram-negative bacteria. All beta-lactams basic structure consists of a beta-lactam ring **(14)**, responsible for the antibacterial activity and variable side chains that account for

the major differences in their chemical and pharmacological properties (Botsoglou & Fletouris, 2001).



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2.8 Pathogenic Bacteria

Humans, animals and the environment are heavily colonized with microbes. Some of these microbes can go through a transition from the colonization state into a state of genuine infection. Microbes capable of causing such infections or microbes that are obligatory infectious agents in humans or animals are called pathogens (Van Belkum, 2006; Pordeus *et al*., 2008). Pathogens may display different levels of invasive potential, from mild to life-threatening (Sadoh *et al*., 2006; Singh, 2009). The risk depends primarily on the disease invoking or sick-drawing potential of the organisms and the more pathogenic a species is the higher the infection risk will be (Martinez & Baquero, 2002). Common pathogenic bacteria and the type of bacterial diseases they cause include *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis* and *Klebsiella pneumoniae.*

2.8.1 *Staphylococcus aureus*

Staphylococcus aureus is facultative anaerobic gram positive cocci, which occur singly in pairs and irregular clusters. It is non-motile, non-spores forming and coagulase positive. It is considered to be the major pathogen that colonises and infects both hospitalized patients with decreased immunity and healthy immune–competent people in the community. This bacterium is found naturally on the skin and in the nasopharynx of the human body. It can cause local infections on the skin, nose, urethra, vagina and gastrointestinal tract, most of which are minor and not life-threatening (John & Lindsay, 2008).

2.8.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative, rod-shaped, asporogenous and monoflagellated bacterium that has an incredible nutritional versatility. It is a very ubiquitous microorganism, for it has been found in environments such as soil, water, humans, animals, plants, sewage and hospitals (Joshua & Lederberg, 2000). *Pseudomonas aeruginosa* is an opportunistic human pathogen because it seldom infects healthy individuals. Instead, it often colonizes immuno-compromised patients, like those with cystic fibrosis, acute leukemia, organ transplants, cancer, AIDS and intravenous-drug addiction (Botzehardt & Doring, 1993). The most serious infections include malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia and septicemia (Bodey *et al*., 1983).

2.8.3 *Escherichia coli*

Escherichia coli are the most prevalent infecting organism in the family of gram-negative bacteria known as enterobacteriaceae (Einstein *et al*., 2000). Some *E. coli* is beneficial, while some cause infections other than gastrointestinal infections, such as urinary tract infections (Peter *et al*., 2002). They are normal inhabitants of the human large intestine and are responsible for diarrheal infections worldwide as well as neonatal meningitis and septicemia (Makvarana *et al*., 2015).

2.8.4 *Bacillus subtilis*

Bacillus subtilis, which is one of the food-poisoning bacteria, is a Gram-positive, facultative, aerobic, sporulating bacillus normally found in soil (Aymerich *et al*., 1986). *Bacillus subtilis* is normally considered as being non-pathogenic; but it has been linked to food-borne illnesses, causing diarrhea, nausea, vomiting, and is associated with rice dishes served in oriental restaurants (Willey *et al*., 2008).

2.8.5 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a nonmotile, rodshaped, gram-negative bacteria. It is an opportunistic pathogen and a frequent cause of nosocomial infection, urinary tract infections, infections of the respiratory tract, septicemial and pneumonia (Carsten & Karen, 2004). It is well known to most clinicians as a cause of community-acquired bacterial pneumonia, occurring particularly in chronic alcoholics (Carpenter, 1990) and showing characteristic radiographic abnormalities (Podschun & Uilmann, 1998) due to a severe pyogenic infection which has a high fatality rate if untreated.

2.9 Spectroscopic tools used in structure elucidation

Spectroscopy is the measurement of the absorption, emission, or scattering of electromagnetic radiation by matter to qualitatively or quantitatively study the matter or physical processes. The interaction of radiation with matter can cause redirection of the radiation and absorption via transitions between the energy levels of the atoms or molecules. Spectroscopy is widely used to study structural and dynamical aspect of molecular systems; it is a reliable tool for the characterization of crystalline materials (Donald *et al.*, 2009).

2.9.1 Thin layer chromatography

Thin layer chromatography is a "solid-liquid adsorption" chromatography. In this method stationary phase is a solid adsorbent substance coated on glass plates. As adsorbent material all solid substances coated on glass plates and the mobile phase travels upward through the stationary phase. The solvent travels up the thin plate soaked with the solvent by means of capillary action, thus, the separation of analytes is achieved. This upward travelling rate depends on the polarity of the material, solid phase, and of the solvent (Kowalska *et al*., 2003). In cases where molecules of the sample are colorless, florescence, radioactivity or a specific chemical substance can be used to produce a visible coloured reactive product so as to identify their positions on the chromatogram. Formation of a visible colour can be observed under room light or UV light. The position of each molecule in the mixture can be measured by calculating the ratio between the distance travelled by the molecule and the solvent. This measurement value is called relative mobility, and expressed with a symbol R_f. R_f value is used for qualitative description of the molecule (Preethi *et al*., 2017).

2.9.2 Mass spectrometry

Mass spectrometry is one of the most powerful analytical tools. It has revolutionized many areas due to the combination of very high sensitivity and the ability to identify and/or obtain structural information of unknown components (Osborn *et al*., 2008). Electrospray ionization (ESI) is the most commonly used ionization method when dealing with liquid samples. Generally the ions derived by ESI-MS process are multiply charged, and the analyte remains intact (no fragmentation) when appropriate instrumental conditions are used. In positive ion mode (when the spraying nozzle is kept at positive potential) the charging generally occurs via protonation (sometimes metalation also), but in negative ion mode (when the spraying nozzle is kept at negative potential) charging occurs via deprotonation of the analyte. The ions observed by mass spectrometry may be quasimolecular ions created by

addition of a hydrogen cation and denoted $[M+H]^+$ or of another cation such as sodium ion, $[M+Na]^+$, or the removal of a hydrogen nucleus, $[M+H]^-$. Multiply charged ions such as $[M+nH]^{n+}$ are often observed. Since the charging of the analyte occurs by transfer of protons, the ionic species detected are not the true molecular ions (which are formed by the loss or gain of the electron), but they are more preferably protonated or deprotonated molecules (Todd, 1991; Pitt, 2009).

2.9.3 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is a type of column chromatography where the column is generally made out of an unreactive metal. The reason for this is that the mobile phase is pushed through the stationary phase at high pressures. The typical pressures used in HPLC range from 500 to 4000 psi. (McMaster & Marvin, 2007). Because of the high pressure required, all HPLC's have a pumping system that is integral to the instruments operation. The pumping system needs to be of a high enough quality that it can consistently produce a high pressure and relatively pulse-free output (Skoog *et al*., 1998). In preparative HPLC the detector is used to determine when to separate the fractions in order to purify compounds. Quantitative HPLC uses the detector to measure the amount of material eluted. The most common type of detector is a UV-Vis absorption detector. These detectors measure the amount of ultraviolent and visible light absorbed by the analyte components as they elute. While variable wavelength detectors and photodiode arrays are capable of measuring more than one wavelength, a commonly analyzed wavelength is 254nm. Absorption is measured at 254nm because compounds with substituted aromatic chromophores usually absorb around this wavelength (Gerber *et al*., 2004). Setting the detector is used to determine when to separate the fractions in order to purify compounds. These detectors measure the amount of ultraviolent and visible light absorbed by the analyte components as they elute. While variable wavelength detectors and photodiode arrays are capable of measuring more than one wavelength, a commonly analyzed wavelength is 254nm. Absorption is measured at 254nm because compounds with substituted aromatic chromophores usually absorb around this wavelength (McMaster & Marvin, 2007).

2.9.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance (NMR) spectroscopy is a powerful and theoretically complex analytical tool that allows the study of compounds in either solution or in the solid state and serves equally in quantitative as in structural analysis, it is very efficient

in gathering structural information concerning molecular compounds. NMR spectroscopy is one of the methods of spectroscopic technique in which under appropriate conditions in a magnetic field, a sample can absorb electromagnetic radiation in the radio frequency (r.f) region at frequencies governed by the characteristics of the sample (David & Churchill, 1999; Francis & Annick, 2007). All nuclei carry a charge, in some nuclei this charge "spins" on the nuclear axis and this circulation of nuclear charge generates a magnetic dipole along the axis. The angular momentum of the spinning charge can be described in terms of quantum spin numbers I: these numbers have values of 0,1/2,1,3/2 and so on (I=0 denotes no spin). The intrinsic magnitude of the generated dipole is expressed in terms of nuclear magnetic moment, μ (Shah *et al*., 2006).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection of plant material

Fresh leaves of *Polyscias fulva* were collected from Kakamega rain forest (0º 10'-0º 21' N 34º 58' E) (Fig. 3). They were identified by a taxonomist Prof. S. T. Kariuki and deposited at the Department of Biological Sciences, Egerton University. The leaves were collected in bags and quickly transported to the laboratory to avoid metabolic transformations. The leaves were then taken to the Centre for Herbal Research at Egerton University where they were dried under shade for three weeks.

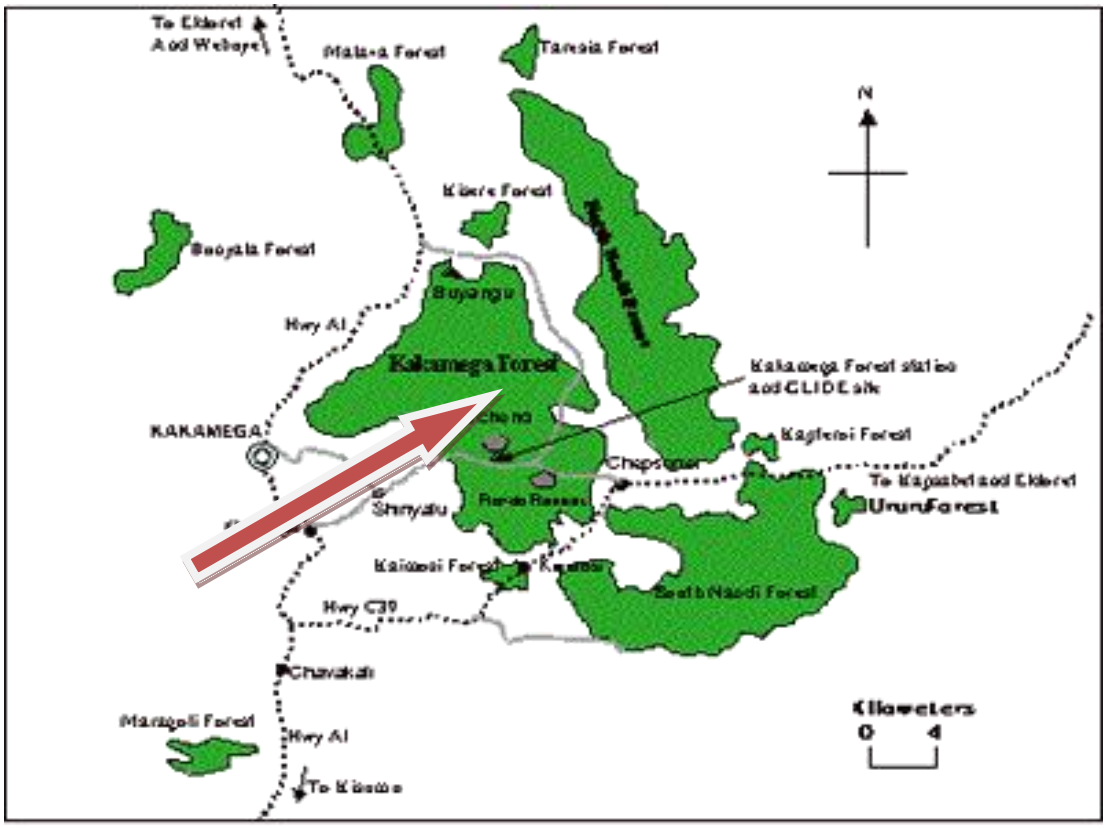


Figure 3: A map of Kakamega rain forest

3.2 Isolation and identification of endophytic fungi

Endophytic fungi were isolated from internal plant tissues using a modification of the method used by Zinniel *et al*. (2002). In this method, the leaves of the selected healthy plants were washed under running tap water to remove any soil or other foreign materials and blotted dry. The leaves were surface sterilized for 3 minute each using 1% sodium hypochlorite followed by 70% ethanol. Thereafter, the leaves were rinsed three times with

sterile distilled water to remove any traces of the disinfectant. The leaves were then cut aseptically into sections approximately 1mm by 4 mm. The surface disinfected leaves were then plated in petri-plates containing Potato Dextrose Agar (PDA) media with 200 mg/L concentration of streptomycin sulphate. The plates were sealed using parafilm and incubated at 25 ºC. The petri dishes were monitored daily to check the growth of endophytic fungal colonies from the plant segments. As and when the hyphal tips emerged out from plant segments they were isolated and sub-cultured and brought to pure culture by serial sub-culturing. The isolates were identified by sequencing the Internal Transcribed Spacer (ITS) region of the ribosomal DNA (rDNA) extracted from the endophytic fungi using automated illumine genome analyzer IIX DNA sequencing machine.

3.3 Screening of fungal endophytes for antibacterial activity

Screening of endophytic fungal isolates for antibacterial activity was done using dual culture assay following the method described by Stadler *et al.* (2004).The endophytic isolates were grown on PDA medium for 20 days at 25 ± 2.0 ºC. Plugs of approximately 7mm were cut using a sterile cork borer and placed in Muller Hinton agar plates that were seeded with 10^5 CFU/ml *S. aureus* ATCC25922 (gram positive bacterium) and *K. pneumoniae* ATCC13883 (gram negative bacterium). To allow for diffusion of secondary metabolites into the agar, the r plates were stored at 4 ºC for 24h and subsequently incubated at 37 ºC overnight. Inhibition zones were measured after 24h and isolates that demonstrated antibacterial activity were selected for further secondary screening. The experiment was done in triplicate.

3.4 Fermentation of endophytes

Endophytic fungi which showed any antibacterial activity were selected for fermentation and testing of their secondary metabolites. The fungi were grown in twenty 500 mL Erlenmeyer flasks containing 90g of parboiled rice in 90 mL distilled water per flask. The rice was autoclaved twice at 120 ºC for 40 minutes. Agar plugs (about 2 × 2cm) were cut from the 7-day-old cultures on PDA and used to inoculate the flasks. One flask, without inoculum, was used as control. After 21 days of incubation at 30 ºC, 150 mL of methanol were added to each flask and the contents allowed to stand overnight at room temperature and then ultrasonicated at 40 ºC for 2 hours. The methanol was then filtered and evaporated under reduced pressure to yield the methanol extract. The methanol extract was then suspended in water and subjected to liquid -liquid partitioning with hexane and ethyl acetate. The resulting

organic layer was evaporated under reduced pressure to produce the hexane and ethyl acetate extracts.

3.5 Fractionation of ethyl acetate extracts

The ethyl acetate extract was fractionated by column chromatography (3×115cm) on Sephadex LH 20 with HPLC grade methanol as eluent. Fractions of equal volume were collected and combined according to the Thin Layer Chromatography (TLC) profiles. Fractions of TLC patterns were grouped together, which yielded eight major fractions namely; F1, F2, F3, F4, F5, F6 and F7. Fraction F4 and F7 were further purified using preparative HPLC.

The procedure for the isolation, fermentation and extraction of the secondary metabolites from endophytes is summarized in Figure 4.

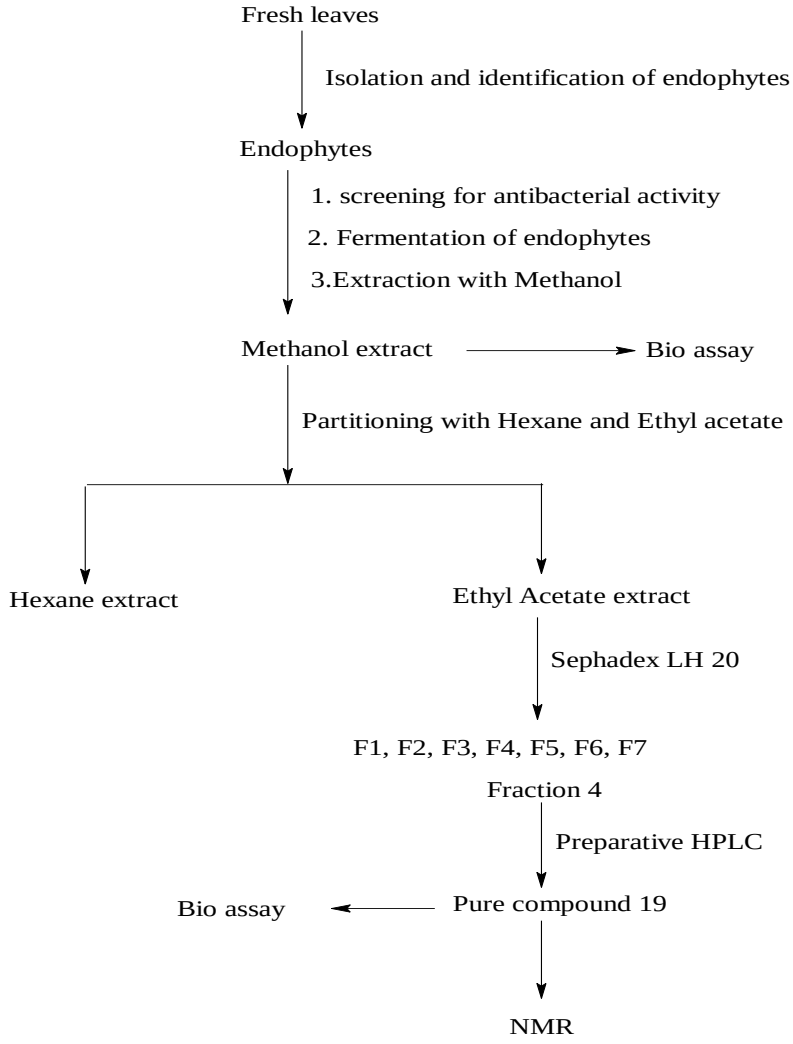


Figure 4: A flow chart summarizing isolation, fermentation and extraction of the secondary metabolites from fungal endophytes

3.6 Extraction and isolation of secondary metabolites from the leaves of *P. fulva*

Extraction was done using methanol solvent. The solvent was distilled first before use since it was of the GPR grade.

3.6.1 Extraction of phytochemicals

Leaves of *P. fulva* were dried under shade and at room temperatures to prevent the loss of labile compounds and to retain their natural active compounds. The leaves were turned over periodically during drying to avoid growth of moulds and ground to fine powder using a Thomas-Wiley mill model 4. Six kilograms of the *P. fulva* ground materials were soaked in methanol at room temperature for 24 hours with periodical shaking. The contents were then filtered through Whatman no. 1 filter paper and the filtrate was concentrated in vacuum at 50°C using Buchi Rotavapor R-205 rotary evaporator. The methanol crude extracts were placed in the fume hood to total dryness.

The procedure for the extraction, isolation and structure elucidation of pure compounds is described and summarized in Figure 5.

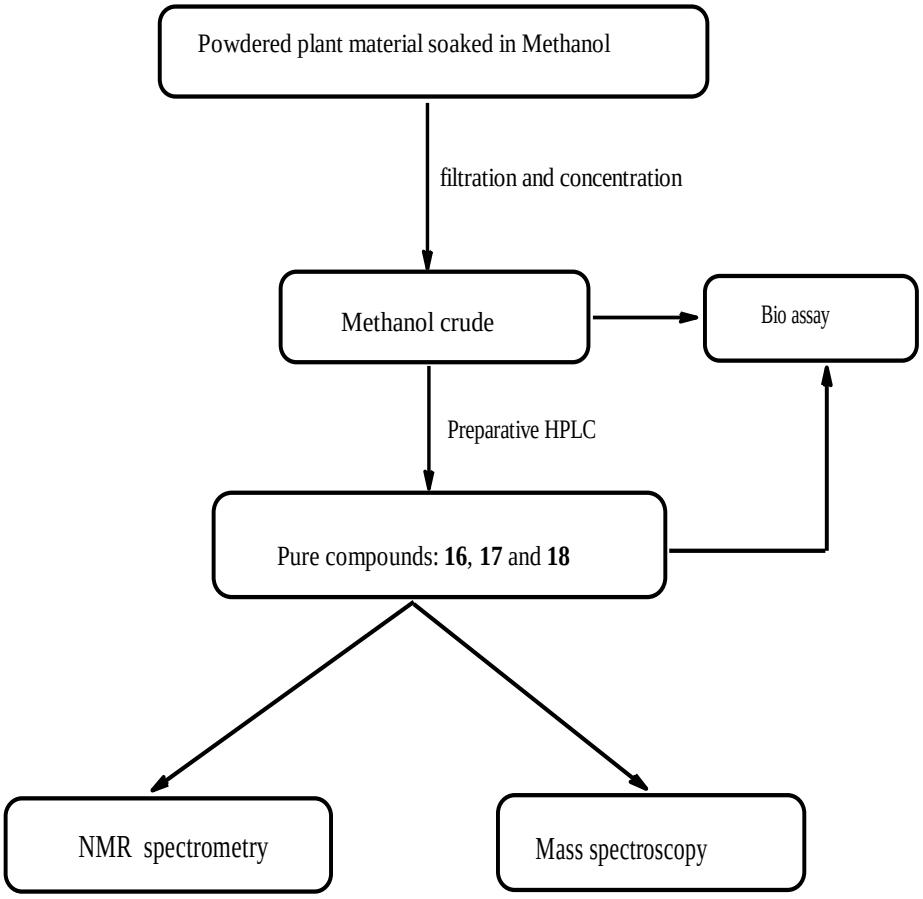


Figure 5: A flow chart summarizing isolation and structure elucidation of secondary metabolites

3.6.2 Preparative High Performance Liquid Chromatography

The fractions F4 and F7 from the Sephadex LH 20 of the *Fusarium* species together with methanol extracts of *P. fulva* were purified using preparative High Performance Liquid Chromatography equipped with uv-vis detector. C-18 column with dimensions of 250 mm by 20mm, 10um together with mobile phase consisting of Acetonitrile: Water 5:95v/v was delivered at a flow rate of 15.000 µl/min and the elution profiles were read at different wavelengths. Fraction F_4 yielded two fractions, F4-1 (as compound **19**) and F4-2 with weights of 7.01mg and 6.04 mg respectively while fraction F7 yielded two fractions each, namely F7-1(1.01 mg) and F7-2 (0.87 mg). The methanol extract of *P. fulva* yielded three major fractions namely PF-1 (as compound **15**), PF-2 (as compound **16**) and PF-3 (as compound **17**). The compounds **16**, **17**, **18** and **19** were divided into two portions each; one portion of each compound was used for 1 and 2D high field NMR spectroscopy and Mass spectroscopy while the other portions were subjected to assays against selected bacteria. The NMR spectroscopic analysis was not done for Fractions F7-1 and F7-2 since the amount obtained was less than 3mg.

3.7 Antibacterial assay of the pure compounds

The disc diffusion method for antibacterial susceptibility testing was carried out according to the standard method by Zaidan *et al*. (2005). The pure extracts were screened for antibacterial activity against *S. aureus* and *K. pneumoniae*. Sterile nutrient agar mixed with bacteria at a concentration of 1×10^6 cfu/ml was poured in petri dishes and allowed to cool. The plant extracts equivalent to 1000 µg, dissolved in methanol were applied to sterile paper discs (6mm diameter). The solvent were then allowed to evaporate and the discs aseptically deposited on the surface of the inoculated agar plates. The plates were incubated for 24 hrs at 37 ^oC. Zones of inhibition were measured in mm after 24 hrs of growth. The negative control used in this experiment was 1% dimethyl sulfoxide (DMSO) whereas 30 µg/disc chloramphenicol discs were used as the positive control.

3.8 Nuclear magnetic resonance (NMR) spectroscopy

The ^1H, ^{13}C, DEPT, HSQC, COSY and HMBC NMR spectra were recorded on the Bruker Advance 500 MHz NMR spectrometer at the Technical University of Berlin, Germany. The readings were done in DMSO and chemical shifts assigned by comparison with the residue proton and carbon resonance of the solvent. Tetramethylsilane (TMS) were used as an internal standard and chemical shifts were given as δ (ppm). The structures were

then determined using ACD NMR manager program to obtain the chemical shifts of proton. The off- diagonal elements were used to identify the spin – spin coupling interactions in the ^{1}H –^{1}H COSY (Correlation spectroscopy). The proton-carbon connectivity, up to three bonds away, was identified using ^{1}H–^{13}C HMBC (Heteronuclear Multiple Bond Correlation) spectrum. The ^{1}H–^{13}C HSQC spectrum (Heteronuclear Single Quantum Coherence) were used to determine the connectivity of hydrogen to their respective carbon atoms.

3.9 Mass spectrometry

The compounds' mass spectra were recorded on Finnigan Tripple Stage Quadrupol Spectrometer (TSQ-70) with electron spray ionization (ESI) method in negative and positive ion mode. In the analysis, Thermo XcaliburQual computer software was used in analysis of the mass chromatograms.

3.10 Data Analysis

Mean inhibition zones were calculated and equality of means was analyzed using one-way analysis of variance (ANOVA). Tukey's Honestly Significant Difference (HSD), a Post-Hoc Analysis, was used to determine if there was any significant difference between the means of the isolates. Data analysis was performed using R statistical software version 3.3.1.

CHAPTER FOUR
RESULTS AND DISCUSSION

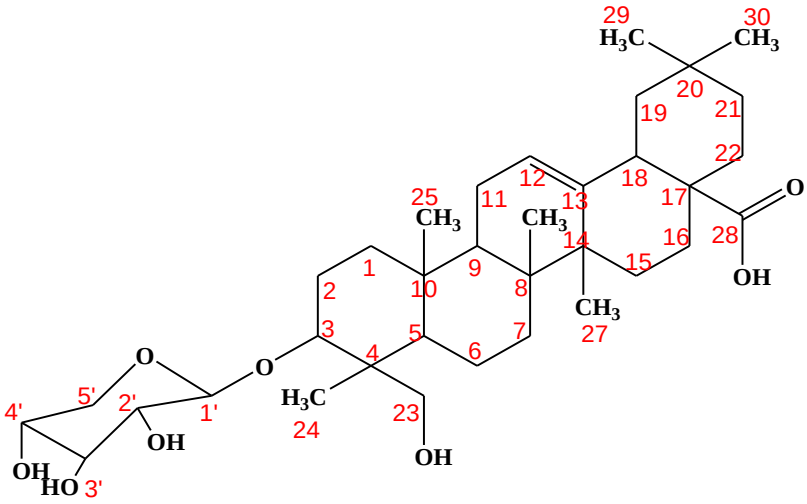
4.1 Isolated compounds

Four compounds were isolated and their structures elucidated. Of the four, three were from the crude methanol extract of the plant *P. fulva* and the remaining one from its endophytic *Fusarium* species PF1.

4.2 Structure elucidation of compound 15

The compound **15** (Figure 6) was obtained from plant extract as a dark brown substance with a weight of 10.27 mg. The 1D and 2D NMR spectral data of compound **15** are summarized in Table 2.The molecular formula of compound **15** was established by ESI - MS as $C_{35}H_{56}O_8$. The ^{1}H-NMR spectrum (Appendix 1) of **15** showed characteristic signals of six methyl groups at δ_H 0.58 (H-24), 0.88 (H-25), 0.71 (H-26), 1.10 (H-27), 0.86 (H-29), 0.87 (H-30), one olefinic proton at δ_H 5.16 (H-12) indicating that the aglycone could be of olean-12-en skeleton, an oxymethine at 3.49 (H-3) and a hydroxymethyl group at δ_H 3.08/3.41 (H-23), which were characteristics signals for the oleanane skeleton with a hydroxyl group at C-23. This compound was identified as an olean-12-ene type pentacyclic triterpene and this was confirmed by comparison of its NMR data with those of known olean-12-ene type derivatives (Mahato & Kundu, 1994; Beaudelaire *et al*., 2014; Maillard *et al.*, 1992), hence 23- hydroxy oleanolic acid was also identified as the aglycone. Additionally, ^{1}H NMR spectra showed the presence of anomeric protons at δ 4.19 (H-1') indicating the presence of a sugar in its structure.

DEPT NMR spectrum (Appendix 2) showed the presence of nine methine (CH) signals at δc-41.2, 46.5, 47.5, 68.0, 71.5, 73.1, 80.2, 105.2 and 122.0. A total of twelve methylene (CH_2) signals at δc – 17.6, 23.0, 23.4, 25.5, 27.6, 32.4, 32.5, 33.7, 38.4, 46.1, 63.1 and 65.5 were also seen. Six methyl (CH_3) signals were identified at δc- 13.4, 16.0, 17.3, 23.8, 26.0 and 33.2. The other missing seven signals were found to be quaternary carbons. It also showed one signal of a carbonyl group at δ 173.0 (C-28) hence the aglycone was identified as oleanolic acid, Also, one anomeric signal at δ 4.19 (H-1') was observed giving HSQC correlations with one anomeric carbon at δ 105.2. In the particular case of triterpenoid saponins with oleanolic acid as aglycone, this carbon (C-3 of the aglycone) resonates at about 80.0 to 89.0 ppm (Kawai *et al*., 1989; Huan *et al*., 1998; Tuyet *et al*., 2009).



15

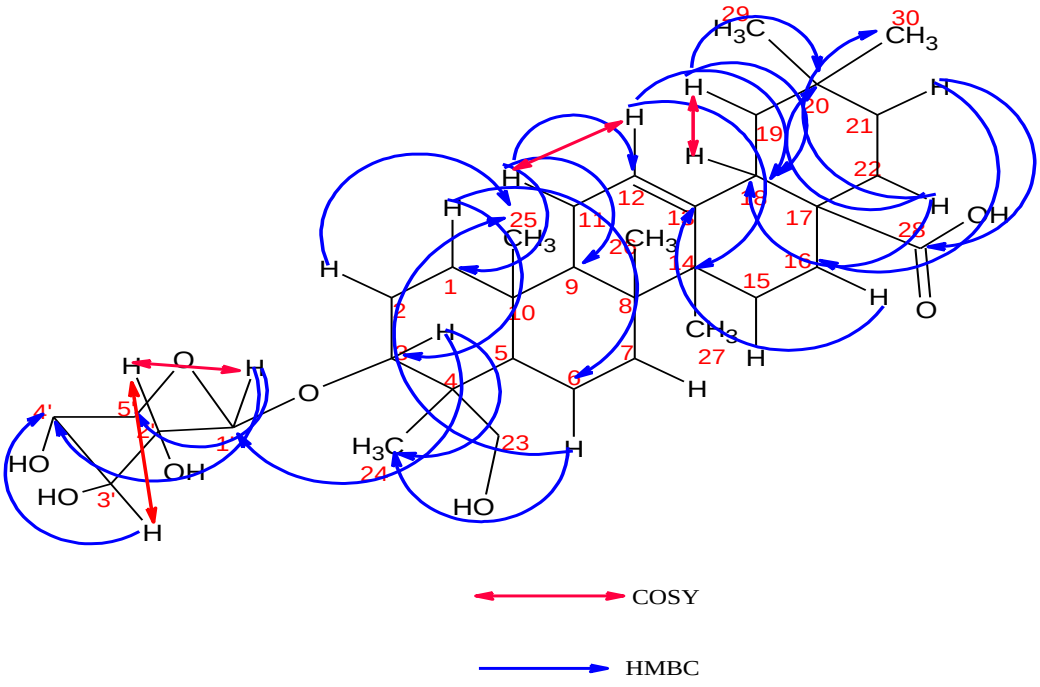


Figure 6: COSY and HMBC correlations of Compound **15**

The HSQC spectrum (Appendix 3) was used to assign protons directly attached to carbon atoms. This spectrum showed correlation between proton δ_H – 0.85/1.49 (C-1), 0.99/1.65 (C-2), 3.49 (C-3), 1.51(C-5),1.17 (C-6), 1.18/1.43 (C-7), 1.18 (C-9), 1.48 (C-11), 5.16 (C-12), 1.81 (C-15), 1.72 (C-16), 2.74 (C-18), 1.05/1.61 (C-19), 2.28 (C-21), 1.61 (C-22), 3.08/3.41 (C-23), 0.58 (C-24), 0.88 (C-25), 0.71 (C-26), 1.10(C-27), 0.86(C-29) and 0.87 (C-30). However, coupling constants could not be determined due to the presence of broad peaks observed in the ^{1}H-NMR spectrum.

The HMBC spectrum (Appendix 4) showed proton correlations with carbon atoms that are two-three bonds away. This gave information on which carbons were next to each other or two-three bonds away from each other. Proton H-1 resonating at δ_H-0.85/1.49 showed correlation with carbon C-3 which is two bonds away and C-6 which is three bonds away. Similarly H-2 absorbing at δ_H-0.99/1.65 correlated with C-25 which is three bonds away while proton H-3 resonating at δ_H -3.49 correlated with C-24 and the anomeric carbon C-1' which are two bonds away. The protons H-5 resonating at δ_H-1.49 correlated with C-1 and a quaternary carbon C-10 and C-25 which are two bonds away. Similarly, proton H-6 absorbing at δ_H-1.17 correlated with C-24 and C-25 which are three bonds away. The proton H-7 resonating at δ_H-1.19/1.43 showed good correlations with methine carbons C-9 and a methyl carbon C-26. Similarly, proton H-9 resonating at δ_H-1.18 which is attached to a methine carbon correlated with quaternary carbons C-10 and C-14 and methyl carbons C-25 and C-26. Similarly, a methylene proton H-11 (δ_H-1.48) correlated with C-1, C-9 and C-12. The proton H-12 absorbing at δ_H-5.16 correlated with a quaternary carbon C-14 and methine carbon C-18. The methylene protons H-15 (δ_H-1.81) correlated with olefinic carbons C-12 and C-13 and H-16 (δ_H-1.72) correlated with a quaternary carbon C-13. A methyl proton H-18 resonating at δ_H-2.74 had good correlations with olefinic carbons C-12 and C-13 and a quaternary carbon C-17. Similarly, proton H-19 (δH-1.05/1.61) correlated with C-18, C-20, C-29 and C-30 while methylene protons H-21 (δH-2.28) correlated with C-16 and C-28 and proton H-22 (δH-1.61) correlated with C-18, C-20 and C-30. Similarly, proton H-23 (δ_H-3.08/3.41) correlated with C-3 and proton H-24 (δ_H 0.58) showed correlations with C-3 and C-23. Likewise, methyl proton H-25 resonating at δ_H- 0.88 correlated C-1,C-9, C-10 and C-11 while proton H-26 (δ_H-0.71) correlated with C-7,C-8, C-11 and C-14. The proton H-27 (δH- 1.10) correlated with C-8, C-13 and C18. Lastly, the protons H-29 (δH-0.86) and H-30 (δH-0.87) correlated with C-19, C-20 and C-21.

Table 2: NMR data of compound **15**

Carbon	DEPT	TYPE	$^{1}H(\delta)$	HMBC	COSY	^{13}C*
1	38.4	CH_2	0.85/1.49	3, 6,		39.9
2	27.6	CH_2	0.99/1.65	25		26.2
3	80.2	CH	3.49	24, 1’		81.1
4	40.9	C	-	-		43.5
5	47.5	CH	1.51	1, 10, 25		47.7
6	17.6	CH_2	1.17	24, 25		18.2
7	32.5	CH_2	1.19/1.43	9, 26		33.0
8	39.3	C	-	-		39.2
9	46.5	CH	1.18	10, 14, 25, 26		48.2
10	36.2	C	-	-		36.8
11	23.0	CH_2	1.48	1, 9, 12	12	23.8
12	122.0	CH	5.16	14, 18	11	123.0
13	147.7	C	-	-		144.2
14	41.0	C	-	-		41.9
15	23.4	CH_2	1.81	12, 13		21.8
16	25.5	CH_2	1.72	13		23.5
17	45.6	C	-	-		46.1
18	41.2	CH	2.74	12, 13, 17	19	42.2
19	46.1	CH_2	1.05/1.61	18, 20, 29, 30	18	47.1
20	30.8	C	-	-		30.8
21	33.7	CH_2	2.28	16, 28		33.9
22	32.4	CH_2	1.61	18, 20, 30		33.1
23	63.1	CH_2	3.08/3.41	3		63.9
24	13.4	CH_3	0.58	3, 23		14.1
25	16.0	CH_3	0.88	1, 9, 10, 11		16.3
26	17.3	CH_3	0.71	7, 8, 11, 14		17.6
27	26.0	CH_3	1.10	8, 13, 18		26.3
28	173.0	C	-	-		176.8
29	33.2	CH_3	0.86	19, 20, 21		33.3
30	23.8	CH_3	0.87	19, 20, 21		23.8
1’	105.2	CH	4.19	3, 4’, 5’	2’	
2’	71.5	CH	3.30	1’	1’, 3’	
3’	73.1	CH	3.31	4’	2’	
4’	68.0	CH	3.60	1’, 3’		
5’	65.5	CH2	3.32/3.65	1’, 3’, 4’		

^{13}C* -Literature (Maillard *et al*., 1992; Njateng *et al*., 2015)

The proton – proton COSY correlations for compound **15** were also determined. COSY spectrum (Appendix 5) gave information on the correlation between protons attached to adjacent carbon atoms. The protons H-11 (δ_H – 1.48) correlated with proton H-12 (δ_H – 5.16) and proton H-18 (δ_H – 2.74) correlated with proton H-19 (δ_H – 1.05/1.61).

The sugar moiety was assigned mainly from the ^{1}H-^{1}H COSY, HSQC and HMBC spectra which allowed the identification of an anomeric proton signal at δ_H- 4.19. Attachment of the sugar molecule at the C-3 of the aglycone was established by the HMBC correlation between the δ_H4.19 (H -1') and δ - 80.25 (C-3). The ^{1}H NMR spectrum of the glycone portion showed presence of three oxymethine protons at δ_H- 3.30, 3.60, 3.31 together with a methylene proton at 3.32/3.65 for H-5' and anomeric proton at δ_H– 4.19. The HMBC spectrum showed proton H-1' absorbing at δ_H-4.19 correlated with C-3, C-4' and C-5' while proton H-2' (δ_H- 3.30) and proton H-3' (δ_H- 3.31) correlated with C-1'and C-4' respectively. Similarly, proton H-4' (δ_H- 3.60) and proton H-5' (δ_H- 3.32/3.65) showed good correlation with C-1', C-3' and C-1', C-3', C-4' respectively. The sugar molecule also showed COSY correlations between protons H-1' (δ_H-4.19) and proton H – 2' (δ_H-3.30) and between proton H – 2' (δ_H-3.30) and proton H- 3' (δ_H -3.60).

Analytical HPLC indicated that compound **15** had a retention time of 6.55 min as shown in Figure 7. The compound absorbs at 500 mAu with a maximum wavelength **(**λmax) of 250 nm as shown by the corresponding UV spectrum (Figure 7). Retention time is the amount of time that a compound spends on the column after it has been injected. Several factors affect the retention time of a compound and this include, the particular size of the column, the stationary phase of the column, the specific solvent mixture composition and the pressure used which in turn affects the flow rate of the solvent mixture. A solvent mixture of acidified Millipore water (H_2O + 0.1% CH_2O_2) and acidified acetonitrile (ACN + 0.1% CH_2O_2) was used. Compound **15** being a saponin is a highly polar compound and was eluted at 6.55 minutes in a gradient 5-100-20 min. Absorption or emission involves the transition of electrons between orbitals. Of immense consideration in this study is the Л → Л* transitions which requires less energy. This transition occurs at longer wavelengths which in some cases can be indicative of the relative extent of conjugation or presence of a chromophoric group by coloration (Olaniyi, 2000). Chromophores are parts of the compound that are responsible for the absorption of light in a compound. The presence of hydroxyl group and carbonyl groups in the compound therefore initiates resonance hence resulting to a shift of themaximum absorption wavelength of 255nm.

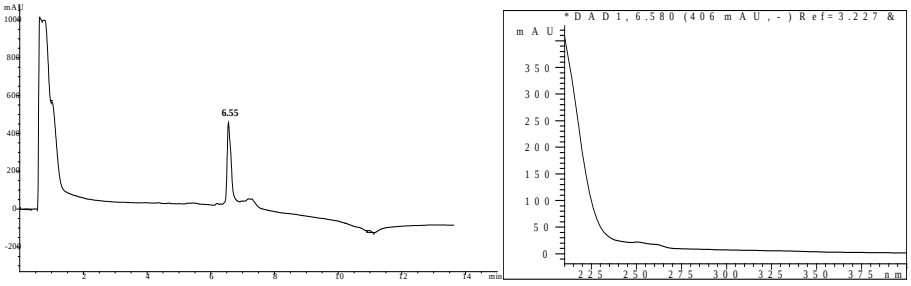


Figure 7: Chromatogram and UV spectrum of compound **15**

The molecular formula of compound **15** was established as $C_{35}H_{56}O_8$ according to ESI-MS which showed ion peaks at m/z 605.40 $[M+H]^+$, 627.39$[M+Na]^+$ and 643.43$[M+K]^+$ upon positive ionization (Figure 8) and at m/z 603.39$[M-H]^-$ and 1207.79$[2M-H]^-$ upon negative ionization (Figure 9). Thus, on the basis of the above evidence and analysis, compound **15** was identified as a monosaccharide triterpenoid saponin of olean-12-en-28-oic acid aglycone with the molecular formula $C_{35}H_{56}O_8$, and established to be 3-O-[xylopyranosyl]-23-hydroxy-oleanolic acid (**15**). This compound has been isolated before from the stem bark of *P. fulva* (Joshi *et al*., 1992; Njateng *et al*., 2015). However, it is isolated for the first time from the leaves of this plant.

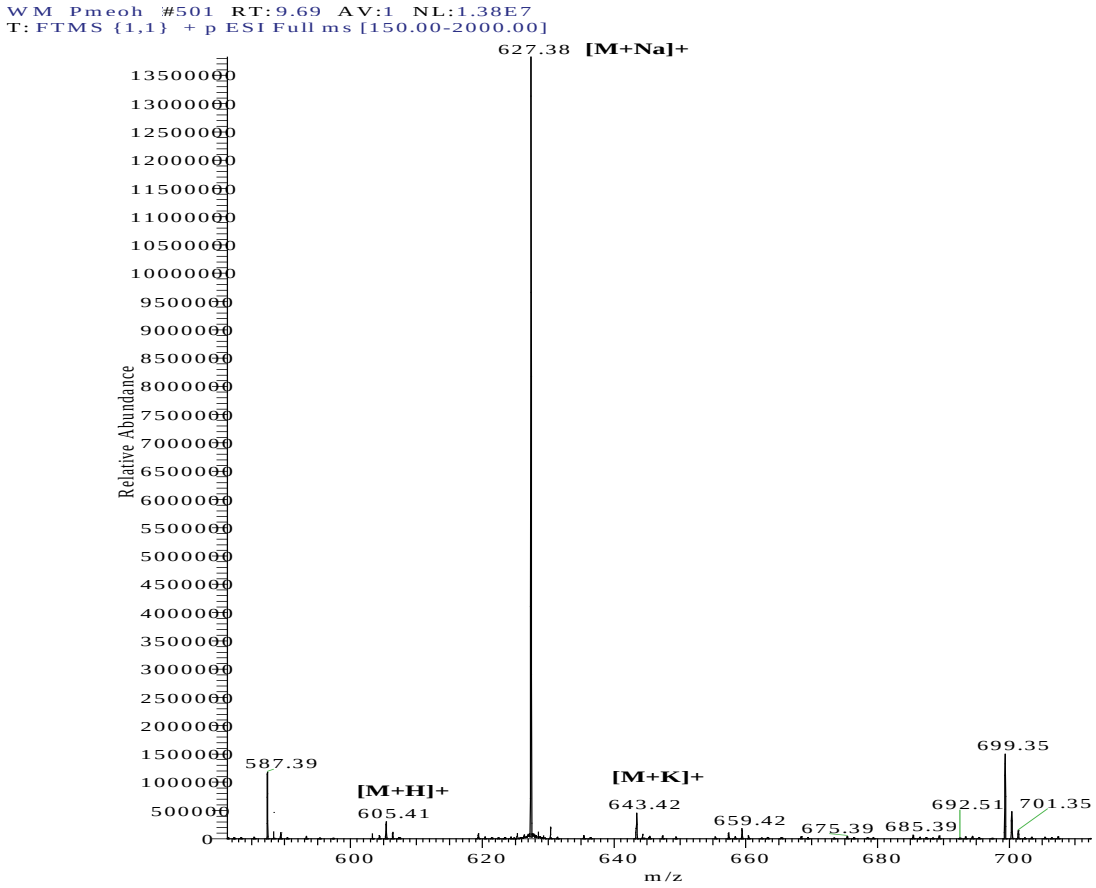


Figure 8: Mass spectrum of compound **15** in positive ionization mode

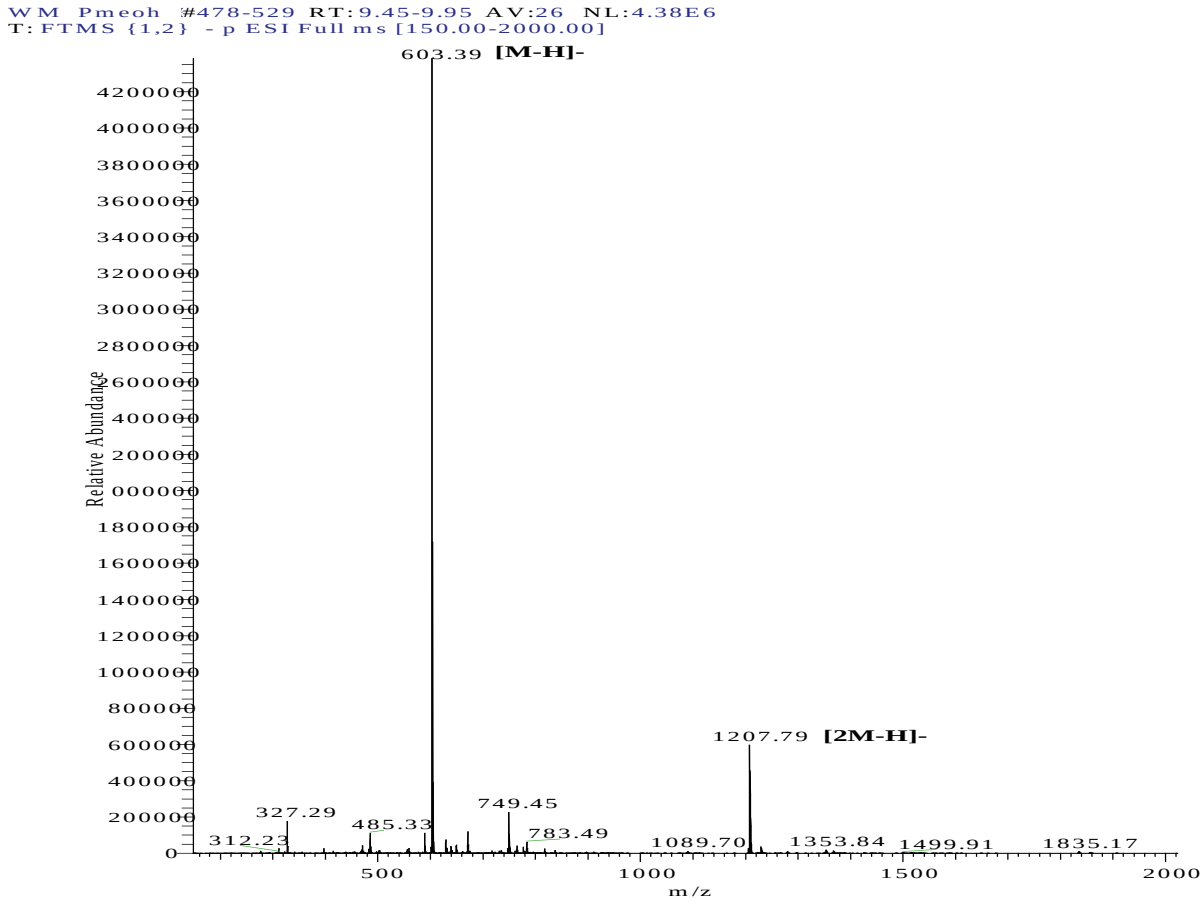


Figure 9: Mass spectrum of compound **15** in negative ionization mode

4.3 Structure elucidation of compound 16

Compound **16** (Figure 10) was obtained from the plant extract as a dark brown substance with a mass of 30.81mg. The 1D and 2D NMR spectral data of compound **16** are summarized in Table 3. Its molecular formula was determined using ESI - MS as $C_{41}H_{66}O_{12}$. The aglycone region in the DEPT NMR spectra showed great similarity to that of compound **15**. The six sp^3 hybrid carbon signals at δc 13.4, 16.0, 17.3, 23.8, 26.0 and 33.2, and the two sp^2 hybrid carbon signals at δc 122.0 and 144.0 (Onoja & Ndukwe, 2013) together with the information from ^1H NMR analysis (six methyl proton singlets at δ_H 0.57, 0.70, 0.87, 0.88, 0.89 and 1.10 and a vinyl proton at δ_H 5.16) indicated that the aglycone possesses an olean-12-ene skeleton.

The HSQC spectrum (Appendix 8) showed correlation between protons absorbing at δ_H-1.49 (C-1), 0.98/ 1.66 (C-2), 3.50 (C-3), 1.52 (C-5), 1.38 (C-6), 1.15(C-7), 1.19 (C-9), 1.91 (C-11), 5.16 (C-12), 1.81 (C-15), 1.72 (C-16), 2.74 (C-18), 1.03 (C-19), 1.11/2.28 (C-21), 1.01 (C-22), 3.09/3.31 (C-23), 0.57 (C-24), 0.87 (C-25), 0.70 (C-26), 1.10 (C-27), 0.88

(C-29) and 0.87 (C-30). The presence of six methyl signals was characteristic signals for the oleanane skeleton with a hydroxyl group at C-23. Additionally, anomeric protons signals in NMR spectrum at δ_H 4.34 and δ_H 5.06 together with carbon signals at δ_C 100.38 and 103.40 in the DEPT NMR data suggested that compound **16** was a glycoside with two sugar units.

The DEPT NMR spectrum (Appendix 7) showed a total of fourteen methine (CH) signals at δc- 41.2, 46.6, 47.5, 66.2, 68.5, 70.8, 70.9, 72.5, 73.3, 74.6, 79.7, 100.3, 103.4 and 122.0. A total of twelve methylene carbons (CH_2) signals absorbing at δc- 17.5, 23.0, 23.3, 25.7, 27.6, 32.3, 32.5, 33.7, 38.6, 46.1, 62.9 and 64.8. It also showed a total of seven methyl (CH_3) signals at δc- 13.4, 16.0, 17.3, 18.2, 23.8, 26.0 and 33.2. The other missing eight signals were found to be quarternary carbons.

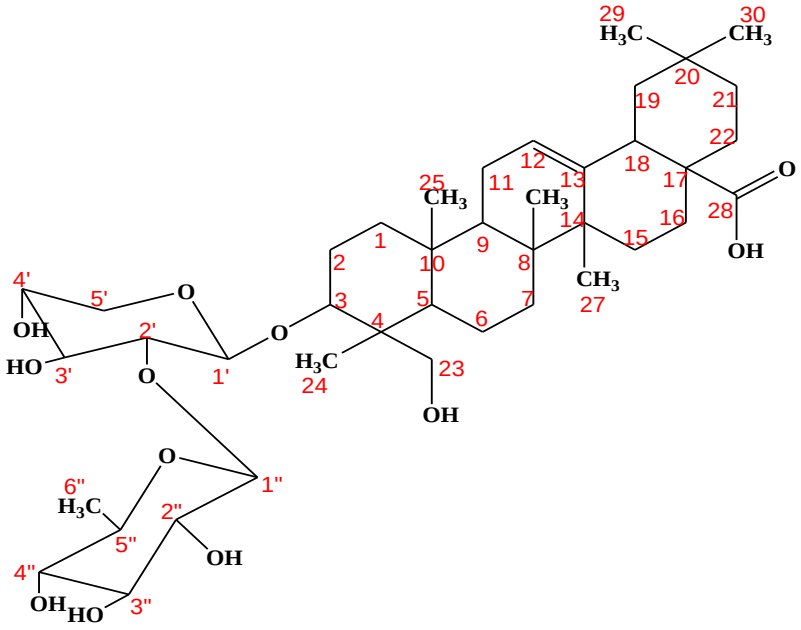
The HMBC spectrum (Appendix 9) was almost similar to the one for compound **15** with the only difference occurring with two sugar moieties substituent at position C-3. The proton H-1 resonating at δ_H 1.49 correlated with methine carbon C-3, a quarternary carbon C-10, methylene carbon C-11 and methyl carbon C-25. The proton H-2 (δ_H 0.98/1.66) correlated with C-1 while proton H-3 resonating at δ_H 3.50 correlated with methine carbons C-24 and anomeric carbon C-1' whereas proton H-5 (δ_H 1.52) correlates with C-1, C-11 and C-25. Proton H-6 and H-7 resonating at δ_H1.38 and δ_H 1.15 correlated with C-1, C-10 and C-27 respectively while H-9 (δ_H1.19) showed correlations with C-6, C-25 and C-27. Similarly, proton H-11 (δ_H1.91) and H-12 (δ_H 5.16) showed correlations with C-5, C-12, C-13 and C-11 C-18 respectively while proton H-15 resonating at δ_H 1.81 showed good correlations with C-9, C-12, C-13 and C-16. Proton H-16 (δ_H1.72) correlated only with C-19 and H-18 (δ_H 2.74) correlated with C-11, C-12, C-13, C-19 and C-28 while H-19 (δ_H1.03) correlated with C-30. Proton H-21 (δ_H 1.11/2.28) showed correlations with C-18 and C-19 while H-22 (δ_H1.01) showed correlations with C-16, C-18 and C-20. The methyl proton H-24 resonating at δ_H 0.57 correlated with a methine carbon C-3 and a methylene carbon C-23 while proton H-25 (δ_H 0.87) showed correlations with C-1, C-5 and C-11. The methyl proton H-26 (δ_H 0.70) correlated only with C-7 and proton H-27 (δ_H 1.10) showed good correlations with C-13 and C-18. Lastly, protons H-29 (δ_H0.88) and H-30 (δ_H0.87) both correlated with C-19, C-20 and C-21 respectively. The proton-proton COSY spectrum (Appendix **9**) showed correlations between protons H-11 (δ_H-1.91) – H-12 (δ_H-5.16) and H-18 (δ_H-2.74) – H-19 (δ_H-1.03).

Table 3: NMR data of compound **16**

Carbon	DEPT	TYPE	$^{1}H(\delta)$	HMBC	COSY
1	38.6	CH_2	1.49	3, 10, 11, 25	
2	27.6	CH_2	0.98/1.66	1	
3	79.7	CH	3.50	1', 24	
4	40.9	C	-	-	
5	47.5	CH	1.52	1, 11, 25	
6	17.5	CH_2	1.38	1, 10	
7	32.5	CH_2	1.15	27	
8	39.3	C	-	-	
9	46.6	CH	1.19	6, 25, 27	
10	36.0	C	-	-	
11	23.0	CH_2	1.91	5, 12, 13	12
12	122.0	CH	5.16	11, 18	11
13	144.0	C	-	-	
14	41.0	C	-	-	
15	23.3	CH_2	1.81	9, 12, 13, 16	
16	25.7	CH_2	1.72	19	
17	45.6	C	-	-	
18	41.2	CH	2.74	11, 12, 13, 19, 28	19
19	46.1	CH_2	1.03	30	18
20	30.6	C	-	-	
21	33.7	CH_2	1.11/2.28	18, 19	
22	32.3	CH_2	1.01	16, 18, 20	
23	62.9	CH_2	3.09/3.31	4, 24	
24	13.4	CH_3	0.57	3, 23	
25	16.0	CH_3	0.87	1, 5, 11	
26	17.3	CH_3	0.70	7	
27	26.0	CH_3	1.10	13, 18	
28	178.6	C	-	-	
29	33.2	CH_3	0.88	19, 20, 21	
30	23.8	CH_3	0.87	19, 20, 21	
1'	103.4	CH	4.34	3, 2'	2'
2'	74.6	CH	3.52	3, 3'	1'
3'	72.5	CH	3.18	1'	
4'	66.2	CH	3.58		
5'	64.8	CH_2	3.32/3.67	1'	
1"	100.3	CH	5.06	4", 3'	2"
2"	70.8	CH	3.67	1", 3", 4",	1"
3"	73.3	CH	3.48		
4"	70.9	CH	3.49		
5''	68.5	CH	3.71	6''	6"
6"	18.2	CH_3	1.07	4'', 3''	5"

Similar to compound **15** the aglycone of compound **16** was confirmed to have a pentacyclic olean-12-ene type triterpene skeleton. The signals of the aglycone's C-12 and C-13 at δ 122.0 and δ 144.0 respectively showed the presence of two olefinic carbons (Mehta *et al.*, 2004; Xu *et al.*, 2010) and a signal at δ 178.6 (C-28) which showed the presence of a carboxlic acid at that position, thus, confirming that the aglycone is of olean-12-ene skeleton and therefore olean-12-en-28-oic acid aglycone.

The presence of two sugar moieties was evidenced in the signals at δ 103.4 and 100.3 confirming the presence of two anomeric carbons. The ^{1}H NMR spectrum (Appendix 6) showed anomeric proton signals at δ_H 4.34 and δ_H 5.06 and one methyl group signal at δ_H 1.07 suggesting the occurrence of rhamnopyranosyl unit. The sugar moieties were assigned mainly from ^{1}H-^{1}H COSY, HMQC and HMBC experiments which allowed the identification of one rhamnopyranose unit with anomeric protons resonating at δ_H5.06 and one xylopyranose unit with the anomeric protons at δ 4.34. In the particular case of a rhamnose the carbon C-6" usually resonates about 17.6-18.8 ppm (Nguyen *et al.*, 2009). The structure of the sugar chain at C-3 was determined by the HMBC correlations. A correlation between proton H-1' signal (δ_H 4.34) and C-3 signal (δ_C 79.7) of the aglycone indicated that this pentose is directly attached to the aglycone. Further correlations were observed between signals of xyl C-2 (74.6) and Rha H-1" (δ 5.06). ^{1}H-^{1}H COSY correlations was also observed between proton H-1' (δ_H4.34) and H-2' (δ_H3.52), proton H-1" (δ_H 5.06) and H-2" (δ_H 3.67) and between proton H-5" (δ_H 3.71) and H-6" (δ_H 1.07).



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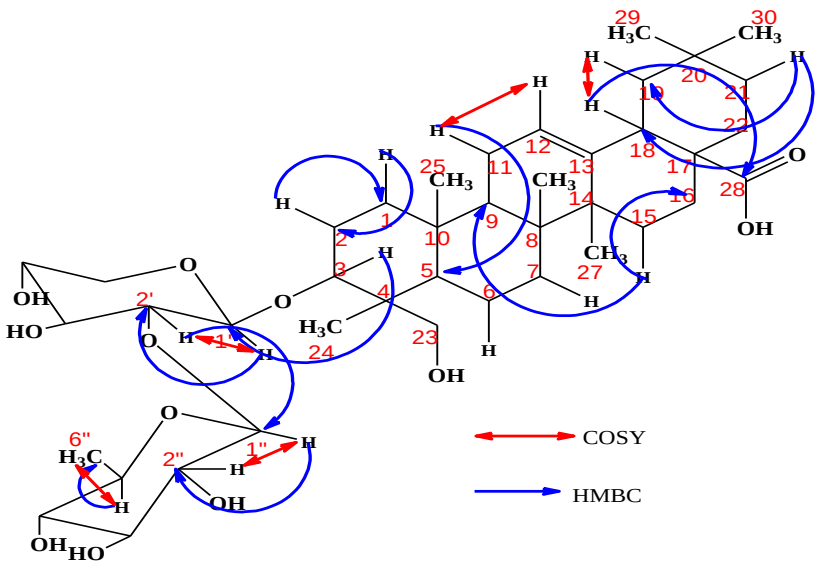


Figure 10: COSY and HMBC correlations of Compound **16**

Compound **16** had a retention time of 6.28 min as shown in Figure 11 and its corresponding UV spectrum indicates that the compound has a maximum wavelength **(**λmax) at 250 nm with absorption of 700 mAu. A column of 100 × 60 mm 5 micron was used and the pressure applied such that the flow rate of the solvent mixture was maintained at 15 ml/min. A solvent mixture of acidified Millipore water (H_2O + 0.1% CH_2O_2) and acidified acetonitrile (ACN + 0.1% CH_2O_2) was used. Compound **16** is a highly polar compound and was eluted at 6.28 minutes in a gradient 5-100-20 min. The compound has carbonyl groups and hydroxyl groups which are responsible for the electronic transitions and hence the absorption of UV light.

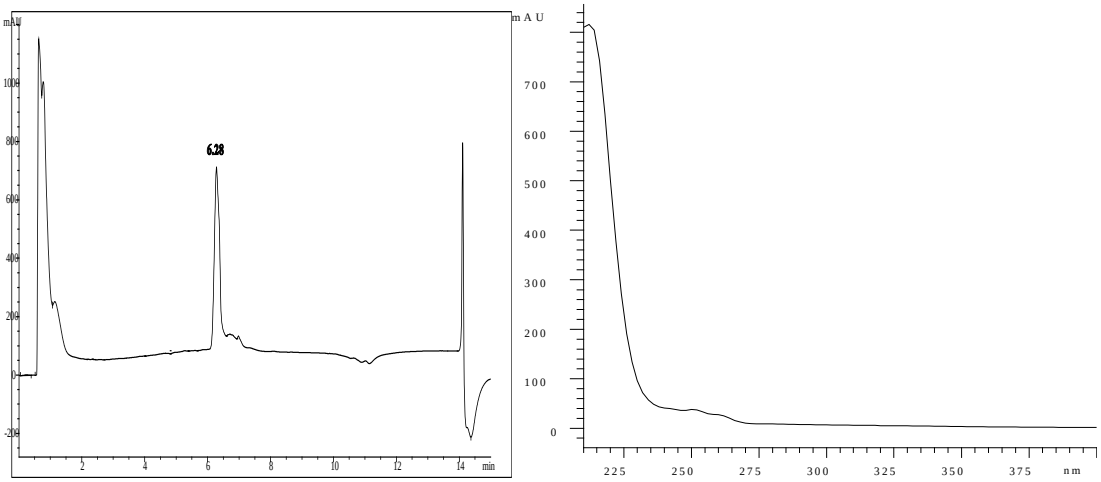


Figure 11: Chromatogram and UV spectrum of compound **16**

According to electrospray ionization - mass spectrometry (ESI-MS) the positive electron mass spectrometry showed peaks at m/z 751 $[M+H]^+$, 773 $[M+Na]^+$, 789$[M+K]^+$ and 1523 $[2M+Na]^+$ upon positive ionization mode (Figure 12) and at 749$[M-H]^-$, 785$[M+Cl]^-$ and 1499$[2M-H]^-$ upon negative ionization mode (Figure 13).

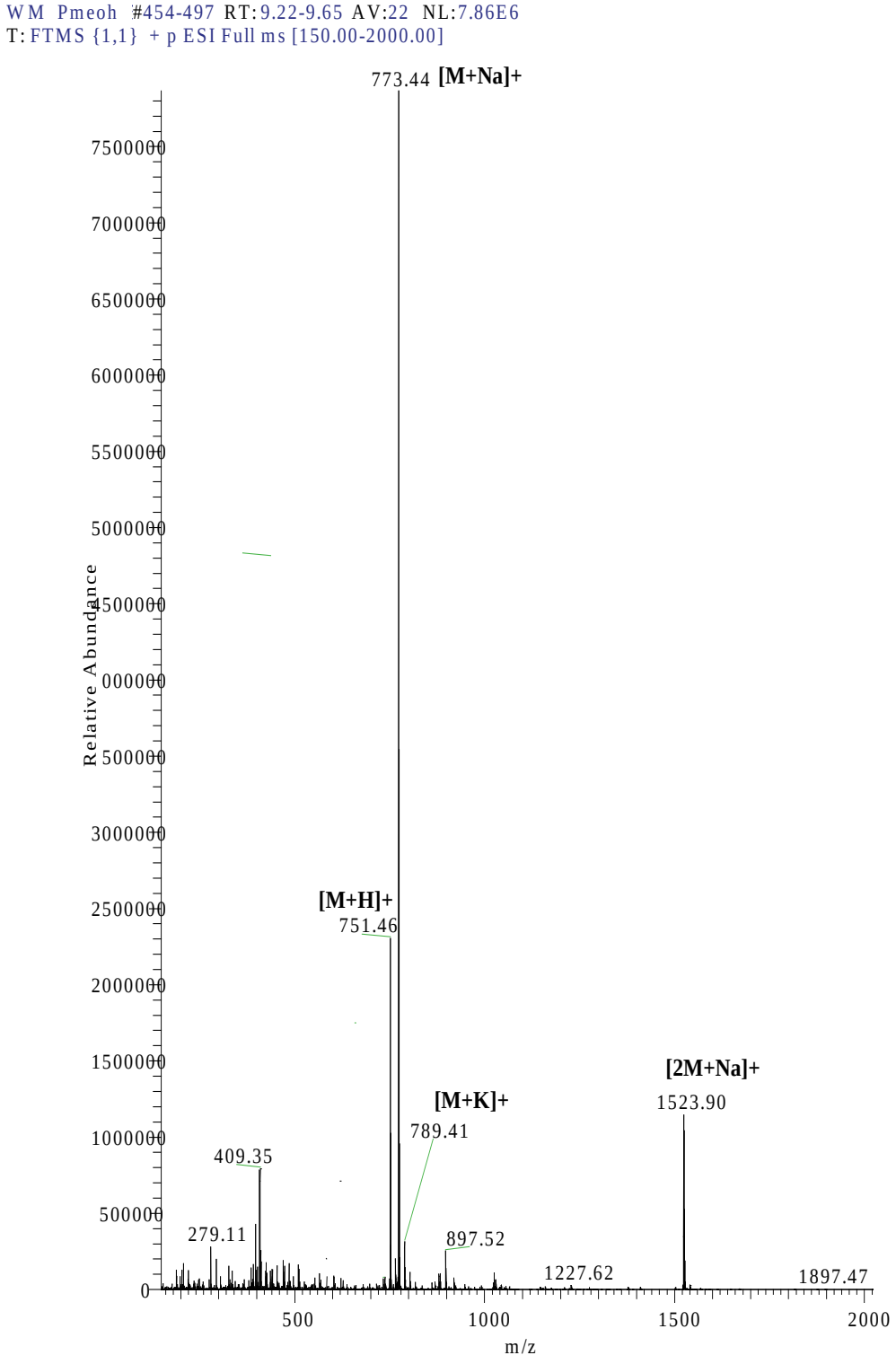


Figure 12: Mass spectrum of compound **16** in positive ionization mode

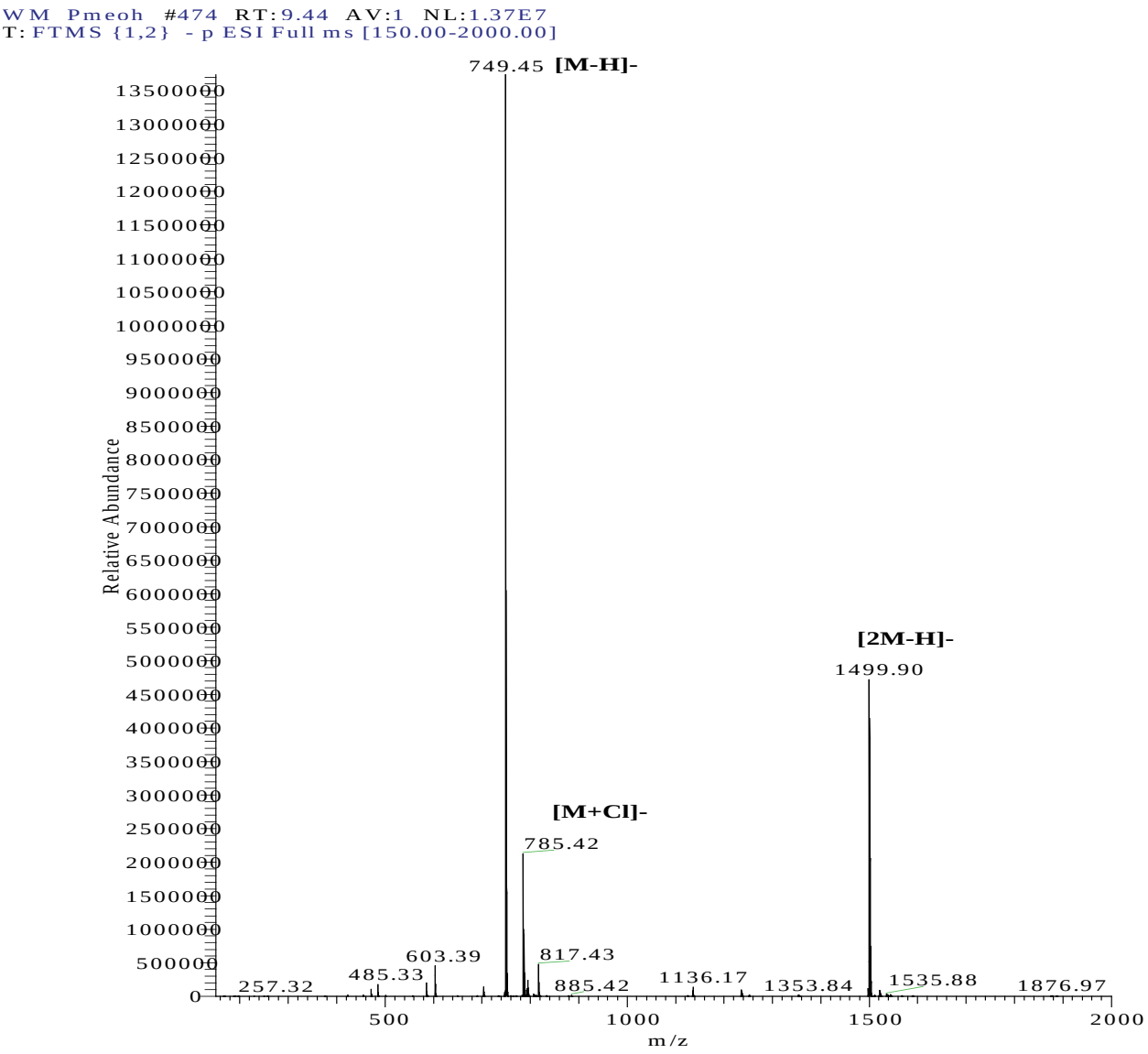


Figure 13: Mass spectrum of compound **16** in negative ionization mode

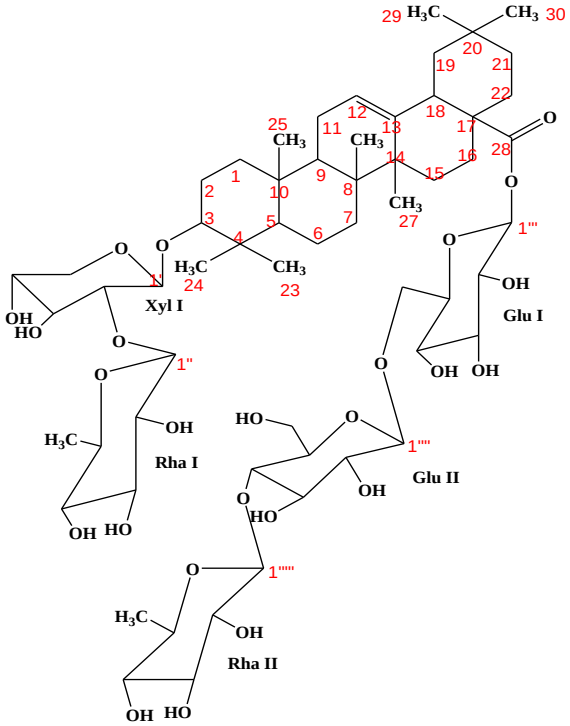
Thus, on the basis of the above evidence and analysis, compound **16** has been identified as a disaccharide triterpenoid saponin of olean-12-en-28-oic acid aglycone with the molecular formula of $C_{41}H_{66}O_{12}$ and has been established to be 3-O-[rhamnopyranosyl (1→2)-xylopyranosyl]-23-hydroxy-oleanolic acid (**16**). This compound has been isolated before from the stem bark of *P. fulva* (Joshi *et al*., 1992; Njateng *et al*., 2015), however, it is isolated for the first time from the leaves of this plant.

4.4 Structure elucidation of compound 17

Compound **17 (**Figure 14**)** was isolated from the plant extract as a brownish amorphous powder. The molecular formula was determined as $C_{59}H_{96}O_{25}$ based on pseudo-

molecular ion peak at 1227[M+Na-H]$^{+}$and 1242[M+K+H]$^{+}$ upon positive ion mode (Figure 16) and at 1203[M-2H]$^{-}$ upon negative ionization mode (Figure 17). The structural elucidation of compound **18** was carried out using 1D and 2D (^{1}H, DEPT, ^{1}H-^{1}H COSY, HSQC and HMBC) NMR experiments and is summarized in Table 4.

The ^{1}H NMR spectrum (Appendix 11) of compound **18** displayed seven methyl signals at δ 0.68, 0.74, 0.86, 0.87, 0.88, 0.93 and 1.07 which is a characteristic of a typical oleanane type triterpene, an oxymethine at δ 3.01 and olefinic protons at δ 5.06 indicating that the aglycone is of olean -12- ene skeleton and therefore olean-12-en-28-oic acid aglycone (Mehta *et al*., 2004; Xu *et al*., 2010). It also displayed five anomeric proton signals at δ 4.30, 4.27, 5.21/5.33, 4.69 and 5.04 signifying the presence of five sugar units.



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The DEPT NMR spectrum (Appendix 12) showed a total of fifty one signals and the other missing eight signals were quarternary signals. Out of the total fifty nine signals, 30 signals were assigned to the oleanolic acid moiety and the remaining 29 signals to the saccharide portion. The DEPT spectra of the aglycone displayed seven methyl signals at δ 15.7, 16.7, 17.1, 23.8, 25.9, 27.8 and 33.2; ten methylene (CH_2) signals at δ 18.1, 22.9, 23.3, 26.1, 27.7, 32.1, 32.6, 33.6, 38.7 and 46.0. It also showed a total of five methine (CH) signals at δ 41.1, 47.5, 55.5, 88.2 and 122.1. The other missing eight signals were quarternary signals. The first carbon was found to resonate at δ 38.7 (C-1), 27.7 (C-2) and 88.2 (C-3).

Quarternary carbons at C-4, C-8, C-10, C-13, C-14, C-17, C-20 and C-28 were found to absorb at δ 40.9, 39.3, 36.2, 143.8, 41.0, 45.6, 30.6 and 175.6 respectively. Carbon 5 were found to absorb at δ 55.5 while C-6, C-7, C-9, C-11, C-12, C-15, C-16, C-18, C-19, C-21 and C-22 were found to resonate at δ 18.1, 32.6, 47.5, 22.9, 122.1, 26.1, 23.3, 41.1, 46.0, 33.6 and 32.1 respectively. Carbons C-23, C-24, C-25, C-26 and C-27 resonates at δ 27.8, 16.7, 15.7, 17.1 and 25.9 while carbon C-29 and C-30 were found to absorb at δ 33.2 and 23.8 respectively.

Analysis through HMBC (Appendix 14) correlations which gave information on the correlations between 2-3 bonds away showed that H-1 (δ 0.85/1.53) correlated with C-2 and C-5 while H-2 (δ 0.87) showed correlations with C-1 and C-3. Proton H-3 (δ 3.01) showed correlations with C-1’ and C-2. Proton H-18 (δ 2.74) showed good correlations with a methine carbon C-12, a quarternary carbon C-13, a methylene carbon C-19 and a methyl

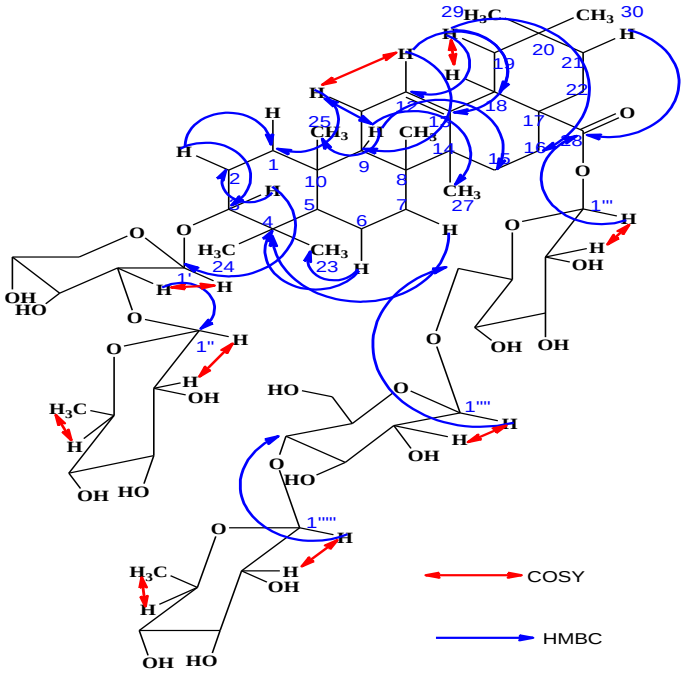


Figure 14: COSY and HMBC correlations of Compound **17**

carbon C-30 and H-19 (1.07/1.61) showed good correlations with methine carbons C-12 and C-18, a quarternary carbon C-13 and a methylene carbon C-16 and C-18. Similarly, proton H-21 (δ 1.32) and H-23 (δ 1.48) showed correlations with C-28 and C-13 respectively. Further confirmation with ^{1}H – ^{1}H COSY correlations showed that there was a strong correlation between protons H-9 (δ 1.47) and H-11 (δ 1.59) and proton H-11(δ 1.59) and H-12 (δ 5.17) and proton H-18 (δ 2.74) and H-19 (δ 1.07/1.61).

Table 4: NMR data of compound **17**

Carbon	DEPT	TYPE	^{1}H (δ)	HMBC	COSY	13C*
1	38.7	CH_2	0.85/1.53	2, 5		38.7
2	27.7	CH_2	0.87	3, 1		26.0
3	88.2	CH	3.01	1', 2		88.0
4	40.9	C	-	-		39.0
5	55.5	CH	0.70/0.73	9, 25		55.0
6	18.1	CH_2	1.47	4, 23		19.0
7	32.6	CH_2	1.15	4	8	33.0
8	39.3	C	-	-	7	40.0
9	47.5	CH	1.47	1, 15, 25, 27		48.0
10	36.2	C	-	-		37.0
11	22.9	CH_2	1.59	1	9, 12	24.0
12	122.1	CH	5.17	9, 18	11	123.0
13	143.8	C	-	-		144.0
14	41.0	C	-	-		42.0
15	26.1	CH_2	0.86	19, 22		28.2
16	23.3	CH_2	1.79	13, 22		23.3
17	45.6	C	-	-		47.0
18	41.1	CH	2.74	12, 13, 19, 30	19	41.6
19	46.0	CH_2	1.07/1.61	12, 13, 16, 18	18	46.2
20	30.6	C	-	-		30.7
21	33.6	CH_2	1.32	28		33.9
22	32.1	CH_2	1.48	13		32.5
23	27.8	CH_3	0.93	1, 3, 24		28.2
24	16.7	CH_3	0.74	2, 3, 5, 23		17.0
25	15.7	CH_3	0.85	1, 4, 5, 6, 9		15.6
26	17.1	CH_3	0.68	7, 9		17.5
27	25.9	CH_3	1.07/1.62	11, 18		26.0
28	175.6	C	-	-		176.5
29	33.2	CH_3	0.87	19, 20, 30		33.0
30	23.8	CH_3	0.86	19, 20		24.0

^{13}C* Literature (Harami *et al*., 2016)

Table 5: NMR data for the sugar portion of compound **17**

Position	**δ_C**	**Type**	**δ_H**
1’	104.2	CH	5.04
2’	76.9	CH	3.23
3’	72.3	CH	3.18
4’	69.7	CH	3.17
5’	64.2	CH	3.67
1’’	103.0	CH	4.27
2’’	72.8	CH	3.57
3’’	74.2	CH	2.99
4’’	75.6	CH	3.07
5’’	67.9	CH	3.59
6’’	18.1	CH_3	1.08
1’’’	94.4	CH	5.21
2’’’	72.4	CH	3.11
3’’’	76.9	CH	3.40
4’’’	70.8	CH	3.47
5’’’	69.0	CH	3.84
6’’’	68.0	CH_2	3.91
1’’’’	100.9	CH	4.69
2’’’’	75.9	CH	3.21
3’’’’	77.3	CH	3.36
4’’’’	70.8	CH	3.67
5’’’’	68.7	CH	3.87
6’’’’	60.4	CH_2	3.42/3.60
1’’’’’	100.4	CH	5.04
2’’’’’	68.4	CH	3.66
3’’’’’	74.9	CH	3.57
4’’’’’	71.1	CH	3.60
5’’’’’	72.6	CH	3.53
6’’’’’	18.2	CH_3	1.09

The sugar portion of **17** contained in the ^{1}H NMR spectrum five anomeric proton signals at δ 5.04, 4.27, 5.21, 4.69, 5.04 and two methyl signals at δ 1.08 and 1.09 suggesting the occurrence of two rhamnose units. The sugar moieties were assigned mainly from ^{1}H-^{1}H COSY, HMQC and HMBC experiments (Table 5) which allowed the identification of one Arabinopyranose unit with the anomeric proton signal at δ 5.04, two glucopyranose units

with anomeric protons resonating at δ 4.69 and 5.21/5.33 and two rhamnopyranose units with anomeric protons resonating at δ 4.27 and 5.04. Considering the δ values of the signals due to C-3 (δ 88.2) and C-28 (δ-175.6) in the DEPT NMR spectrum, saponin **17** was a 3, 28 bisdesmoside, thus, it has been identified as a bisdesmosidic pentasaccharide triterpenoid saponin of olea-12-en-28-oic acid aglycone with the molecular formula, $C_{63}H_{102}O_{26}$. The structure of the sugar chain at C-3 was unambiquously defined by the HMBC correlations (Appendix 14) (Mehta *et al.*, 2004). A correlation between C-3 signal (δ 88.2) of the aglycone and Ara H-1 signal (δ 4.30) indicated that this pentose was directly attached to the aglycone. Further correlations were observed between signals of Xyl C-2' (δ 76.9) and Rha I H-1 (δ 4.27). ^{1}H-^{1}H COSY (Appendix 15) correlations was also observed between proton H-1 (δ 4.27) and H-2 (δ 3.57). The structure of the oligosaccharide chain at C-28 was identified from the HMBC correlations between signals of Glc I C-4 (δ 69.0) and Glc II H-1 (δ 4.69), Glc II C-4 (70.8) and Rha II H-1(δ 5.04).

Analytical HPLC indicated that compound **17** had a retention time of 5.26 min as shown in Figure 15. The compound absorbs at 800 mAu with a maximum wavelength (λmax) of 250 nm and 280nm as shown by the corresponding UV spectrum**.** From the retention time obtained for compound **17,** it exhibited the most polarity at a recovery time of 5.26 min as compared to compound **15** and **16**.

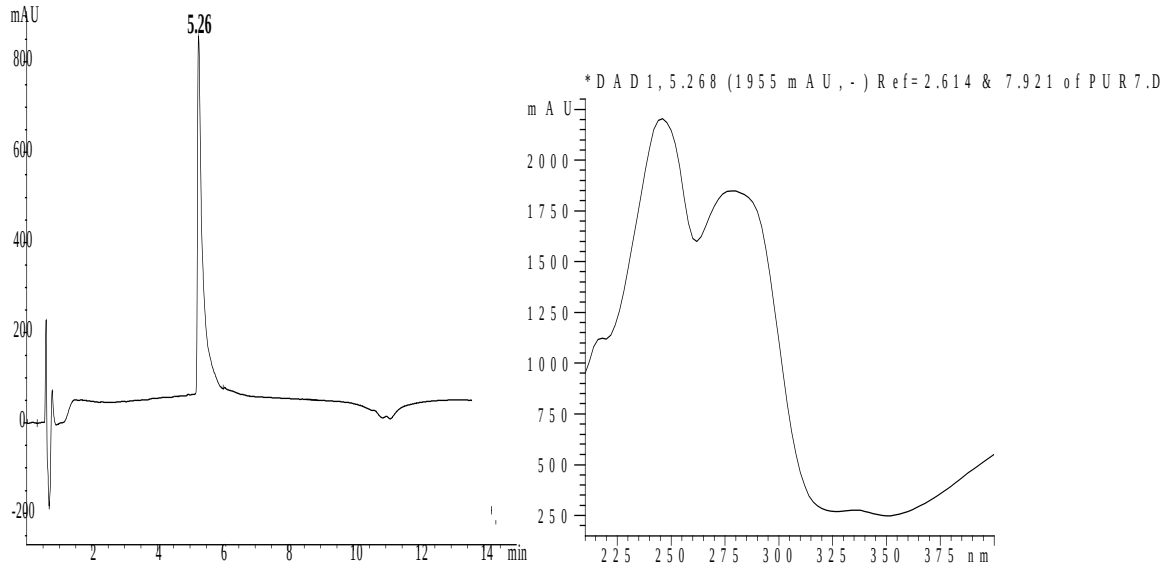


Figure 15: Chromatogram and UV spectrum of compound **17**

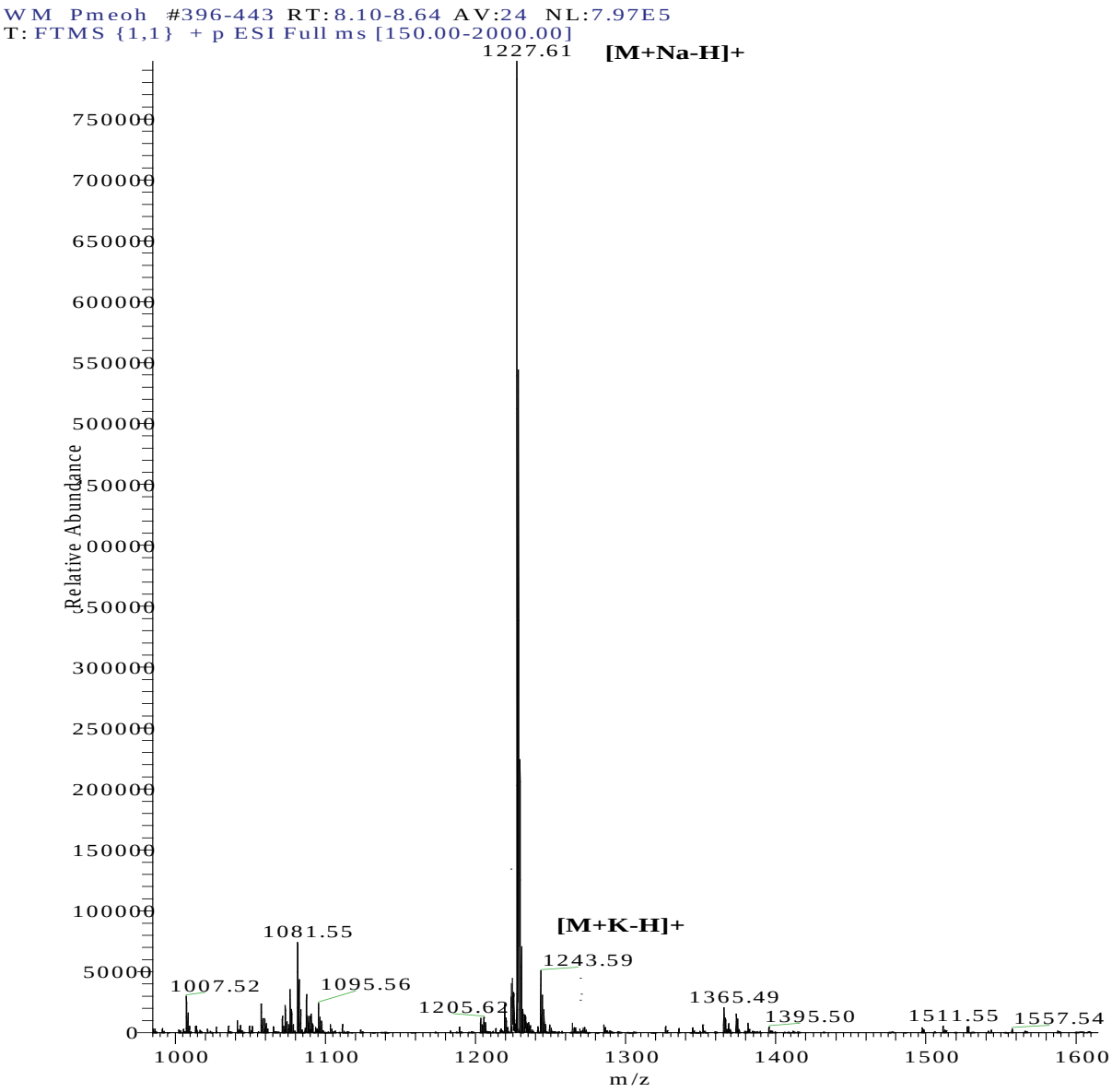


Figure 16: Mass spectrum of compound **17** in positive ionization mode

Correlating signals due to Glc I H-1 (δ 5.21/5.33) and aglycone C-28 (δ 175.6) provided a definitive evidence of an ester linkage between a trisaccharide chain and the aglycone. Thus, on the basis of the above evidence and analysis, compound **17** was identified as a pentasaccharide triterpenoid saponin of olean-12-en-28-oic acid aglycone with the molecular formula of $C_{59}H_{96}O_{25}$ and has been established to be 3-O-[rhamnopyranosyl-(1→2)-xylopyranosyl]-olean-12-en-28-O-[rhamnopyranosyl-(1→4)-glucopyranosyl-(1→6)glucopyranosyl]ester (**17**).

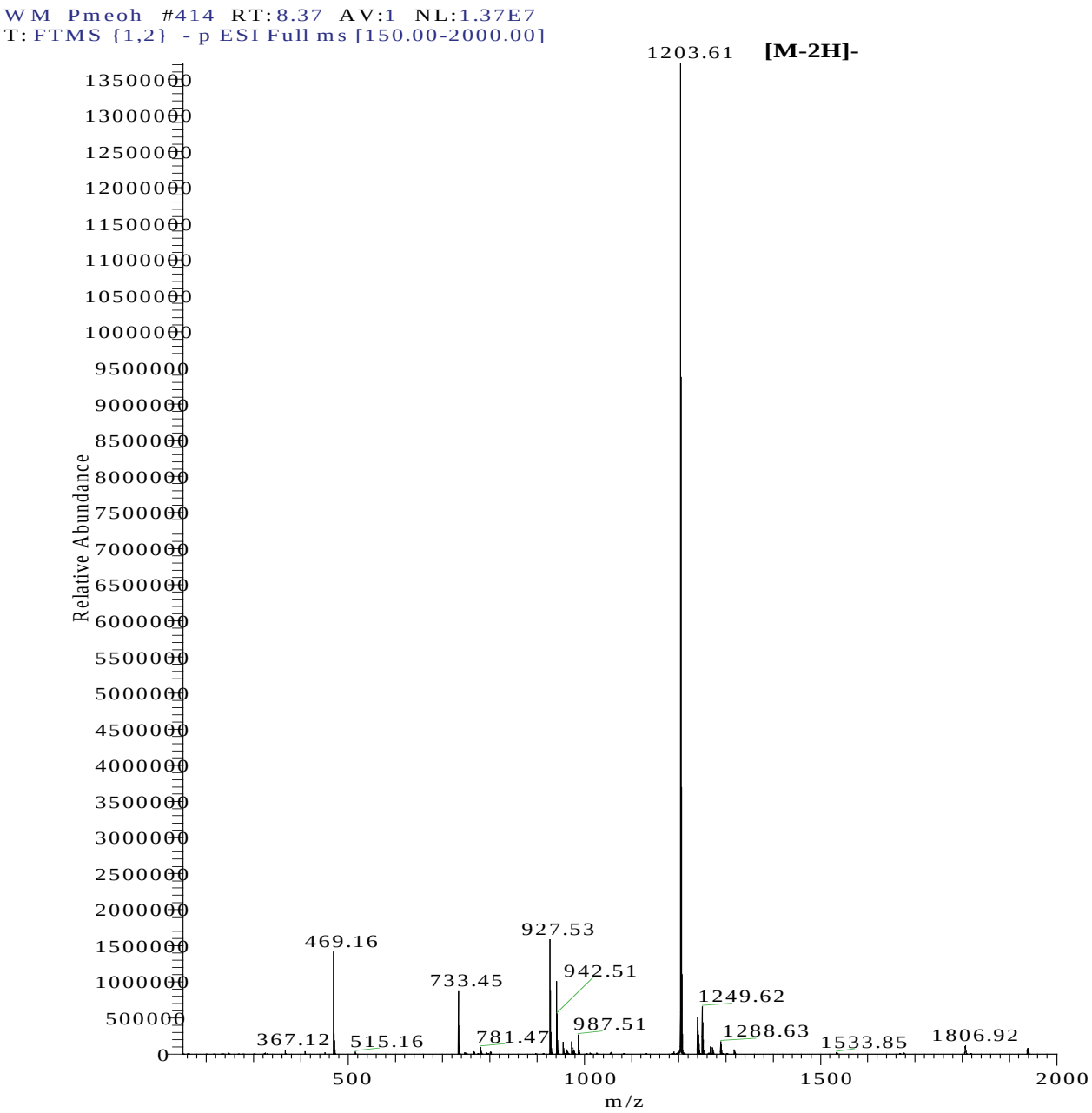


Figure 17: Mass spectrum of compound **17** in negative ionization mode

This compound was isolated for the first time from this plant However, it has the same skeleton with Hederasaponin B isolated from the leaves of *Hedera helix L.* (Nanyoung *et al.*, 2017), the only difference occurring at C-23. The hydroxymethyl group at C-23 in Hederasaponin B was substituted with a methyl group in compound **17**.

4.5 Structure elucidation of compound 18

Compound **18** (figure 18) was obtained from the ethyl acetate extract of the rice culture of *Fusarium* species PF1 as a dark yellow substance. The molecular formula of compound **18**

was established as $C_8H_8O_3$. This was deduced from ESI-MS analysis, where a molecular ion peak at m/z 153$[M+H]^+$, and 198$[M+2Na]^+$ upon positive ionization mode (Figure 19) was observed. The structural elucidation of compound **18** was carried out using 1D- and 2D- (^{1}H, DEPT, ^{1}H-^{1}H COSY, HSQC and HMBC) NMR experiments and is summarized in Table 6.

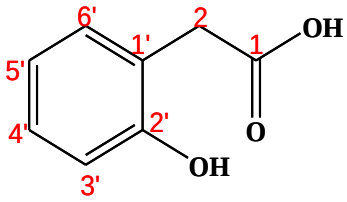
The ^{1}H NMR spectrum (Appendix 16) of **18** displayed four methine signals at δ 6.76/6.78, 6.71, 7.04 and 7.06/7.08 and one methylene signal at δ 3.45. The DEPT NMR (Appendix 17) data revealed the presence of 6 aromatic/olefinic carbons and one methylene group. The other missing three signals were found to be quarternary carbons.

Table 6: NMR data for compound **18**

Carbon	**DEPT**	**TYPE**	**^{1}H (δ)**	**HMBC**	**COSY**	**13C***
1	173.2	C	-	-		183.5
2	36.1	CH_2	3.45	1		42.7
1’	122.1	C	-	-		127.0
2’	155.6	C	-	-		156.9
3’	115.2	CH	6.76/6.78	2’, 5’	4’	118.8
4’	128.2	CH	7.04	2’, 1’	3’, 5’	130.9
5’	119.1	CH	7.06/7.08	1’, 2’, 3’ 4’, 6’	4’	123.3
6’	131.4	CH	-	1, 1’, 2’,		133.8

^{13}C* literature (Amar *et al.*, 2012)

The HSQC spectrum (Appendix 18) was used to assign protons directly attached to carbon atoms. This spectrum showed correlation between proton δ_H – 3.45 (C-2), 6.76/6.78 (C-3’), 7.04 (C-4’), 6.71 (C-5’) and 7.06/7.08 (C-6’). The HMBC spectrum (Appendix 19) showed proton correlations with carbon atoms that are two-three bonds away. Proton H-2 resonating at δ_H-3.45 showed correlation with carbon C-1 which is two bonds away and proton H-3’ (δ 6.76/6.78) correlates with C-2’ which is two bonds away and a methine carbon C-5’ which is three bonds away. Similarly H-4’ absorbing at δ_H-7.04 correlated with C-2’ and C-1’ which is two bonds away while proton H-5’ resonating at δ_H -6.71 correlated with C-1’, C-2’, C-3’, C-4’ and C-6’. The protons H-6’ resonating at δ_H-7.06/7.08 showed good correlations with C-1, C-1’ and C-2’. Further analysis was done using ^{1}H-^{1}H COSY (Appendix 20) which showed good correlations between protons H-3’ and H-4’ absorbing at δ_H 6.76/6.78 and δ_H7.04 and protons H-4’ and H-5’ absorbing at δ_H 7.04 and δ_H 6.71 respectively.



18

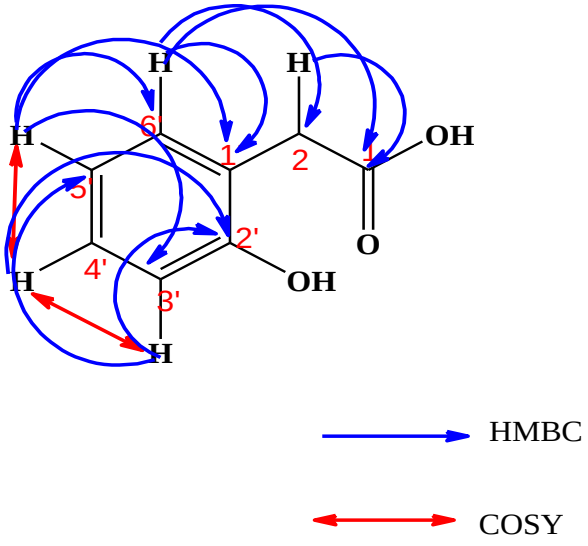


Figure 18: Structure of compound **18** with and without HMBC and COSY correlations**.**

Analytical HPLC indicated that compound **18** had a retention time of 3.15 min as shown in Figure 20. The compound absorbs at 240 mAu with a maximum wavelength (λmax) of 220 nm and 275 nm as shown by the corresponding UV spectrum**.** From the retention time obtained for compound **18,** it exhibited the most polarity at a recovery time of 3.15 min. The compound has carbonyl group and a benzyl ring which are responsible for the electronic transitions and hence the absorption of UV light.

The compound was established as a Phenolic compound by comparing the DEPT NMR spectral data of the compound with reported similar phenolic compounds (Amar *et. al*., 2012). The signals at C-2 and C-1 at δ 36.12 and δ 173.29 respectively indicated that the compound is an acetic acid. Thus, on the basis of the above evidence and analysis, compound **18** has been identified as an acetic acid derivative with a molecular formula, $C_8H_8O_3$, and has been established as (2-hydroxyphenyl) acetic acid (**18**).

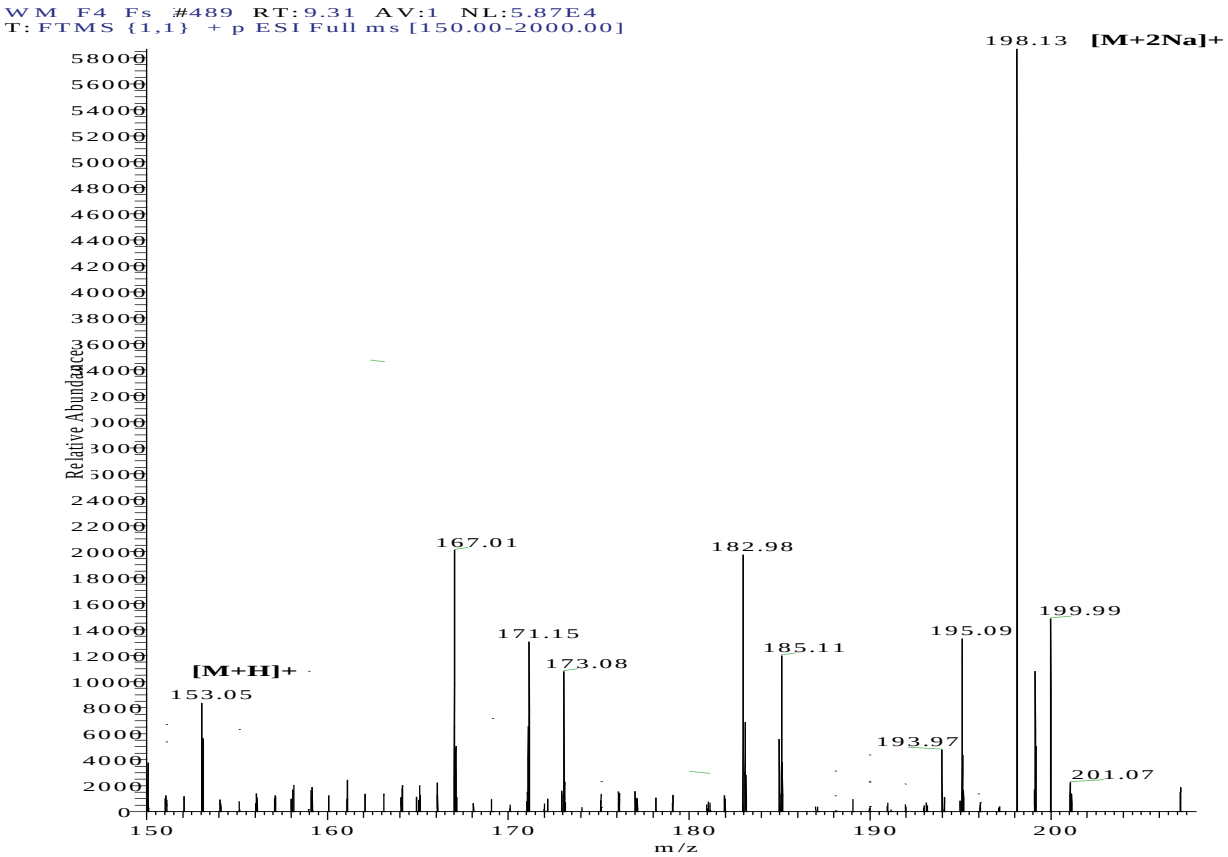


Figure 19: Mass spectrum of compound **18** in positive ionization mode

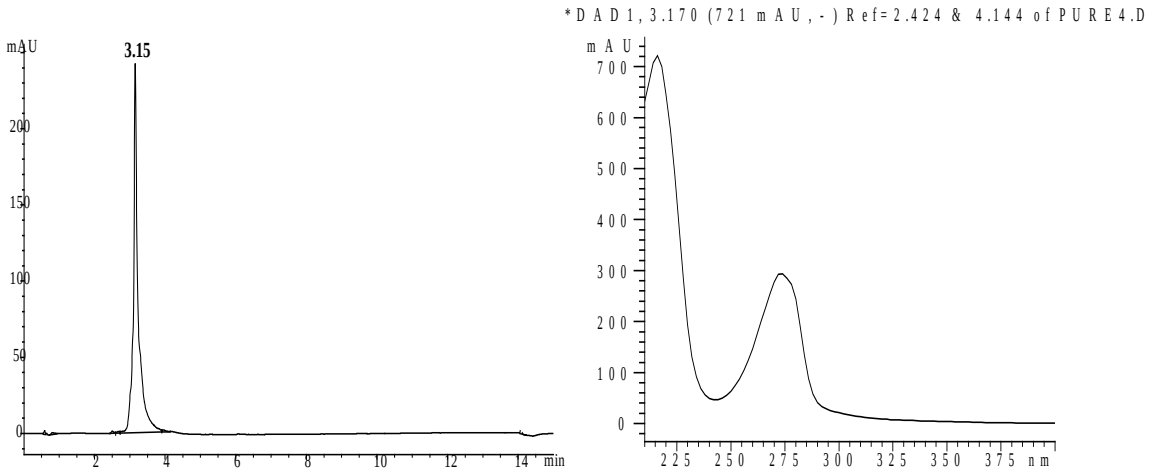


Figure 20: Chromatogram and UV spectrum of compound **18**

4.6 Antagonistic assay of fungal endophytes against test human pathogenic bacteria

Two endophytes were isolated from the fresh leaves of *P. fulva* and identified as *Fusarium* sp. PF1 and *Fusarium* sp. PF2. Antagonistic screening of the endophytic fungal isolates was done using the dual culture assay method against pathogenic bacteria *K. pneumoniae* and *S. aureus*. *Fusarium* sp. PF1 and *Fusarium* sp. PF2 demonstrated

antagonism against both gram negative bacteria (*K. pneumoniae*) and gram positive bacteria (*S. aureus*) (Plate 2). *Fusarium* sp. PF2 was more active against *K. pneumoniae* bacteria but had no activity against *S. aureus. Fusarium* species PF1 showed activity on both *K. pneumoniae* and *S. aureus* with inhibition zones of 8.7 ± 0.57mm and 7.0 ± 1.00mm respectively as shown in table 7.

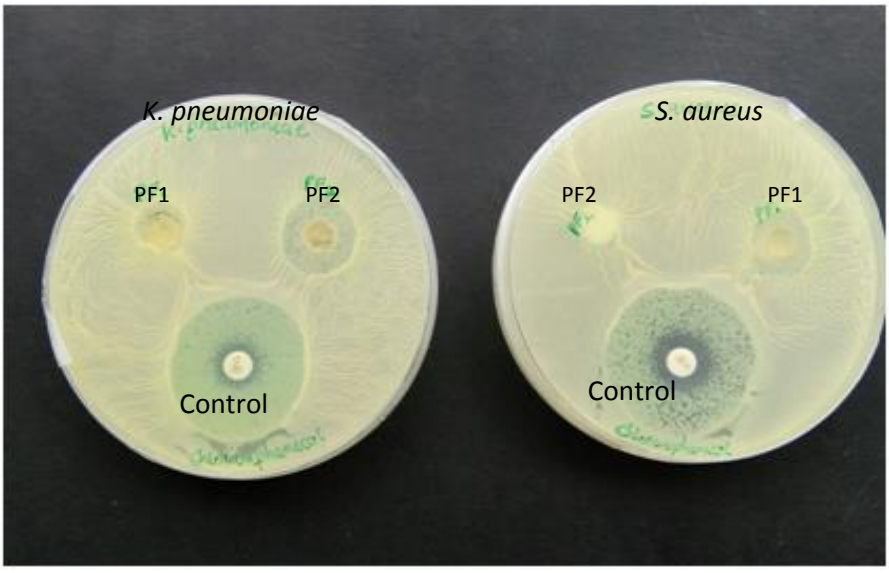
Table 7: Inhibition diameters (mm) for the fungal endophytes against test organisms

	Test organisms (diameter in mm, n=3)	
Endophyte/treatment	***K. pneumoniae***	***S. aureus***
Fusarium sp. PF1	8.7 ± 0.57^{c}	7.0 ± 1.00^{b}
Fusarium sp. PF2	11.3 ± 0.57^{b}	0.0 ± 0.00^{c}
Chloramphenicol	25.0 ± 0.00^{a}	27.0 ± 0.00^{a}

Within a column, extracts with different letter (s) are significantly different (α =0.05, Tukey's test)

The inhibition zones of *Fusarium* sp. PF1 and *Fusarium* sp. PF2 against *K. pneumonia*e were significantly smaller in comparison to chloramphenicol. Similarly, the inhibition zones of *Fusarium* sp. PF1 and *Fusarium* sp. PF2 against *S. aureus* were significantly smaller than that of chloramphenicol.

Plate 2: Antagonistic activity of endophytic *Fusarium* sp, against *K. pneumoniae* and *S. aureus*



Fusarium species have been reported as endophytes from several plants with diverse biological activity (Shiono *et al*., 2007; Kour *et al*., 2008; Deng *et al*., 2009). According to Swathi *et al*. (2013), *Fusarium* species isolated from marine water samples showed good antibacterial activities against gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella. pneumoniae*) and gram positive bacteria (*Bacillus subtilis, Staphylococcus epidermis*). Extracts from *Fusarium* species isolated as endophytes have been reported to demonstrate antibacterial activity against a range of pathogenic microorganisms. Most of these biologically active extracts are composed of mycotoxins (Xu *et al*., 2010; Wang *et al*., 2011). However, in a few cases, there has been documentation of non-mycotoxin secondary metabolites that are active against pathogenic microorganisms (Tegos *et al*., 2002; Wang *et al*., 2014). As observed in this study, bioactivity of *Fusarium* species (PF1 and PF2) could possibly be due to mycotoxin (toxic secondary metabolite produced by fungi or such non-mycotoxin secondary metabolites. The results obtained in this study suggest that these endophytes have the potential to be sources for novel bioactive products.

4.7 Antibacterial activity of the extracts and pure compounds against human pathogens

Secondary metabolites isolated from *Fusarium* species PF1 and *P. fulva* leaf extracts were tested for antibacterial activity against gram negative bacteria (*K. pneumoniae*) and gram positive bacteria (*S. aureus*) using disc diffusion assay test. The inhibition zones were measured and recorded (Table 8).

The crude extract and the isolated compounds generally demonstrated antibacterial activities. The most sensitive bacterium was *S. aureus*. Compound **18** which was obtained from the fungal endophyte *Fusarium* sp. PF1 was found to be the most active compound against both *K. pneumoniae* and *S. aureus* with inhibition zones of 11.7 ± 2.51mm and 12.0 ± 3.60mm respectively. Compound 18 was the least active against both *K. pneumoniae* and *S. aureus* with inhibition zones of 7.3 ± 0.57mm and 7.0 ± 1.00mm respectively.

The antibacterial properties of the crude extract and fractions can be explained by the presence of active secondary metabolites detected in them. Among the compounds isolated from the crude methanol extract, compound **15** was more active on the two tested microorganisms. This difference in activity may be attributed to the presence of hydroxyl group in position 23 and presence of 3-O-[xylopyranosyl] group in the compound (Njateng *et al*., 2017).

Compound **16** that result from the addition of 3-O-[rhamnopyranosyl] group to compound **15** was less active. This modification may have slightly reduced the antibacterial activity of compound **16**. The substitution of position C-23 with a methyl group and the addition of O-[rhamnopyranosyl-(1→4)-glucopyranosyl-(1→6)-glucopyranosyl] group in position 28 to compound **17** could have contributed to its being less active against both bacteria compared to compounds **15** and **16** (Njateng *et al*., 2017). According to Pavithra *et al*. (2010), saponins possess antimicrobial activities. Compound **18** which is a phenolic compound was the most active. Its antibacterial activity could be attributed to the presence of hydroxyl groups (Teke *et al*., 2011).

Table 8: Inhibition diameters (mm) for the isolated extracts against test organisms

	Test organisms (diameter in mm, n=3)	
Extracts/treatment	***K. pneumoniae***	***S. aureus***
Methanol crude	9.3 ± 0.57^{bc}	10.3 ± 1.52^{bc}
Compound **15**	8.0 ± 1.00^{c}	10.0 ± 1.73^{bc}
Compound **16**	7.7 ± 0.57^{c}	7.3 ± 0.57^{bc}
Compound **17**	7.3 ± 0.57^{c}	7.0 ± 1.00^{c}
Compound **18**	11.7 ± 2.51^{b}	12.0 ± 3.60^{b}
Chloramphenicol	16.0 ± 0.00^{a}	17.0 ± 0.00^{a}
1% DMSO	0.0 ± 0.00^{d}	0.0 ± 0.00^{d}

Within a column, extracts sharing the same letter(s) are not significantly different while those with different letter (s) are significantly different ($\alpha = 0.05$, Tukey's test)

The mean inhibition zones of extracts in comparison with chloramphenicol are represented in Table 8. The comparison showed that all the extracts were significantly less active than chloramphenicol. The mean inhibition diameters of compounds **15**, **16** and **17** against *K. pneumoniae* were not significantly different (Appendix 21) while compounds **15**, compound **17** and the methanol extract were not significantly different against *S. aureus* (Appendix 22).

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

i. Known compounds **15, 16** and **17** was obtained from the dried leaves of *P. fulva* while compound **18** was isolated from the fungal endophytes *Fusarium sp*. PF1.

ii. The structures of compounds **15**, **16**, **17** and **18** were successfully determined using a combination of spectroscopic techniques that include 1 and 2D high field NMR spectroscopy and Liquid Chromatography-Mass Spectrometry (LC-MS)

iii. Compounds **15**, **16**, **17** and **18** together with the methanol crude extract showed antibacterial activities against both *K. pneumonia*e and *S. aureus* while the cultures of Endophytic *Fusarium* spp. PF1 demonstrated antibacterial activity against both human pathogens and PF2 was only active against *K. pneumoniae*.

In summary, results from this study indicate that the three Oleanane triterpene saponins obtained from the dried leaves of *P. fulva* together with 2-hydroxyphenyl acetic acid obtained from the fungus *Fusarium* species (PF1) are potential sources of antibacterial agents and also validate the traditional use of the plant for its antibacterial activity.

5.2 Recommendations

i. Further research should be done on isolation and characterization of compounds from roots and stems of the medicinal plant *P. fulva.*

ii. Further structure determination should be done on the unidentified fractions.

iii. Further evaluation of the isolated compounds for activity against a wide range of other pathogenic bacteria.

iv. Further exploration of the bioactive secondary metabolites from the leaves of *P. fulva* and its endophytic fungi and their possible contribution as lead molecules for antibiotics discovery.

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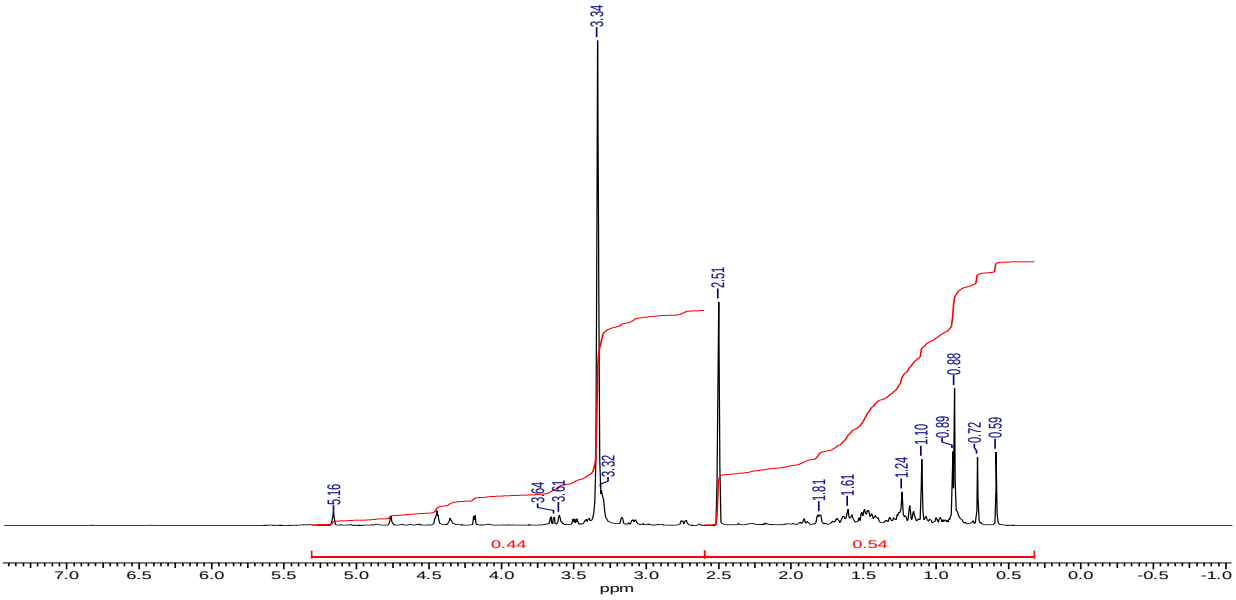
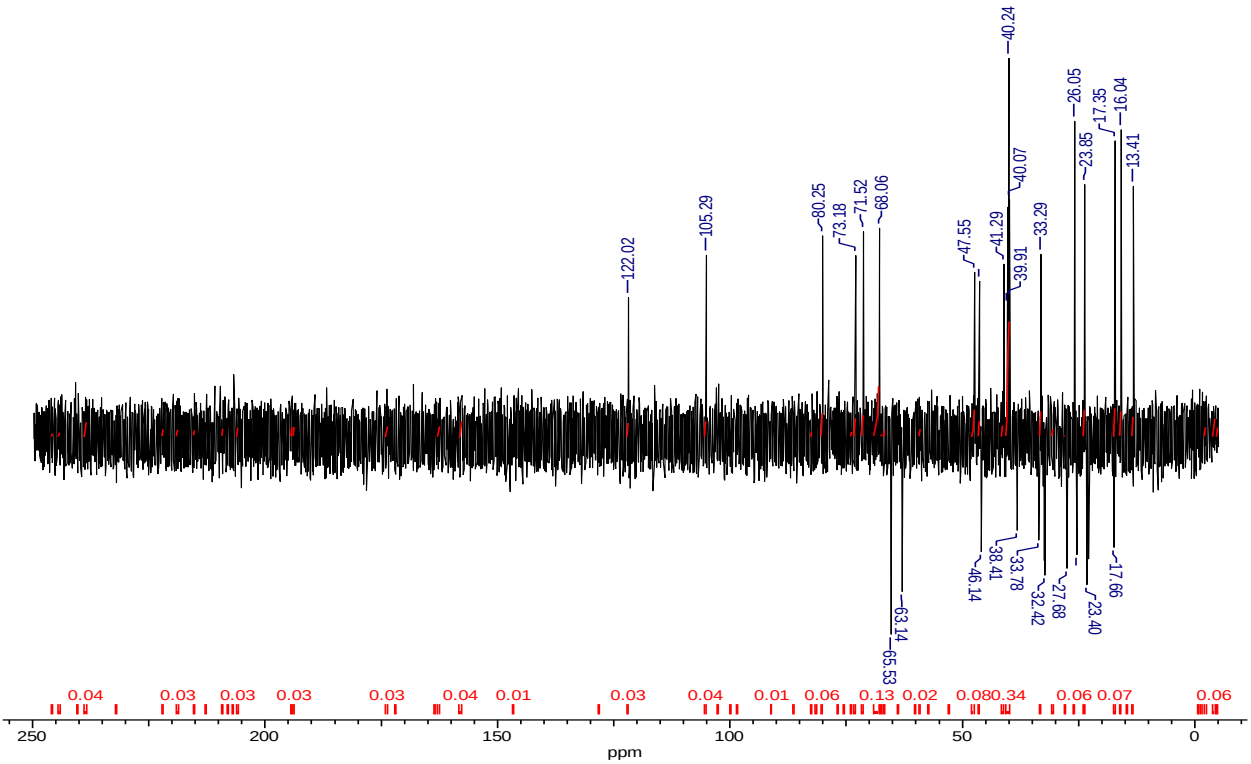
APPENDICES

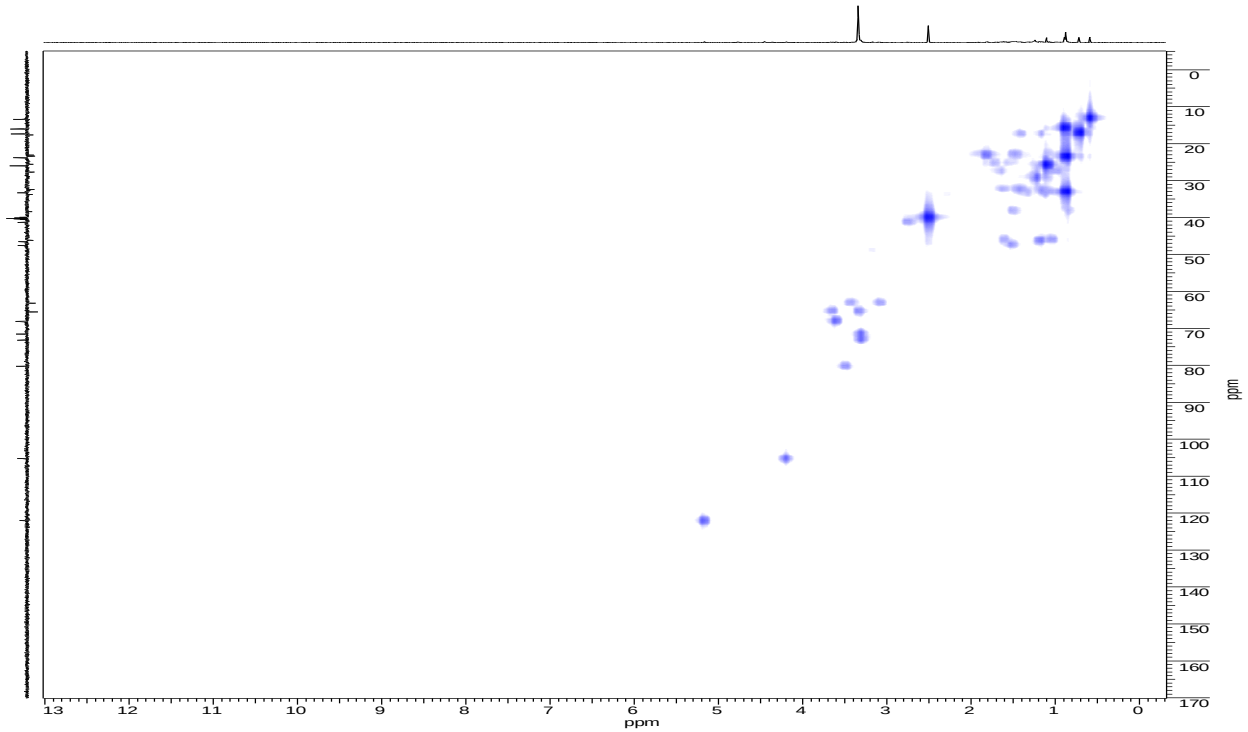
Appendix 1: ^{1}H-NMR of Compound 15



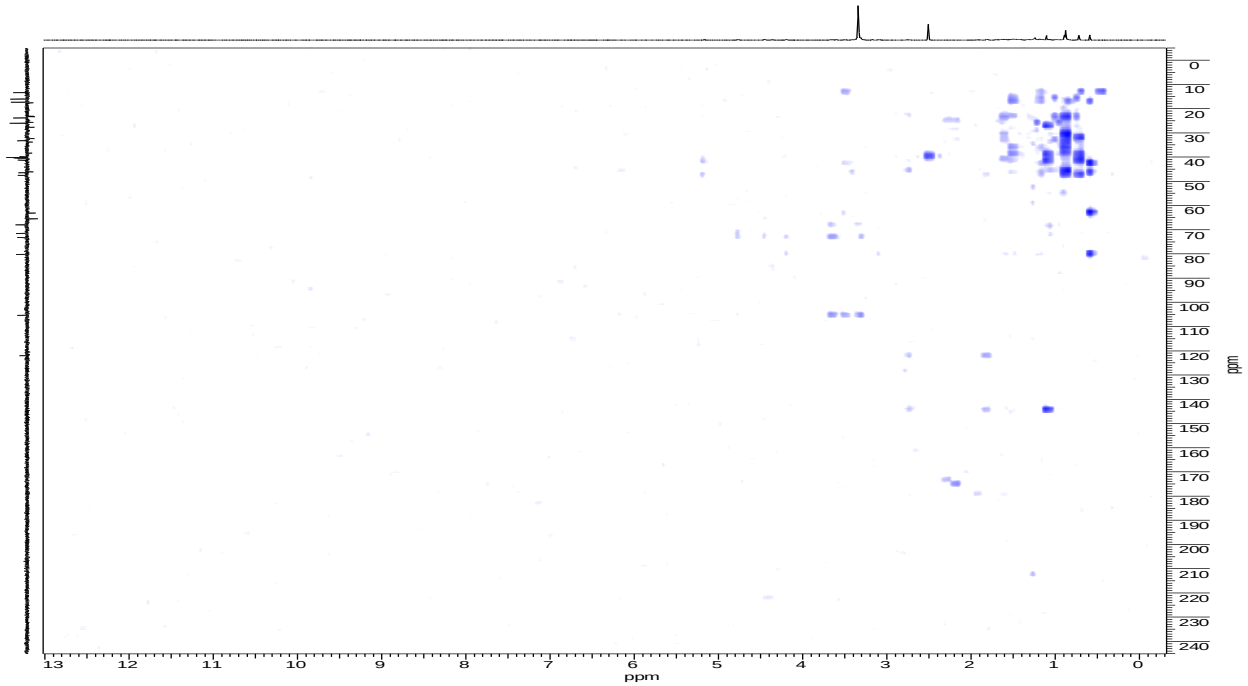
Appendix 2: DEPT-NMR spectrum of compound 15



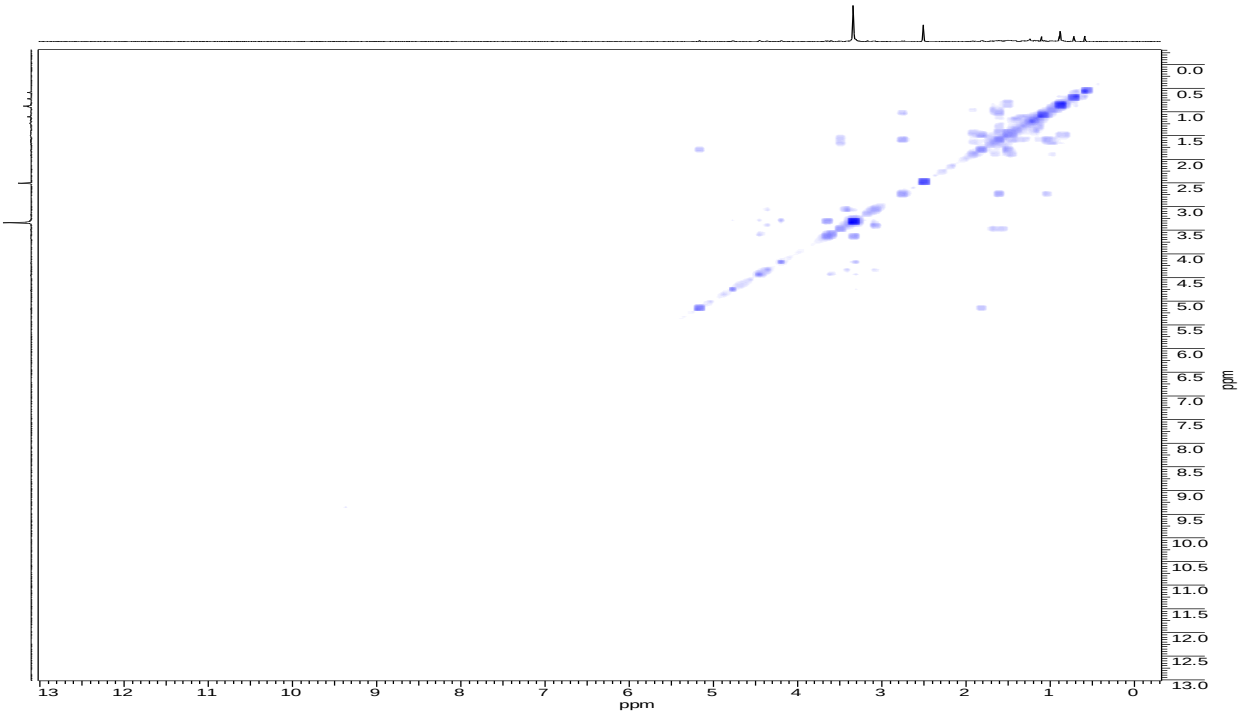
Appendix 3: HSQC spectrum of compound 15



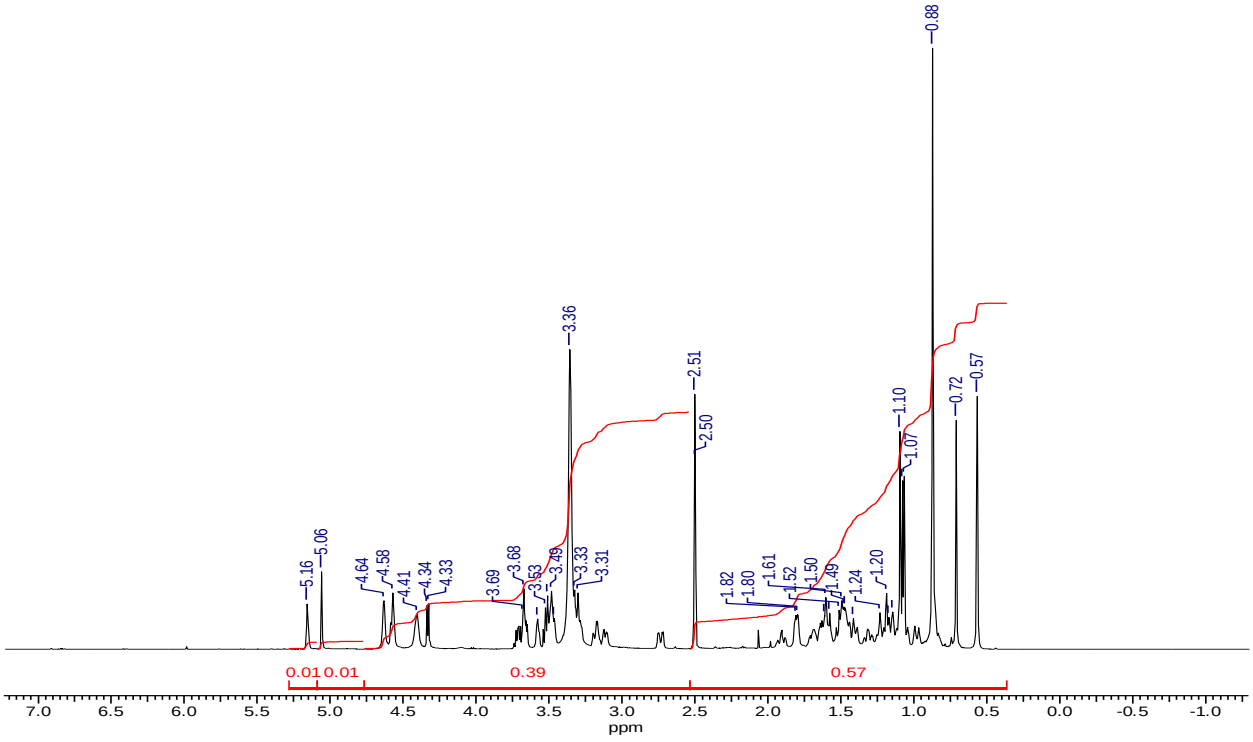
Appendix 4: HMBC spectrum of compound 15



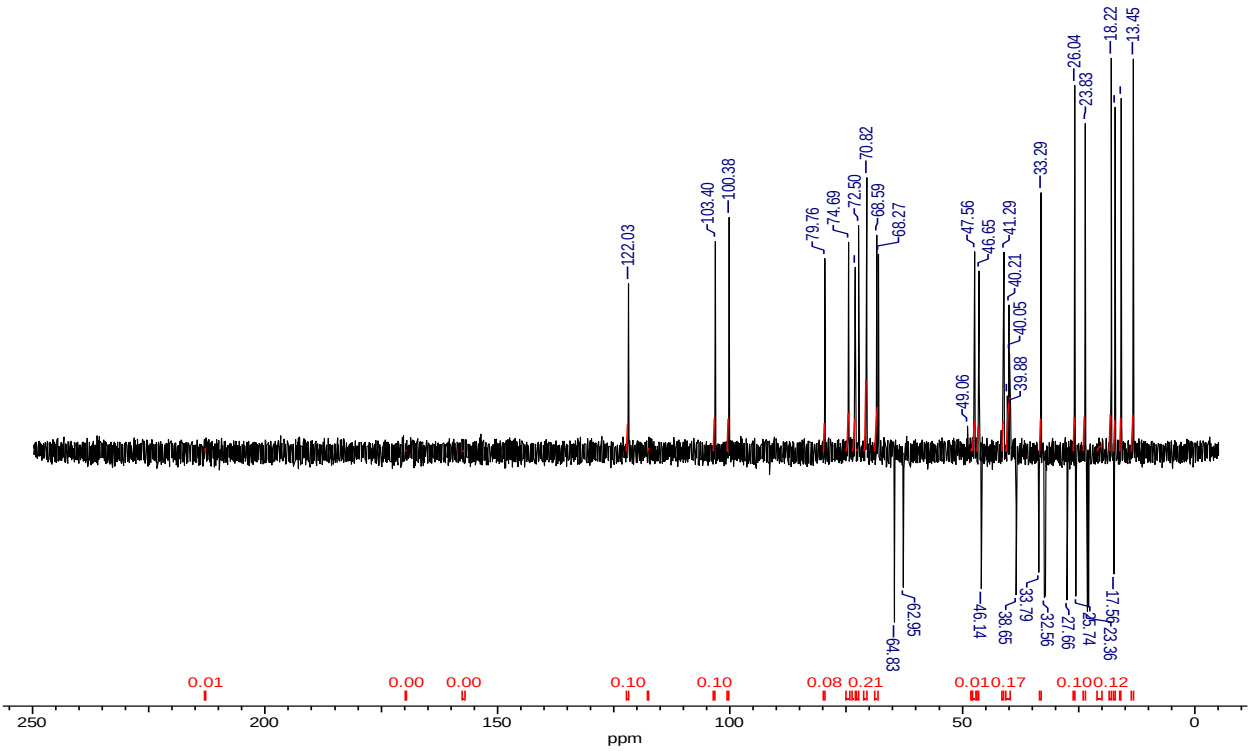
Appendix 5: ^{1}H-^{1}H NMR of compound 15



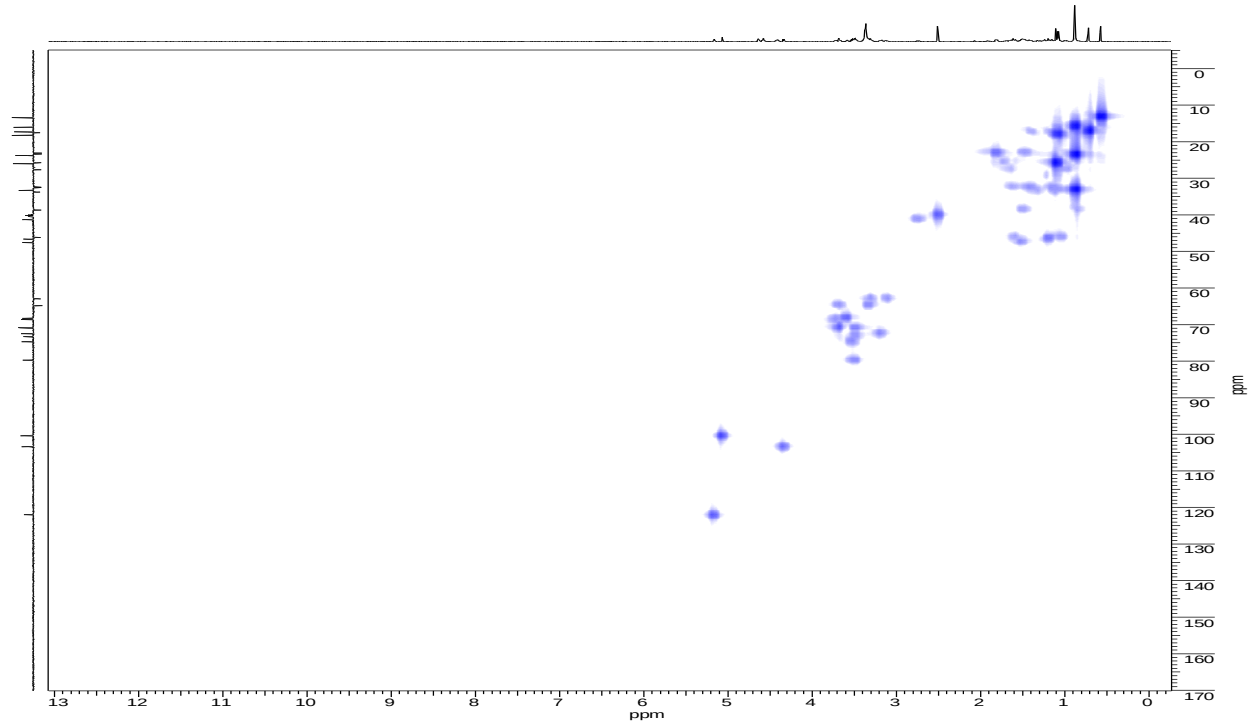
Appendix 6: ^{1}H-NMR spectrum of compound 16



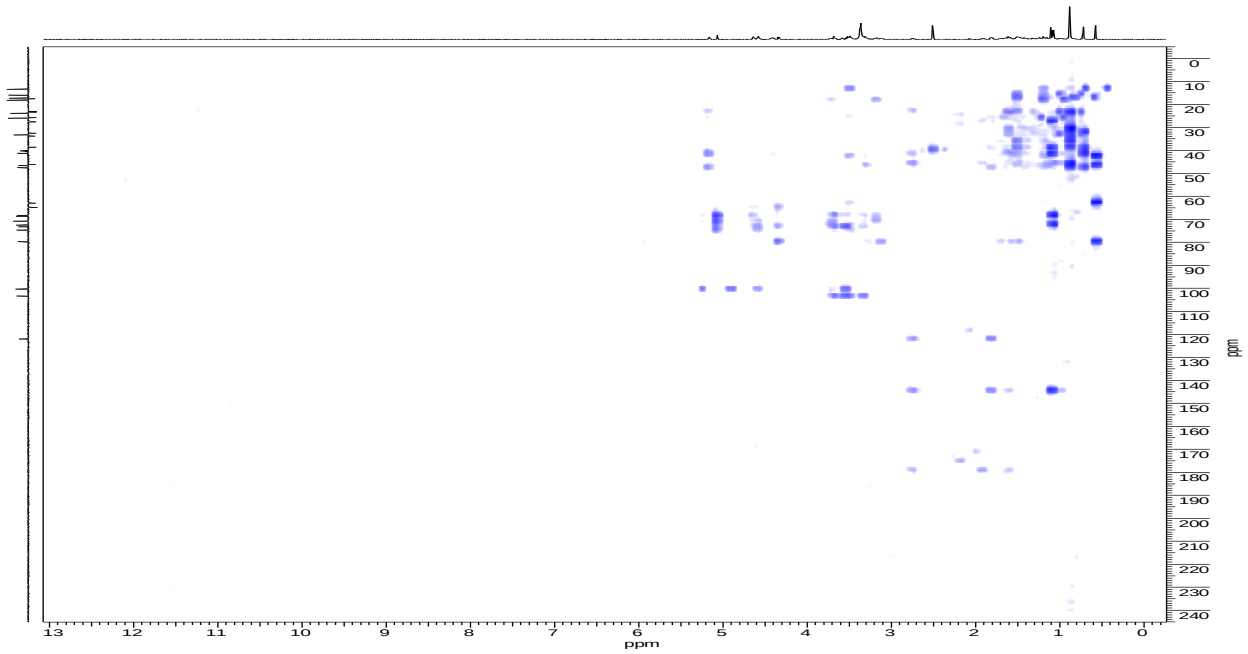
Appendix 7: DEPT-NMR spectrum of compound 16



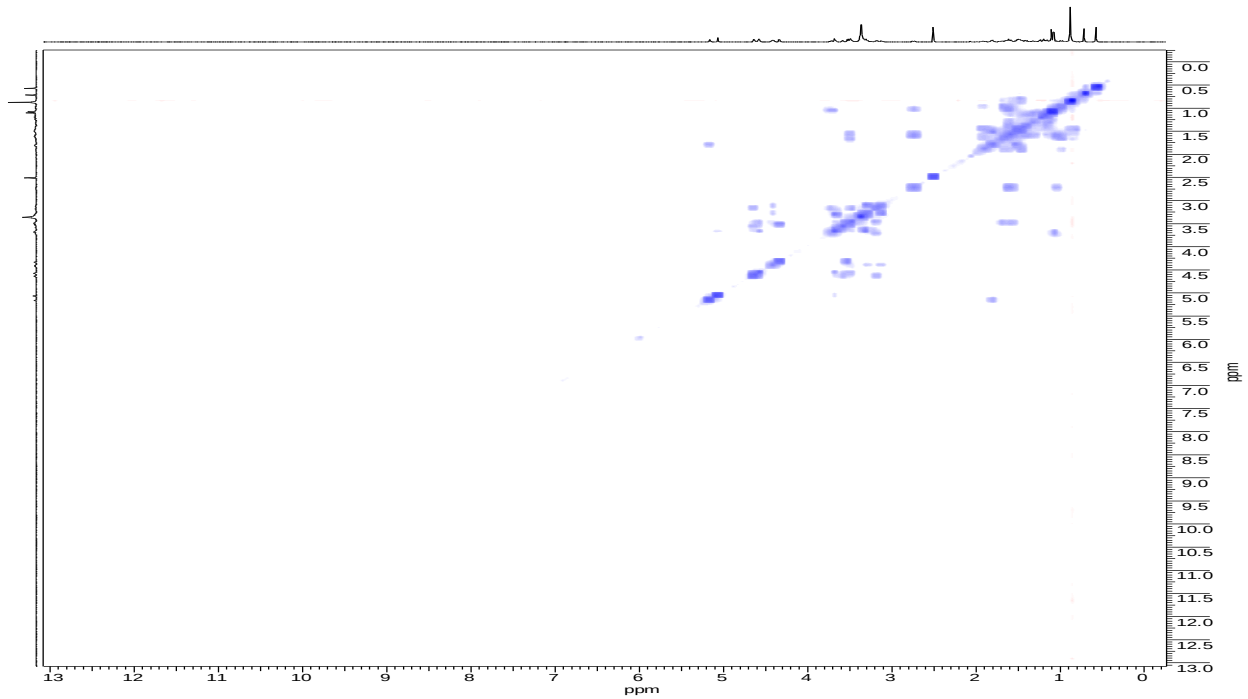
Appendix 8: HSQC spectrum of compound 16



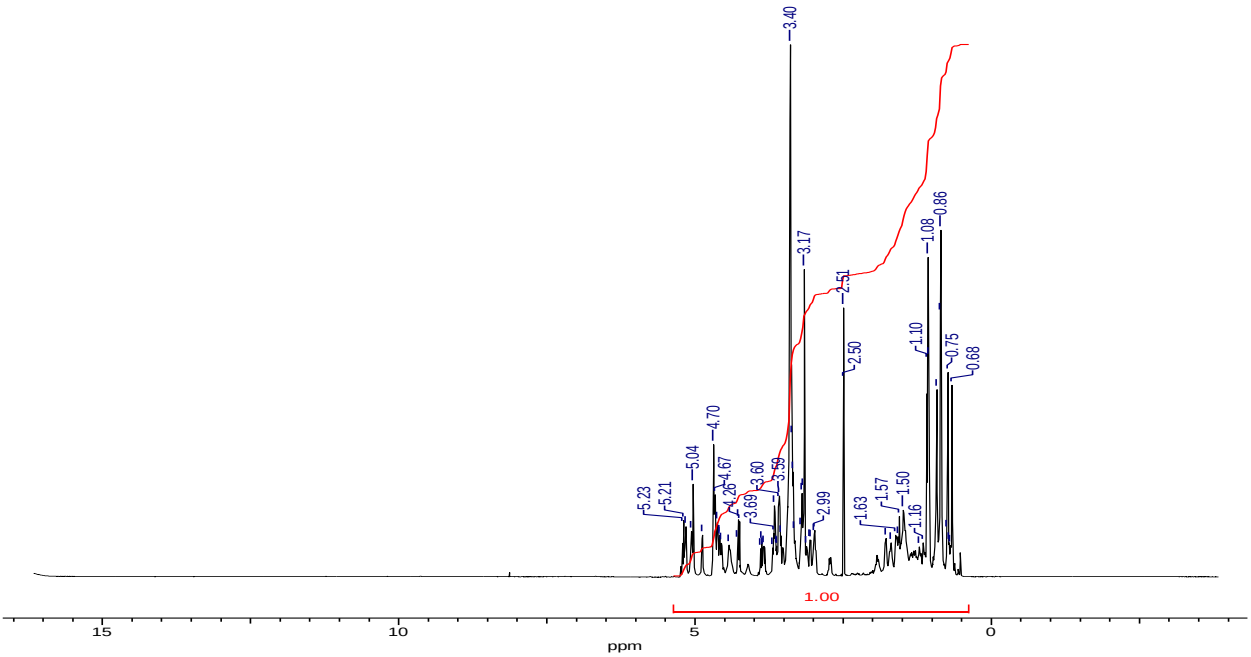
Appendix 9: HMBC spectrum of compound 16



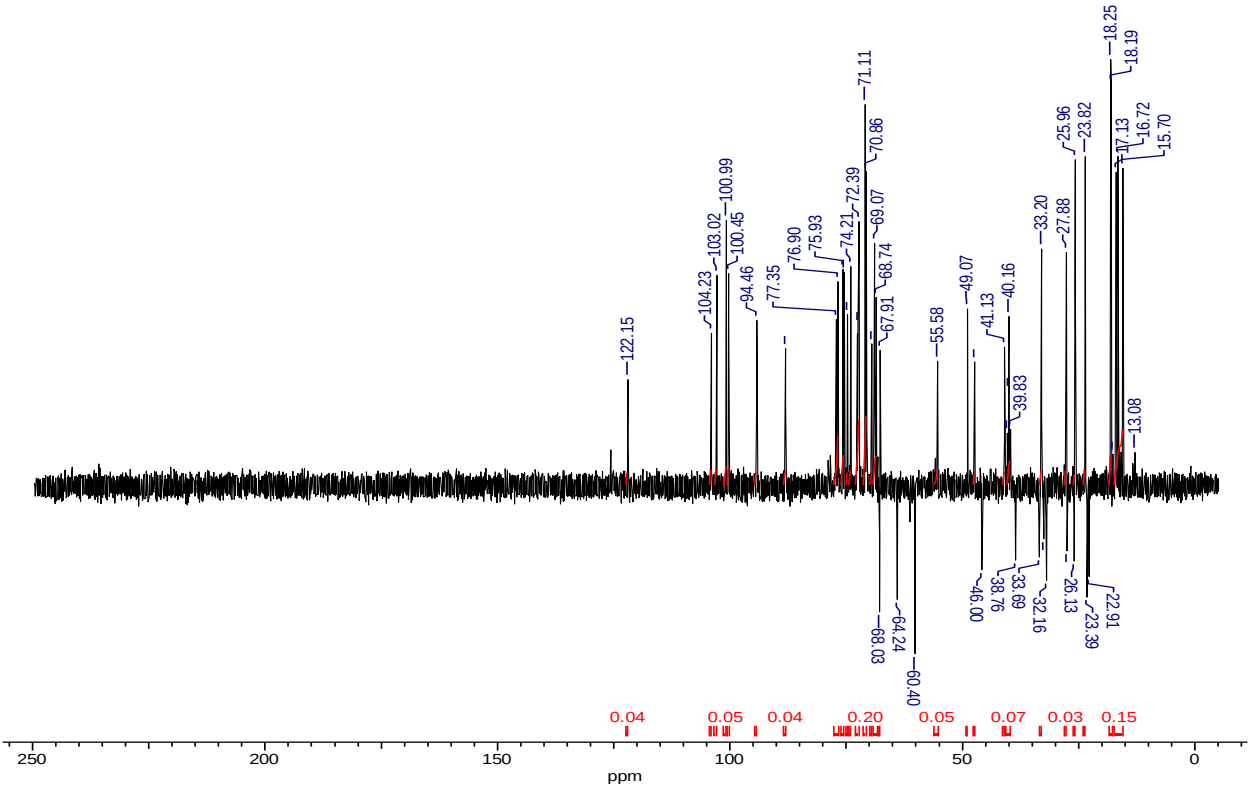
Appendix 10: ^{1}H-^{1}H NMR spectrum of compound 16



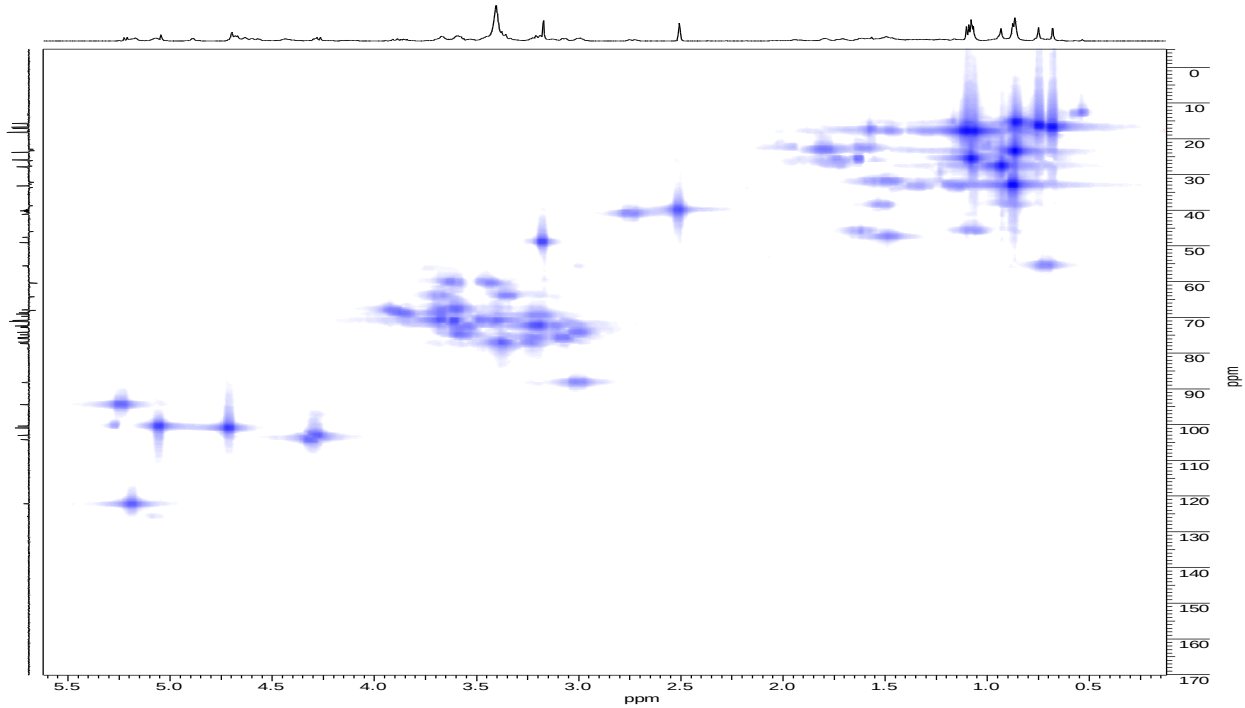
Appendix 11: ^{1}H-NMR spectrum of compound 17



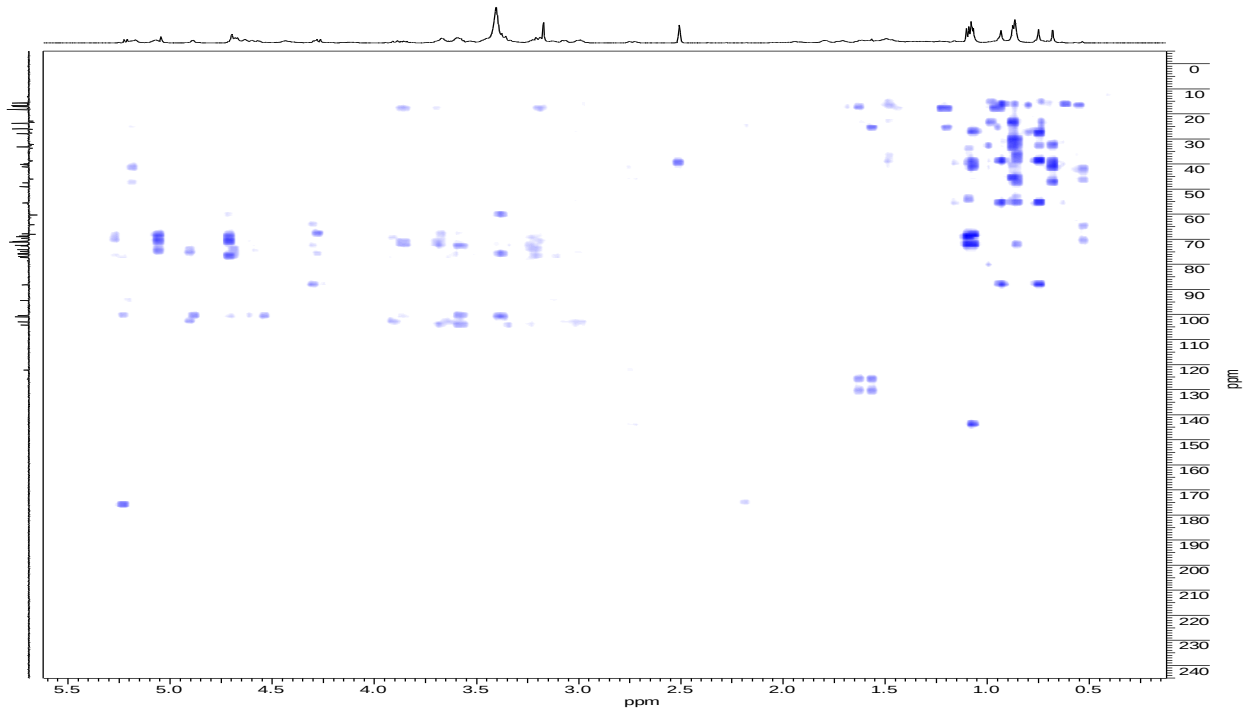
Appendix 12: DEPT-NMR spectrum of compound 17



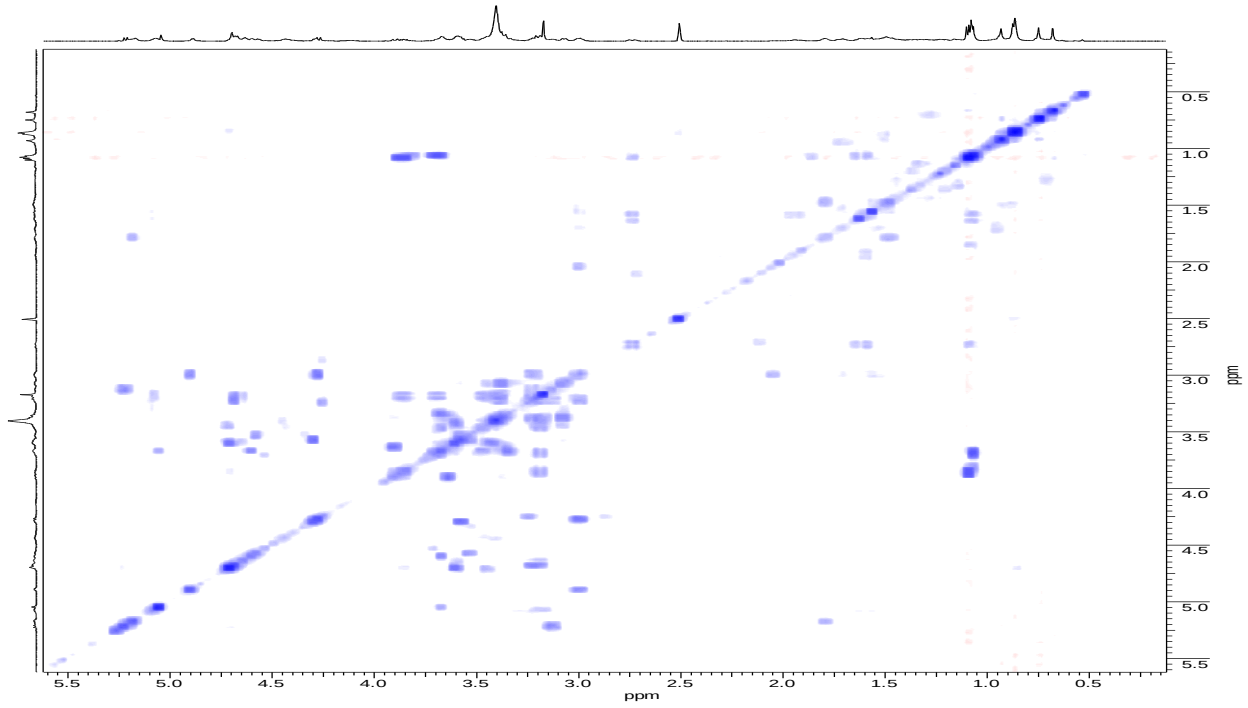
Appendix 13: HSQC spectrum of compound 17



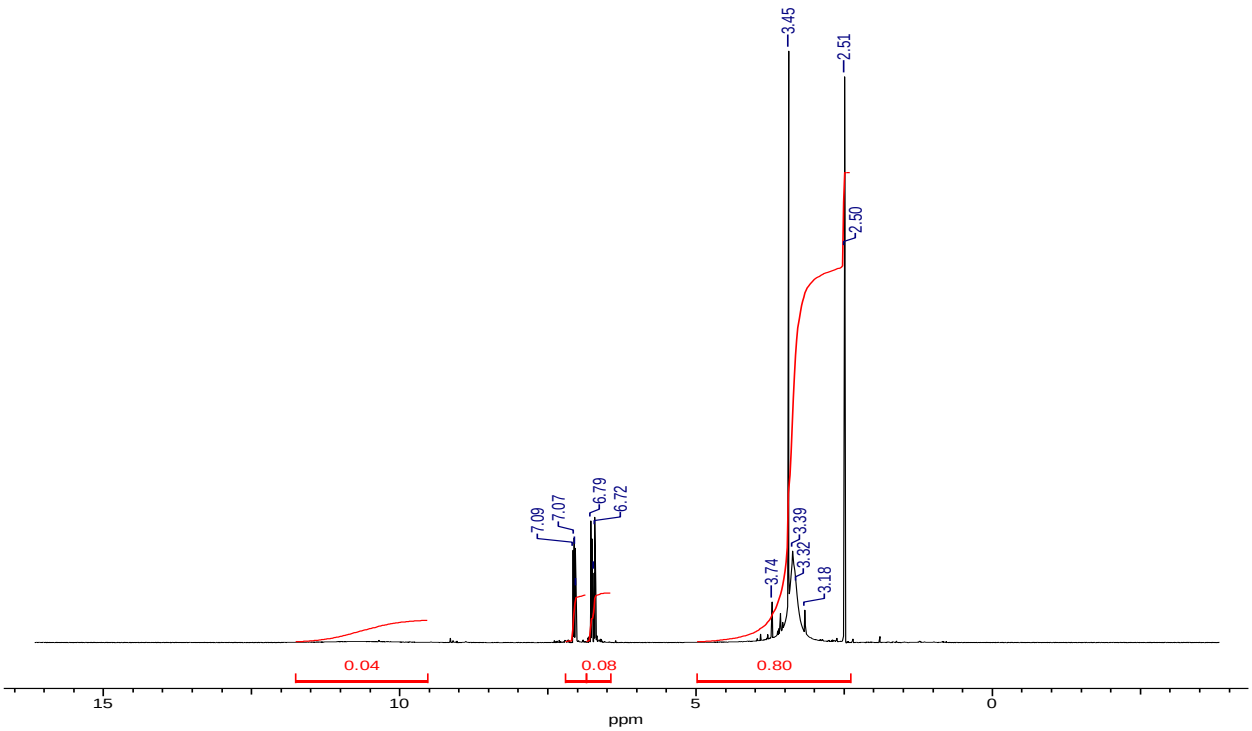
Appendix 14: HMBC spectrum of compound 17



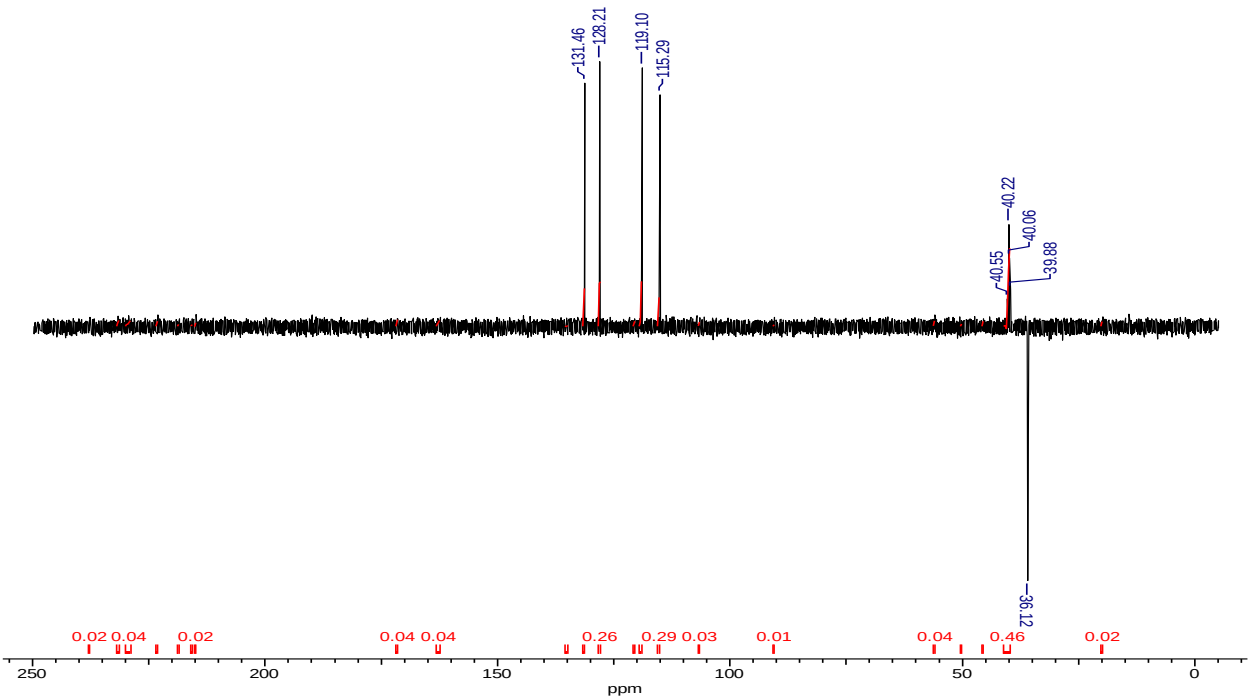
Appendix 15: ^{1}H-^{1}H NMR spectrum of compound 17



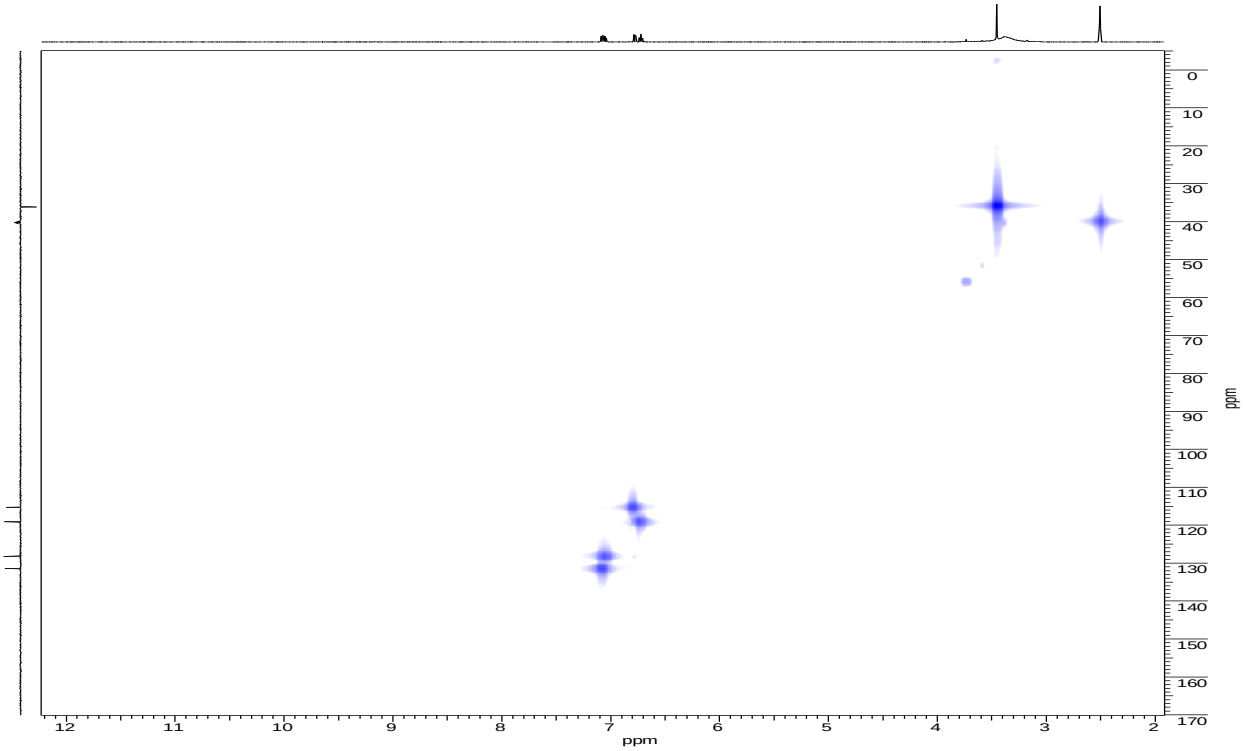
Appendix 16: ^{1}H- NMR spectrum of compound 18



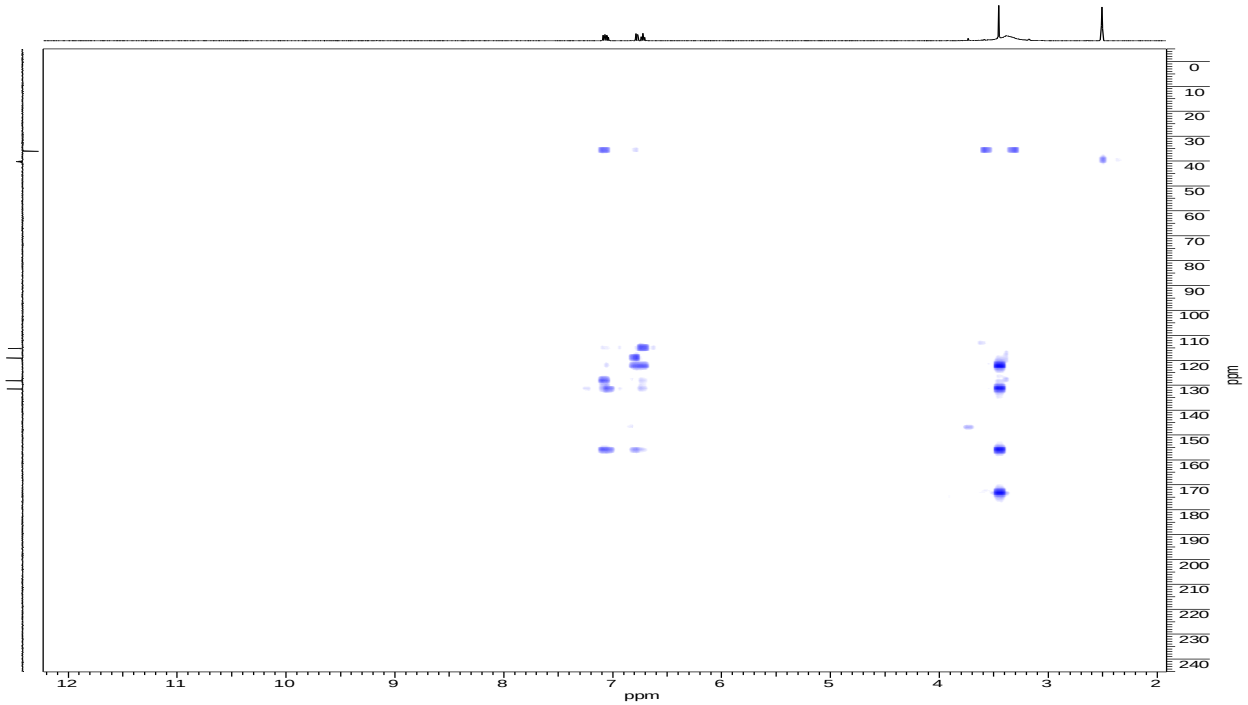
Appendix 17: DEPT- NMR spectrum of compound 18



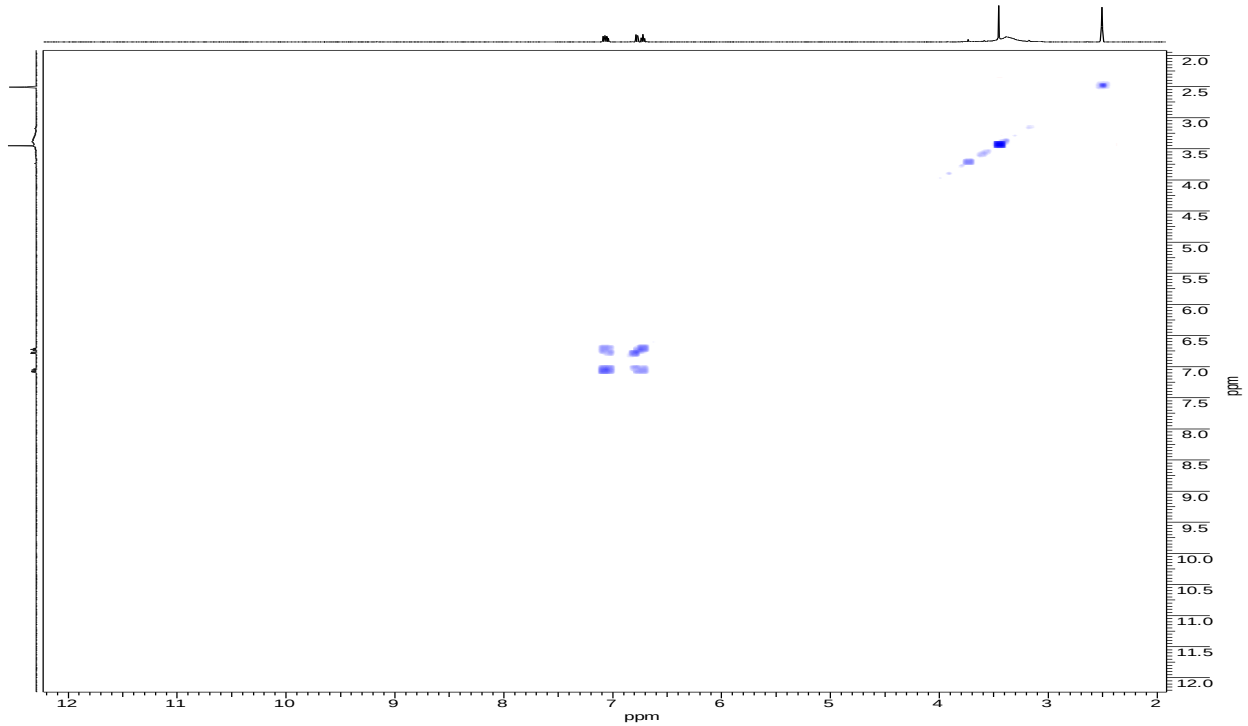
Appendix 18: HSQC spectrum of compound 18



Appendix 19: HMBC spectrum of compound 18



Appendix 20: ^{1}H-^{1}H NMR spectrum of compound 18



Appendix 21: Table showing significance difference of extracts against *K. pneumoniae*

```
ONEWAY pathogen BY Extracts
  /MISSING ANALYSIS
  /POSTHOC=TUKEY BTUKEY LSD ALPHA (0.05).
```

ANOVA

pathogen

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	421.143	6	70.190	58.960	.000
Within Groups	16.667	14	1.190		
Total	437.810	20			

Post Hoc Tests

Homogeneous Subsets

pathogen

	Extracts	N	Subset for alpha = 0.05			
			1	2	3	4
Tukey HSD[a]	7	3	.00			
	4	3		7.33		
	3	3		7.66		
	2	3		8.00		
	1	3		9.33	9.33	
	5	3			11.66	
	6	3				16.00
	Sig.		1.000	.743	.054	1.000
Tukey B[a]	7	3	.00			
	4	3		7.33		
	3	3		7.66		
	2	3		8.00		
	1	3		9.33		
	5	3			11.66	
	6	3				16.00

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 22: Table showing significance difference of extracts against S. *aureus*

ONEWAY pathogen BY Extracts

/MISSING ANALYSIS

/POSTHOC=TUKEY BTUKEY LSD ALPHA (0.05).

Oneway

ANOVA

pathogen

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	479.238	6	79.873	29.427	.000
Within Groups	38.000	14	2.714		
Total	517.238	20			

Homogeneous Subsets

pathogen

			Subset for alpha = 0.05			
	Extracts	N	1	2	3	4
Tukey HSD[a]	7	3	.00			
	3	3		7.00		
	4	3		7.33	7.33	
	2	3		10.00	10.00	
	1	3		10.33	10.33	
	5	3			12.00	
	6	3				17.00
	Sig.		1.000	.339	.070	1.000
Tukey B[a]	7	3	.00			
	3	3		7.00		
	4	3		7.33		
	2	3		10.00	10.00	
	1	3		10.33	10.33	
	5	3			12.00	
	6	3				17.00

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.