ISOLATION OF SECONDARY METABOLITES FROM ENDOPHYTIC FUNGI OF *Syzygium cordatum* (MYRTACEAE) FOR THE CONTROL OF BEAN BACTERIAL PATHOGENS

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DECLARATION AND APPROVAL

Declaration

This thesis is my original work and has not been submitted for the conferment of a degree or award of a diploma in this or any other university:

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DEDICATION

To my daughter Chepkoech Towett and my siblings

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ABSTRACT

Common bean (*Phaseolus vulgaris L.*) is the most consumed leguminous crop among all communities in Kenya. Its productivity is declining due to bacterial infections; common bacterial blight and halo blight. Synthetic agrochemicals are currently used in curbing these bacterial infections in beans but studies have reported lots of problems associated to their usage. Medicinal plants host important microorganism (endophytes) that mutualistically correlate with the host plant and are considered a prolific source of important secondary metabolites that can be used as leads in production of drugs. *S. cordatum* is among these medicinal plants that host important endophytic fungi. This study was carried out to evaluate bioactive endophytic fungi isolated from *S. cordatum* plant and their extractives against bean bacterial pathogens; *Pseudonomas syringae pv phaseolicola* and *Xanthonomas axonopodis pv phaseoli*. Endophytic fungi were isolated from sterilized leaves and stem bark plated on Potato Dextrose agar (PDA) modified with 20 mg/l of streptomycin sulphate, incubated and sub-cultured to their pure fungal cultures. Seven (7) pure cultures were isolated from the plant materials, however, three (3) of them were identified. All isolated fungal endophytes were active against the test organism, with SC-S (9), SC-S (11) and SC-L (7) all identified as *Diaporthe* species showing the highest antibacterial activities against the selected test organism. The three (3) most active fungal endophytes were subjected to fermentation on rice media and secondary metabolites extraction done using methanol. Methanol crude extracts were then suspended in water then thereafter partition between hexane and ethyl acetate yielding hexane and ethyl acetate crude extracts. Purification of SC-S (11) ethyl acetate crude extract on column chromatographic techniques thereafter in preparative High Performance Liquid Chromatography (HPLC) yielded three new compounds; Two geometric isomers, named (*2Z,4Z, 8Z*)- 5-MethylDec-2, 4, 8-triene-1, 6, 7-triol (**20**), (*2E*,*4E*, *8E*)- 5-MethylDec-2, 4, 8-triene-1, 6, 7-triol (**21**) and one phenolic derivate; 2-methyl-5-(1-(3-methylaziridin-2-yl)ethyl)phenol (**22**). Similarly, SC-S (9) yielded one pure compound named; 3-mthoxy-5-mehtylnaphalene-1, 7-diol (**23**). The pure compounds were identified using 1D and 2D NMR analysis and molecular mass confirmation done using High Resolution Mass Spectrometer (HRMS). Agar disc diffusion assay was used to evaluate antibacterial activities of extracts. Agar disc diffusion assay was used to evaluate antibacterial activities of extracts. It showed that all the fungal extracts had activities against *X. axonopodis pv phaseoli* with F2 of *Diaporthe sp3*, showing the least MIC value of 2.5 mg/ml corresponding to zones of inhibition of 8.00 ± 0.58 mm. A weak antibacterial activity of fungal extracts was recorded against *P. syringae pv phaseolicola* with F4 of *Diaporthe sp3* showing the lowest MIC value of 1.25 mg/ml corresponding to zones of inhibition of 7.33 ± 0.33 mm. The results from the study showed a diverse source of antibacterial secondary metabolites from endophytic fungi housed by most medicinal plants and the potentiality of these compounds to be used as leads in control of bean bacterial pathogens especially *X. axonopodis pv phaseolicola.* Therefore toxicological and cytotoxicity tests should be done to evaluate their safety in crop protection application.

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LIST OF ABBREVIATIONS AND ACRONYMS

^{13}C	Carbon-13 NMR
^{1}H	Proton NMR
BBS	Bacterial Brown Spot
CBB	Common Bacterial Blight.
CFU	Colony Forming Unit
COSY	Correlation Spectroscopy
DMSO	Dimethyl Sulfoxide.
HPLC	High Pressure Liquid Chromatography.
IR	Infrared.
LSD	Leads Significant Differences
MIC	Minimum Inhibitory Concentration.
MLND	Maize Lethal Necrotic Disease
NMR	Nuclear Magnetic Resonance.
NOESY	Nuclear Over Hauser Enhanced Spectroscopy
DNA	Deoxyribonucleic Acid
dNTP	Deoxy Nucleotide Triphosphate
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
ODS	Octa Decyl Silane
PDA	Potato Dextrose Agar.
Psp	*Pseudonomas syringae pv phaseolicola*
PTLC	Preparative Thin Layer Chromatography.
Pv	Pathovar
SDA	Sabouraud Dextrose Agar.
SPSS	Statistical Package for Social Sciences
TLC	Thin Layer Chromatography.
UV	Ultra Violet.
Xap	*Xanthonomas axonopodis pv phaseoli*

DEFINATION OF TERMS

Agrochemicals	Chemical compositions used in crop production
Anti phytopathogens	Substance that can act against plant pathogens
Anti-bacterial	Agent that inhibits or destroys the growth of bacterial
***Cis*- isomer**	Form of geometric isomer that has the priority groups on the same side of the plane
Endophytes	An organism especially fungus that lives inside plant's tissues
In vitro	Biological process that occurs under a controlled environment outside the organism always in small scale
In vivo	Biological process always carried out in the living organisms (models)
MIC	The least concentration of antibiotics required to suppress development of pathogens
Pathogens	Disease causing organisms
***Trans*- isomer**	Form of geometric isomers that have priority groups on the opposite sections of the plane
Zone of inhibition	Area around antibiotic disc having no bacterial growth

CHAPTER ONE

INTRODUCTION

1.1. Overview

This chapter evaluates the background studies in relation to the current study; it clearly brings out the problem statement as well as the key objectives.

1.2. Background of the Study

Agriculture in Kenya dominates the economy, having about 25 % contribution to the GDP (Mohajan, 2014). Most of the areas in Kenya are growing maize (*Zea mays L* and common beans (*Phaseoli vulgaris L*), which are the most staple food for mid and low level income earners in Kenya (Beddington *et al.*, 2012; Vermeulen *et al.*, 2012). The beans are grown mostly as an intercrop with maize and it is a major staple food crop for people of all income categories, as a major source of proteins for the poor who cannot afford other alternative sources.

The country consumes approximately 450,000 tons of beans against a local production level of between 150,000 and 200,000 tons harvested from about 800,000 hectares. Apart from providing the cheapest source of protein, bean is also the fastest and most reliable means to generate incomes among the locals in Kenya (Okoth andSiameto, 2011). Microbes are known to be serious pathogens that cause acute effects on plants and in 2012, the entrances of the well-known maize lethal necrotic disease (MLND) caused a serious drop in maize production, especially in the Rift-valley regions, hence creating an increase dependence on beans as the only cheap alternative food crop for the natives (Beyene *et al.*, 2017). The bean production on the other hand have been also adversely affected by bacteria; *Pseudonomas sryingae* pv *Phaseolicola* and *Xanthonomas axonopodis pv phaseoli* which causes Halo blight and common bacterial blight respectively (McGrane andBeattie, 2017). These have

therefore led to a drop in food crop production in Kenya (Naseri andAnsari Hamadani, 2017; Tock *et al.*, 2017; Torres *et al.*, 2017).

The use of synthetic agrochemicals such as copper based agrochemicals or standard antibiotics like streptomycin have in turn increase the productivity in bean farming because of their applications on foliage and infected seeds respectively (Nderitu *et al.*, 2007). This method however, has been proven to cause harmful effects to the environment, direct users and consumers (Hahn, 2014). Cases such as; the occurrences of pesticide residue on the farms, resistance developed by the pathogens have also been reported (Hahn, 2014). This then has prompted an alternative search for anti-phytopathogenic compounds from natural sources which includes; medicinal plants, bacterial and fungal endophytes.

Medicinal plants encompass endophytes, which are host to the plants and do produce bioactive secondary metabolites, which help them defend their host territories from incursion by other microbes and epiphytes. These endophytes do reside in the plant tissues and are among a group of fungi who up-to date haven't been clearly understood, even though they are important to the host plant and community (Mausse-Sitoe *et al.*, 2016; Rho *et al.*, 2018).

Syzygium cordatum is among these medicinal plants species that hosts important endophytic fungi, a plant that grows to a maximum of 20 m and native to high altitude areas of Zimbabwe and Kenya highlands (Orwa *et al.*, 2009; Mausse-Sitoe *et al.*, 2016). The plant's leaves and fruits have been traditionally used in the treatment of diarrhea and other microbial attacks in plants (Sibandze *et al.*, 2010; Tian *et al.*, 2017).

Recently endophytes have been viewed as a source of potent natural products which can be used as anti-cancer agents in pharmaceutical and in agriculture as bio-control agents (Aly *et al.*, 2010; Alvin *et al.*, 2014). Researchers have proven that, plant based anti-phytopathogenic and pesticides are the safer alternatives because they are environmentally friendly, cheaply sourced and are considered specific in action (Crozier *et al.*, 2008; Akula andRavishankar, 2011). This study was based on evaluating anti-phytopathogenicity of endophytic fungi that are host to *S. cordatum* plant and secondary metabolites isolated from its fungal endophytes against selected bean pathogens *P. syringae pv phaseolicola and X. axonopodis pv phaseoli*).

1.3. Statement of the Problem

Kenya depends hugely on agricultural activities, as a source of livelihood to her citizens. Common bean (*P. vulgaris L.*) is among the food crop that is generally consumed by the natives as a cheap source of proteins, vitamins and essential minerals. The productivity of common beans has been to the expected level in recent past, but currently due to increasing population, biotic and abiotic factors, its productivity of 150,000 tons is not up to the demand of 450,000 tons annually. Bacterial infections; common bacterial blight and halo blight caused by *X. axonopodis pv phaseoli* and *P. syringae pv phaseolicola* destroy both the leaves and the seed pods of bean crop, hence partly contributes to the drop in productivity of beans nationally. The use of copper-based foliar spray and standard antibiotics such as streptomycin sulphate is currently utilized for bacterial control in the leaves and seeds of bean crops. Studies have revealed that these synthetic agrochemicals are harmful to the environment, consumers and other organisms within the ecosystem due to their non-biodegradability. This study then investigated a safer source of antibacterial from natural sources.

1.4. General Objective

The broad objective of the study was to isolate secondary metabolites from endophytic fungi of *S. cordatum* and study their antibacterial activities against selected bacterial pathogens of beans

1.5. Specific Objectives

The following objectives guided the study;

i. To isolate and identify the endophytic fungi of *S. cordatum* leaves and stems.
ii. To test antibacterial activities of endophytic fungal cultures, crude extracts and pure compounds against selected bacterial bean pathogens
iii. To characterize the purely isolated bioactive compounds of *S. cordatum* endophytic fungi using spectroscopic techniques (NMR and MS).

1.6. Hypotheses of the Study

The fungal endophytes culture, crude ethyl acetate extracts, fractions from column chromatography and pure compounds of *S. cordatum* endophytic fungi have no antibacterial activities against *Pseudomonas syringae pv phaseolicola* and *Xanthonomas axonopodis pv phaseoli*.

1.7. Justification of the Study

Common bean (*P. vulgaris L*) is one of the most consumed food crop in Kenya and in sub-Saharan African as a whole. It is consumed as a cheap alternative source of proteins and iron for middle and low-income earners, because they cannot afford other sources like meat, which are relatively expensive. The farming of Common beans (*P. vulgaris L.*) is therefore essential to the country's economy and by extension the country's food security. The infection of bacterial pathogens *P. syringae pv phaseolicola* and *X. axonopodis pv phaseoli* have led to a drastic drop on the productivity of this food crop especially in Kenya, in 2012 alone a 30 % drop was

registered by common farmers in Kenya (Mangeni *et al.*, 2014). Chemical control, which includes; the use of copper based foliar spray for infected leaves and streptomycin for infected seeds, have been generally used by most Kenyan farmers. This method though is considered expensive to the common farmers and an environmental hazard due to its non-biodegradability nature.

S. cordatum hosts fungal endophytes, which are considered a prolific source of antibiotics, agrochemicals and pharmaceuticals; however, research has not been exhaustively done to evaluate the anti-phytopathogenicity of secondary metabolites produced by the host fungal endophytes against bean pathogens. This is therefore, necessary to do an extensive evaluation of secondary metabolites from fungal endophytes of higher plants, which can be used in control of phytopathogens especially those that are detrimental to common bean (*P. vulgaris L.*). Hence, there was a need for a research to be done to explore other less toxic and environmentally friendly sources that can be used in the management of these persistent phytopathogenic in common beans. The study will significantly enhance the productivity in common beans and reduce environmental pollution caused by synthetic agrochemicals especially in Kenya.

1.8. Significance of the Study

The findings from this study are paramount, because it chiefly contributes to the Kenyan's bid four agenda specifically on agriculture and industrialization. The usage of synthetic agrochemicals has been adversely affected with lots of drawbacks, which includes, environmental pollution, resistance developed by pathogens and pesticide residue in crops. This therefore has rendered food security in the county at stake; the study therefore tries to bring an alternative source of agrochemicals from natural

sources and tests its applicability in bacterial pathogens in common beans, this is because natural sources are regarded as eco-friendly and specific in action.

1.9. Scope of the Study

The study was carried out to isolate and identify endophytes and their bioactive compounds present in *S. cordatum* leaves and stem barks collected from Mt. Elgon forest.

1.10. Limitation of the Study

The main limitation of the study was that, the experiments were conducted only on *in vitro* analysis. Secondly, the study was also narrowed to solving bacterial pathogenic diseases in common beans, without giving consideration to other pathogenic diseases. Thirdly, the study fully concentrates on *Diaporthe* species host to the plant *S. Cordatum* and not other fungal strains within the plant tissues. These limitations therefore warrant another study to effect its actualization.

1.11. Assumptions of the Study

The plant materials collected from the same plant at the same proximity are assumed to house the same endophytic fungi.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

This chapter will make a review of an in-depth literature search related to the research in question. In other words, it creates a research gap that needs to be filled through a scientific adoption of related theories or modifications of the existing ones. This, therefore, involves a search on the botanical classification of the *S. cordatum* , its geographical distribution, the endophytes present in it and more so related bioactive composition of Myrtaceae family and genus *Syzygium*.

2.2. The Myrtaceae Family

The family includes about 5930 species which are native to tropical and temperate zones of the world biodiversity. Myrtaceae family is divided into; Myrtoideae which have plump fruits and opposite whole leaves as their distinguishing characteristics (Govaerts *et al.*, 2008).

The genera of *Myrtoideae* can be very hard to differentiate in the non-appearance of developed fruits. They are found universal in sub-tropical and tropical regions but mainly in the Australia and Malaysia. On the other hand, sub-family Leptospermoideae (about 80 genera) has dry, dehiscent fruits (capsules) and leaves arranged alternately and are typically found in Australia and its environs (Govaerts *et al.*, 2008).

Johnson and Briggs (1984) challenged the division of Myrtaceae into *Leptospermoideae* and *Myrtoideae* but they acknowledged other 14 tribes within Myrtaceae and found *Myrtoideae* to be polyphyletic. A group of authors in 2008 confirmed the baccate (plump) fruits and as such, the two-subfamily taxonomy does not precisely depict the history of the Myrtaceae family. Thus most of the scientists

have resorted to using analysis that was done by Wilson *et al in 2001* as a preliminary stage on the study of the family.

2.3. Genus *Syzygium*

Syzygium is a genus of flowering plants that belongs to the Myrtaceae family and it comprises of 1200-1800 plant species (Christenhusz andByng, 2016) which natively extent from Africa and Madagascar through Asia (Tuiwawa *et al.*, 2013). Though most of the plant species are also found in Malaysia and Australia with plant species which has not been taxonomically identified (Tuiwawa *et al.*, 2013). *Syzygium aromaticum* is regarded as the most important species because its flowers can be used as spices, some of the comestible species of the genus are planted all over the world's ecosystem and several have become bellicose classes in some island ecosystems. Various species of this genus bears fruits that can be consumed by humans (Biffin *et al.*, 2006).

Syzygium and *Eugenia* were sometime entangled together but the later discovered that the genus has its premier particular assortment in the neotropics, though numerous classes previously classified as *Eugenia* is today encompassed in *Syzygium*, although the former name is still being used in agriculture. In April 2016 a group of researchers was formed to look into the producing the monograph for the genus *Syzygium* working group (Simsek *et al.*, 2010).

2.4. *Syzygium cordatum* (Water Berry)

It's an evergreen water-loving tree, which grows to a height of (10-15m), the tree is mainly found in the forests or swampy spots or in an open grassland high altitude of forest zones, just like the Mt. Elgon forest (Hitimana *et al.*, 2004). The plant has elliptic circular blue leaves with white fragrant flowers and oval dark purple berries when ripe (Plate 2.1) (Orwa *et al.*, 2009)

Plate 2.1: Photographs showing leaves and berries of *S. cordatum* (De Wet *et al.*, 2010).

2.4.1. Traditional Uses of the plant *S. cordatum*

This tree is known for its many uses. The fleshy fruit is edible, slightly acid in flavor, and is eaten by children, monkeys, bush-babies and birds (Sibandze *et al.*, 2010). The ripe fruits were used in brewing fermented drinks and the pounded stem-bark used as a fish poison (Maroyi, 2018). In central Africa, the tree is known as a remedy for stomach ache and diarrhea (Sibandze *et al.*, 2010). It was also used to treat air-borne related diseases like tuberculosis (Orwa *et al.*, 2009)

2.4.2. Distribution and Habitat of *S. cordatum*

Syzygium cordatum is found in lengthways stream-banks eastwards from Cape through KwaZulu-Natal and northwards to Mozambique (Pillay *et al.*, 2013). It grows in forest margins, in the bush, in riverine thicket and forest, and in the open grass and sometimes high country and forested areas of high altitudes in Kenya (Orwa *et al.*, 2009).

2.5. Biological Activities of Some *Syzygium* species

Biological activities of *Syzygium* species extracts have been recently studied. The variety of secondary metabolites extracted from *Syzygium* species, explains the

diversity of their biological activities. Among the diverse biological activities, antibacterial studies are the most reported. Antibacterial activity was found to be common to all species in all extracts followed by antidiabetic then antimalarial. Antidiarrheal was reported in methanolic extracts of *cordatum* and *guineense* Maliehe *et al.* (2015), while antioxidant activity was reported in leaves hydro distillates of *cumini* and *guineense*. Antibacterial compounds are mainly lipophilic and will partition from an aqueous phase into bacterial membrane structures, causing expansion of the membranes, increased fluidity, disordering of the membrane structure and inhibition of membrane-embedded enzymes. The biological activities of *Syzygium* species that have been studied and documented are listed in Table 2.1

Table 2.1:

Biological activities of some *Syzygium* species extracts

Plant species	Part	Extracts	Biological activities	Reference
Syzygium alternifolium	leaves	methanolic	antibacterial	Pushpangadan (2017)
Syzygium cordatum	leaves	methanolic	antibacterial	Maliehe *et al.* (2015)
	fruit pulps	methanolic	antidiarrheal	Sibandze *et al.* (2010)
	seeds	methanolic	antibacterial	Maliehe *et al.* (2015)
	roots	methanolic	antibacterial antidiarrheal	Maliehe *et al.* (2015) Sibandze *et al.* (2010)
Syzygium cumini	leaves	aqueous methanolic hydro-alcoholic	anti-allergic anti-microbial antioxidant antibacterial	Pushpangadan (2017)
Syzygium guineense	leaves	aqueous	antimalarial	Tadesse and Wubneh (2017)
		hydro distillation	antibacterial antioxidant	Ayele and Engidawork (2010)
	stem bark	methanolic	antihypertension	Tankeu *et al.* (2016)

2.6. Secondary Metabolites of Myrtaceae Family

Some of these compounds isolated the family myrtaceae includes; isoprenoid (**1**), a mono-terpenoid essential oil, (Simsek *et al.*, 2010), 1,8-cineol (**2**), Linalool (**3**) and α-

pinene (**4**) which represent the major fraction of essential oils, (Verdeguer *et al.*, 2009). On the other hand, Palmitic acid (**5)** and arachidic acid (**6**) were also isolated from Myrtaceae family and it represents the major fractions of saturated hydrocarbons, (Simsek *et al.*, 2010). Oleic (**7**) and linolenic acid (**8**) fraction isolates represent the unsaturated hydrocarbons from the plants in Myrtaceae family (Simsek *et al.*, 2010). Other compounds that were also isolated include Gallic acid (**9**) and myricetin-3-*O*-β-glucoside (**10**) (Sharma, 2018).

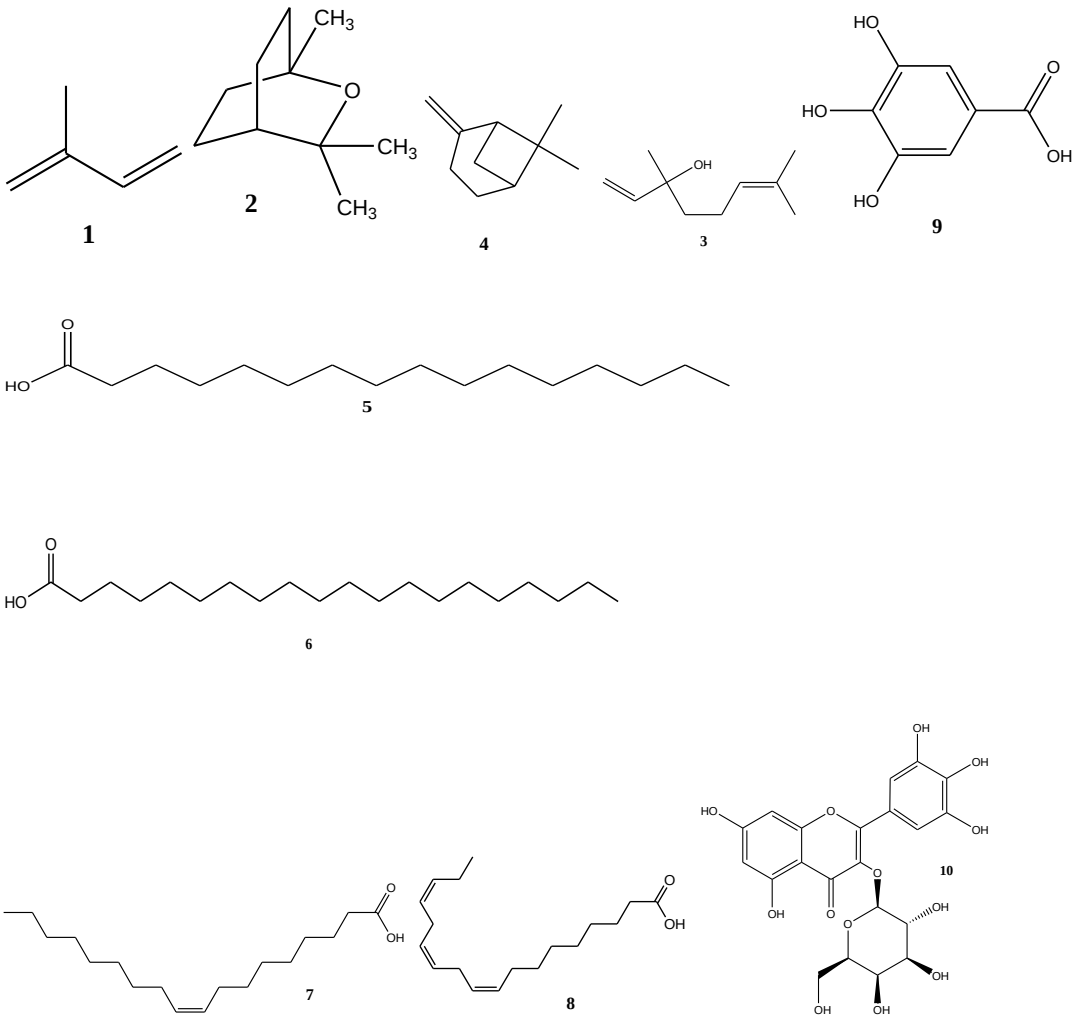

Figure 2.1: Chemical structures of some bioactive compounds of Myrtaceae family.

2.7. Secondary Metabolites Previously Isolated from *Syzygium cordatum* Plant

Some of the secondary metabolites isolated from the plant *S. cordatum* include; friedelin (**11**), gallic acid (**8**) and ellagic acid (**12**) all isolated from the wood bark. Leucocyanidin (**13**) and leucodelphinidin (**14**) were isolated from both the leaves and the bark (Chalannavar *et al.*, 2011). Hydro distillation of *S. cordatum* fresh leaves yielded 1.13 % essential oil, with 60 different compounds in it. 6, 10, 14-trimethylpentadecane-2-one (14.4 %) (**15**) and 2, 3-butanediol diacetate (13.3 %) (**16**) were found to be the major constituents of the essential oil (Chalannavar *et al.*, 2011).

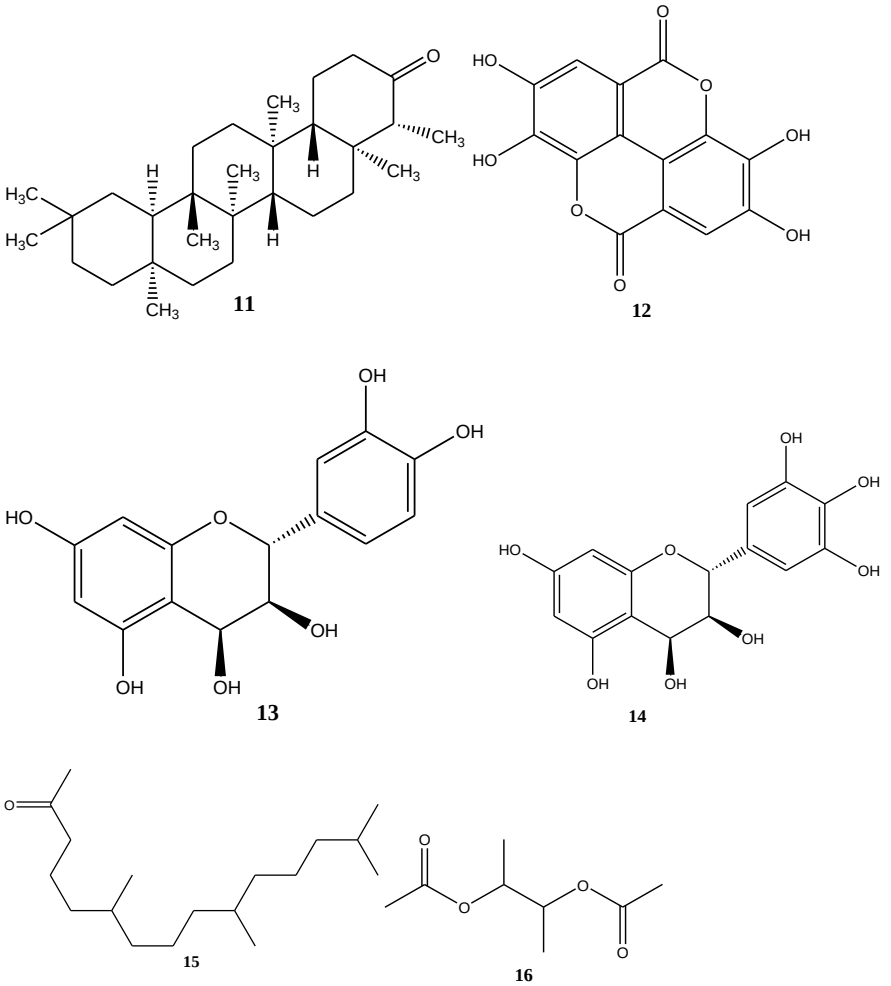

Figure 2.2: Some of the secondary metabolites isolated from *S. cordatum* plant

2.8. Common Beans (*Phaseolus vulgaris L.)*

Common beans (Plate 2.2) are one of the staple food crops consumed by most of the people in East Africa countries and Latin America. It constitutes a significant amount of proteins and other essential micro-nutrients (Petry *et al.*, 2015). In Kenya, common beans are grown mainly in regions; Rift-valley, Nyanza, Central and Eastern province (Katungi *et al.*, 2010). However, the crop has been affected by persistent pathogenic diseases, hence reducing its dependability by common users. Some of these pathogenic effects are discussed in subsequent sub-topics.

Plate 2.2: A photograph of common bean (*Phaseolus vulgaris L.*) (Petry *et al.*, 2015)

2.8.1. Bean pathogens

Phytopathogens are disease-causing agents in plants and it includes fungi, oomycetes, bacteria, viruses, viroids, phytoplasmas, protozoa, nematodes and s crounging plants (Horsfall, 2012). Some of these diseases are Halo blight (caused by *P. syringae*, bacterial brown spot (caused by *P. syringae*) and common bacterial blight caused by (*Xanthonomas campestris* or *X. axonopodis* (Schwartz, 2011).

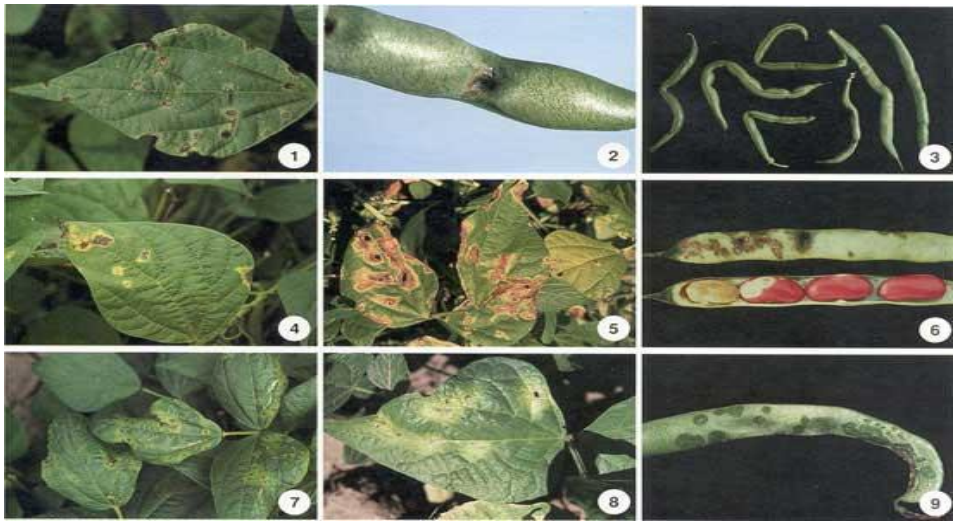

Plate 2.3: Photographs showing pathogenic effects in the bean plant (Schwartz, 2011).

2.8.2. Symptoms and signs

i. Bacterial Brown Spot (BBS)

The condition starts with water-soaked like leaves with characteristic necrotic brown spots about 3-8 mm in diameter, frequently with a thin, long-winded buttery margin (Plate 2.3- (1). The scratches may broaden, merge and drop out forming a tattered appearance with Recessed brunette acnes on the pods (Plate 2.3- (2). On the early stage of development, this infection can cause the pod twisted at the affected points (Plate 2.3- (3) (McGrane andBeattie, 2017).

ii. Common Bacterial Blight (CBB)

Leaf symptoms initially appear as water-soaked spots that become necrotic, light brown lesions of irregular shape with distinct, bright yellow margins (Plate 2.3- (4). These gashes expand to 10 mm or more (Plate 2.3- (5) and may kill the leaflet. Comparable water-soaked acnes formed on hulls and expand into rosy tanned grazes (Plate 2.3- (6). In moist weather, buttery microbial fluid-like droppings may be present on the base of the bean pod. Infected developing seeds may abort or shrivel and discolour as they mature (Plate 2.3 - (6) (McGrane andBeattie, 2017).

iii. Halo Blight

This infection at early stages seem as minor water-soaked colouration on the base of the developing leaves and ultimately emerging into plentiful minor, reddish-brown scratches on the leaves (Plate 2.3 - (7). Greenish-yellow coronae with varied size successively appear around the base of the pod (Plate 2.3- (7 and 8). At the advanced stage of this infection may completely cause streak and ultimate death of the new flora. Hull indicators originally seem as small water-soaked plugs and lines on its surface (Plate 2.3- (9**)** (Schwartz, 2011; McGrane andBeattie, 2017).

2.8.3. Diseases' cycle

i. Common Bacterial Blight (CBB)

The disease can persist in/on a kernel and the infected seed is the origin of the causative agents. The dregs of the bean plant are also chief sources of this pathogenic bacterium and commonly persist on the surface deposits of the residue than in the soil debris. This bacterium infection can be shifted to a new zone via seeds exchange from infected zones and secondarily spread through wind, rain or other agricultural activities like irrigation. Common bacterial blight increases are ideal at high temperature and humid conditions. This then affects the yield of the beans if the infection occurs at early stages (Vidaver, 2012).

ii. Halo blight

It is primarily spread by using infected bean seeds. The bacterium *P. syringae* persistently survive for a period of more than 4 years in the seed, and just a single seed is enough to cause a serious epidemics (Tock *et al.*, 2017).

2.9. Pathogenic Control in Common Beans (*Phaseolus vulgaris L*)

Pathogenic control in beans lately, entails the use of synthetic agrochemicals. These agrochemicals include; organophosphates (OPs), chlorinated hydrocarbons (CHCs)

and carbamates (Cs) (Hahn, 2014). The mentioned agrochemicals have been used for years in control of pathogenic diseases in beans. Chemical control in common beans have gained good acceptance in its control but a series of problems associated with them have been reported, i.e. these chemicals possess toxins that are lethal to human, they are non-biodegradable hence a major environmental hazard and that the pathogens have developed resistance towards them, hence rendered less-effective (Hahn, 2014). An alternative search for potent anti-bacterial from natural sources is therefore necessary. These sources include endophytes; microorganisms that reside inside the tissues of healthy plants and are reported to produce bioactive compounds that can be isolated and utilize as a potent source of anti-bacterial.

2.10. Endophytes

An endophyte is a bacterial or fungal micro-organism, which spends the whole or part of its life cycle colonizing inter-cellular inside the healthy tissues of the host plant without causing any immediate, overt negative effects (Ibáñez *et al.*, 2017). The most frequently isolated endophytes are fungi (Hyde andSoytong, 2008). Examination of plant material can lead to the isolation and identification of these endophytes (fungi and bacteria) (Alvin *et al.*, 2014). The fungi isolated are often host specific (Hyde andSoytong, 2008), 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. The number of these species potentially associated with plants can reach several hundred. Furthermore, it has been reported, that endophytes are found in marine algae, mosses and ferns (Raghukumar, 2008).

These endophytes exist inside the tissues of nearly all healthy plants and they create a symbiotic to pathogenic kind of relationship with the host plant (Aly *et al.*, 2010). They work together with the host plant by producing useful substances, such as

bioactive secondary metabolites, that prevent the plant from being infested by fungi and pests (Aly *et al.*, 2010; Fuchs *et al.*, 2017). Endophytes may also produce excess substances of probable uses too many industries (Aly *et al.,* 2010). Endophytes do provide greater prospects on the development of new drugs that can be used in fighting diseases in people, plants and animals (Aly *et al.*, 2010; Fuchs *et al.*, 2017).

2.10.1. Endophytic fungi

Fungal endophytes are defined as a group of organisms capable of living in host plants tissue without causing any symptoms (Messelink, 2017; Noor *et al.*, 2018). Endophytic fungi have been divided into four classes based on; host range, type of tissue(s) occupied, the establishment in planta and assortment in planta, broadcast and suitability benefits. Endophytic fungi are extremely ubiquitous; it is thought that the vast majority of plant species in natural ecosystems harbor fungal endophytes (Noor *et al.*, 2018). Endophytic fungi are estimated to be represented by at least one million species residing in plants (Noor *et al.*, 2018).

2.10.2. Isolation of endophytes

Endophytes vary in distribution depending on the biotic, abiotic and experimental factors. They are known to colonize the host plant either locally or systematically (Guo *et al.*, 2008). Their study then can be done through a direct observation method or a caltiva method. Direct observation method involves the use of microscopic techniques such as the use of Light or Electron microscope in the visualization of mycelia and other physical properties. The method is used in the study of endophytes that may not grow on standard nutrient media like PDA or SDA. The morphological information obtained on the above method may not satisfactorily place the fungi into any taxonomical level this is because most of the fungi do not form spores. The method, therefore, may not be used in the identification of fungal endophytes (Alvin

et al., 2014). Caltiva method, on the other hand, has been useful in isolation and identification of endophytes. The method involves surface sterilization of the plant materials using standard disinfectants like ethanol or sodium hypochlorite to remove epiphytes and other microbes on the surface of the materials. The time under which the disinfection process should last depends on the thickness of the plant material, the host plant, tissue age or sensitivity to the sterilants. Even though the process is time-consuming but it is very crucial in avoiding contamination of fungal isolates on the growth medium. The surface sterilized materials are the aseptically sectioned into small portions, blotted dry then transferred into growth media such as PDA (Potato Dextrose Agar), SDA (Sabouraud Dextrose Agar) or MEA (Malt Extract Agar). In the method, the growth media is usually modified with standard antibiotics such as Chloramphenicol, Streptomycin sulphate, tetracycline and many others to prevent the development of bacteria on the media (Messelink, 2017; Shiono, 2017; Rho *et al.*, 2018).

2.10.3. Identification of fungal endophytes

Endophytes have a growing importance in the field of pharmaceutical industries as a source of antibiotics and as bio control agents, this then enhances the need for identification, as they are isolated from the host plants. Morphological and molecular identifications are the two that have been adopted for the study and identification of endophytes (Aly *et al.*, 2010).

i. Morphological identification

The method encompasses observation of physical features of endophytes. Generally, it involves the observation of hyphae, colour, texture, margins and elevation of the media by use of Microscopic techniques. The method may not be effective in the identification of fungal endophytes because most of them do not form spores hence

may not be effectively placed on their respective taxonomic keys. The method, therefore, may not be independently used for identification of fungal endophytes (Guo *et al.*, 2008).

ii. Molecular Identification

Molecular identification method takes into account the genetic information of the fungi, which are very specific to each organism. For this reason, therefore, this method is considered the most effective way of identifying non-sporulating fungal endophytes. In the method, DNA extraction from a 7-day old culture is extracted using BIO BASIC EZ -10 Spin column mini-prep kit according to manufactures instructions (Bio Basic Inc.). Ribosomal internal transcribed spacer (ITS) regions, which are regarded as the most conserved regions, are always targeted because they are useful in analysis and identification of lower taxonomic levels. PCR machine is then used for amplification by use of a set of primers like ITS1F and ITS4. The PCR products from amplification procedures are passed through Gel electrophoresis for viewing and confirmation of the amplified bands. The amplicons thereafter have to be sequenced to give the nucleotide order, which will be placed on gene bank such as BLAST (Basic Local Alignment Search Tool) for identification of fungal cultures. This method is regarded as the most appropriate for identification of fungal endophytes (Guo *et al.*, 2008).

2.10.4. Large scale fermentation of endophytes

Fermentation is a biotechnological process that involves the conversion of substrates into simple compounds in the presence of an inoculum such as. bacterial or fungal (Abdel-Banat *et al.*, 2010). In the process, carbon (IV) oxide and ethanol alongside secondary metabolites are produced. Abiotic factors such; pH, temperature, media content, culture duration and ventilation within the bioreactor are among the factors

that affect the quality and level of secondary metabolites produced. The metabolites produced ranges from peptide enzymes and antibiotics which are substrate specific (Kusari *et al.*, 2012).

The fermentation process involves inoculum preparation, media sterilization then inoculation of inoculum onto sterile media. This can be achieved through solid or liquid substrate fermentation (Gaden Jr, 1959).

i. Liquid fermentation

This involves the introduction of inoculum on sterilized liquid media like broth or molasses. This method is preferred for bacterial endophytes because they require high moisture content, for their development. Other liquid substrates that can be used include; fruit extracts, soluble sugars or wastewater (Kusari *et al.*, 2014).

ii. Solid state fermentation

This involves the introduction of inoculum on a solid media like; rice, wheat, bagasse or synthetic solid media. This is one of the oldest methods in the history of biotechnology to be practically used in fermentation of microorganisms. In the method, the substrates are utilized steadily by the microorganisms hence can produce a wide range of useful secondary metabolites. The method is considered one of the best techniques in the fermentation of endophytic fungi (Kusari *et al.*, 2014).

2.11. Extraction, Purification and Identification of Secondary Metabolites

Secondary metabolites are chemical compounds that are produced by bacteria, fungi or plants and are not used in the normal growth, development or reproduction of the organism (Crozier *et al.*, 2008). The chemical compounds are mainly used by organisms for self-defense. Extraction of these organic secondary metabolites is done using a mixture of organic solvents such as; hexane, ethyl acetate, dichloromethane, methanol and many others. The order at which extraction is done depends on the

polarity of the target compounds in the sample. In most cases, Methanol is preferred in first line extraction of these metabolites because it exhaustively extracts almost all the targeted secondary metabolites. Mechanical methods like ultra-sonication can also be used to enhance the extraction of these metabolites, followed by filtration then concentration under a reduced pressure using rotary evaporator to remove solvents used in extractions. The solid crude extracts obtained after concentration are then portioned between organic solvents with increasing polarity. This is done mostly to get rid of fatty acid within the sample. Mid-polar compounds are preferred due to their chemistry to easily interact and can be used as lead compounds in the formulation of antibiotics. On completion of extraction purification of these secondary metabolites is vital to specifically isolate compounds present in the sample (Azmir *et al.*, 2013). This can be achieved with purification techniques like column chromatography, Gas-chromatography (GC) or High Performance Liquid Chromatography (HPLC).

2.11.1. Column chromatography

This purification technique involves eluding of a single compound from crude mixtures. In the method, the extract passes through an evenly packed silica gel (stationary phase) which are eluded with solvents mixtures determined through intense TLC analysis. The interaction between the sample with the stationary phase (silica gel) and its solubility in the mobile phase (solvent mixtures) is what brings about the separation of compounds. The pooling together of fractions thereafter is achieved through spotting of the eluded fraction on a TLC plate then visualized using UV-light or other illuminating agents such as iodine Vapor and many others (Guillarme *et al.*, 2010).

2.11.2. Gas-Chromatography (GC)

Gas chromatography is a separation technique that is used in the separation of compounds that can be vaporized without decomposition. In the method, the use of a carrier gas usually an inert gas such as helium or nitrogen is used as mobile phase and layers of liquid or polymer mounted on an inert solid or tubing (columns) acts as the stationary phase. The principles of separation are similar to that of a column chromatography only that in GC the mobile phase is the inert gas and a stationary phase is a liquid composition. Flame ionization detectors (FIDs) are used to monitor separation and are thereafter outputted as chromatographs. The machine also can be fitted with an auto-sampler or coupled with a Mass spectrometer. GC has a database that can combine information on separation and mass in predicting the probable compounds present in the sample. This method is suitable for in separation and analysis of volatile compounds like essential oils (McNair andMiller, 2011).

2.11.3. High Preforming Liquid Chromatography (HPLC)

This is a strong analytical technique is used in separation of compounds, identifying and quantifying the composition of analyte (Snyder *et al.*, 2012). The machine is fitted with a strong pump that pushes the solvents containing the sample through micro-tubes packed with an adsorbent (stationary phase) (Snyder *et al.*, 2012). In this method, the principle is based on the interaction of compounds present in the sample with the stationary phase and its solubility in the mobile phase (solvent mixtures). This technique is phase into; normal phase which is fitted with a polar stationary phase and reverse phase fitted with a non-polar stationary phase. The interplay on the propositions and the gradient of solvent mixtures brings about a good separation of compounds. HPLC can either be analytical; used for profiling or preparative; used when the composition of the sample is not known. Preparative HPLC is fitted with a

fraction collector which is programmed to collect a given section in the chromatograph displayed in the computer system (output devices) (Moldoveanu andDavid, 2012; Adhami *et al.*, 2015).

2.11.4. Nuclear Magnetic Resonance spectroscopy (NMR)

Nuclear Magnetic Resonance spectroscopy is an analytical technique that is chiefly used in the structure determination of compounds (Stothers, 2012). The method utilizes the magnetic properties found within the nuclei. The nuclei upon spinning generate a spin-magnet with a magnetic moment which gets align into two spin states (+1/2 and -1/2), when an external magnetic field is applied (De Graaf, 2019). The energy differences between the two states can be utilized to give useful chemical information. 1D or 2D NMR experiments are run when identifying the chemical information of compounds. 1D experiment include ^{13}C NMR, ^{1}H NMR and DEPT which are used in identifying the number of carbon and hydrogen present in a compound and the type of carbon atom in it respectively. On the other hand, 2D NMR experiments include; HSQC used in identifying connectivity of hydrogen to their respective carbon atoms, HMBC shows the correlation of Proton and carbon that are 2-3 bonds away and COSY which shows a correlation of protons in the neighboring carbon atoms. All the NMR experiments are carried out in Deuterated solvents such as DMSO or $CDCl_3$ to avoid proton interferences in the solvent, TMS is also mixed with the sample to act as an internal standard because it absorbs at a chemical shift of 0 ppm (Ludwig andViant, 2010; Holzgrabe, 2017).

2.11.5. Mass spectrometry

Mass Spectrometry is a strong analytical technique used in quantification of unknown compounds within a sample. The technique is based on ionization of into several ions forms, which are separated according to their specific mass to charge ration (m/z) and

recorded as relative abundances of each ion form. The information therein is useful in identification of molecular masses of compounds and hence their structure determinations (Bjarnholt *et al.*, 2014).

2.12. Secondary Metabolites Previously Isolated from Endophytes

Endophytes are recently regarded as a prolific source of useful bioactive compounds. Compounds isolated from endophytes have been known to possess; Anti-cancer, anti-bacterial, anti-fungal, anti-oxidant and anti-viral activities (Aly *et al.*, 2010). compounds ergosta-5, 7, 22-trien-3β-ol (**17)** and 9-*O*-methylfusarubin (**18**) both isolated from the endophyte *Fusarium proliferatum* showed 100 % cytotoxicity against the brine shrimp *Artemia salina* at 100 µg/Ml (Dame *et al.*, 2016). Mycophenolic acid (**19)** isolated from fungus *Phomopsis longicolla* host to seaweed *Bostrychia radicans showed significant activities against Staphylococcus aureus* and *Staphylococcus saprophyticus with MIC values of 1 and 2 µg/ml respectively (Erbert et al., 2012), hence can be used as antibiotics*

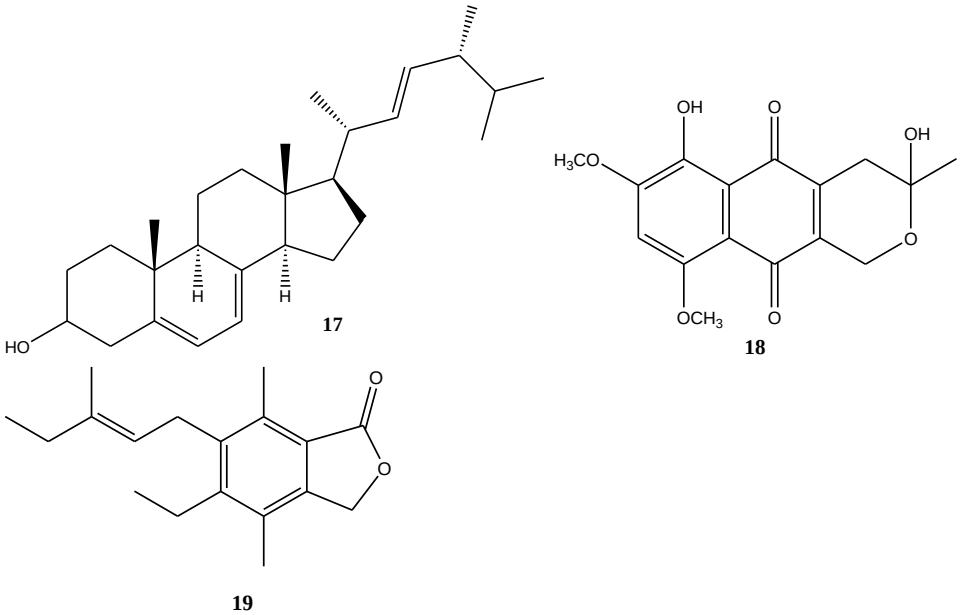

Plate 2.4: Some of the chemical compounds isolated from endophytes

The discussions above have therefore given an insight of the bacterial pathogens that have been reported to cause serious effects in beans (Hahn, 2014). The currently used agrochemicals are reported to cause adverse effects to the environment (soil, water, food and the users) because most of which are non-biodegradable. The bacterial pathogens have also developed a serious resistance towards them and similarly are quite expensive to the common farmers here in Kenya (Kretschmer *et al.*, 2009; Hahn, 2014). Therefore, there is a justifiable reason to search for the most effective alternative anti-phytopathogenic from natural sources, hence the basis of this study.

CHAPTER THREE

METHODOLOGY

3.1. Overview

This chapter addresses methods that were used in achieving the specific objectives of the study.

3.2. Research Design

Experimental research design was used, which involves the use of various analytical laboratory techniques.

3.3. Samples and Sampling Procedures

Simple random sampling method was used in section of plants, where the leaves and stem barks were collected. The leaves and the stem berks were also collected in random from the identified plants. The isolation of fungal endophytes was conducted thereafter.

3.4. Collection of Plant Materials

Fresh leaves and stem bark of *S. cordatum plant were collected from Mt. Elgon forest (01* ^{0} 08'00'' N 34 ^{0} 35'00'' E or 1.13333 ° N, 34.583333 ° E), taken to the Botany Department of Egerton University for scientific identification by a taxonomist. Isolation of fungal endophytes was thereafter done within eight hours after collection of plant materials to avoid drying up at the biotechnology lab of Egerton University

3.5. Experimental Procedures

The following are the main experimental procedures, which aided in actualization of the specific objectives of the study.

3.5.1. Media preparation

A concentration of 39 g/L of Potato Dextrose Agar (PDA), 65 g/L of Sabouraud Dextrose Agar (SDA) and 38 g/L of Muller Hinton Agar (MHA) was prepared. The media was sterilized at 15 lbs, 121 °C for 15 minutes, removed and allowed to cool to 45 °C, then plated on sterile Petri dishes in a laminar flow. A concentration of 200 mg/L of streptomycin sulphate was added to SDA and PDA plates after sterilization to inhibit bacterial growth for endophytic fungal isolation and serial sub-culturing procedures.

3.5.2. Isolation of endophytic fungi

Endophytic fungi were isolated from internal plant tissues using the method used by Tian *et al.* (2017). In this method, the leaves and stem of the selected healthy plants were washed in running tap water to remove any soil or other foreign materials and blotted dry. The leaves/stems were then sectioned to approximately 1 mm by 4 mm sizes. The sectioned plant materials were then surface sterilized for 5 minutes in 10 ml of 1 % sodium hypochlorite followed by 10 ml 70 % ethanol. Thereafter the materials were rinsed three times with sterile distilled water to wash off disinfectants. The surface disinfected materials were then plated in petri-plates containing Potato Dextrose Agar (PDA) amended with 200 mg/L concentration of streptomycin sulphate. The plates were sealed using Para film and incubated at 25 ± 2 ºC in a light chamber. The petri dishes were daily checked to monitor the growth of fungal endophytes from the plant materials (Plate 3.1). On the emerging of the vegetative parts of the fungi from plant segments, isolation and sub-culturing were done bringing them to pure culture by series of serial sub-culturing.

Plate 3.1: A photograph of sprouting fungal endophytes from stem bark of *S. cordatum*

3.5.3. Pre-Screening of endophytes for antibacterial activities

Antimicrobial activity of isolated endophytic fungi against bean bacterial pathogens was done using the method explained by Arya and Sati (2011). Where; the pathogenic bacteria; *P. syringae pv phaseolicola* (gram-negative bacterium) *and X. axonopodis pv phaseoli* (gram-negative bacterium) were inoculated in 50 ml conical flasks containing nutrient agar medium and incubated at 37 ± 2 ^{0}C for 24 hrs. After 24 hours, one loop of each pathogenic culture was transferred to Erlenmeyer flasks containing distilled water and shaken until the turbidity of bacterial suspension is comparable to the turbidity of 0.5 McFarland's standard solution ($1.5 \times 10^{\wedge 8}$ CFU/ml) which was prepared by mixing 0.05mL of 1.175 % Barium chloride ($BaCl_2.2H_20$) in 9.95 mL of 1 % Sulphuric acid). 100 µL of each suspended bacteria ($1.5 \times 10^{\wedge 8}$ CFU/ml) were inoculated in petri dishes containing Muller Hinton agar using sterile micro-dispenser. The six-millimeter diameter of 7 day actively growing mycelial plugs from Potato Dextrose Agar (PDA) plates was cut using a sterile cork-borer and placed on the surface of the Muller Hinton agar media. These plates were sealed with Para film and incubated at 37 ± 2 °C for 24 hours for complete diffusion of antimicrobial compounds and observed for the zone of inhibitions. The zones of inhibition were measured in millimeters using a ruler scale. The experiment was performed in triplicates.

3.5.4. Large scale fermentation of fungal endophytes

This was done in a solid media experiments which were carried out in 21, 500 mL Erlenmeyer flasks containing 90 g of parboiled rice in 90 mL distilled water per flask, it was then autoclaved at 120 ºC for 40 minutes for each fungal strain. Agar plugs (6 mm diameter) were cut from the 7-day-old original cultures on PDA agar and placed on the surface of sterile rice media**.** One flask, without inoculum, was kept for control use. After 21 days of incubation, 200 mL of methanol was added to each flask then followed by ultrasonic extraction using Ultra-sonic cleaner. The methanol was then filtered and evaporated using Rotary evaporator under reduced pressure to yield the methanol extract. The methanol extract was then be subjected to liquid-liquid partitioning with hexane (to remove fatty acids) and ethyl acetate. The resulting organic layer was evaporated under reduced pressure to produce the hexane and ethyl acetate extracts.

3.5.5. Chromatographic analysis of ethyl acetate fractions

A good solvent system for running the column was determined first using a series of TLC analysis. The solvents employed were those used during extraction, (hexane, ethyl acetate and methanol). The ethyl acetate crude of the identified active fungal strains SC-L(7), SC-S(9) and SC-S(11) all identified as *Diaporthe* species, were subjected to column chromatography using silica gel with mesh size 70-230 STM supplied by Scharlau Lab chemical supplies Limited. All the samples were reconstituted with a little amount of distilled ethyl acetate to make paste-like slurry. The samples were then loaded into an evenly packed silica gel column using a clean micro-pipette. For SC-S (9) and SC-S (11) a mobile phase of methanol: ethyl acetate: hexane (2: 5: 3) was used, while for SC-L (7) a mobile phase of methanol: ethyl acetate (1:9) was used. In each case, fractions from the column were collected in glass

test-tubes. All the eluded fractions were monitored through TLC analysis and using UV lamp for spots visualization then subsequently grouped thereafter. Test-tubes with identical fractions were pooled together. SC-S (9) and SC-S (11) yielded four fractions, named F1-F4, while SC-L (7) yielded two fractions named F1- F2. All the fractions were screened for antibacterial activities against selected bean bacterial pathogens. Those that showed good activities were further taken for MIC analysis and further purified using preparative HPLC.

3.5.6. Purification of active fractions using PHPLC

Preparative High Performance Liquid Chromatographic system (Shimadzu-UFLC prominence), fitted with an auto-sampler (Model- SIL 20AC HT) and UV-Visible detector (Model-SPD 20A) was used in the separation of compounds. Dry samples obtained from column chromatography were re-dissolved in HPLC grade methanol each to make a concentration of 20 mg/ml. The prepared solutions were centrifuged using Bio-Cote centrifuge, to enhance sedimentation of solids that may block the column. 150 µl of centrifuged samples were then loaded onto an Auto-sampler. This separation was performed on Kromasil reverse phase ODS C18 5 µm column (4.6 × 250 mm). Gradient separation was performed using mobile phase A (100 % Millipore water) and mobile phase B (100 % HPLC grade methanol). The separation condition was set as follows; 10 % of B in A at injection time, 80 % of B in A at 30^{th} minute, 100 % of B at 31^{st} to 37^{th} minute then normalized to 10 % of B in A at 38^{th} minute to 45^{th} minute where the separation process was stopped, and the collected fractions removed and later concentrated under a reduced pressure in Rotary evaporator to yield pure compounds. Oven temperature was maintained at 40 °C and a flow rate of 3 ml/minute. Chromatographic separations were monitored at absorbance ranges of 220-420 nm. Both Millipore water and methanol were of analytical grade supplied by

Scharlau Lab supplies limited. F2 of SC-S (11) yielded three pure compounds, recorded as compound **20, 21** and **22** respectively, while F3 of SC-S(9) yielded one pure compound recorded as compound **23**. High field 1D and 2D NMR spectroscopy and mass spectrometer were performed for Compounds **20**, **21, 22 and 23**.

3.6. *In vitro* Analyses

3.6.1. Antibacterial assay for crude extracts, fractions and pure compounds

Paper disc diffusion inhibition was used to screen for antibacterial activities of crude ethyl acetate extracts, fractions from column chromatography and the purified compounds. 100 µ of bacterial suspensions ($1.5 \times 10^{\wedge 8}$ CFU/ml) was homogeneously spread on sterile Mueller Hinton agar (38 g/l) in Petri dishes. The ethyl acetate fungal extracts, fractions from column chromatography and pure compounds were prepared by dissolving them in 0.1 % DMSO in distilled sterile water. The sterile paper disc was soaked in 50 mg/ml concentration of the prepared extracts then placed at the Centre of MHA plates containing the bean bacterial pathogens. A sterile disc dipped in 0.1 % DMSO was used as a negative control, while standard chloramphenicol antibiotic was used as a positive control for the anti-bacterial activities. The plates were sealed using para film and inoculated at 37 ± 2 °C for 24 hours, after which the zones of inhibitions were measured in millimeters using a ruler scale. This experiment was done in triplicates.

3.6.2. Determination of Minimum Inhibitory Concentration (MIC)

The extracts and the pure compounds showing anti-bacterial activity in the pre-screening assay were evaluated for their Minimum inhibitory concentration (MIC) using a method explained by Balouiri *et al.* (2016), whereby; serial dilutions of the identified extracts and compounds with appreciable inhibition zones was dissolved using 0.1 % dimethyl sulfoxide (DMSO) in distilled sterile water. The crude extracts

and the purified compounds were diluted to the following serial geometric dilutions: 75 %, 50 % and 25 %. The subsequent minimum inhibition zones were used to determine the array for MIC analyses. In all cases, the cultured plates were incubated at 37 ± 2 °C for 24 hours. The lowest concentration able to induce inhibition zones was considered as the MIC. The experiment was performed in triplicates.

3.7. Structure Elucidation

3.7.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{1}H, ^{13}C, DEPT, HSQC, COSY and HMBC NMR spectra were recorded on the Bruker Advance 500 MHz NMR spectrophotometer at the Technical University of Berlin, Germany. The readings were done in Deuterated DMSO and chemical shifts assigned by comparison with the residue proton ^{1}H and carbon, ^{13}C resonance of the solvent. Tetramethyl-silane (TMS) was used as an internal standard and chemical shifts were given as δ (ppm). 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). HSQC spectrum (Heteronuclear Single Quantum Coherence) was used to determine the connectivity of hydrogen to their respective carbons atoms.

3.7.2. Mass spectrometry

The compound's mass spectra were recorded on FinniganTripple Stage Quadrupole Spectrometer (TSQ-70) with electron spray ionization (ESI) method in the analysis. Thermo X Calibur Qual computer software was used in the analysis of the mass chromatograms.

3.8. Statistical Analysis

Comparison of means for antibacterial activities was done using SPSS version 25.0 and the most bioactive endophytes or secondary metabolites was selected based on the antibacterial as shown by the size of inhibition zones. The difference in the mean inhibitory effect of each fungal extract was determined using one-way ANOVA; Where correlation of their antibacterial activities against positive and negative control were performed through Tukey's Honestly Significant Difference (HSD) test, at $P < 0.05$, Turkey's test). NMR data of pure compounds were analyzed using Bruker topspin 3.5 software.

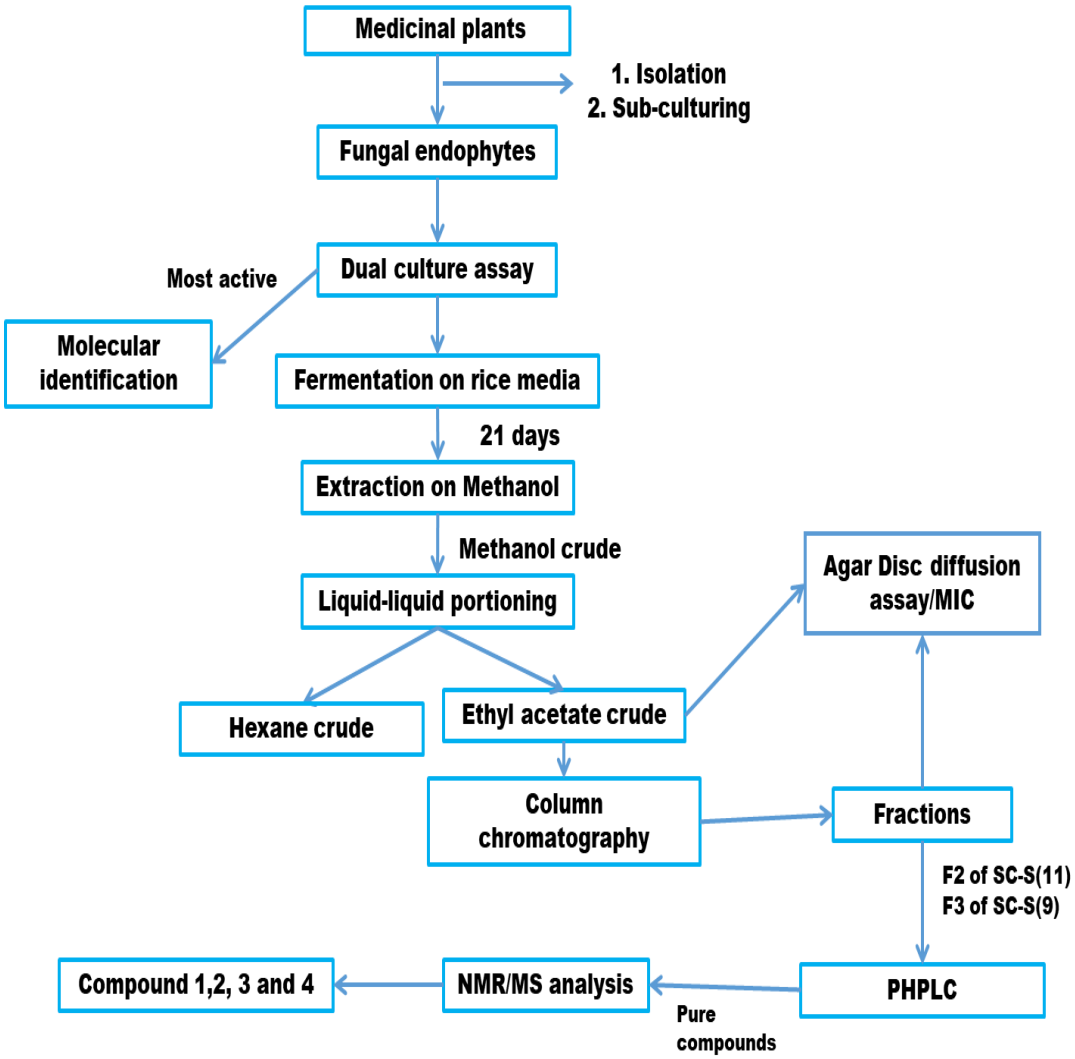

Figure 3.1: Summary of methodology

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Introduction

This chapter addresses the research data and a comprehensive discussion supporting the experimental data.

4.2. Isolation and Identification of Endophytes

A total of 7 endophytes were isolated from *S. cordatum plant* (Plate 4.1). After antagonistic assay, three most active endophytes; SC-S (9), SC-S (11) and SC-L (7) (Plate 4.1) were taken for molecular identification at the Institute of Earth and Life (Louvain-la-Neuve) Belgium.

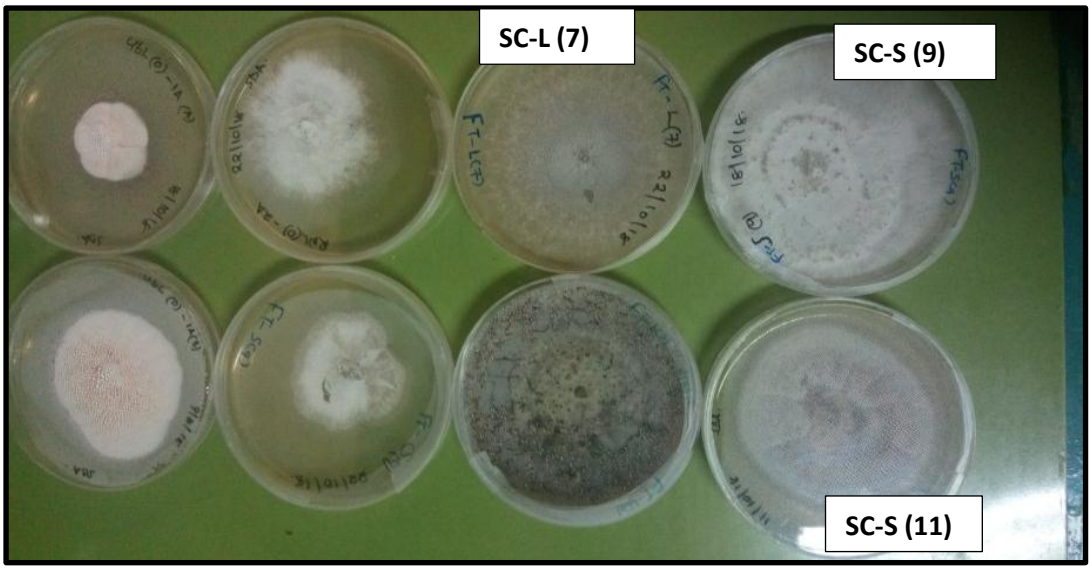

Plate 4.1: Fungal endophytes isolated from the plant *S. cordatum*

In this study, fresh leaves and stem bark of *S. cordatum* plant were used in isolation of endophytic fungi in PDA media. Plant tissues house varied fungal or bacterial endophytes that are known to mutualitically relate with the host plant (Messelink, 2017). They penetrate into plant tissues through cuticles and coexist inside the plant tissues (Noor *et al.*, 2018). Most of these endophytic fungi are non-sporulators the fungal endophytes then makes morphological identification less effective for in placing them on their respective taxonomical levels (Raviraja, 2005).

Molecular identification was used in identifying the three most active fungal endophytes: SC-S (9), SC-S (11) and SC-L (7). The results showed that they all belong to genus *Diaporthe* of phylum *Ascomycota* kingdom fungi. SC-S (9) and SC-S (11) showed a close correlation with *Diaporthe sophorae* while SC-L (7) was closely related to *Diaporthe phaseolorum* according to BLAST information (Appendix 1). *Ascomycota* are generally referred to as "sac-fungi" because of their ability to form sac-like structure (Gomes *et al.*, 2013). These findings are in agreement with other studies which showed that, most fungal endophytes isolated from medicinal plants belong to the phylum *Ascomycota* (Thangadurai *et al.*, 2016). On the other hand, genus *Diaporthe* is the most encountered genera of fungal endophytes in several host plants. The genus is known to be a source of enzymes and bioactive secondary metabolites having anti-bacterial, anti-cancer and anti-fungal activities. In the past, plants in the genus *Diaporthe* have been known to produce biochemical that deters herbivores, hence can be used as a bio control agent (Radji *et al.*, 2011; Gomes *et al.*, 2013; Li *et al.*, 2015).

As compared to this study, endophytic fungi of genus *Diaporthe* has also been isolated from plants such as acacia, *Maytenus ilicifolia, Berberis vulgaris* having substantial anti-bacterial and antifungal activities (Aly *et al.*, 2010). Therefore, this shows that endophytic fungi are source of anti-bacterial compounds.

4.3. Antagonistic Assay

In this study, the antagonistic assay of isolated fungal endophytes from the leaves and the stem bark of *S. cordatum* plant with bean bacterial pathogens; *X. axonopodis pv phaseoli and P. syringae pv phaseolicola,* were statistically used to access their dual activities (Appendices 2 and 3). All the isolated endophytes showed activities against the two bacterial pathogens. SC-S (9), SC-S (11) and SC-L (7) all identified to be in

the genus *Diaporthe* showed appreciably higher activities against both *X. axonopodis pv phaseoli and P. syringae pv phaseolicola* (Table 2). Of the three, fungus SC-L (7) had the largest zones of inhibition of 20.00 ± 0.00 mm against *X. axonopodis pv phaseoli* and 17.33 ± 1.45 mm against *P. syringae pv phaseolicola.* Based on their activities the three endophytic fungi were selected for large-scale fermentation in solid media. Leven's test showed a non-uniformity of the variance in their zones of inhibitions of isolated fungi against selected bean bacterial pathogens, with a p-value of 0.978 (Appendices 2 and 3). The activities of endophytic fungi SC-S (9), SC-S (11) and SC-L (7) had no significant difference with that of chloramphenicol (Table 4.1, Plate 4.2).

Table 4.1:

Inhibition zones (mm) for fungal endophytes isolated from the plant *S. cordatum* against selected bean bacterial pathogens.

Fungal endophytes		**Zone of inhibition (mm)**	
isolation code	**Identity**	***P. syringae pv phaseolicola***	***X. axonopodis pv phaseoli***
SC-S(3)	-	9.00 ± 1.00^{a}	13.00 ± 1.53^{a}
SC-S(4)	-	$10.67 \pm 0.67^{a,b}$	12.33 ± 0.33^{a}
SC-S(5)	-	7.00 ± 0.58^{a}	12.00 ± 1.15^{a}
SC-S(7)	-	$11.33 \pm 0.67^{a,b}$	11.33 ± 0.88^{a}
SC-S(8)	-	8.33 ± 1.20^{a}	12.17 ± 2.74^{a}
SC-S(9)	*Diaporthe sp2*	14.00 ± 1.15^{b}	17.00 ± 0.58^{b}
SC-S(11)	*Diaporthe sp3*	$15.67 \pm 0.88^{b,c}$	17.67 ± 0.67^{b}
SC-L(7)	*Diaporthe sp1*	$17.33 \pm 1.45^{b,c}$	$20.00 \pm 0.00^{b,c}$
Chloramphenicol		$20.33 \pm 0.33^{b,c}$	25.00 ± 1.00^{c}

The values given represent the mean of the three experiments ± S.E. within the column; those sharing the same letter (s) are not significantly different in the dual assay. While those with different letter(s) are significantly different in the dual assay (P <0.05, Turkey's test)

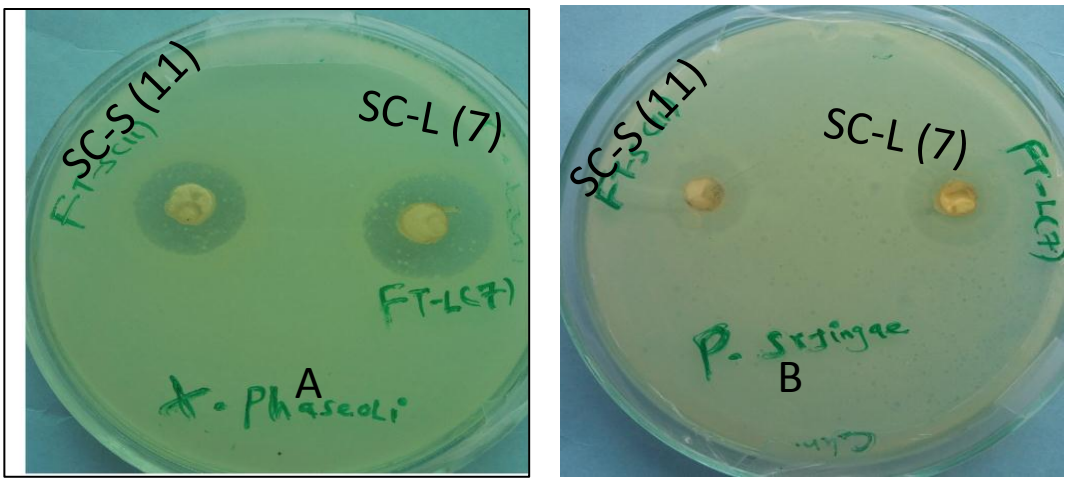

Plate 4.2: Pictorials showing dual culture assay of some fungal endophyte against *Xap* (A) and *Psp* (B)

4.4. Extraction and Purification of Secondary Metabolites

Secondary metabolites from the fermented most active endophytic fungi in dual culture assay (Table 4.1) of *Syzygium cordatum* coded; SC-S (9), SC-S (11) and SC-L (7) were extracted using methanol to yield their respective methanol crude extracts. SC-L (7) methanol crude extract after portioning between hexane and ethyl acetate yielded 2.82 g and 2.11 g of crude extracts respectively. SC-S (9) methanol crude extracts yielded 2.01 g hexane crude and 1.82 g ethyl acetate crude extract. On the other hand, SC-S (11) methanol crude extract yielded 2.03 g hexane extract and 1.92 g ethyl acetate extract.

Anti-bacterial compounds possess mid to high polarity; in this case, ethyl acetate extracts of all the strains were taken for bioassay-guided fractionation. SC-L (7) ethyl acetate extract after fractionation in column chromatography yielded two fractions named; F 1(80.12 mg) and F2 (100.22 mg). SC-S (9) yielded four tractions named; F1 (10 mg), F2 (30.45 mg), F3 (100.18) and F4 (40.98 mg), SC-S (11) had a similar separation profile in column chromatography under the same solvent mixtures, its ethyl acetate extract yielded four fractions named; F1 (10.12 mg), F2 (80.43 mg), F3

(42.14 mg) and F4 (30.02 mg). F3 of SC-S (9) and F2 of SC-S (11) due to their appreciable activities against bean bacterial pathogens; *P. syringae pv phaseolicola and X. axonopodis pv phaseoli* were selected for further purification in preparative High performance liquid chromatographic system (Shimadzu-UFLC prominence), fitted with an auto sampler (model- SIL 20AC HT) and UV-Visible detector (model-SPD 20A). F2 of SC-S (11) yielded three compounds named; SC-S (11)-F2-2 (compound **22**) which appeared as cream yellow solid and two similar compounds SC-S (11)-F2-1(compound **20** and **21**) both appeared as brown solids at room temperature. Whereas, F3 of SC-C (9) yielded one compound, recorded as SC-S (9)-F3-1 (compound **23**).

4.5. Minimum Inhibitory Concentration (MIC) determination

4.5.1. MIC assay against *X. axonopodis pv phaseoli*

Secondary metabolites present in all the extracts showed activities against bacterium *X. axonopodis pv phaseoli (*Table 4.2*).* F1 and F3 of SC-S (9) and F2 of SC-S (11) showed palpable MIC values of 1.25 mg/ml (7.67 ± 0.33 mm), 1.25 mg/ml (7.00 ± 0.00 mm) and 2.50 mg/ml (10.67 ± 0.58 mm) against *Xap* respectively, other MIC values are shown in (Table 4.2). The information above shows that the extracts contains mixture of secondary metabolites that can yield lead compounds to be used in the formulation of agrochemicals to be used in anti-phytopathogenic control in beans (*P. vulgaris L.*).

4.5.2. MIC assay against *P. syringae pv phaseolicola*

Most of the fungal extracts did not show noticeable activity against the bacterium *P. syringae pv phaseolicola*. F1 and F2 of SC-S (9) and ethyl acetate crude, F1 and F2 of SC-S (11) showed appreciable MIC value of 3.75 mg/ml (10.33 ± 0.33 mm), 2.50

mg/ml (7.00 ± 0.00 mm), 1.25 mg/ml (7.33 ± 0.33 mm), 1.25 mg/ml (9.33 ± 0.67 mm) and 2.50 mg/ml (7.00 ± 0.00 mm) other MIC values are shown in (Table 4.2**)**.

Table 4.2:

Zones of inhibitions (mm) of serially diluted endophytic extracts against bean bacterial pathogens

			X. axonopodis pv phaseoli	***P. syringae pv phaseolicola***
Endophytes	**Extracts**	**serial dilution**	**ZoI ± S.E**	**ZoI ± S.E**
SC-L(7) *Diaporthe sp1*	EA	100%	8.67 ± 0.33 [b]	8.67 ± 0.33 [b]
		75%	**7.33 ± 0.33 [b]**	7.67 ± 0.33 [b]
		50%	0.00 ± 0.00 [a]	7.00 ± 0.00 [b]
		25%	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
	F1	100%	11.00 ± 1.00 [b,c]	0.00 ± 0.00 [a]
		75%	10.33 ± 0.33 [b,c]	0.00 ± 0.00 [a]
		50%	**7.00 ± 0.00 [b]**	0.00 ± 0.00 [a]
		25%	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
	F2	100%	13.00 ± 0.58 [c]	10.67 ± 0.33 [b,c]
		75%	10.67 ± 0.33 [b,c]	**9.00 ± 0.58 [b]**
		50%	8.33 ± 0.33 [b]	0.00 ± 0.00 [a]
		25%	**7.00 ± 0.00 [b]**	0.00 ± 0.00 [a]
SC-S(9) *Diaporthe sp2*	EA	100%	12.00 ± 0.58 [b,c]	**7.00 ± 0.00 [b]**
		75%	11.00 ± 0.58 [b,c]	0.00 ± 0.00 [a]
		50%	9.67 ± 0.33 [b]	0.00 ± 0.00 [a]
		25%	**7.00 ± 0.00 [b]**	0.00 ± 0.00 [a]
	F1	100%	14.33 ± 0.33 [c]	12.00 ± 0.33 [c]
		75%	12.33 ± 0.33 [b,c]	**10.33 ± 0.33 [b,c]**
		50%	9.67 ± 0.33 [b]	0.00 ± 0.00 [a]
		25%	**7.67 ± 0.33 [b]**	0.00 ± 0.00 [a]
	F2	100%	13.33 ± 0.67 [c]	12.33 ± 0.33 [c]
		75%	11.00 ± 0.58 [b,c]	11.00 ± 0.58 [b,c]
		50%	9.67 ± 0.33 [b]	7.00 ± 0.00 [b]
		25%	**7.33 ± 0.33 [b]**	0.00 ± 0.00 [a]
	F3	100%	12.33 ± 0.88 [b,c]	10.33 ± 0.88 [b,c]
		75%	11.00 ± 0.58 [b,c]	7.33 ± 0.33 [b]

		50%	9.00 ± 0.58 [b]	0.00 ± 0.00 [a]
		25%	**7.00 ± 0.00 [b]**	0.00 ± 0.00 [a]
	F4	100%	11.67 ± 0.88 [b,c]	0.00 ± 0.00 [a]
		75%	10.00 ± 0.58 [b,c]	0.00 ± 0.00 [a]
		50%	8.00 ± 0.58 [b]	0.00 ± 0.00 [a]
		25%	**7.00 ± 0.00 [b]**	0.00 ± 0.00 [a]
SC-S(11) *Diaporthe sp3*	EA	100%	11.67 ± 0.88 [b,c]	12.67 ± 0.67 [c]
		75%	10.00 ± 0.58 [b,c]	10.67 ± 0.67 [b,c]
		50%	**8.00 ± 0.58 [b]**	10.00 ± 0.58 [b,c]
		25%	0.00 ± 0.00 [a]	7.33 ± 0.33 [b]
	F1	100%	9.00 ± 0.58 [b]	13.33 ± 1.20 [c]
		75%	8.33 ± 0.88 [b]	11.67 ± 0.33 [b,c]
		50%	**7.67 ± 0.67 [b]**	10.33 ± 0.88 [b,c]
		25%	0.00 ± 0.00 [a]	9.33 ± 0.67 [b]
	F2	100%	14.00 ± 0.88 [c]	10.67 ± 0.33 [b,c]
		75%	12.00 ± 0.58 [b,c]	10.33 ± 0.68 [b,c]
		50%	10.67 ± 0.58 [b,c]	7.00 ± 0.00 [b]
		25%	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
	F3	100%	12.33 ± 0.88 [b,c]	0.00 ± 0.00 [a]
		75%	10.00 ± 1.73 [b,c]	0.00 ± 0.00 [a]
		50%	9.00 ± 0.58 [b]	0.00 ± 0.00 [a]
		25%	**7.33 ± 0.33 [b]**	0.00 ± 0.00 [a]
	F4	100%	10.67 ± 0.88 [b,c]	0.00 ± 0.00 [a]
		75%	9.67 ± 0.67 [b]	0.00 ± 0.00 [a]
		50%	8.33 ± 0.88 [b]	0.00 ± 0.00 [a]
		25%	**7.33 ± 0.33[b]**	0.00 ± 0.00 [a]
Chloramphenicol			23.33 ± 0.88 [d]	20.67 ± 0.33 [d]
Negative control			0.00 ± 0.00 [a]	0.0 0.00 [a]

The values given are the mean of the three experiments ± Standard error (S.E). The highlighted values show the zones of inhibition (ZoI) regarded to give MIC values of the fungal extracts. Extracts sharing the same letter (s) within the columns are not significantly different in their anti-bacterial activities. While those with a different letter(s) are significantly different in their activities (P<0.05, Turkey's test). The coding, EA representing ethyl acetate crude extracts of the fungal endophytes while F

(1-4) represents fractions eluded from column chromatographic analyzes. The red colored zones of inhibition represents MIC values of extracts at different geometric dilutions

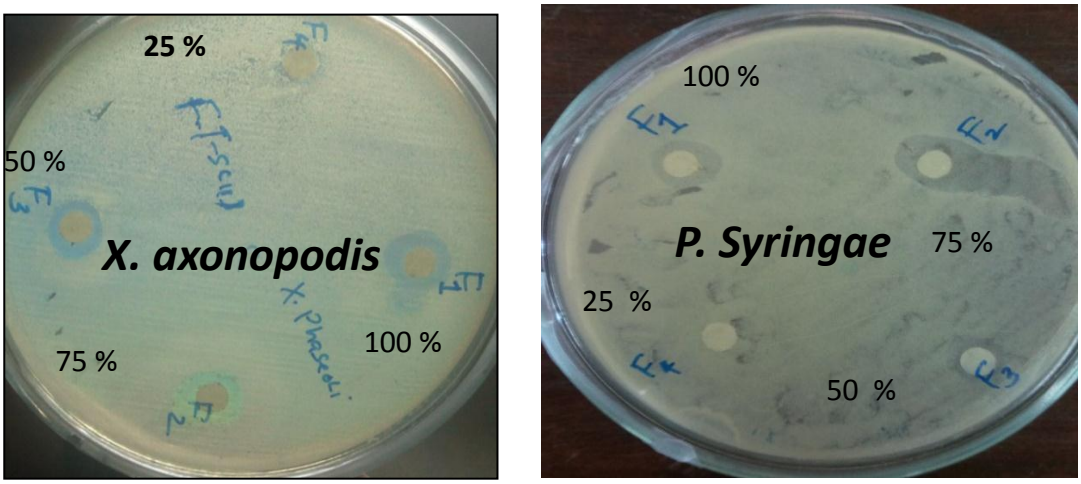

Plate 4.3: Photographs of serial dilution assay of fungal extracts against selected bean bacterial pathogens

**Plate 4.3 represents antibacterial activities of fraction 2 of Diaporthe sp3 against X. axonopodis pv phaseoli and P. syringae pv phaseolicola at different geometric dilutions of F1-100 % (5 mg/ml), F2-75 % (3.75 mg/ml), F3- 50 mg/ml (2.50 mg/ml) and F4- 25 % (1.25 mg/ml).*

Antibacterial results of this study, correlate with a research study done by Wanga *et al.* (2018). Whereby, the ethyl acetate of fungal endophyte *Fusarium solani* isolated from *Markhamia lutea* which showed a considerable antibacterial activity against *X. axonopodis pv phaseoli* with a zone of inhibition of 14.00 ± 0.00 mm.

Fractions yielded on column chromatography were all active against bacterium *X. axonopodis pv phaseoli* , F3 and F1 of SC- S- (9) and F2 of SC- S (11) showed the highest activities against *X. axonopodis pv phaseoli* with their MIC value as 1.25 mg/ml, 1.25 mg/ml and 2.50 mg/ml respectively.

Bacterium, *P. syringae pv phaseolicola* was very resistant to most of the fungal extracts, it is only ethyl acetate crude extract of SC- S (11) and SC-S (9) which

showed a significant activity at different concentrations. Of the fractions, F1 and F3 of SC-S (9) and F1 and F2 of SC-S (11) which had significant activities against this bacterium (Table 2). The results obtained were in agreement with that of Wanga *et al.* (2018) where most of the fungal and plant extracts showed minimal activities against bean bacterial pathogen *P. syringae pv phaseoli* . However; Arunachalam *et al.* (2010) found that plant extractives from *Proposis juliflora* were highly active against plant pathogen *P. syringae pv phaseoli* with a MIC value of 1.25 mg/ml.

The resistivity of *P. syringae pv phaseoli* bacterium is associated with the many modes under which the bacterium can easily mutate. Secondly, is due to the presence of E-flux pump; an internally developed mechanism, which is substrate specific, and functions to prevent the accumulations of antibacterial drugs within their systems, which in turn prevents them from reaching the target sites.

4.6. Characterization of Secondary Metabolites

In this study, **four** pure compounds were isolated; three (3) from endophytic fungi *Diaporthe sp3* and one from *Diaporthe sp2*. The pure isolates were characterized using spectroscopic techniques which include; 1D, 2D NMR experiments and mass spectrometer.

4.6.1. Compound 20

Compound **20** was obtained as brown solid at room temperature. Its molecular mass was established to be 198.13 amu, from MS data, corresponding to molecular ion at *m/z* 221.26 ($C_{11}H_{18}O_3$ + Na) (Fig 4.1) and a molecular formula of $C_{11}H_{18}O_3$, which indicates double bond equivalence of 3, corresponding to the three double bonds presence in the aliphatic chain. This compound was identified as an aliphatic enol compound based on its characteristic absorption on both 1D and 2D NMR data. ^{1}H NMR spectrum (Appendix 4), showed presence of methylene and methyl protons with

different multiplicities resonating at; at δ_H 4.00 and 4.13 (H-1), δ_H 5.66 (H-2), δ_H 6.40 (H-3), δ_H 6.00 (H-4), δ_H 3.98 (H-6), δ_H 4.29 (H-7), δ_H 5.34 (H-8), δ_H 5.45 (H-9), δ_H 1.57 (H-10) and δ_H 1.75 (H-5'), which corresponded to carbon signals in the ^{13}C NMR spectrum at; δ_C 61.4 (C-1), δ_C 129.4 (C-2), δ_C 127.5 (C-3), δ_C 128.0 (C-4), δ_C 76.7 (C-6), δ_C 68.4 (C-7), δ_C 131.5 (C-8), δ_C 125.3 (C-9), δ_C 18.2 (C-10) and δ_C 13.4(C-5'), in the HSQC spectrum respectively. The coupling constant of protons at δ_H 5.66 (H-2) and δ_H 6.40 (H-3) was found to be (7.24 and 1.74) while those at δ_H 5.34 (H-8), δ_H 5.45 (H-9) was found to be 2.14 and 7.06 Hz, this indicates that the $\triangle^{2,8}$ in compound **20** is a *Z* (*cis*) isomer (Table 4). This therefore is evidence that compound **20,** has a small dihedral angle between protons found at the stilbene alkenes at C-2 and C-3, C-8 and C-9.

^{13}C NMR and DEPT spectral information showed presence of hydroxylated aliphatic alkene carbon system, with a total of 11 carbon signals having, one methylene carbon at δ_C 61.4 (C-1), seven methine carbon at δ_C 68.4 (C-7), δ_C 76.7 (C-6), δ_C 125.3 (C-9), δ_C 127.5 (C-3), δ_C 128.0 (C-4), δ_C 129.4 (C-2) and δ_C 131.5 (C-8), two methyl carbons at δ_C 13.4 (C-11) and δ_C 18.2 (C-10) and a quaternary carbon at δ_C 138.9 (C-5).

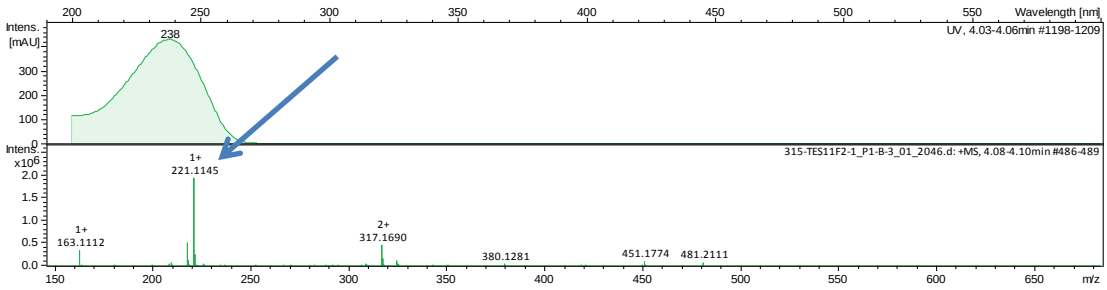

Figure 4.1: Mass spectrum of compound 20

COSY spectrum (Appendix 6) showed correlation of; H-2 with H-3, H-3 with H-4, H-7 with H-6 and H-8 while H-9 with H-10. While the HMBC spectrum (Appendix 7)

showed that, proton resonating at δ_H 6.00 (H-4) correlates with C-2, C-3, C-5 and C-6, while that resonating at δ_H 3.98 correlates with C-4, C-5, C-7 and C-8. Other COSY and HMBC spectral information is summarized in Fig 4.2 and Table 4.3. Compound **20** was assigned IUPAC name (*2Z, 4Z, 8Z*)-5-methyldec-2, 4, 8-triene-1, 6, 7-triol and trivial name as *Z*-cordatenol.

Table 4.3:

NMR data of compound 20

No.	**^{13}C NMR δ_C ppm**	**TYPE**	**HSQC δ_H ppm**	3J H-H **Hz**	**COSY(^{1}H/^{1}H)**	**HMBC**
1	61.4	CH_2	4.00,4.13		-	2,3
2	129.4	CH	5.66	7.24	3	3,4
3	127.5	CH	6.40	1.76	2,4	2,4,5
4	128.0	CH	6.00	6.08	3	2,3,5,6
5	138.9	C	-		-	-
6	76.7	CH	3.98		7	4,5,7,8
7	68.4	CH	4.29		6,8	5,6,8
8	131.5	CH	5.34	2.14	7,9	7,9,10
9	125.3	CH	5.45	7.06	8	7,8,10
10	13.4	CH_3	1.57		9	7,8,9
11	18.2	CH_3	1.75		-	5

Information coined from Table 4.3 was collectively used in developing structure for compound **20** as shown below.

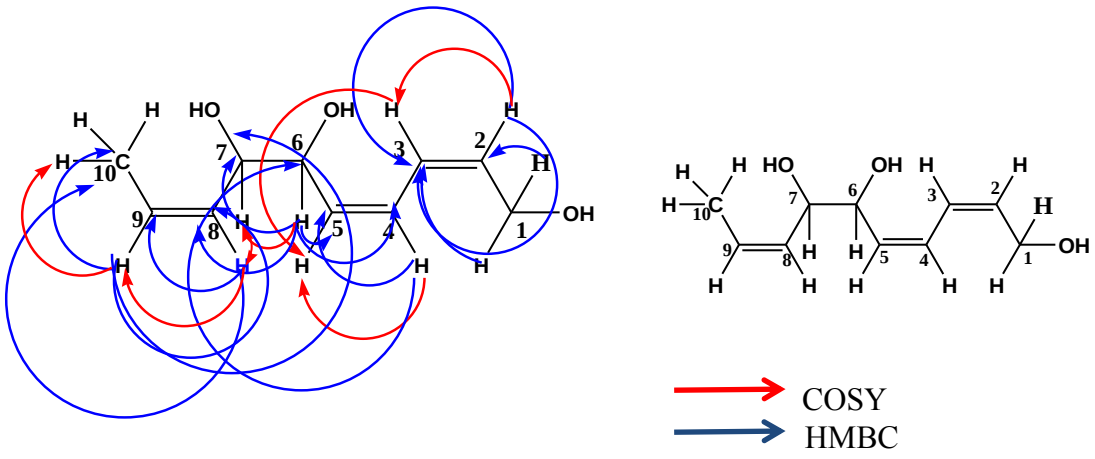

Figure 4.2: Structure, HMBC-COSY correlations of Compound 20

4.6.2. Compound 21

This compound was isolated together with Compound **20,** from F2 of SC- S (11) (*Diaporthe sp3.)* as brown solid at room temperature. The two compounds are geometric isomers having difference on the spatial arrangement of atoms at the alkenes sections. Its mass was established to be 198.13 amu from MS data, corresponding to molecular ion at *m/z* 221.26 ($C_{11}H_{18}O_3$ + Na) (Fig 4.3) and a molecular formula of $C_{11}H_{18}O_3$, which indicates double bond equivalence of 3, conforming to the three double bonds presence in the aliphatic chain. The compound was identified as an *E*-isomer of compound **20**, which they both belong to an enol group of compounds. ^{1}H NMR spectrum (Appendix 8) showed the presence of methylene and methyl protons with different multiplicities resonating at; δ_H 4.02 and 4.06 (H-1), δ_H 6.04 (H-2), δ_H 6.34 (H-3), δ_H 6.34 (H-4), δ_H 3.90 (H-6), δ_H 3.87(H-7), δ_H 5.49 (H-8), δ_H 5.53 (H-9) , δ_H 1.62 (H-10) and δ_H 1.71 (H-11) which corresponded to carbon signals resonating at; δ_C 56.6 (C-1), δ_C 125.2 (C-2), δ_C 124.8 (C-3), δ_C 122.5 (C-4), δ_C 77.3 (C-6), δ_C 73.7 (C-7), δ_C 132.2 (C-8), δ_C 125.1 (C-9), δ_C 17.6 (C-10) and δ_C 12.8 (C-11) in the HSQC spectrum respectively. In addition, ^{1}H NMR showed a *Trans* vicinal correlations of protons attached to C-2 and C-3, C-8 and C-9 with $^3J_{H-H}$ coupling constants of (11.88 and 11.00), (11.24 and 11.36) Hz respectively. These higher coupling constants indicates that the double bonds in compound **21,** are *trans* or this compound is *E*-isomer.

^{13}C NMR and DEPT spectral information showed presence of hydroxylated aliphatic alkene carbon system, with a total of 11 carbon signals, with one methylene carbon at δ_C 56.6 (C-1), seven methine carbon at δ_C 125.2 (C-2), δ_C 124.8 (C-3), δ_C 122.5 (C-4), δ_C 77.3 (C-6), δ_C 73.7(C-7), δ_C 132.2 (C-8) and δ_C 125.1 (C-9), two methyl carbons at δ_C 12.8 (C-11) and δ_C 17.6 (C-10) and a quaternary carbon at δ_C 126.2 (C-5).

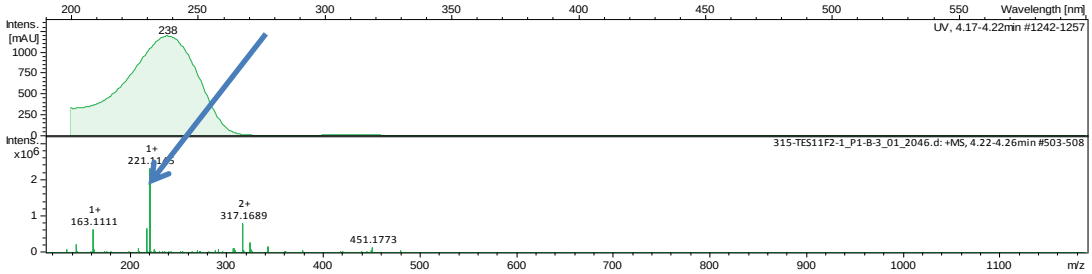

Figure 4.3: Mass spectrum of compound 21

COSY spectrum (Appendix 10), showed that protons resonating at δ_H 5.53 (H-9) correlates with that resonating at δ_H 1.62 (H-10). HMBC spectrum (Appendix 11), showed that, proton at δ_H 4.02 and 3.06 (H-1) correlates with C-2, proton at δ_H 6.34 correlates with C-5, C-6 and C-11 while that resonating at δ_H 5.49 (H-8) correlates with C-6, C-7, C-9 and C-10. Other COSY and HMBC spectral information is summarized in Table 4.4 and Fig 4.4. Compound **21** was assigned IUPAC name (*2E, 4E, 8E*)-5-methyldec-2, 4, 8-triene-1, 6, 7-triol and a trivial name as *E*-cordatenol a geometric isomer of compound **20.**

Table 4.4:

NMR data of compound 2

No.	**^{13}C NMR δ_C ppm**	**TYPE**	**HSQC δ_H ppm**	**$^3J_{HH}$ Hz**	**COSY(^{1}H/^{1}H)**	**HMBC**
1	56.6	CH_2	4.02, 4.06			2
2	125.2	CH	6.04	11.88		1,3
3	124.8	CH	6.34	11.00		4,5
4	122.5	CH	6.34	11.00		5,6,11
5	126.2	C	-			-
6	77.3	CH	3.90			7,8
7	73.7	CH	3.87			8,9
8	132.2	CH	5.49	11.24		6,7,9,10
9	125.1	CH	5.53	11.36	10	7,8,10
10	17.6	CH_3	1.62		9	8,9
11	12.8	CH_3	1.71			5

Information coined from Table 4.4 was collectively used in developing structure for compound **21** as shown below

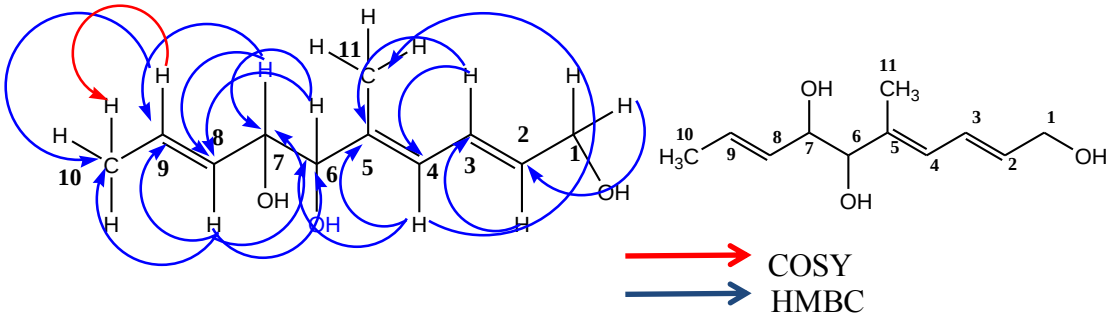

Figure 4.4: Structure, HMBC-COSY correlations of Compound 21

Compound **20** and **21** were isolated as geometric isomers with different orientations of atom attachments in C-2, C-3, C-8 and C-9. Generally, *cis* isomers have lower $^{3}J_{HH}$ coupling constants, which typically ranges between 6-10 Hz while that of *trans* isomers have a higher $^{3}J_{HH}$ coupling values which ranges between 11-18 Hz. The higher coupling values in *trans* isomers is generally attributed to a large dihedral angle, which in most cases is 180□, while that in *cis* is due to a small dihedral angle which is about 0-60□ (Jenkins, 2009). The two compounds belong to an enol group of compounds formally referred to as alkenols (Kawauchi andAntonov, 2013). Enols are those compounds represented with hydroxylation on an olefin's carbon chain; they are regarded as reactive compounds or intermediates. In plant biological systems, they are synthesized via a substrate level phosphorylation with the help of enzyme enolase. The compounds are known to undergo keto-enol tautomerism which involves auto-conversion of enol compounds to ketones; this is so to help in its stabilities (Kawauchi andAntonov, 2013). The activity of the two compounds **20** and **21** can be attributed to the presence of sp^2 hybridized carbon atoms and hydroxylation on the olefin carbon chain, which imparts more nucleophilicity to the compounds, these two parameters creates more active sites within the two molecules.

4.6.3. Compound 22

Compound **22,** was isolated from F2 of SC-S (11)-*Diaporthe sp3* as a cream yellow solid at room temperature. its mass was established to be 191.0 amu based on MS

data, corresponding to a molecular ion at 175.0 m/z ([$C_{12}H_{17}NO - NH_3$] + H) (Fig 4.5) and molecular formulae of $C_{12}H_{17}NO$, which indicates a double bond equivalence of 5 one aromatic ring, three double bonds within the aromatic ring and one aziridine ring at the side substituent.

^{1}H-NMR spectrum (Appendix 12), showed three characteristic protons of an aromatic compound resonating at; δ_H 6.90 (H-3), δ_H 6.59 (H-4), δ_H 6.50 (H-6) which corresponded to carbon signals resonating at δ_C 129.1 (C-3), δ_C 112.7 (C-4), δ_C 113.8 (C-6) in the HSQC spectrum respectively. Five methyl protons resonating at; δ_H 2.67 (H-1′), δ_H 3.07 (H-2′), δ_H 2.77 (H-3′), δ_H 0.76 (H-4′) and δ_H 1.15 (H-5′) which corresponded to carbon signals resonating at, δ_C 32.0 (C-1′), δ_C 38.7 (C-2′), δ_C 43.7(C-3′), δ_C 12.1 (C-4′) and δ_C 23.2 (C-5′) in the HSQC spectrum respectively, one benzylic proton resonating at δ_H 2.03 (H-7) which corresponded to carbon signal resonating at δ_C 15.9 (C-7) in the HSQC spectrum.

^{13}C NMR and DEPT (Appendix 14 and 15), showed that, compound **22** has at a total of 12 carbon atoms, with, six methine carbon at δ_C 129.1 (C-3), δ_C 112.7 (C-4), δ_C 113.8 (C-6), δ_C 32.0 (C-1′), δ_C 38.7 (C-2′), and δ_C 43.7(C-3′), three methyl carbons at δ_C 15.9 (C-7), δ_C 12.1 (C-4′) and δ_C 23.2 (C-5′) and three quaternary carbons at δ_C 152.9 (C-1), δ_C 121.2 (C-2) and δ_C 140.6 (C-5). High carbon absorbance at C1 (δ_C 152.9) is due to hydroxylation at C-1 which makes it to absorbs at high field because of de-shielding effects caused by oxygen connected to C-1

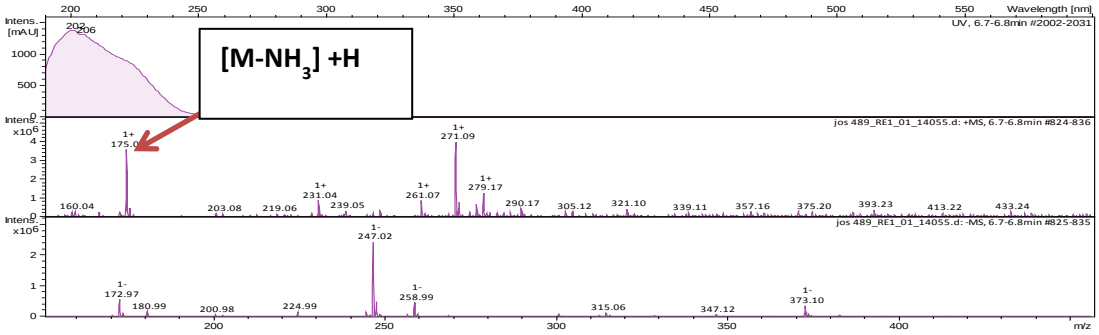

Figure 4.5: Mass spectrum of compound 22

The COSY spectrum (Appendix 16), showed that proton resonating at δ_H 2.67 (H-1') correlates with δ_H 1.15 (H-5') while a proton resonating at δ_H 2.77 (H-3') correlates with δ_H 0.76 (H-4'). HMBC spectrum (Appendix 17) revealed that protons resonating at; δ_H 6.90 (H-3) correlates with C-1, C-4, C-5 and C-7, H-4 (δ_H 6.59) correlates with C-1, C-2 and C-3 while H-6 (δ_H 6.50) correlates with C-1, C-1', C-2 and C-3. Other COSY and HMBC correlations are summarized in Table 4.5 and Fig 4.6 below.

The IUPAC name assigned to compound **22** was 2-methyl-5-(1-(3-methylaziridin-2-yl) ethyl) phenol and a trivial name as cordenol coined from the species name of the host plant and ol functional group representing phenol group of compounds.

Table 4.5:

NMR data of compound 22

	^{13}C $\delta_{C\ ppm}$	Type	HSQC $\delta_{H\ ppm}$	COSY($^1H/^1H$)	HMBC
1	152.9	C	-	-	-
1'	32.0	CH	2.67	5'	-
2	121.2	C	-	-	-
2'	38.7	CH	3.07		
3	129.1	CH	6.90		1,4,5,7
3'	43.7	CH	2.77	4'	
4	112.7	CH	6.59		1,2,3
4'	12.1	CH_3	0.76	3'	2',3'
5	140.6	C	-	-	-
5'	23.2	CH_3	1.15		1,2',5
6	113.8	CH	6.50		1,1',2,3
7	15.9	CH_3	2.03		1,2,3

Information coined from Table 4.3 was collectively used in developing structure for compound **20** as shown below.

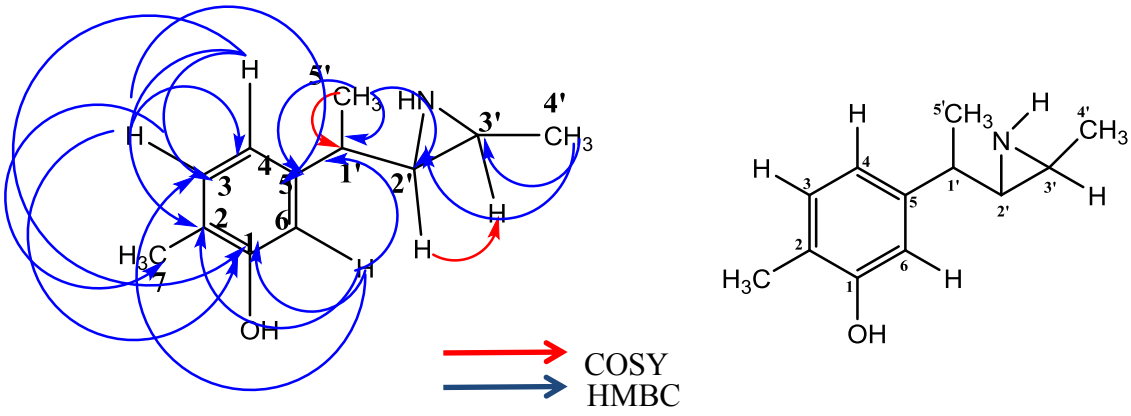

Figure 4.6: Structure, HMBC-COSY correlations of Compound 22

Compound **22**, is among the phenolic derivatives, which comprises a second largest group of secondary metabolites isolated from plant and their endophytes (Rajput *et al.*, 2018). Phenols and phenol derivatives are biosynthesized through condensation of acetic acid (acetic acid pathway) or metabolism of phosphorylated sugars through Skimmic acid and aromatic amino acid (Skimmate pathway) (Hennessy, 2014; Heleno *et al.*, 2015). Phenol derivatives such as carvacrol (5-isopropyl-2-

methylphenol) isolated from the essential oil of thyme leaves, thymol (2-isoprophyl-5-methylphenol) isolated from the essential oil of thyme and aregona plants and eugenol (4-allyl-2-methoxyphenol) isolated from essential oils of clove, rose plants. These compounds are known for their aromatherapy, antioxidant, antifungal and antibacterial activities because of hydroxylation within their aromatic rings (Reboredo-Rodríguez *et al.,* 2018). Compound **22** is among these phenolic derivatives, having an aziridine ring as part of a substituent. Aziridine is a nitrogen-containing functional group in a 3-membered strained ring, their biosynthetic pathways are not straightforward due to regiospecificity and streospecificity in it (Znati *et al.,* 2018). The presence of aziridine within the ring offers a molecule a useful property that can be utilized as an active intermediate in the synthesis of drugs or agrochemicals (Gopalan *et al.,* 2017) .

4.6.4. Compound 23

Compound **23,** was isolated as a white solid at room temperature from endophytic fungus *Diaporthe sp2* (SC-S (9) having molecular mass of 204 and a molecular formulae of $C_{12}H_{12}O_3$. It has hydrogen deficiency index of seven, corresponding to; two fused aromatic rings and five carbon double bonds within the aromatic system.

^{1}H NMR (Appendix 18) reveals that compound **23** had four aromatic protons resonating at; δ_H 6.59 (H-2), δ_H 7.21 (H-4), δ_H 6.70 (H-6) and δ_H 6.62 (H-8) corresponding to carbon signals resonating at δ_C 99.0(C-2), δ_C 103.0 (C-4), δ_C 118.1 (C-6) and δ_C 101.7 (C-8) in the HSQC spectrum respectively. A highly de-shielded methyl proton resonating at δ_H 2.73 (H-11) corresponding to carbon signal at δ_C 25.0 (C-11) in the HSQC spectrum. One methoxy proton resonating at δ_H 3.90 corresponding to carbon signal at δ_C 55.8 in the HSQC spectrum.

^{13}C NMR and DEPT spectra (Appendix 20), revealed that compound **23** had a total of 12 carbon atoms of which; four were methine carbons at δ_C 99.0(C-2), δ_C 103.0 (C-4), δ_C 118.1 (C-6) and δ_C 101.7 (C-8), one methyl carbon at δ_C 25.0 (C-11), one methoxylated carbon at δ_C 55.8 and six quaternary carbon at, δ_C 164.7 (C-1), δ_C 166.1 (C-3), δ_C 138.2 (C-5), δ_C 152.8 (C-7), δ_C 98.4 (C-9) and δ_C 108.2 (C-10).

HMBC spectrum (Appendix 21) showed that proton resonating at δ_H 6.59 (H-2) correlates with carbon signals at C-1, C-3, C-4 and C-9 while that resonating at δ_H 7.21 (H-4) correlates with C-2, C-3, C-9 and C-10. In addition methoxy protons resonating at δ_H 3.90 correlates with C-3. Other HMBC correlations are shown in Table 4.6 and Fig 4.7 below. Data from NMR experiments gave compound **23** and IUPAC name as 3-methoxy-5-methylnapthalene-1, 7-diol.

Table 4.6:

NMR data of compound 23

S/No	^{13}C δ_C ppm	DEPT	HSQC δ_H ppm	HMBC
1	164.7	C	-	-
2	99.0	CH	6.59	1,3,4,9
3	166.1	C	-	-
4	103.0	CH	7.21	2,3,9,10
5	138.2	C	-	-
6	118.1	CH	6.70	8,10,11
7	152.8	C	-	-
8	101.7	CH	6.62	6,7,10
9	98.4	C	-	-
10	108.2	C	-	-
11	25.0	CH_3	2.73	5,6,10
O-CH_3	55.8	CH_3	3.90	3

Information coined from Table 4.6 was collectively used in developing structure for compound **23** as shown below.

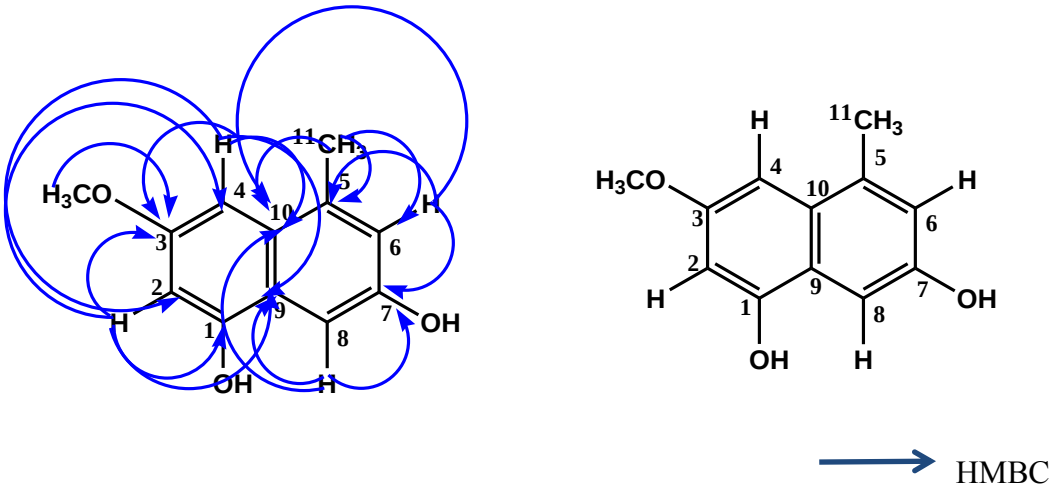

Figure 4.7: Structure and HMBC correlations of compound 23

Compound **23**, is a naphalene derivative with, hydroxyl, methyl and methoxy groups attached to it. Naphalene are group of volatile organic compounds that contains two fused benzene ring with a skeletal molecular formulae of $C_{12}H_8$. It has been used in production of phthalic anhydride that acts as a moth repellant, this is so because it is more reactive than benzene (Daisy *et al.*, 2002). Its derivatives are known to possess strong antimicrobial, insecticidal and vermicidal activities (Würthner andStolte, 2011).

Muscodor albus an endophytic fungus isolated from *Terminalia prostrata*, produces volatile organic compounds such as naphthalene having significant antimicrobial activities (Kouipou andBoyom, 2019). Other napthalene derivatives such as 2-napthtol, 1-iodonapthalene among others have also been isolated from endophytic fungus *M. albunus* with noticeable antimicrobial activities against series of plant pathogens (Strobel, 2011).

Endophytic fungi produce volatile organic compounds such as naphthalene and its derivatives to restrict the growth of other fungal or bacterial competitors within their environment especially in the host plant (Zhi-Lin *et al.*, 2012; Kouipou andBoyom, 2019). It is evident therefore that compound **23** being one of these naphthalene

derivatives possess strong antimicrobial activities especially towards plant pathogens. This was in line with bioassay results obtained after screening of fungal extracts against bean bacterial pathogens *P. syringae pv phaseolicola* and *X. axonopodis pv phaseoli*, whereby, fraction 3, that yielded compound **23** had a significant antibacterial activities which corresponds to zones of 12.33 ± 0.88 and 10.33 ± 0.88 mm against *Xap* and *Psp* respectively. This is so because compound **23** have naphthalene ring which act as a strong nucleophile hence enhances destabilization of bacterial cell membranes, which in turn inhibits their growth. This compound is new and therefore through the present study it is reported for the first time.

On the other hand, significant antibacterial activities of F2 of SC-S(11) *Diaporthe sp3*, against the test organisms, with corresponding zones of inhibition of 10.67 ± 0.33 mm and 14.00 ± 0.88 mm against *Psp* and *Xap* respectively, is chiefly attributed to the presence of these bioactive secondary metabolites. The three compounds isolated from this fungus and named as compound **20**, **21** and **22** are also new and through these findings, they are reported for the first rime. These results provide scientific validity and credence to the use of this plant in the treatment of bean infection caused by some of the bacteria used in this study and highlights the usefulness of endophytic fungi from leaves and stem bark of S. *cordatum* in the treatment of bacterial infections.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1. Introduction

This chapter makes a summary of all research findings from this study, investigator's point of view, recommendations, and research gap that has to be filled through extensive research studies.

5.2. Summary

A total of seven (7) endophytic fungi were isolated from leaves and stem bark of *S. cordatum* plant. However, *Diaporthe sp1* (SC-L (7)), *Diaporthe sp2 (SC*-S (9) and *Diaporthe sp3 (*SC-S (11) showed probable antagonism against bean bacterial pathogens, *P. syringae pv phaseolicola* and *X. axonopodis pv phaseoli* with *Diaporthe sp1* having largest zone of inhibition of 17.33 ± 1.45 and 20.00 ± 0.00 mm against the tests organisms respectively. These antibacterial activities are attributed to the mixture of secondary metabolites produced by these fungal endophytes. Extractives from fungal endophytes *Diaporthe* species were all active against *X. axonopodis pv phaseoli*, with F2 of *Diaporthe sp3* showing significant activity at a concentration of 2.50 mg/ml corresponding to a zone of inhibition of 10.67 ± 0.58 mm. Minimal antibacterial activities were recorded against *P. syringae pv phaseolicola*, this is so because the bacterium is known to contain an E-flux pump which prevent the accumulations of antibacterial drugs within their systems hence removing them from reaching the target sites.

Three **(3)** new compounds were isolated from fungal endophyte Diaporthe *sp3*, two geometric isomers named as compounds **20 (***2Z, 4Z, 8Z*)-(5-methyldec-2, 4, 8-triene-1, 6, 7-triol) and compound **21 (***2E, 4E, 8E*)-(5-methyldec-2, 4, 8-triene-1, 6, 7-triol) which appeared as brown solids at room temperature. One phenolic derivative with an

aziridine site chain, named as compound **22** (2-methyl-5-(1-(3-methylaziridin-2-yl) ethyl phenol) which appeared as creamy yellow solid at room temperature was isolated from the fungal extracts. Bioactivity of Compound **20** and **21** is attributed to hydroxylation on the aliphatic alkene chains whereas in compound **22,** hydroxylation of benzene ring and site chain aziridine ring enhances its activity. On the other hand, F3 of *Diaporthe sp2* yielded one **new** compound, which was isolated as a white solid at room temperature; a naphthalene derivative which was named as; 3-methoxy-5-methylnapthalene-1, 7-diol (compound **23).** Bioactivity of this compound is associated to the presence of naphthalene ring, which enhances its nucleophilicity and presence of hydroxyl and methoxy groups attached to the main ring, which also act as ring activators, these will in turn, enhances its reactivity.

5.3. Conclusions

The study reveals that *S. cordatum* hosts important endophytic fungi of genus *Diaporthe* and phylum Ascomycota. *Diaporthe* species isolated from the tissue of *S. cordatum* plant showed appreciable anti-bacterial activities against bean bacterial pathogens, *P. syringae pv phaseolicola* and *X. axonopodis pv phaseoli*, Extractives from fungal endophyte *Diaporthe*, showed a noticeable anti-bacterial activity against *Xap* but dismally against *P. syringae pv phaseolicola.*

Four **new** compounds; compound, **20 (***2Z, 4Z, 8Z*)-(5-methyldec-2, 4, 8-triene-1, 6, 7-triol), compound **21 (***2E, 4E, 8E*)-(5-methyldec-2, 4, 8-triene-1, 6, 7-triol), compound **22** (2-methyl-5-(1-(3-methylaziridin-2-yl) ethyl phenol) and compound **23** (3-methoxy-5-methylnapthalene-1,7-diol) were isolated from Fungal extracts after a series of purifications on column chromatography and HPLC techniques respectively.

5.4. Recommendations

i. Further study to be done on isolation and identification of other classes of fungal endophytes that are host to *S. cordatum* plant.

ii. Further studies on alternative molecular identification markers such as elongation factor-1 α-gene, β-tubulin should be used as opposed to the known ITS region.

iii. Toxicological and cytotoxicity studies on extractives from *Diaporthe* species. fungal endophytes should be done in order to enhance their safety and applications as antibacterial.

5.5. Suggestions for Further Research

Diaporthe species host to *S. cordatum* have shown a good antagonism against bean bacterial pathogens, *P. syringae pv phaseolicola* and *X. axonopodis pv phaseoli,* evident that they contain mixture of secondary metabolites that act against the pathogens. A research study therefore, has to be done on how the fungal endophytes can be introduced as inoculum into the bean seeds to enhance their defense against the pathogens. The study will also check on optimum conditions for inoculum development as well as the expected side-effects of the inoculum to the seeds and by extension to the planting environment. This will enhance bio-control agent's development as oppose to the currently used chemical control methods which have been marred with lots of problems.

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APPENDICES

Appendix 1: ITS consensus sequence of the isolated fungal endophytes

S/N	Isolation code	DNA coding	Species identity
1.	SC-S(9)	TCCGTTGGTGAACCAGCGGAGGG ATCATTGCTGGAACGCGCCCCAG GCGCACCCAGAAACCCTTTGTAA ACTTATACCTTACTGTTGCCTCGG CGCAGGCCGTCCCCTATGGGGTCC CTTGGAAACAAGGAGCAGCCGGC CGGCGGCCAAGTTAACTCTGTTTT TAAACTGAAACTCTGAGTACAAA ACATAAATGAATCAAAACTTTCA ACAACGGATCTCTTGGTTCTGGCA TCGATGAAGAACGCAGCGAAATG CGATAAGTAATGTGAATTGCAGA ATTCAGTGAATCATCGAATCTTTG AACGCACATTGCGCCCTCTGGTAT TCCGGAGGGCATGCCTGTTCGAGC GTCATTTCAACCCTCAAGCCTGGC TTGGTGTTGGGGCACTGCCTGTAA AAGGGCAGGCCCTGAAATATAGT GGCGAGCTCGCCAGGACTCCGAG CGTAGTAGTTAAACCCTCGCTTTG GAAGGCCTGGCGGTGCCCTGCCG	*Diaporthe sp.* Related to *Diaporthe sophorae*

		TTAAACCCCAACTTCTGAAAATTT	
		GACCTCGGATCAGGTAGGAATAC	
		CCGCTGAACTTAAGCATATCAA	
2.	SC-S(11)	TCCGTTGGTGAACCAGCGGAG	*Diaporthe sp.*
		GGATCATTGCTGGAACGCGCC	Related to *Diaporthe sophorae*
		CCAGGCGCACCCAGAAACCCT	
		TTGTAAACTTATACCTTACTGT	
		TGCCTCGGCGCAGGCCGTCCC	
		CTATGGGGTCCCTTGGAAACA	
		AGGAGCAGCCGGCCGGCGGCC	
		AAGTTAACTCTGTTTTTAAACT	
		GAAACTCTGAGTACAAAACAT	
		AAATGAATCAAAACTTTCAAC	
		AACGGATCTCTTGGTTCTGGCA	
		TCGATGAAGAACGCAGCGAAA	
		TGCGATAAGTAATGTGAATTG	
		CAGAATTCAGTGAATCATCGA	
		ATCTTTGAACGCACATTGCGCC	
		CTCTGGTATTCCGGAGGGCAT	
		GCCTGTTCGAGCGTCATTTCAA	
		CCCTCAAGCCTGGCTTGGTGTT	
		GGGGCACTGCCTGTAAAAGGG	
		CAGGCCCTGAAATATAGTGGC	
		GAGCTCGCCAGGACTCCGAGC	

		GTAGTAGTTAAACCCTCGCTTT GGAAGGCCTGGCGGTGCCCTG CCGTTAAACCCCAACTTCTGA AAATTTGACCTCGGATCAGGT AGGAATACCCGCTGAACTTAA GCATATCAA		
3.	SC-L(7)	TCCGTTGGTGAACCAGCGGAG GGATCATTGCTGGAACGCGCT TCGGCGCACCCAGAAACCCTT TGTGAACTTATACCTTACTGTT GCCTCGGCGTCAGGCCGGCCT TGTCACCAAGGCCCCTCGGAG ACGAGGAGCAGCCCGCCGGCG GCCAAGTTAACTCTTGTTTTTA CACTGAAACTCTGAGAAATAA ACATAAATGAATCAAAACTTT CAACAACGGATCTCTTGGTTCT GGCATCGATGAAGAACGCAGC GAAATGCGATAAGTAATGTGA ATTGCAGAATTCAGTGAATCA TCGAATCTTTGAACGCACATTG CGCCCTCTGGTATTCCGGAGG GCATGCCTGTTCGAGCGTCATT TCAACCCTCAAGCCTGGCTTG GTGATGGGGCACTGCTTCCGC	*Diaporthe sp.*	Related to *Diaporthe phaseolorum*

AAGGGAGCAGGCCCTGAAATC

TAGTGGCGAGCTCGCCAGGAC

CCCGAGCGCAGTAGTTAAACC

CTCGCTCTGGAAGGCCCTGGC

GGTGCCCTGCCGTTAAACCCC

CAACTCTTGAAAATTTGACCTC

GGATCAGGTAGGAATACCCGC

TGAACTTAAGCATATCAATAG

GCGGAGG

Appendix 2: One- Way ANOVA summary of zones of inhibition of isolated fungal endophytes against *P. syringae pv phaseolicola*

Descriptive						
Antibacterial activity against *P. syringae*						
	N	**Mean**	**Std. Deviation**	**Std. Error**	**95% Confidence Interval for Mean**	
					Lower Bound	**Upper Bound**
SC-S(3)	3	9.0000	1.73205	1.00000	4.6973	13.3027
SC-S(4)	3	10.6667	1.15470	.66667	7.7982	13.5351
SC-S(5)	3	7.0000	1.00000	.57735	4.5159	9.4841
SC-S(7)	3	11.3333	1.15470	.66667	8.4649	14.2018
SC-S(8)	3	8.3333	2.08167	1.20185	3.1622	13.5045
SC-S(9)	3	14.0000	2.00000	1.15470	9.0317	18.9683
SC-S(11)	3	15.6667	1.52753	.88192	11.8721	19.4612
SC-L(7)	3	17.3333	2.51661	1.45297	11.0817	23.5849
Chloramphenicol	3	20.3333	.57735	.33333	18.8991	21.7676
Total	27	12.6296	4.52470	.87078	10.8397	14.4195

Test of Homogeneity of Variances/ Levene Statistic					
		Levene Statistic	**df1**	**df2**	**Sig.**
Antibacterial activity against *P. syringae*	Based on Mean	.978	8	18	.483
	Based on Median	.362	8	18	.927
	Based on Median and with adjusted df	.362	8	13.445	.923
	Based on trimmed mean	.924	8	18	.520

ANOVA				
Antibacterial activity of fungal endophytes isolated from *S. cordatum* plant against *P. syringae*				
	Sum of Squares	**Df**	**Mean Square**	**F**

Between Groups	(Combined)		484.296	8	60.537	22.701
	Linear Term	Contrast	364.089	1	364.089	136.533
		Deviation	120.207	7	17.172	6.440
Within Groups			48.000	18	2.667	
Total			532.296	26		

Post Hoc Tests

Multiple Comparisons						
Dependent Variable: Antibacterial activity against *P. syringae*						
Tukey HSD						
(I) Endopytic fungi isolated from *S. cordatum* plant	(J) Endopytic fungi isolated from *S. cordatum* plant	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
SC-S(3)	SC-S(4)	-1.66667	1.33333	.933	-6.3385	3.0051
	SC-S(5)	2.00000	1.33333	.842	-2.6718	6.6718
	SC-S(7)	-2.33333	1.33333	.711	-7.0051	2.3385
	SC-S(8)	.66667	1.33333	1.000	-4.0051	5.3385
	SC-S(9)	-5.00000*	1.33333	.031	-9.6718	-.3282
	SC-S(11)	-6.66667*	1.33333	.002	-11.3385	-1.9949
	SC-L(7)	-8.33333*	1.33333	.000	-13.0051	-3.6615
	Chloramphenicol	-11.33333*	1.33333	.000	-16.0051	-6.6615
SC-S(4)	SC-S(3)	1.66667	1.33333	.933	-3.0051	6.3385
	SC-S(5)	3.66667	1.33333	.198	-1.0051	8.3385
	SC-S(7)	-.66667	1.33333	1.000	-5.3385	4.0051
	SC-S(8)	2.33333	1.33333	.711	-2.3385	7.0051
	SC-S(9)	-3.33333	1.33333	.294	-8.0051	1.3385
	SC-S(11)	-5.00000*	1.33333	.031	-9.6718	-.3282
	SC-L(7)	-6.66667*	1.33	.002	-	-1.9949

			333		11.3385	
	Chloramphenicol	-9.66667*	1.33333	.000	-14.3385	-4.9949
SC-S(5)	SC-S(3)	-2.00000	1.33333	.842	-6.6718	2.6718
	SC-S(4)	-3.66667	1.33333	.198	-8.3385	1.0051
	SC-S(7)	-4.33333	1.33333	.081	-9.0051	.3385
	SC-S(8)	-1.33333	1.33333	.981	-6.0051	3.3385
	SC-S(9)	-7.00000*	1.33333	.001	-11.6718	-2.3282
	SC-S(11)	-8.66667*	1.33333	.000	-13.3385	-3.9949
	SC-L(7)	-10.33333*	1.33333	.000	-15.0051	-5.6615
	Chloramphenicol	-13.33333*	1.33333	.000	-18.0051	-8.6615
SC-S(7)	SC-S(3)	2.33333	1.33333	.711	-2.3385	7.0051
	SC-S(4)	.66667	1.33333	1.000	-4.0051	5.3385
	SC-S(5)	4.33333	1.33333	.081	-.3385	9.0051
	SC-S(8)	3.00000	1.33333	.417	-1.6718	7.6718
	SC-S(9)	-2.66667	1.33333	.562	-7.3385	2.0051
	SC-S(11)	-4.33333	1.33333	.081	-9.0051	.3385
	SC-L(7)	-6.00000*	1.33333	.007	-10.6718	-1.3282
	Chloramphenicol	-9.00000*	1.33333	.000	-13.6718	-4.3282
SC-S(8)	SC-S(3)	-.66667	1.33333	1.000	-5.3385	4.0051
	SC-S(4)	-2.33333	1.33333	.711	-7.0051	2.3385
	SC-S(5)	1.33333	1.33333	.981	-3.3385	6.0051
	SC-S(7)	-3.00000	1.33333	.417	-7.6718	1.6718
	SC-S(9)	-5.66667*	1.33333	.011	-10.3385	-.9949
	SC-S(11)	-7.33333*	1.33333	.001	-12.0051	-2.6615
	SC-L(7)	-9.00000*	1.33333	.000	-13.6718	-4.3282

	Chloramphenicol	-12.00000*	1.33333	.000	-16.6718	-7.3282
SC-S(9)	SC-S(3)	5.00000*	1.33333	.031	.3282	9.6718
	SC-S(4)	3.33333	1.33333	.294	-1.3385	8.0051
	SC-S(5)	7.00000*	1.33333	.001	2.3282	11.6718
	SC-S(7)	2.66667	1.33333	.562	-2.0051	7.3385
	SC-S(8)	5.66667*	1.33333	.011	.9949	10.3385
	SC-S(11)	-1.66667	1.33333	.933	-6.3385	3.0051
	SC-L(7)	-3.33333	1.33333	.294	-8.0051	1.3385
	Chloramphenicol	-6.33333*	1.33333	.004	-11.0051	-1.6615
SC-S(11)	SC-S(3)	6.66667*	1.33333	.002	1.9949	11.3385
	SC-S(4)	5.00000*	1.33333	.031	.3282	9.6718
	SC-S(5)	8.66667*	1.33333	.000	3.9949	13.3385
	SC-S(7)	4.33333	1.33333	.081	-.3385	9.0051
	SC-S(8)	7.33333*	1.33333	.001	2.6615	12.0051
	SC-S(9)	1.66667	1.33333	.933	-3.0051	6.3385
	SC-L(7)	-1.66667	1.33333	.933	-6.3385	3.0051
	Chloramphenicol	-4.66667	1.33333	.050	-9.3385	.0051
SC-L(7)	SC-S(3)	8.33333*	1.33333	.000	3.6615	13.0051
	SC-S(4)	6.66667*	1.33333	.002	1.9949	11.3385
	SC-S(5)	10.33333*	1.33333	.000	5.6615	15.0051
	SC-S(7)	6.00000*	1.33333	.007	1.3282	10.6718
	SC-S(8)	9.00000*	1.33333	.000	4.3282	13.6718
	SC-S(9)	3.33333	1.33333	.294	-1.3385	8.0051
	SC-S(11)	1.66667	1.33333	.933	-3.0051	6.3385
	Chloramphenicol	-3.00000	1.33	.417	-7.6718	1.6718

			333			
Chloramphenicol	SC-S(3)	11.33333*	1.33333	.000	6.6615	16.0051
	SC-S(4)	9.66667*	1.33333	.000	4.9949	14.3385
	SC-S(5)	13.33333*	1.33333	.000	8.6615	18.0051
	SC-S(7)	9.00000*	1.33333	.000	4.3282	13.6718
	SC-S(8)	12.00000*	1.33333	.000	7.3282	16.6718
	SC-S(9)	6.33333*	1.33333	.004	1.6615	11.0051
	SC-S(11)	4.66667	1.33333	.050	-.0051	9.3385
	SC-L(7)	3.00000	1.33333	.417	-1.6718	7.6718

*. The mean difference is significant at the 0.05 level.

Appendix 3: One- Way ANOVA summary of zones of inhibition of isolated fungal endophytes against *X. axonopodis pv phaseoli*

Descriptive						
Antibacterial activity against *X. axonopodis pv phaseoli*						
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
SC-S(3)	3	13.0000	2.64575	1.52753	6.4276	19.5724
SC-S(4)	3	12.3333	.57735	.33333	10.8991	13.7676
SC-S(5)	3	12.0000	2.00000	1.15470	7.0317	16.9683
SC-S(7)	3	11.3333	1.52753	.88192	7.5388	15.1279
SC-S(8)	3	12.1667	4.75219	2.74368	.3616	23.9718
SC-S(9)	3	17.0000	1.00000	.57735	14.5159	19.4841
SC-S(11)	3	17.6667	1.15470	.66667	14.7982	20.5351
SC-L(7)	3	20.0000	.00000	.00000	20.0000	20.0000
Chloramphenicol	3	25.0000	1.00000	.57735	22.5159	27.4841
Total	27	15.6111	4.81251	.92617	13.7073	17.5149

Test of Homogeneity of Variances					
		Levene Statistic	df1	df2	Sig.
Antibacterial activity against X. axonopodis	Based on Mean	2.342	8	18	.064
	Based on Median	1.425	8	18	.252
	Based on Median	1.425	8	6.776	.330

	and with adjusted df				
	Based on trimmed mean	2.288	8	18	.069

ANOVA					
Antibacterial activity against *X. axonopodis pv phaseoli*					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	523.000	8	65.375	14.864	.000
Within Groups	79.167	18	4.398		
Total	602.167	26			

Post Hoc Tests

Multiple Comparisons						
Dependent Variable: Antibacterial activity against *X. axonopodis pv phaseoli*pv phaseoli *X. axonopodis pv phaseoli*						
Tukey HSD						
(I) Endopytic fungi isolated from *S. cordatum* plant	(J) Endopytic fungi isolated from *S. cordatum* plant	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
SC-S(3)	SC-S(4)	.66667	1.71234	1.000	-5.3331	6.6665
	SC-S(5)	1.00000	1.71234	.999	-4.9998	6.9998
	SC-S(7)	1.66667	1.71234	.984	-4.3331	7.6665
	SC-S(8)	.83333	1.71234	1.000	-5.1665	6.8331
	SC-S(9)	-4.00000	1.71234	.372	-9.9998	1.9998
	SC-S(11)	-4.66667	1.71234	.206	-10.6665	1.3331
	SC-L(7)	-7.00000*	1.71234	.015	-12.9998	-1.0002
	Chloramphenicol	-12.00000*	1.71234	.000	-17.9998	-6.0002

SC-S(4)	SC-S(3)	-.66667	1.71234	1.000	-6.6665	5.3331
	SC-S(5)	.33333	1.71234	1.000	-5.6665	6.3331
	SC-S(7)	1.00000	1.71234	.999	-4.9998	6.9998
	SC-S(8)	.16667	1.71234	1.000	-5.8331	6.1665
	SC-S(9)	-4.66667	1.71234	.206	-10.6665	1.3331
	SC-S(11)	-5.33333	1.71234	.105	-11.3331	.6665
	SC-L(7)	-7.66667*	1.71234	.007	-13.6665	-1.6669
	Chloramphenicol	-12.66667*	1.71234	.000	-18.6665	-6.6669
SC-S(5)	SC-S(3)	-1.00000	1.71234	.999	-6.9998	4.9998
	SC-S(4)	-.33333	1.71234	1.000	-6.3331	5.6665
	SC-S(7)	.66667	1.71234	1.000	-5.3331	6.6665
	SC-S(8)	-.16667	1.71234	1.000	-6.1665	5.8331
	SC-S(9)	-5.00000	1.71234	.148	-10.9998	.9998
	SC-S(11)	-5.66667	1.71234	.073	-11.6665	.3331
	SC-L(7)	-8.00000*	1.71234	.005	-13.9998	-2.0002
	Chloramphenicol	-13.00000*	1.71234	.000	-18.9998	-7.0002
SC-S(7)	SC-S(3)	-1.66667	1.71234	.984	-7.6665	4.3331
	SC-S(4)	-1.000	1.71234	.999	-6.9998	4.9998

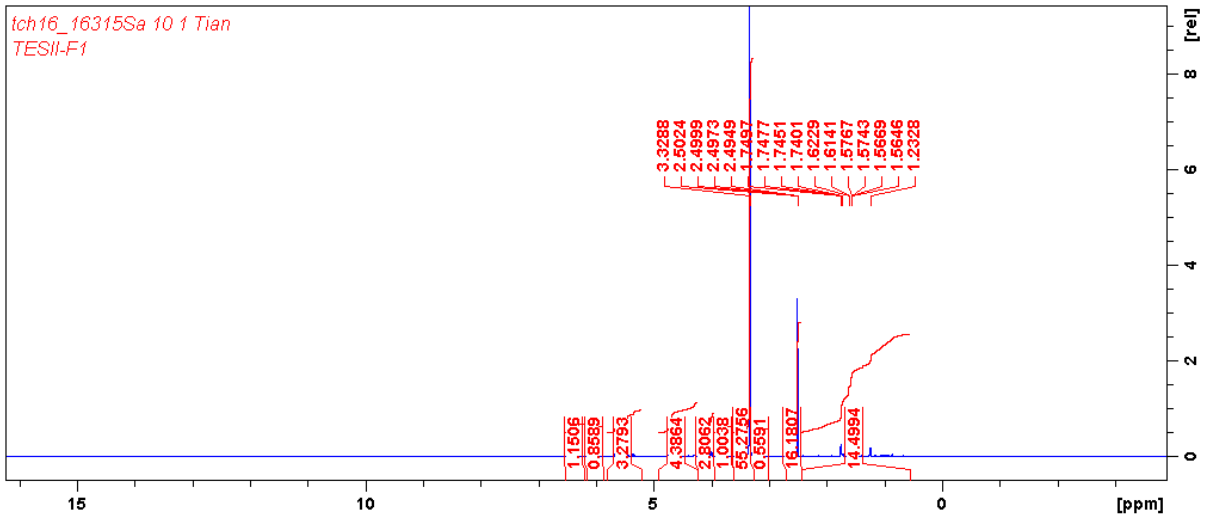
		00				
	SC-S(5)	-.66667	1.71234	1.000	-6.6665	5.3331
	SC-S(8)	-.83333	1.71234	1.000	-6.8331	5.1665
	SC-S(9)	-5.66667	1.71234	.073	-11.6665	.3331
	SC-S(11)	-6.33333*	1.71234	.034	-12.3331	-.3335
	SC-L(7)	-8.66667*	1.71234	.002	-14.6665	-2.6669
	Chloramphenicol	-13.66667*	1.71234	.000	-19.6665	-7.6669
SC-S(8)	SC-S(3)	-.83333	1.71234	1.000	-6.8331	5.1665
	SC-S(4)	-.16667	1.71234	1.000	-6.1665	5.8331
	SC-S(5)	.16667	1.71234	1.000	-5.8331	6.1665
	SC-S(7)	.83333	1.71234	1.000	-5.1665	6.8331
	SC-S(9)	-4.83333	1.71234	.175	-10.8331	1.1665
	SC-S(11)	-5.50000	1.71234	.087	-11.4998	.4998
	SC-L(7)	-7.83333*	1.71234	.006	-13.8331	-1.8335
	Chloramphenicol	-12.83333*	1.71234	.000	-18.8331	-6.8335
SC-S(9)	SC-S(3)	4.00000	1.71234	.372	-1.9998	9.9998
	SC-S(4)	4.66667	1.71234	.206	-1.3331	10.6665
	SC-S(5)	5.00000	1.71234	.148	-.9998	10.9998
	SC-S(7)	5.66667	1.71234	.073	-.3331	11.6665

	SC-S(8)	4.83333	1.71234	.175	-1.1665	10.8331
	SC-S(11)	-.66667	1.71234	1.000	-6.6665	5.3331
	SC-L(7)	-3.00000	1.71234	.710	-8.9998	2.9998
	Chloramphenicol	-8.00000*	1.71234	.005	-13.9998	-2.0002
SC-S(11)	SC-S(3)	4.66667	1.71234	.206	-1.3331	10.6665
	SC-S(4)	5.33333	1.71234	.105	-.6665	11.3331
	SC-S(5)	5.66667	1.71234	.073	-.3331	11.6665
	SC-S(7)	6.33333*	1.71234	.034	.3335	12.3331
	SC-S(8)	5.50000	1.71234	.087	-.4998	11.4998
	SC-S(9)	.66667	1.71234	1.000	-5.3331	6.6665
	SC-L(7)	-2.33333	1.71234	.898	-8.3331	3.6665
	Chloramphenicol	-7.33333*	1.71234	.010	-13.3331	-1.3335
SC-L(7)	SC-S(3)	7.00000*	1.71234	.015	1.0002	12.9998
	SC-S(4)	7.66667*	1.71234	.007	1.6669	13.6665
	SC-S(5)	8.00000*	1.71234	.005	2.0002	13.9998
	SC-S(7)	8.66667*	1.71234	.002	2.6669	14.6665
	SC-S(8)	7.83333*	1.71234	.006	1.8335	13.8331
	SC-S(9)	3.00000	1.71234	.710	-2.9998	8.9998
	SC-S(11)	2.33333	1.71234	.898	-3.6665	8.3331
	Chloramphenicol	-5.00000	1.71234	.148	-10.9998	.9998
Chloramphenicol	SC-S(3)	12.00000*	1.71234	.000	6.0002	17.9998
	SC-S(4)	12.66	1.71234	.000	6.6669	18.6665

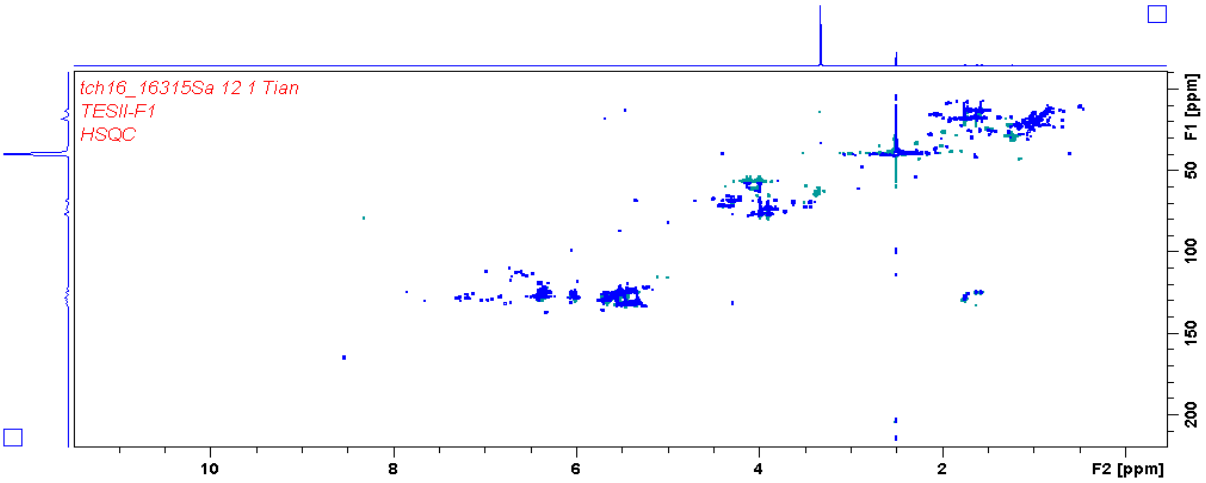
		667*				
	SC-S(5)	13.00000*	1.71234	.000	7.0002	18.9998
	SC-S(7)	13.66667*	1.71234	.000	7.6669	19.6665
	SC-S(8)	12.83333*	1.71234	.000	6.8335	18.8331
	SC-S(9)	8.00000*	1.71234	.005	2.0002	13.9998
	SC-S(11)	7.33333*	1.71234	.010	1.3335	13.3331
	SC-L(7)	5.00000	1.71234	.148	-.9998	10.9998

*. The mean difference is significant at the 0.05 level.

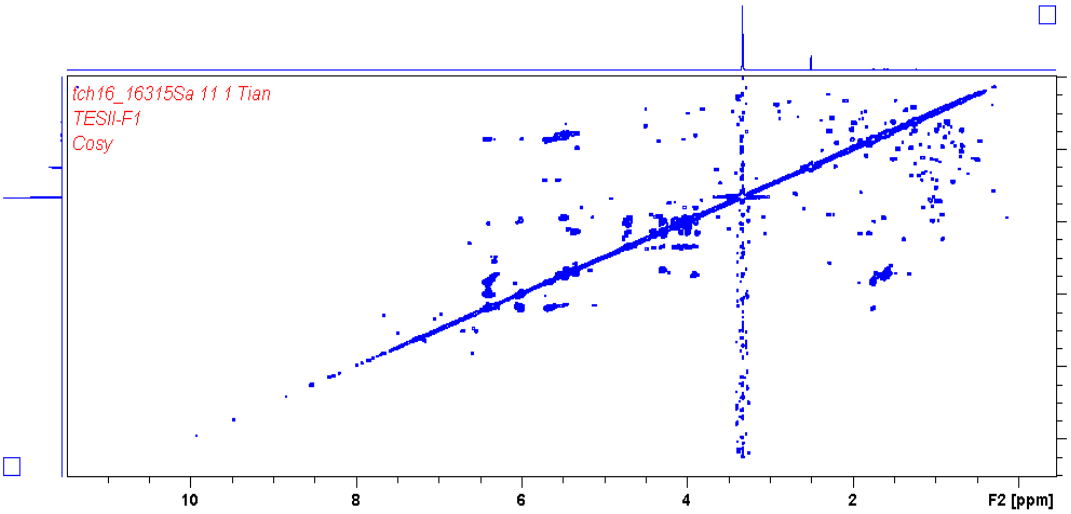
Appendix 4: ^{1}H NMR of compound 20



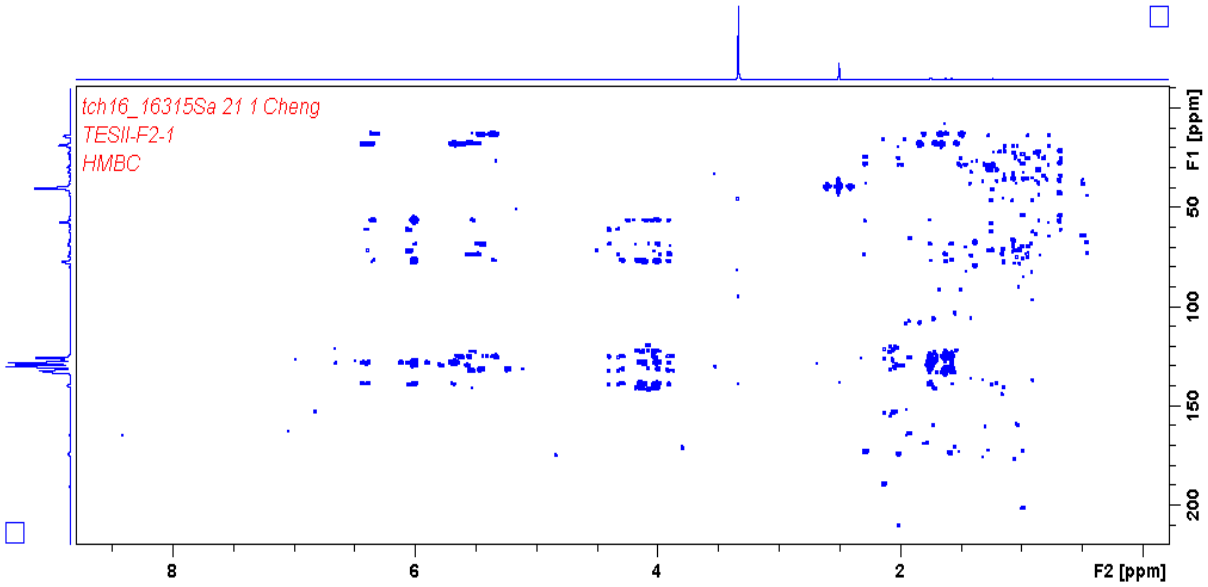
Appendix 5: HSQC of compound 20



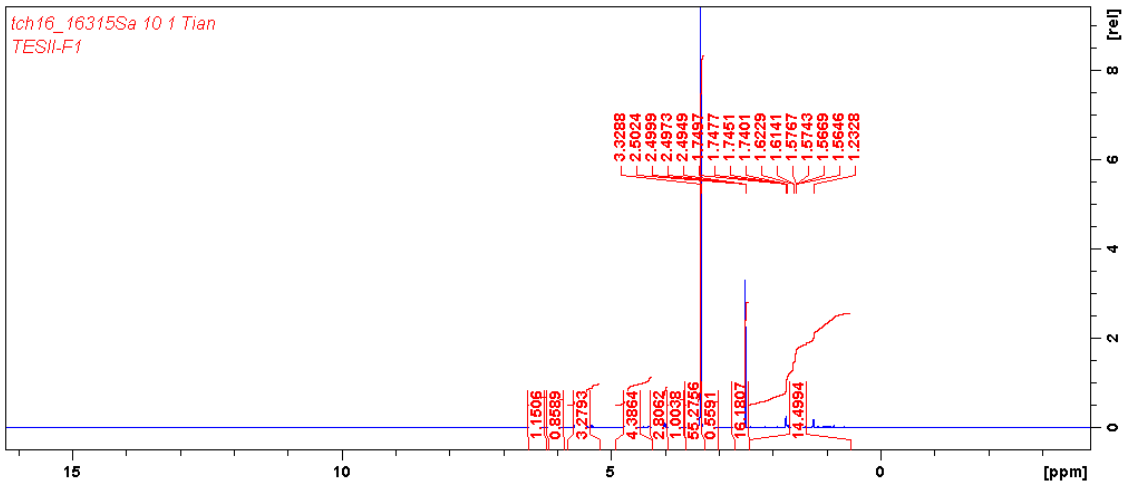
Appendix 6: COSY of compound 20



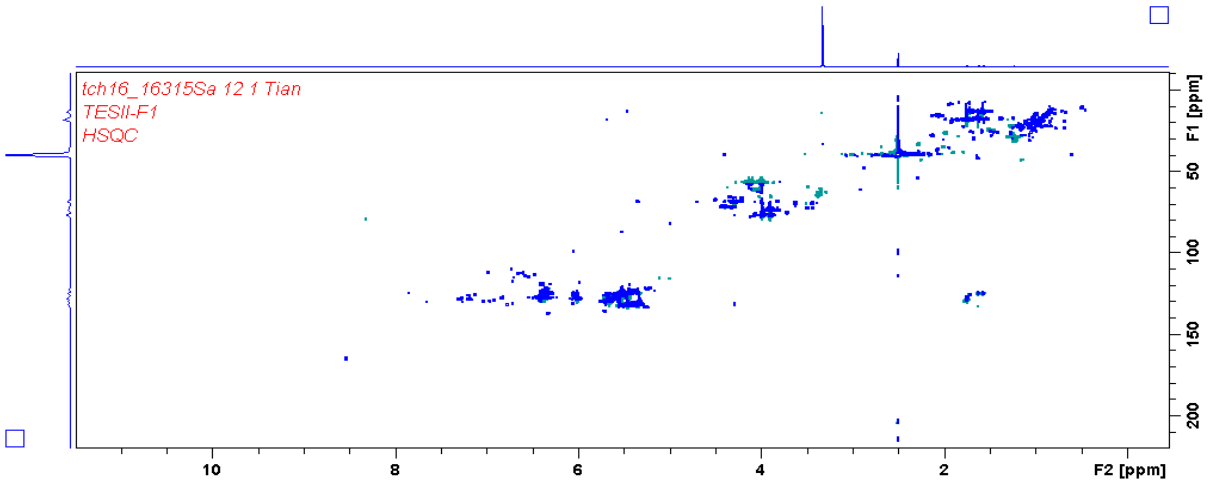
Appendix 7: HMBC of compound 20



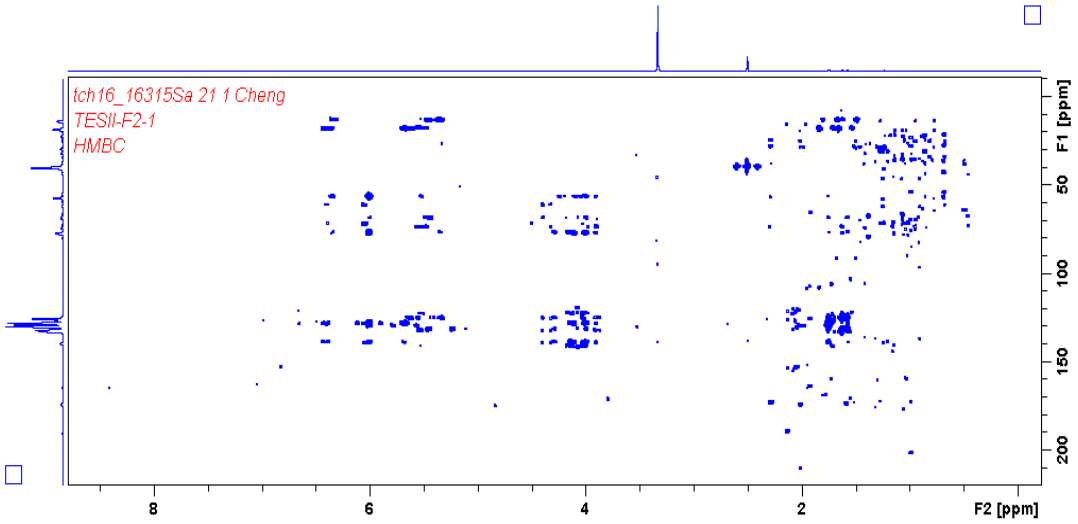
Appendix 8: ^{1}H NMR of compound 21



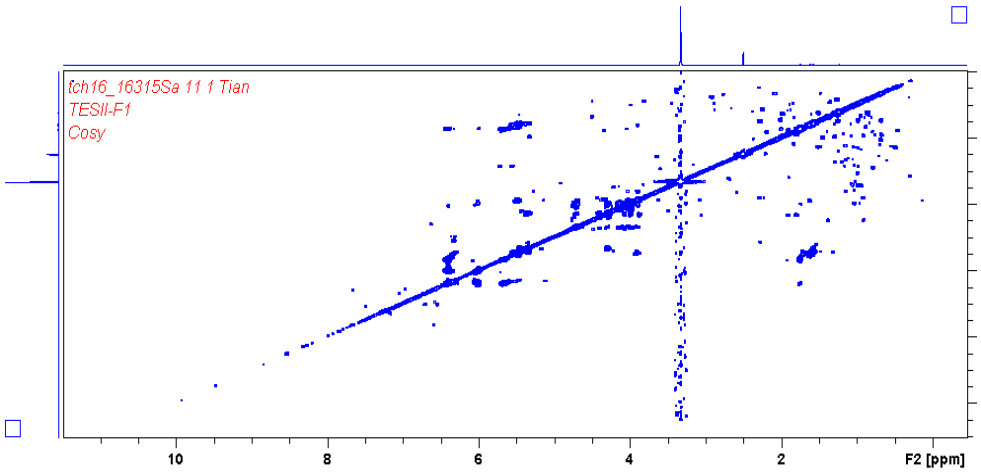
Appendix 9: HSQC of compound 21



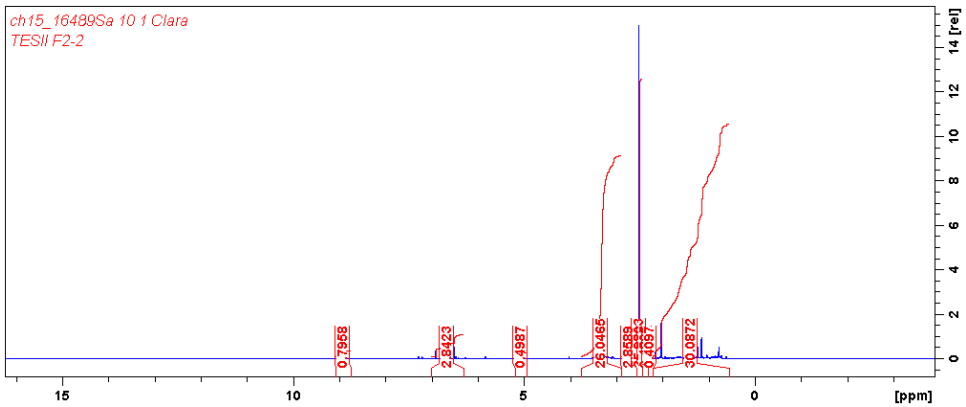
Appendix 10: HMBC of compound 21



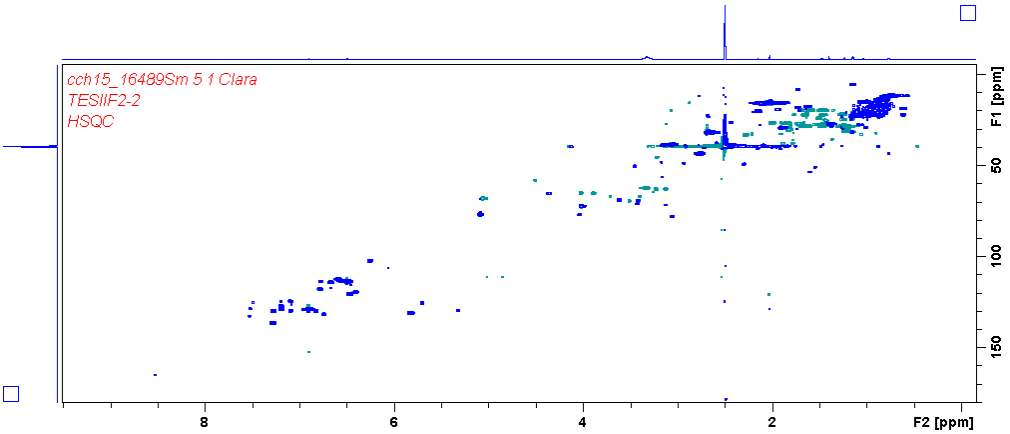
Appendix 11: COSY of compound 21



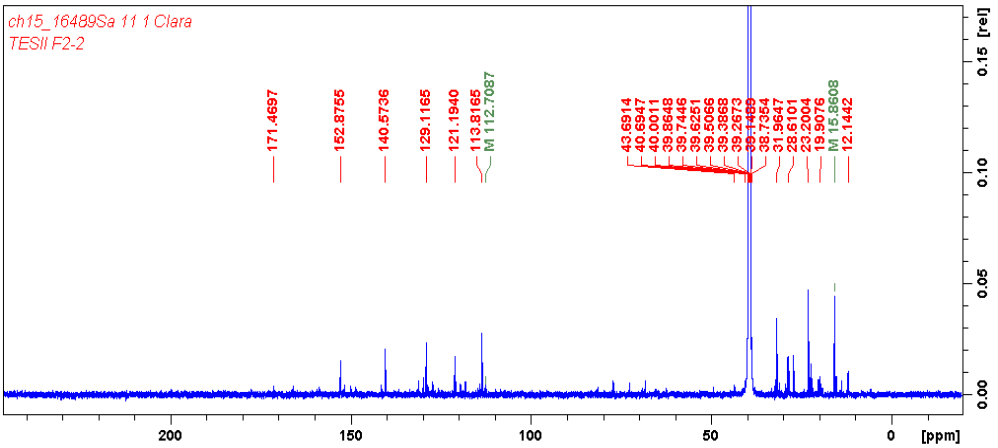
Appendix 12: ^{1}H NMR of compound 22



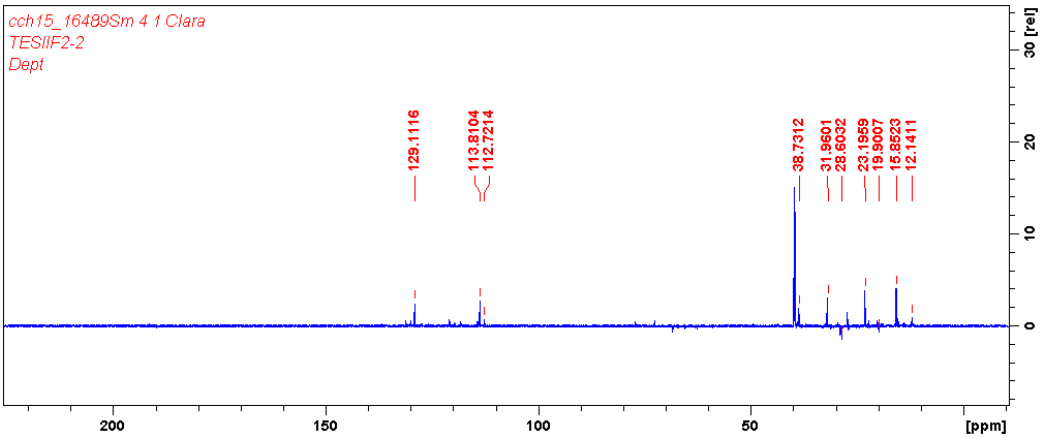
Appendix 13: HSQC of compound 22



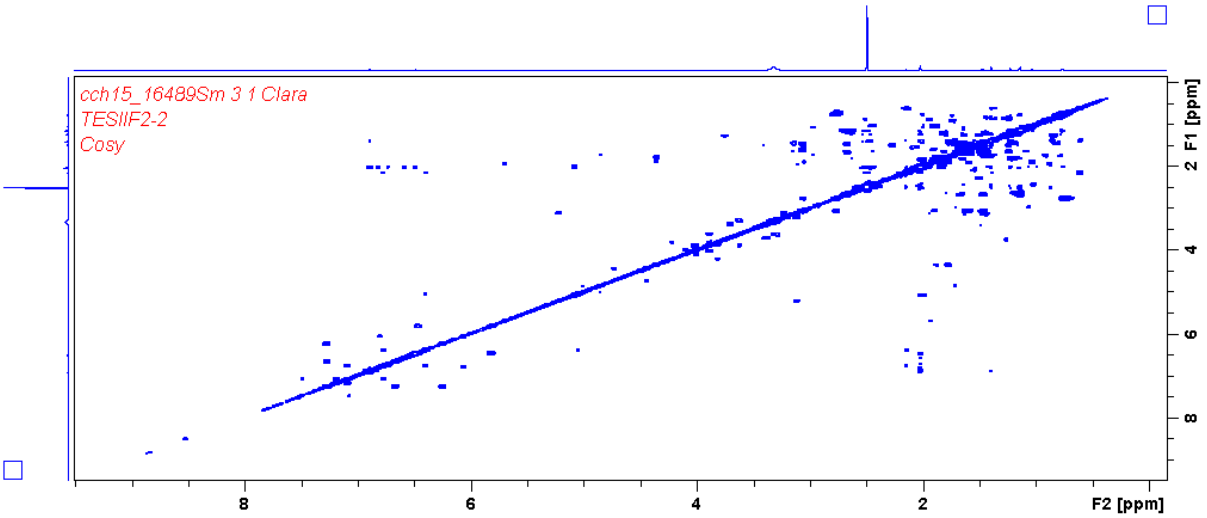
Appendix 14: ^{13}C NMR of compound 22



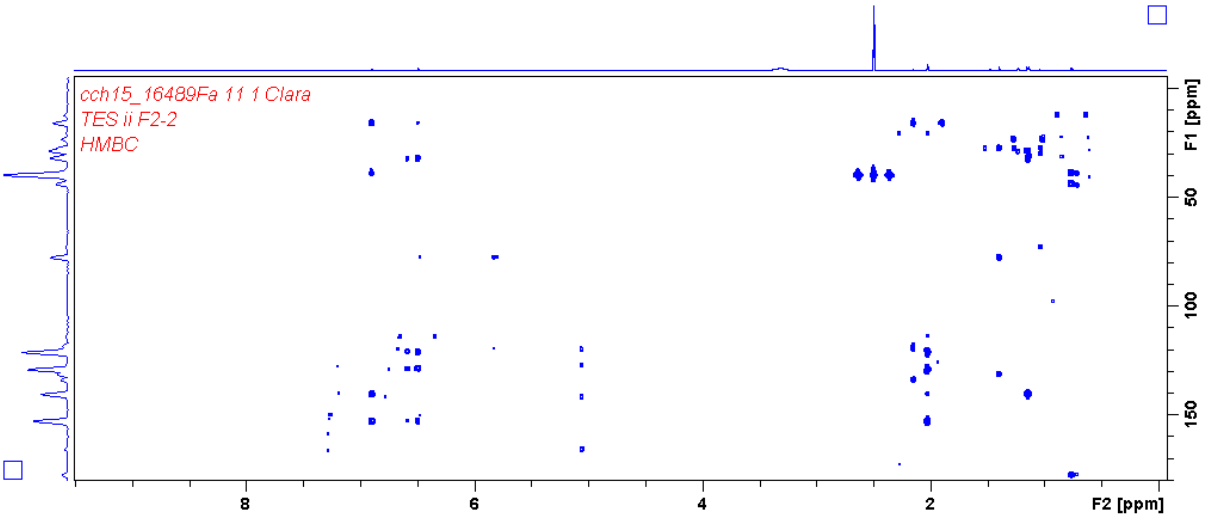
Appendix 15: DEPT-135 analysis of compound 22



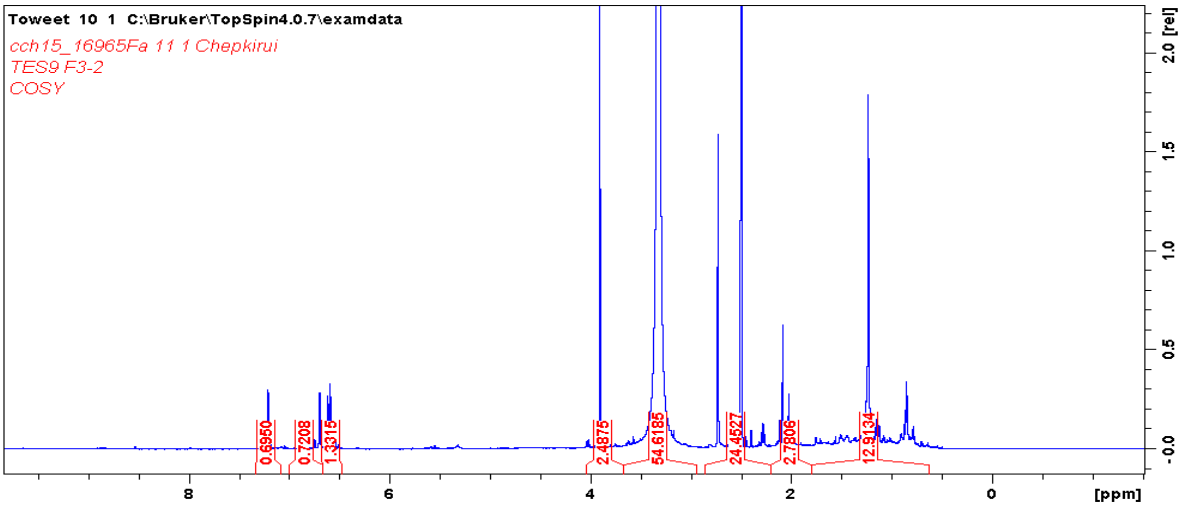
Appendix 16: $^{1}H/^{1}H$ correlation of compound 22



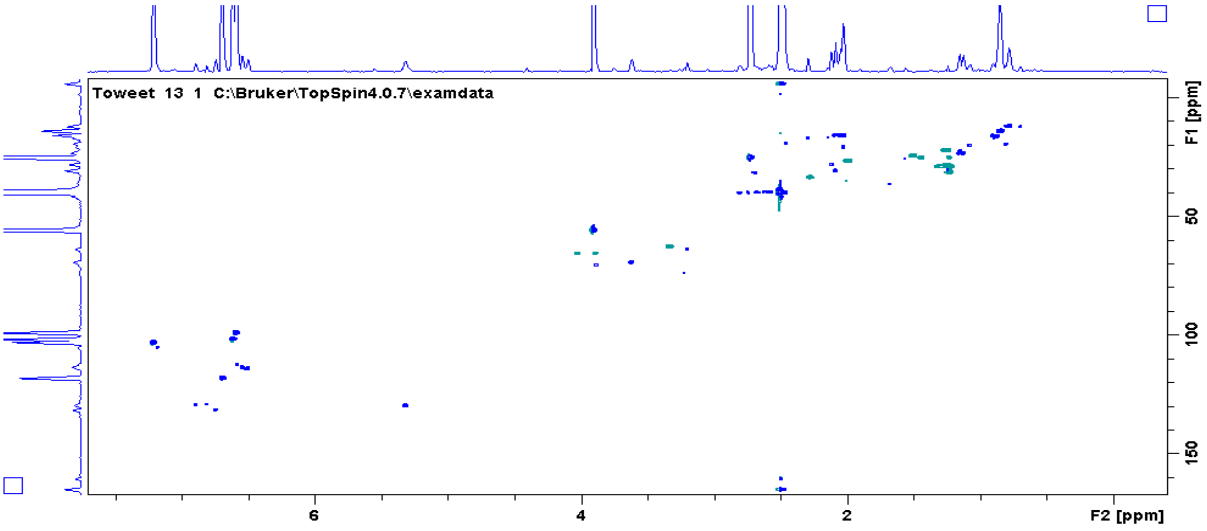
Appendix 17: HMBC correlations of compound 22



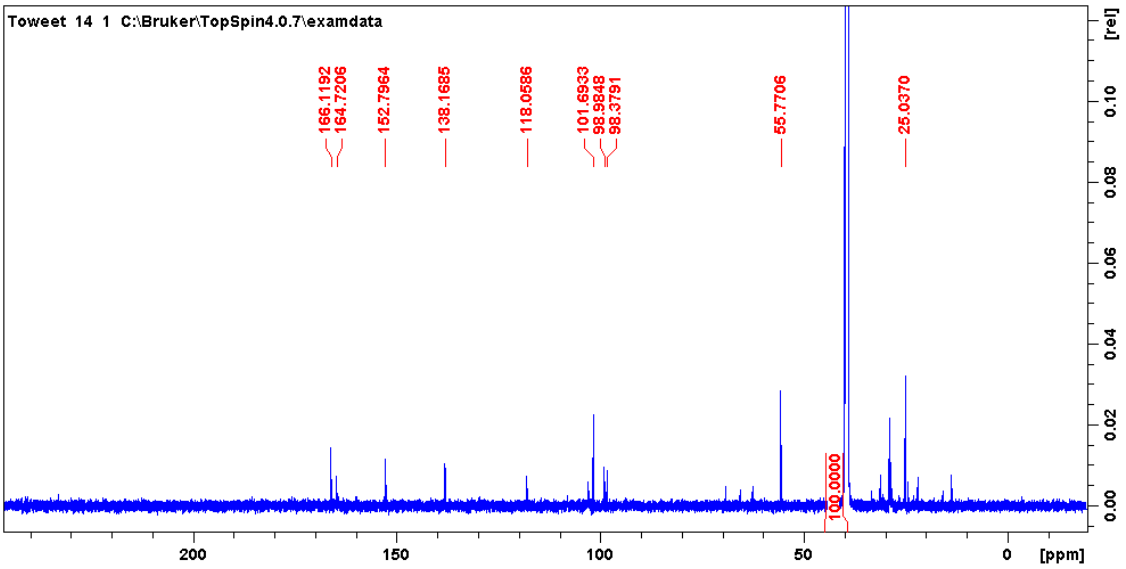
Appendix 18: ^{1}H NMR of compound 23



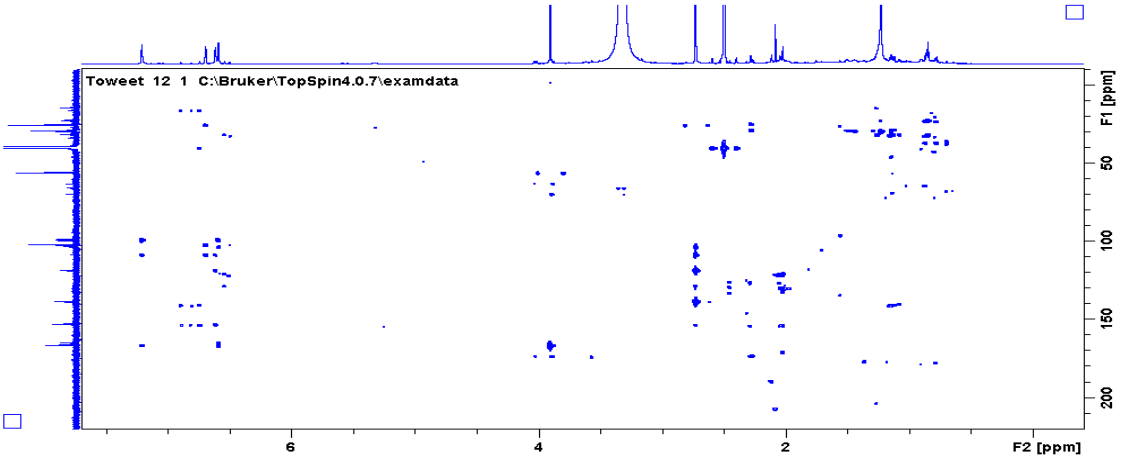
Appendix 19: HSQC of compound 23



Appendix 20: ^{13}C NMR of compound 23



Appendix 21: HMBC spectrum of compound 23



Appendix 22: NACOSTI Research Permit

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone:+254-20-2213471,
2241349,3310571,2219420
Fax:+254-20-318245,318249
Email: dg@nacosti.go.ke
Website : www.nacosti.go.ke
When replying please quote

NACOSTI, Upper Kabete
Off Waiyaki Way
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No. **NACOSTI/P/19/98230/30461**

Date: **29th May, 2019.**

Erick Kipngetich Towett
University of Kabianga
P.O. Box 2030 - 20200
KERICHO.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Isolation of secondary metabolites from Endophytic fungi of Syzygium cordatum (Myrtaceae) for the control of bean bacterial pathogens."*** I am pleased to inform you that you have been authorized to undertake research in **Nakuru County** for the period ending **27th May, 2020.**

You are advised to report to **the County Commissioner and the County Director of Education, Nakuru County** before embarking on the research project.

Kindly note that, as an applicant who has been licensed under the Science, Technology and Innovation Act, 2013 to conduct research in Kenya, you shall deposit **a copy** of the final research report to the Commission within **one year** of completion. The soft copy of the same should be submitted through the Online Research Information System.

BONIFACE WANYAMA
FOR: DIRECTOR-GENERAL/CEO

Copy to:
The County Commissioner
Nakuru County.

The County Director of Education
Nakuru County.

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Two New Aliphatic Alkenol Geometric Isomers and a Phenolic Derivate from Endophytic Fungus *Diaporthe* sp. Host to *Syzygium cordatum* (Myrtaceae)

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Abstract

Fungal endophytes are regarded as a prolific source of secondary metabolites with desirable antibacterial, anticancer, antifungal and antidiarrheal properties. In this study, the fungal endophyte *Diaporthe* sp. host to the plant *Syzygium cordatum* yielded three novel antibacterial compounds after extraction with ethyl acetate and purification using column chromatography and preparative high-performance liquid chromatography. The eluted fraction that yielded the three compounds showed significant antibacterial activity against selected common bean bacterial pathogens; *Pseudomonas syringae* and *Xanthomonas axonopodis* with their corresponding zones of inhibition of 10.7 mm and 14 mm, respectively. The structural determination of three compounds was achieved using spectral information from 1D and 2D NMR as well as mass spectrometry. Two enol geometric isomers that were accorded trivial names as *Z*-cordatenol, *E*-cordatenol, and a phenolic derivative was accorded a trivial name as α-cordenol. Bioactivity of *Z*-cordatenol and *E*-cordatenol was attributed to hydroxylation on the aliphatic alkene chains, whereas, in α-cordenol, hydroxylation of the benzene ring and side chain aziridine ring enhanced its activity. These results are evident that endophytic fungi are rich sources of secondary metabolites that can be utilized to control phytopathogens.

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Introduction

Common bean (*Phaseolus vulgaris L.)* as a cheap alternative source of proteins and other essential minerals, is the most consumed leguminous crop among the Kenyans, who cannot afford other sources like meat and fish. Kenyans consume approximately 450,000 tons of beans against the local production between 150,000 and 200,000 tons harvested from about 800,000 hectares [1]. The declining level of bean's productivity is attributed to bacterial pathogens *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli*, which causes halo blight and common bacterial blight (CBB), respectively. These bacterial diseases in beans can be easily noticed by water-soaked coloration and red-brown scratches on the leaves, which subsequently affects the beans hence reducing their productivity [2, 3]. Currently, chemical control methods have been used to manage these bacterial pathogenic diseases in beans. This involves the use of copper based foliar spray and antibiotics like streptomycin sulphate for controlling the effects on the leaves and seeds, respectively. The usage of these pesticides has been reported to cause environmental pollution, which includes the adverse effects on water bodies, soil and food [4]. These bacterial pathogens have also developed resistance towards the synthetic agrochemicals hence reducing their effectiveness in plant applications [5]. Therefore, there is a need to look for an alternative source of anti-phytopathogenic compounds from natural sources, including the use of extracts isolated from plants and endophytic fungi.

The tropical ecosystem is home to most plant and animal species worldwide. The ecosystem by extension, is the major source of bioactive compounds whereby most of the medicinal plants are found in it [6]. Currently, due to the extinction of some plant species and abiotic stress, plants' potential of producing secondary metabolites is not to the expected level [7]. Researchers then shifted their concentrations to exploring secondary metabolites isolated from microorganisms (fungal or bacterial) that reside inside tissues of healthy plants, generally referred to as endophytes. The endophytic fungi are known to produce secondary metabolites that help them to defend their host plant territories against epiphytes and other plant pathogens [8]. Some of the isolated secondary metabolites from endophytic fungi of higher plants have desirable anti-bacterial, anti-cancer, anti-inflammatory, anti-diabetic and anti-viral activities [8]. *Syzygium cordatum* (water berry) of Myrtaceae family is among these plants that host important endophytes [9]. This plant grows to a maximum of 20 m and native to high altitude areas of Zimbabwe and Kenya highlands [6]. The concoction of leaves has been traditionally used in the treatment of stomach ache and diarrhea [10]. The hydro-distillates from the leaves have also been used to treat airborne related diseases like tuberculosis [11]. Due to long term co-hesitant of the fungal endophytes and the plant in a mutualistic relationship, the exchange of genetic materials is therefore possible; hence can synthesize similar secondary metabolites [8]. This study was aimed at evaluating the antibacterial activities of endophytic fungi isolated from *S. cordatum* against selected bacterial pathogens of beans.

Materials and Methods

Collection of plant materials

A research permit was sorted from the National Commission for Science, Technology and Innovation (NACOSTI). Later, fresh leaves and stem bark of *S. cordatum* plant were collected from Mt. Elgon forest (01^0 08'00'' N 34 ^{0} 35'00'' E or 1.13333 ° N, 34.583333 ° E). The samples were taken to the Botany Department of Egerton University for scientific identification. Isolation of fungal endophytes was thereafter done within eight hours after the collection of plant materials to avoid drying.

Isolation of endophytic fungi

The endophytic fungi were isolated from internal plant tissues using the method of Tian et al. [12] with some modifications. The leaves and stem bark of the selected healthy plants were washed in running tap water to remove any soil or other foreign material and blot dry. The leaves and the stem were then sectioned to approximately 1-4 mm size. The sectioned materials were surface sterilized for 5 minutes in 10 ml of 1% sodium hypochlorite followed by 20 ml of 70% ethanol. Thereafter, the materials were adequately rinsed with sterile distilled water to wash off disinfectants. The materials were then placed in petri plates containing potato dextrose agar (PDA) medium amended with 200 mg/l of streptomycin sulphate. The plates were sealed using Parafilm and incubated at 25±2ºC in a light chamber. The growth of mycelia was monitored, which thereafter, were used to generate pure cultures of the fungal endophytes on PDA medium.

Screening and identification of fungal endophytes against bacterial pathogens

Antimicrobial activity of isolated endophytic fungi was determined against common bean bacterial pathogens using the method of Arya and Sati [13] with slight modifications. The pathogens *P. syringae and X. axonopodis* were inoculated in 50 ml conical flask containing nutrient broth medium and incubated at 37±2°C. After 24 hours, pathogen cultures were transferred separately to Erlenmeyer flasks containing sterilized water and shaken until the turbidity of bacterial suspension was comparable to the turbidity of McFarland's standard solution (0.05 ml of 1.175% barium chloride ($BaCl_2.2H_20$) in 9.95 ml of 1 % sulphuric acid). Around 100 µl of suspended bacteria (1.5×10^8 CFU/ml) were inoculated in Petri dishes containing Muller Hinton agar (MHA) using a sterile micro-dispenser. The six-millimeter diameter plugs of actively growing mycelia of fungal endophytes from PDA plates were cut using a sterile cork-borer and placed on the surface of the Muller Hinton agar medium containing the bacterial pathogens. These plates were sealed with Parafilm and incubated at 37±2°C. The zones of inhibition were then measured in mm using a ruler scale after 24 hours of incubation. The experiment was performed in triplicates.

The fungal endophyte that showed the highest antibacterial activity against selected bacterial pathogens was selected and coded as SC-S-11. The molecular method of identification was employed for SC-S-11, where the genomic DNA was extracted and the ITS (Internal Transcriptase Spacer) region was amplified using PCR method. The ITS region was sequenced by Sanger sequencing protocol and compared to known sequences in NCBI GeneBank using BLAST at >98% similarity. The ITS region sequence was submitted to GenBank with accession number: JF773672.1.

Fermentation and extraction of antibacterial metabolites

The fungal endophyte that showed the highest activity against selected bacterial pathogens was selected for solid fermentation. In the method, ten 500 ml Erlenmeyer flask containing 90g of parboiled rice in 90 ml distilled water per flask were autoclaved at 121±2ºC for 40 minutes. Agar plugs (six-millimeter diameter) were cut from the 7-day-old fungal culture on PDA agar then placed on the surface of sterile rice media. One flask without inoculum was used as a control. After 21 days of incubation, 200 ml of methanol was added to each flask and the contents were ultra-sonicated for 90 minutes at 30±2ºC to enhance extractions of secondary metabolites from the endophytic fungi. The methanol extracts were filtered and evaporated under reduced pressure to yield their respective crude extracts. The methanol crude extract was then suspended in water, followed by liquid-liquid partitioning between hexane and ethyl acetate. Around 200 ml of resulting organic layers was evaporated separately, under reduced pressure to yield hexane and ethyl acetate crude extracts. Hexane crude extracts were discarded, whereas the ethyl acetate crude extract was used for subsequent procedures.

Column chromatography

A series of thin layer chromatography analysis (TLC) was done to ascertain the best solvent mixture to be used in eluding the columns, after which hexane: ethyl acetate: methanol (3:5:2) as mobile phase was reached on due to good separation pattern. Dry ethyl acetate crude extract of *Diaporthe* sp. metabolites was reconstituted in the little amount of distilled ethyl acetate and then loaded on the surface of an evenly packed silica gel column using a clean micropipette. Silica gel (70-230 ASTM) supplied by Scharlau Lab supplies limited was used. Columns of 50 cm length and 20 mm diameter were used. The collected fractions that showed similar patterns on TLC analysis were pooled together. Ethyl acetate crude yielded four fractions, named as F1-F4. F2 of this fungal extract was further purified using preparative high-pressure liquid chromatography (HPLC) due to its significant antibacterial activity against the test organisms.

High performance liquid chromatography

Preparative high-performance liquid chromatography system (Shimadzu-UFLC prominence), fitted with an auto sampler (Model- SIL 20AC HT) and UV-visible detector (Model-SPD 20A) was used to separate the compounds. Dry samples obtained from column chromatography were re-dissolved in HPLC grade methanol each to make a concentration of 20 mg/ml. The prepared solutions were centrifuged using Bio-Cote centrifuge, to enhance the sedimentation of solids that may block the column. 150 µl of samples were loaded onto an auto-sampler. This separation was performed on the Kromasil reverse phase C18 5 µm column (4.6 × 250 mm). Gradient separation was performed using mobile phase A (100 % Milli pore water) and mobile phase B (100 % HPLC grade methanol). Both Milli pore water and methanol were of analytical grade supplied by Scharlau Lab supplies

limited. The separation conditions were set as follows: 10% of B in A at injection time, 80% of B in A at 30th minute, 100% of B at 31st to 37th minute then normalized to 10% of B in A at 38th minute to 45th minute where the separation process was stopped. Chromatographic separations were monitored at the absorbance range of 220-420 nm. The collected fractions were concentrated under a reduced pressure in a rotary evaporator to yield pure compounds. Oven temperature at 40°C and a flow rate of 3 ml/minute were maintained. F2 of SC-S-11 yielded three pure compounds, recorded as compounds 1, 2 and 3, respectively.

Antibacterial assay for crude extract, fractions and pure compounds

The paper disc diffusion assay was used to screen for anti-bacterial activities of crude ethyl acetate extracts, fractions from column chromatography and the purified compounds. 100 µl of bacterial pathogen suspensions (1.5×10^8 CFU/ml) was homogeneously spread on sterile Mueller Hinton agar (38 g/l) in Petri dishes. The ethyl acetate fungal extracts, fractions from column chromatography analysis and pure compounds were prepared by dissolving them in 1% DMSO in distilled sterile water. The sterile paper disc was soaked in 5 mg/ml concentration of the prepared extracts, then placed at the center of MHA plates containing the bean bacterial pathogens. A sterile disc dipped in 1% DMSO was used as a negative control, while standard chloramphenicol antibiotic was used as a positive control. The plates were sealed using Parafilm and inoculated at 37± 2°C for 24 hours, after which the zones of inhibitions were measured in mm using a ruler scale. This experiment was done in triplicates.

^{1}H, ^{13}C and ^{2}D NMR spectroscopy and mass spectroscopy

The ^{1}H, ^{13}C and all 2D NMR spectroscopy were recorded on advance Bruker 500 MHz NMR spectrometer. The spectra were referenced according to the deuterochloroform signal at δH 7.24 (for 1H NMR spectra) and δC 77.0 (for ^{13}C NMR spectra). 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). HSQC spectrum (Heteronuclear Single Quantum Coherence) was used to determine the connectivity of hydrogen to their respective carbon atoms. The compound's mass spectra were recorded on Finnigan Tripple Stage Quadrupol Spectrometer (TSQ-70) with an electron spray ionization (ESI) method in the analysis. Thermo X Calibur Qual computer software was used in the analysis of the mass chromatograms. Merck silica gel 60 (0.040-0.063 mm) was used for column chromatography and Merck 20 × 20 cm silica gel 60 F254 aluminum sheets were used for thin-layer chromatography. The TLC plates were analyzed under UV (254 and 366 nm) before being sprayed and developed with a [1:2:97] anisaldehyde: concentrated sulphuric acid: methanol spray reagent and then heated.

Data analysis

The comparison of means was done using SPSS version 21.0 and the most bioactive secondary metabolites were selected based on the antibacterial activity as shown by the size of inhibition zones. The difference in the mean inhibitory effect of each fungal extract was determined using one-way ANOVA. Tukey's Honestly Significant Difference (HSD) and a Post-Hoc analysis were used to determine if there was any significant difference between the means of the isolates and the positive control.

Results and Discussion

The endophytic fungus was isolated from the stem bark tissues of *S. cordatum* plant and given the code as SC-S-11. Molecular information reveals that SC-S-11 belongs to the genus *Diaporthe* of phylum *Ascomycota*, Kingdom Fungi. It showed a close correlation with *Diaporthe sophorae* according to BLAST (Basic Local Alignment Search Tool) information. Genus *Diaporthe* is the most encountered genera of fungal endophytes in several host plants. The genus is known to be a source of enzymes and bioactive secondary metabolites having anti-bacterial, anti-cancer and anti-fungal activities. In the past, plants of genus *Diaporthe* have been known to produce biochemical that deter herbivores, hence can be used as a biocontrol agent [14-16]. As compared to this study, endophytic fungi of genus *Diaporthe* have also been isolated from plants such as acacia, *Maytenus ilicifolia, Berberis vulgaris* having palpable anti-bacterial and antifungal activities [8].

Dual culture assay

Diaporthe sp. SC-S-11 showed a good antagonism against selected common bean bacterial pathogens *X. axonopodis and P. syringae* with corresponding zones of inhibition of 17.67 mm and 1.67 mm, respectively. These results were not significantly

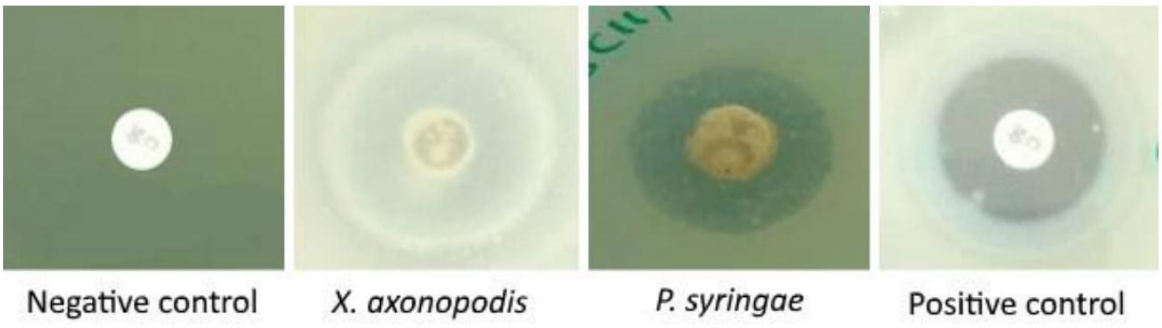

Fig. 1 Antibacterial activity of *Diaporthe* sp. SC-S-11 against *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli.* The standard antibiotic chloramphenicol was used as a positive control.

different at $P>0.05$ with that of standard antibiotic chloramphenicol having zones of inhibitions as 25 mm and 20 mm against *X. axonopodis and P. syringae*, respectively (Fig. 1). The antibacterial activity is attributed to the production of secondary metabolites by strain SC-S-11. *Diaporthe* fungal species have been known to possess good antagonism with bacterial pathogens. For instance, Li et al. [16] showed an appreciable antibacterial efficacy of *Diaporthe* sp. LG23 against human pathogens *Streptococcus pyogenes* and *Pseudomonas aeruginosa*. These results were also in agreement with that of Tong et al. [17] where endophytic fungus *Diaporthe* sp. isolated from traditional herb *Orthosiphon stamieus* was found to have good anti-candida activity against *Candida albican*, an opportunistic pathogen that causes nosocomial infections, especially in the USA. This shows that endophytic fungi of genus *Diaporthe* are a good source of bioactive secondary metabolites that can be used in the formulation of antibiotics or agrochemicals.

Extraction and purification of secondary metabolites

The secondary metabolites from the liquid culture of endophytic fungus SC-S-11 were extracted using methanol. SC-S-11 methanol after portioning between hexane and ethyl acetate solvents yielded 2.03 g hexane extract and 1.92 g ethyl acetate extract. Antibacterial compounds possess mid to high polarity; in this case, the ethyl acetate portion was taken for fractionation using column chromatography, which yielded four fractions named; F1 (10.12 mg), F2 (80.43 mg), F3 (42.14 mg) and F4 (30.02 mg). The fraction F2 was selected for further purification due to its appreciable antibacterial activity against *P. syringae and X. axonopodis*.

MIC determination of active metabolites

MIC assay was performed for ethyl acetate extract and its fractions obtained after column chromatography. In this method, a stock solution of 5 mg/ml (100%) was prepared. Later, three serially diluted concentrations of 3.75 mg/ml (75%), 2.50 mg/ml (50%) and 1.25 mg/ml (25%) were prepared. The agar disc diffusion assay was then performed for all the solutions against selected bean bacterial pathogens and the results are tabulated in Table 1. The secondary metabolites present in all the solutions showed activity against *X. axonopodis* but poor anti-bacterial activity was noticed against *P. syringae.* The fraction F2 showed palpable MIC values of 2.50 mg/ml against *X. axonopodis* and 1.25 mg/ml against *P. syringae*. On the other hand, ethyl acetate crude extract was also active against the test organisms with MIC values of 2.50 mg/ml against *X. axonopodis* and 1.25 mg/ml against *P. syringae* (Table 1). The results of this study correlate with a study done by Wanga et al. [18] where the ethyl acetate of fungal endophyte *Fusarium solani* isolated from *Markhamia lutea* showed antibacterial activity against *X. axonopodis* with an inhibition zone of 14 mm, while dismal activity was recorded against *P. syringae.* The resistivity of *P. syringae* is associated with action modes under which the bacterium can easily mutate. Secondly, the presence of an e-flux pump; an internally developed mechanism, which is a substrate specific and functions to prevent the accumulations of antibacterial drugs within their system and prevents them from reaching the target sites [19].

Characterization of antibacterial compounds

Three new compounds were isolated from the ethyl acetate extract of SC-S-11 and named as compounds 1, 2 and 3. Compound 1 was obtained as a brown solid at room temperature. Its molecular mass was

Table 1 Inhibition zones (mm) of serially diluted ethyl acetate extracts and collected fractions against common bean bacterial pathogens.

Treatments	Extracts	Serial dilution	*X. axonopodis* pv. *phaseoli*	*P. syringae* pv. *phaseolicola*
	Ethyl acetate	100%	11.67±0.88 ^{b,c}	12.67±0.67 ^{c}
		75%	10.00 ±0.58 ^{b,c}	10.67±0.67 ^{b,c}
		50%	8.00±0.58 ^{b}	10.00±0.58 ^{b,c}
		25%	0.00±0.00 ^{a}	7.33±0.33 ^{b}
	F1	100%	9.00±0.58 ^{b}	13.33±1.20 ^{c}
		75%	8.33±0.88 ^{b}	11.67±0.33 ^{b,c}
		50%	7.67±0.67 ^{b}	10.33±0.88 ^{b,c}
		25%	0.00 ±0.00 ^{a}	9.33±0.67 ^{b}
Diaporthe sp.	F2	100%	14.00±0.88 ^{c}	10.67±0.33 ^{b,c}
SC-S-11		75%	12.00±0.58 ^{b,c}	10.33±0.68 ^{b,c}
		50%	10.67±0.58 ^{b,c}	7.00±0.00 ^{b}
		25%	0.00 ±0.00 ^{a}	0.00 ±0.00 ^{a}
	F3	100%	12.33±0.88 ^{b,c}	0.00±0.00 ^{a}
		75%	10.00±1.73 ^{b,c}	0.00±0.00 ^{a}
		50%	9.00±0.58 ^{b}	0.00±0.00 ^{a}
		25%	7.33±0.33 ^{b}	0.00±0.00 ^{a}
	F4	100%	10.67±0.88 ^{b,c}	0.00 ±0.00 ^{a}
		75%	9.67±0.67 ^{b}	0.00±0.00 ^{a}
		50%	8.33±0.88 ^{b}	0.00±0.00 ^{a}
		25%	7.33±0.33^{b}	0.00±0.00 ^{a}
Chloramphenicol	-	-	23.33±0.88 ^{d}	20.67±0.33 ^{d}
Negative control	-	-	0.00±0.00 ^{a}	0.00±0.00 ^{a}

The values given are the mean of the three replicates ± standard error (S.E). Values sharing the same letter (s) within the columns are not significantly different in their anti-bacterial activities (P<0.05, Turkey's test).

established to be 198.13 amu from MS data, corresponding to molecular ion at *m/z* 221.26 $(M+Na)^+$ (Fig 2) and a molecular formula of $C_{11}H_{18}O_3$, which indicates double bond equivalence of 3, corresponding to the three double bonds presence in the aliphatic chain. This compound was identified as an aliphatic enol compound based on its characteristic absorption on both 1D and 2D NMR data. ^{1}HNMR spectrum showed the presence of methylene and methyl protons with different multiplicities resonating at δ_H 4.00 and 4.13 (H-1), δ_H 5.66 (H-2), δ_H 6.40 (H-3), δ_H 6.00 (H-4), δ_H 3.98 (H-6), δ_H 4.29 (H-7), δ_H 5.34 (H-8), δ_H 5.45 (H-9), δ_H 1.57 (H-10) and δ_H 1.75 (H-5'), which corresponds to carbon signals at δ_C 61.4 (C-1), δ_C 129.4 (C-2), δ_C 127.5 (C-3), δ_C 128.0 (C-4), δ_C 76.7 (C-6), δ_C 68.4 (C-7), δ_C 131.5 (C-8), δ_C 125.3 (C-9), δ_C 18.2 (C-10) and δ_C 13.4(C-5'), respectively. The coupling constant of protons at δ_H 5.66 (H-2) and δ_H 6.40 (H-3) was found to be 7.24 and 1.74 while those at δ_H 5.34 (H-8), δ_H 5.45 (H-9) were found to be 2.14 and 7.06 Hz. This implies that the $\Delta^{2,8}$ in compound 1 is a *Z* (*cis*) isomer (Table 2). This, therefore, is evidence that compound 1 has a small dihedral angle between protons found at the stilbene alkenes at C-2 and C-3, C-8 and C-9. ^{13}C NMR and DEPT spectral information showed presence of hydroxylated aliphatic alkene carbon system with a total of 11 carbon signals having one methylene carbon at δ_C 61.4 (C-1), seven methine carbon at δ_C 68.4 (C-7), δ_C 76.7 (C-6), δ_C 125.3 (C-9), δ_C 127.5 (C-3), δ_C 128.0 (C-4), δ_C 129.4 (C-2) and δ_C 131.5 (C-8), two methyl carbons at δ_C 13.4 (C-11) and δ_C 18.2 (C-10), and a quaternary carbon at δ_C 138.9 (C-5).

The COSY spectrum showed the correlation of H-2 (δ_H 5.66) with H-3 (δ_H 6.40), H-3 (δ_H 6.40) with H-4 (δ_H 6.00), H-7 (δ_H 4.29) with H-6 (δ_H 3.98) and H-8 (δ_H 5.34) while H-9 (δ_H 5.45) with H-10 (δ_H 1.57). While the HMBC spectrum showed that proton resonating at δ_H 6.00 (H-4) correlates with C-2 (δ_C 129.4), C-3 (δ_C 127.5), C-5 (δ_C 138.9) and C-6 (δ_C 76.7), while that resonating at δ 3.98 correlates with C-4 (128.0), C-5 (δ_C 138.9), C-7 (δ_C 68.4) and C-8 (δ_C 131.5). Other COSY and HMBC spectral information is summarized in Fig 3 and Table 2. Compound 1 was assigned an IUPAC name as (*2Z, 4Z, 8Z*)-5-methyldec-2, 4, 8-triene-1, 6, 7-triol and trivial name as *Z*- cordatenol coined from the species name of the host plant and enol functional group.

Compound 2 was similar to compound 1, it was as a brown solid at room temperature, having close absorptions on NMR data. The two compounds are geometric isomers having a difference in the spatial arrangement of atoms at the alkene sections. Its mass was established to be 198.13 amu from MS data, corresponding to molecular ion at *m/z* 221.26 $(M+Na)^+$ (Fig 2) and a molecular formula of $C_{11}H_{18}O_3$, which indicates double bond equivalence of 3,

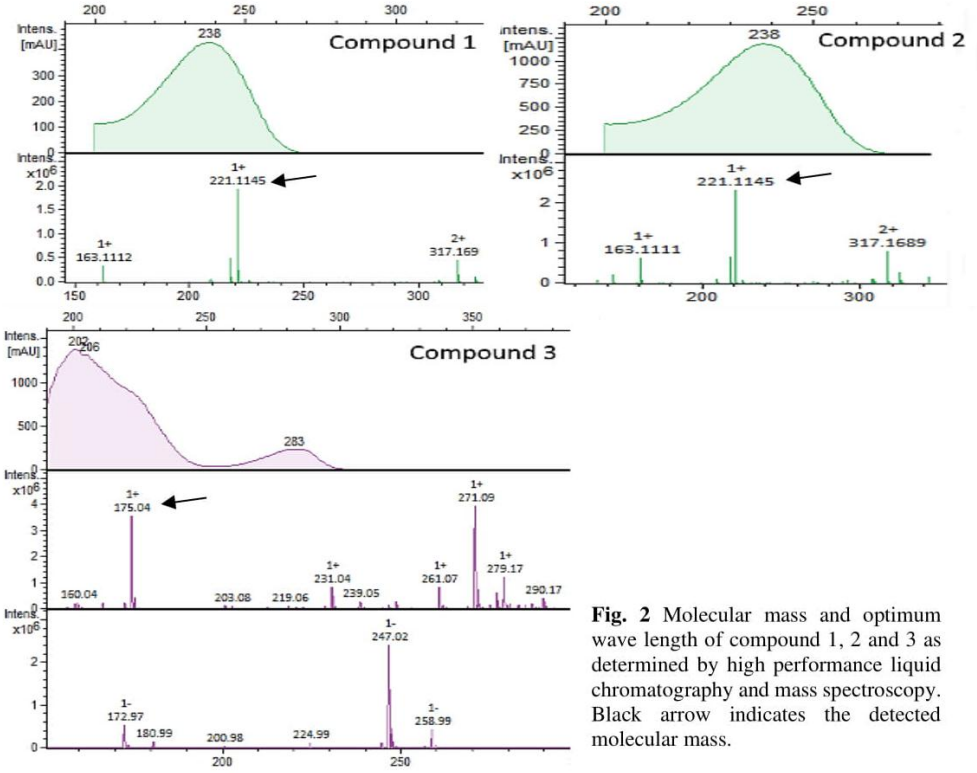

Fig. 2 Molecular mass and optimum wave length of compound 1, 2 and 3 as determined by high performance liquid chromatography and mass spectroscopy. Black arrow indicates the detected molecular mass.

corresponding to the three double bonds presence in the aliphatic chain. The compound 2 was identified as an *E* isomer of compound 1, and both belong to an enol group of compounds. ^{1}H NMR spectrum showed the presence of methylene and methyl protons with different multiplicities resonating at δ_H 4.02 and 4.06 (H-1), δ_H 6.04 (H-2), δ_H 6.34 (H-3), δ_H 6.34 (H-4), δ_H 3.90 (H-6), δ_H 3.87(H-7), δ_H 5.49 (H-8), δ_H 5.53 (H-9) , δ_H 1.62 (H-10) and δ_H 1.71 (H-11), which corresponds to carbon signals resonating at δ_C 56.6 (C-1), δ_C 125.2 (C-2), δ_C 124.8 (C-3), δ_C 122.5 (C-4), δ_C 77.3 (C-6), δ_C 73.7(C-7), δ_C 132.2 (C-8), δ_C 125.1 (C-9), δ_C 17.6 (C-10) and δ_C 12.8 (C-11), respectively. In addition, ^{1}H NMR showed *trans* vicinal correlations of protons attached to C-2 and C-3, C-8 and C-9 with $^{3}J_{H\text{-}H}$ coupling constants 11.88, 11.00, 11.24 and 11.36 Hz, respectively. These higher coupling constants prequalifies the double bond in compound 2, as a *trans* or an *E*-isomer. ^{13}C NMR and DEPT spectral information showed the presence of hydroxylated aliphatic alkene carbon system with a total of 11 carbon signals having one methylene carbon at δ_C 56.6 (C-1), seven methine carbon at δ_C 125.2 (C-2), δ_C 124.8 (C-3), δ_C 122.5 (C-4), δ_C 77.3 (C-6), δ_C 73.7(C-7), δ_C 132.2 (C-8) and δ_C 125.1 (C-9), two methyl carbons at δ_C 12.8 (C-11) and δ_C 17.6 (C-10) and a quaternary carbon at δ_C 126.2 (C-5).

The COSY spectrum showed the correlation proton at δ_H 5.53 (H-9) and δ_H 1.62 (H-10). HMBC spectrum shows a correlation of proton and carbons that are 2-3 bonds away, it revealed that proton at δ_H 4.02 and 3.06 (H-1) correlates with C-2 (δ_C 125.2), proton at δ_H 6.34 correlates with C-5 (δ_C 126.2), C-6 (δ_C 77.3) and C-11 (δ_C 12.8), while that resonating at δ_H 5.49 (H-8) correlates with C-6 (δ_C 77.3), C-7 (δ_C 73.7), C-9 (δ_C 125.1) and C-10 (δ_C 17.6). Other COSY and HMBC spectral information is summarized in Table 3 and Fig 3. Compound 2 was assigned an IUPAC name (*2E, 4E, 8E*)-5-methyldec-2, 4, 8-triene-1, 6, 7-triol and a trivial name as *E*-cordatenol a geometric isomer of compound 1.

Compounds 1 and 2 are geometric isomers with different orientations of atom attachments in C-2, C-3, C-8 and C-9. Generally, *cis* isomers have lower $^{3}J_{HH}$ coupling constants, which typically ranges between 6-10 Hz, while that of *trans* isomers have higher $^{3}J_{HH}$ coupling values, which ranges between 11-18 Hz. Basically, the higher coupling values in

Table 2 Nuclear magnetic resonance spectroscopy analysis of compound 1.

No.	^{13}C NMR δ_C ppm	Type	HSQC δ_H ppm	Coupling constant	COSY(^{1}H/^{1}H)	HMBC
1	61.4	CH_2	4.00, 4.13	-	-	2,3
2	129.4	CH	5.66	7.24	3	3,4
3	127.5	CH	6.40	1.76	2,4	2,4,5
4	128.0	CH	6.00	6.08	3	2,3,5,6
5	138.9	C	-	-	-	-
6	76.7	CH	3.98	-	7	4,5,7,8
7	68.4	CH	4.29	-	6,8	5,6,8
8	131.5	CH	5.34	2.14	7,9	7,9,10
9	125.3	CH	5.45	7.06	8	7,8,10
10	13.4	CH_3	1.57	-	9	7,8,9
11	18.2	CH_3	1.75	-	-	5

Table 3 Nuclear magnetic resonance spectroscopy analysis of compound 2.

No.	^{13}C NMR δ_C ppm	TYPE	HSQC δ_H ppm	$^3J_{HH}$ Hz	COSY(^{1}H/^{1}H)	HMBC
1	56.6	CH_2	4.02, 4.06	-	-	2
2	125.2	CH	6.04	11.88	-	1,3
3	124.8	CH	6.34	11.00	-	4,5
4	122.5	CH	6.34	11.00	-	5,6,11
5	126.2	C	-	-	-	-
6	77.3	CH	3.90	-	-	7,8
7	73.7	CH	3.87	-	-	8,9
8	132.2	CH	5.49	11.24	-	6,7,9,10
9	125.1	CH	5.53	11.36	10	7,8,10
10	17.6	CH_3	1.62	-	9	8,9
11	12.8	CH_3	1.71	-	-	5

Table 4 Nuclear magnetic resonance spectroscopy analysis of compound 3.

	13C	Type	HSQC	COSY(^{1}H/^{1}H)	HMBC
1	152.9	C	-	-	-
1'	32.0	CH	2.67	5'	-
2	121.2	C	-	-	-
2'	38.7	CH	3.07		
3	129.1	CH	6.90		1,4,5,7
3'	43.7	CH	2.77	4'	
4	112.7	CH	6.59		1,2,3
4'	12.1	CH_3	0.76	3'	2',3'
5	140.6	C	-	-	-
5'	23.2	CH_3	1.15	-	1,2',5
6	113.8	CH	6.50	-	1,1',2,3
7	15.9	CH_3	2.03	-	1,2,3

trans isomers are generally attributed to a large dihedral angle, which in most cases is 180º, while that in *cis* is due to a small dihedral angle which is about 0-60º [20]. The two compounds belong to an enol group of compounds formally referred to as alkenols [21]. Enols are compounds represented with the hydroxylation of olefin's carbon chain; they are regarded as reactive compounds or intermediates. In plant biological systems, they are synthesized via a substrate level phosphorylation with the help of the enzyme amylase. These compounds are known to undergo tautomerism involving auto-conversion of enol compounds to ketones; which helps in their stability [21]. The activity of the two compounds 1 and 2 can be attributed to the presence of sp^2 hybridized carbon atoms and hydroxylation on the olefin carbon chain, which imparts more nucleophilicity to the compounds. The two parameters render the compounds more reactive sites within the molecule.

Compound 3 was isolated from fraction F2 as a cream yellow solid at room temperature. Its mass was established to be 191.0 amu based on MS data, corresponding to a molecular ion at 175.0 *m/z* ($[M–NH_3]+H)^+$ (Fig 2) and a molecular formula of $C_{12}H_{17}NO$, which indicates a double bond

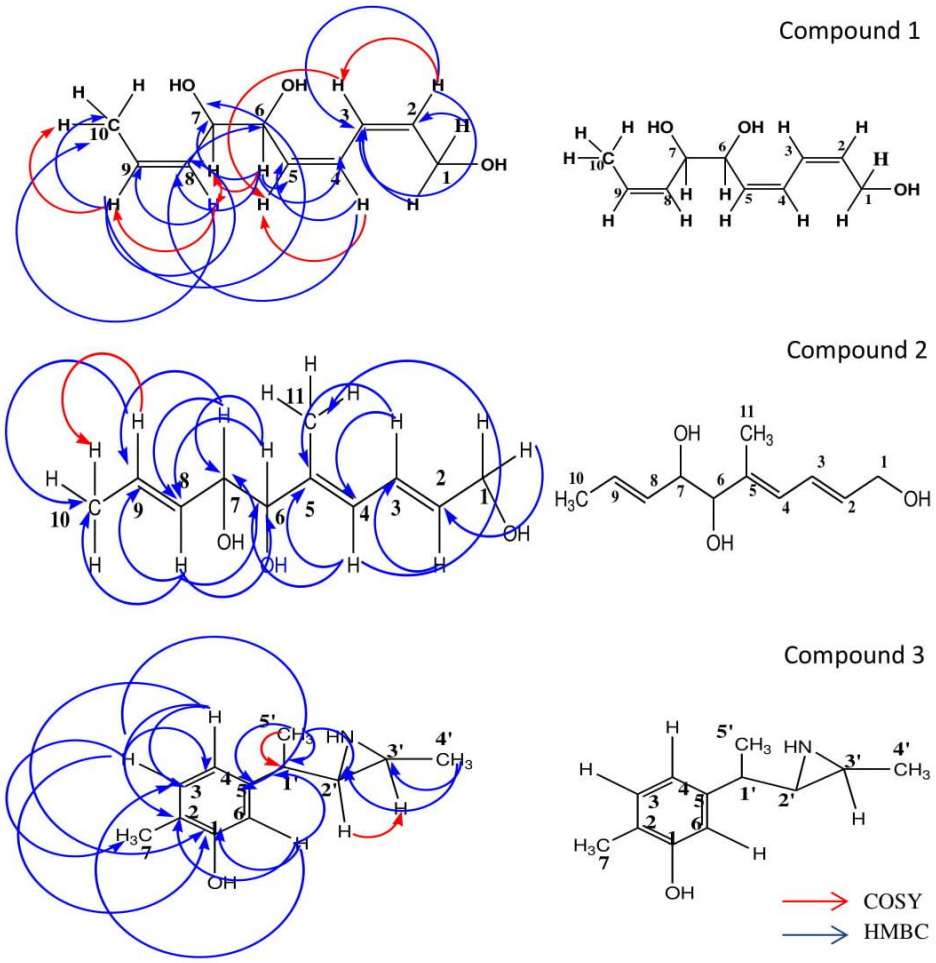

Fig. 3 Molecular structure and HMBC-COSY correlations of compound 1, 2 and 3.

equivalence of 5; one aromatic ring, three double bonds within the aromatic ring and one aziridine ring at the side substituent. ^{1}H NMR spectrum reveals that compound 3 contains three aromatic protons resonating at δ_H 6.90 (H-3), δ_H 6.59 (H-4) and δ_H 6.50 (H-6), which corresponds to carbon signals resonating at δ_C 129.1 (C-3), δ_C 112.7 (C-4) and δ_C 113.8 (C-6), respectively. Five methyl protons resonating at δ_H 2.67 (H-1'), δ_H 3.07 (H-2'), δ_H 2.77 (H-3'), δ_H 0.76 (H-4') and δ_H 1.15 (H-5'), which corresponds to carbon signals resonating at δ_C 32.0 (C-1'), δ_C 38.7 (C-2'), δ_C 43.7(C-3'), δ_C 12.1 (C-4') and δ_C 23.2 (C-5'), respectively, 1 benzylic proton resonating at δ_H 2.03 (H-7) which corresponded to carbon signal resonating at δ_C 15.9 (C-7). ^{13}C NMR and DEPT spectral information were used in identifying the number and the type of carbon atoms present in the compound. Compound 3 had at a total of 12 carbon atoms, with, six methine carbon at δ_C 129.1 (C-3), δ_C 112.7 (C-4), δ_C 113.8 (C-6), δ_C 32.0 (C-1'), δ_C 38.7 (C-2'), and δ_C 43.7(C-3'), three methyl carbons at δ_C 15.9 (C-7), δ_C 12.1 (C-4') and δ_C 23.2 (C-5') and three quaternary carbons at δ_C 152.9 (C-1), δ_C 121.2 (C-2) and δ_C 140.6 (C-5).

The COSY spectrum showed the correlation of neighboring protons that proton resonating at δ_H 2.67 (H-1') correlates with δ_H 1.15 (H-5'), while a proton resonating at δ_H 2.77 (H-3') correlates with δ_H 0.76 (H-4'). HMBC spectrum showed a correlation of proton and carbons that are 2-3 bonds away; this spectral information reveals that protons resonating at H-3 (δ_H 6.90) correlate with C-1, C-4, C-5 and C-7, at H-4 (δ_H 6.59) correlate with C-1, C-2 and C-3, while at H-6 (δ_H 6.50) correlate with C-1, C-1', C-2 and C-3. The other COSY and HMBC correlations are summarized in Table 4 and Fig 3. The IUPAC

name assigned to compound 3 was 2-methyl-5-(1-(3-methylaziridin-2-yl) ethyl) phenol and a trivial name as α-cordenol coined from the species name of the host plant and "ol" functional group representing a phenol group of compounds. Compound 3 is among the phenolic derivatives, which comprise the second largest group of secondary metabolites isolated from plant and their endophytes [22]. Phenol and phenol derivatives are biosynthesized through condensation of acetic acid (acetic acid pathway) or metabolism of phosphorylated sugars through skimmic acid and aromatic amino acid (skimmate pathway) [23, 24]. Phenol derivatives, such as carvacrol (5-isopropyl-2-methylphenol) isolated from the essential oil of thyme leaves, thymol (2-isoprophyl-5-methylphenol) isolated from the essential oil of thyme and aregona plants and eugenol (4-allyl-2-methoxyphenol) were isolated from the essential oils of clove and rose plants. These compounds are known for their aromatherapy, antioxidant, antifungal and antibacterial activities because of hydroxylation within their aromatic rings [25]. Compound 3 is among these phenolic derivatives, having an aziridine ring as part of a substitute. Aziridine is a nitrogen-containing functional group in a 3-membered strained ring, their biosynthetic pathways are not straightforward due to regiospecificity and stereospecificity [26]. The presence of aziridine within the ring offers molecule a useful property that can be utilized as an active intermediate in the synthesis of drugs or agrochemicals [27]. The significant antibacterial activity of compounds produced by SC-S-11 against bean bacterial pathogens *P. syringae* and *X. axonopodis* is chiefly attributed to the presence of these three bioactive secondary metabolites. This then shows a route of sourcing agrochemicals from natural sources like endophytic fungi.

Conclusions

The study reveals that *S. cordatum* plant hosts an important endophytic fungus of genus *Diaporthe*. *Diaporthe* sp. isolated from the stem bark tissues of *S. cordatum* showed appreciable anti-bacterial activity against bean bacterial pathogens, *P. syringae* and *X. axonopodis*, which is attributed to the mixture of secondary metabolites. The extraction and purification of active metabolites showed three new compounds, two geometric isomers named as (*2Z, 4Z, 8Z*)-(5-methyldec-2, 4, 8-triene-1, 6, 7-triol) and (*2E, 4E, 8E*)-(5-methyldec-2, 4, 8-triene-1, 6, 7-triol) and one phenolic derivative with an aziridine side chain, named as (2-methyl-5-(1-(3-methylaziridin-2-yl) ethyl phenol). The identification of new antibacterial compounds showed the potential of using endophytic fungi as a biocontrol agent to control bean pathogens and its active compounds as antibacterial agents for other bacterial diseases.

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Conflict of interests

The authors have no conflict of interest.

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