CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF SECONDARY METABOLITES EXTRACTED FROM *Leucas martinicensis* (Jacq.) R.Br AND ITS ENDOPHYTIC FUNGI

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DECLARATION AND RECOMMENDATION

Declaration

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

Signature: …… Date: ……

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Recommendation

This thesis has been submitted with our approval as supervisors for examination according to Egerton University regulations.

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DEDICATION

To my son Michael, my siblings and parents: Mr. and Mrs. Obare for their constant support and perseverance through this study.

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ABSTRACT

Modern drugs currently in use today have been produced from plants and endophytes. Medicinal plants and endophytes appear to be the best bet for sourcing of novel bioactive compounds. *Leucas martinicensis* was selected based on its traditional uses against infections. This study therefore, was to determine the bioactivity of secondary metabolites from *L. martinicensis* and its endophytes against *Escherichia coli, Proteus vulgaris, Salmonella typhi, Staphylococcus aureus* and *Klebsiella pneumoniae.* Prescreening of the isolated fungal endophytes was done using dual culture assay. Secondary metabolites from endophytes and leaves were extracted using methanol and ethyl acetate, the extracts were then subjected to antibacterial assays against the test bacteria. A total of three fungal endophytes were isolated belonging to the genus *Nigrospora, Epicoccum* and *Diaporthe*. *Nigrospora* isolate had the highest activity against all test bacteria during dual culture assay whereas *Epicoccum* had the least. Ethyl acetate fractions obtained from *Diaporthe* and *Nigrospora* showed activity against test bacteria however, activity was lower than the positive control (chloramphenicol at 30 µg/disc). Furthermore, Minimum Inhibitory Concentration (MIC) assay for *Diaporthe* and *Nigrospora* fractions tested showed increased activity against test bacteria with increase in fraction concentration. Chloramphenicol also produced higher activity than all fractions however, its activity was not significantly ($p<0.05$) different from fraction 3 (19 mm) of *Nigrospora* isolate. Both methanol and ethyl acetate extracts from *L. martinicensis* leaves showed activity against all test bacteria however, the activity of the positive control was higher and similar trend was obtained with MIC assay. Purification of fractions from endophytes and leaves produced; 4, 7- dihydroxy-9-methoxy-1-methylchromen-6-one (**5**), 4, 7, 9- trihydroxy-1-methylchromen-6-one (**6**), 2, 8-dimethyl (2-methlyethenyl) benzo-4- acrylic acid (**7**). Lack of antibacterial activity in pure compounds could be due to interaction of two or more compounds. The results obtained from this study clearly demonstrate that secondary metabolites from *L. martinicensis* leaves and its endophytes can further be exploited to develop antibacterial drugs.

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LIST OF ABREVIATIONS

BLAST	Basic Local Alignment Tool
CFU	Colony Forming Unit
COSY	Correlation Spectroscopy
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ESI	Electron Spray Ionization
ITS	Internal Transcribed Spacer
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
NCBI	National Centre for Biotechnology Information
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
TLC	Thin Layer Chromatography
SPSS	Statistical Packages for Social Sciences
TMS	Tetramethylsilane
DEPT	Distortionless Enhancement Polarization Transfer
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation

MDR	Multi Drug Resistance
MSSA	Methicillin Sensitive *Staphylococcus aureus*
IAA	Indole-3-acetic acid
NCI	National Cancer Institute

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Discovery and use of antibiotics in the 20th century brought relief to health care system, with the hope of minimizing infectious diseases (WHO, 2014). Since their discovery, the antimicrobial drugs have proven to be effective for control of fungal and bacterial infections (Tamma *et al.*, 2017). However, in the recent past bacterial pathogens like *Klebsiella pneumoniae*, *staphylococcus aureus, Escherichia coli* among others have become resistant to the available first line antimicrobial drugs in the market (Brown & Wright, 2016). Furthermore, prolonged misuse of antimicrobial drugs has also brought about the emergence of multidrug-resistant microbes which are difficult to treat (Friedman *et al.*, 2016). This has resulted to increased mortality, healthcare costs and morbidity (Huttner *et al.*, 2013). Control and prevention of these multi-drug resistant infections requires new antimicrobial agents, new vaccines, wise use of existing antimicrobial drugs and public effort to minimize rate of transmission (WHO, 2014). The problem of bacterial resistance was initially addressed by continuous discovery of new classes of antimicrobial drugs such as macrolides, glycopeptides and aminoglycosides as well as chemical derivatives of existing drugs (Laxminarayan *et al.*, 2016). Unfortunately, the discovery of new antibiotics has stalled and a few have been discovered over the last century (Shallcross *et al.*, 2015). Unless the problem of antimicrobial drug resistance is resolved, the dangers of previously manageable and treatable microbial diseases will re-emerge and persist (Strachan & Davies, 2017).

Alternative sources of novel antimicrobial compounds from natural sources should be exploited to establish a pipeline of new antimicrobial drugs. Indigenous medicinal plants and their endophytes offer attractive opportunity for discovery of novel antimicrobials (Balouiri *et al.*, 2016), or lead molecules for development of new antimicrobial drugs (Cragg & Newman, 2013). *Leucas martinicensis* L. (white wort) is an unbranched aromatic plant with a mint-like aroma that

belongs to order Lamiales and family Lamiaceae/Labiatae. *L. martinicensis* is mainly found in different parts of Asia, India and Africa especially in Kenya, Nigeria, Zimbabwe, Botswana and Mozambique (Nondo *et al.*, 2017). Plants of the genus *Leucas* have been widely used for traditional medicine and they are therefore, a potential source for the discovery of new drugs or lead molecules (Chouhan & Singh, 2011).

Endophytes are either bacteria or fungi that inhabit plants tissues without causing any symptoms of a disease (Khianngam *et al.*, 2013). Currently, fungal endophytes are seen as outstanding sources of bioactive natural products, because they occupy millions of unique biological niches and grow in an unusual environment (Liang *et al.*, 2012). Production of secondary metabolites from fungal endophytes is not random, but it seems to be correlated with its ecological niche. The metabolic interaction of fungal endophytes with their host favours the synthesis of biologically active secondary metabolites (Khianngam *et al.*, 2013). Additionally, fungal endophytes protect their host from herbivory by producing secondary metabolites having antagonistic activity, while the host provides nutrients to the fungal endophytes (Higgins *et al.*, 2014). Thus, this study aimed to explore *L. martinicensis* and its fungal endophytes as potential source of novel antimicrobial compounds or lead molecules.

1.2 Statement of the Problem

Bacterial infections have become a threat to the health care system because drugs used in their clinical management have become resistance (Jernigan *et al.*, 2020). The problem of AMR has been exacerbated by insufficient attention towards developing new antimicrobials and the high costs associated with development of new drugs (Tacconelli *et al.*, 2018). Sadly, pharmaceutical companies prefer to invest in drugs that are used in management of chronic infections, because these drugs are taken for longer periods of time or decades, hence profits (Jackson *et al.*, 2018). Since medicinal plants and fungal endophytes play an important role in drug discovery and development they are therefore recognized as sources of active secondary metabolites. Natural products can be used as alternative antimicrobial agents due to their low toxicity to human cells and limited effects on the environment. Therefore, there is dire need for accelerated discovery and development of new, effective and cheaper antimicrobial compounds or lead molecules from medicinal plants and fungal endophytes.

1.3 Objectives

1.3.1 General Objective

To investigate antibacterial activity of secondary metabolites from the medicinal plant *L. martinicensis* and its associated endophytic fungi.

1.3.2 Specific Objectives

i. To characterize endophytic fungi isolated from *L. martinicensis.*
ii. To determine the antibacterial activity of secondary metabolites from the leaves of *L. martinicensis* and endophytic fungi.
iii. To elucidate the secondary metabolites from *L. martinicensis* and its endophytic fungi.

1.4 Hypotheses

i. Fungal endophytes from *L. martinicensis* have similar morphological and molecular characteristics.
ii. Secondary metabolites from *L. martinicensis* and its endophytic fungi do not have antimicrobial activities.
iii. Secondary metabolites from *L. martinicensis* and its endophytic fungi do not have similar structures.

1.5 Justification

Many antimicrobial compounds currently in the market have been isolated from natural sources such as endophytic fungi and medicinal plants. Endophytic fungi and medicinal plants are known to accumulate antimicrobial secondary metabolites that help them survive in adverse environmental conditions. Compounds innately produced by medicinal plants and endophytes are known to possess antimicrobials, antifungals, anti-carcinogens, immunosuppressants or antioxidants characteristics. Plants and endophytic fungi are therefore the dominant sources of novel antimicrobial compounds. Secondary metabolites isolated from endophytic fungi and medicinal plants with antimicrobial potential have not yet been exhausted this far, hence there are still novel antimicrobial compound or lead molecules yet to be discovered. WHO promotes and endorses the addition of herbal drugs in national health care programs because they are time tested and accessible at a price within the reach of a common man and thus considered to be much safer

than synthetic drugs. Therefore, this study aims to identify, isolate and characterize bioactive compounds isolated from *L. martinicensis* leaves and its associated endophytic fungi.

CHAPTER TWO

LITERATURE REVIEW

2.1 Medicinal Plants

In many developing countries, large population relies on medicinal plants and traditional medical practitioners to meet their health care needs (Kigen *et al.,* 2013). Surprisingly, developed countries have also embraced the use of medicinal plants as herbal remedies or nutraceuticals for management of infectious diseases as well as for development of numerous drugs (Rasool Hassan, 2012). Medicinal plants make an important contribution to the Sustainable Development Goal 3 (SDGs) to ensure that all people in the world lead a sustainable productive life (Balangcod *et al.,* 2012). Additionally, medicinal plants have a promising future since their medicinal properties could be used in the treatment of present or future diseases since most of them have not yet been investigated (Silva & Fernandes Júnior, 2010). Medicinal plants that have been used as a source of medical products and drugs that have been isolated from them include *Artemisia annua* which is well known for isolation of an antimalarial drug artemisinin (Efferth, 2017), the broad bean plant that is known for isolation of antibacterial drug chloramphenicol (Mohammed, 2018). *Cephaelis ipecacuanha* which is known for isolation of isoquinoline alkaloid emetine; an amoebicidal drug that is used for the treatment of abscesses, bark of Cinchona tree that naturally harbors quinine which was used in the treatment of malaria. Additionally, Madagascar periwinkle was used in the isolation of antileukaemic alkaloids, vinblastine and vincristine (Samuelsson & Bohlin, 2017).

2.2 *Leucas martinicensis*

Leucas martinicensis L. is an erect, unbranched aromatic annual plant which grows to a height of 50-100 cm. This plant is mainly found in the tropical and subtropical Africa to the Indian sub-continent (Regina *et al.*, 2015). The leaves are opposite, entire with spiky lobes, oval-shaped ends, and petiolated (Figure 1). In Kenya, traditionally the plant is used in management of diarrhoea, whereas crushed leaves are used to treat fevers, snake bites, and a decoction against roundworm in children. The crushed leaves are also to applied to wounds, sores especially those of the eyes and nose as well as treatment of chronic skin diseases such as scabies (Das *et al.*, 2012).

Figure 1: *Leucas martinicensis* growing in undisturbed section of Mt Elgon natural forest in Kenya.

2.3 Secondary Metabolites from the Genus *Leucas*

A number of secondary metabolites that have been isolated and identified from the genus *Leucas* include phenolics, steroids, triterpenes, tannins and alkaloids (Chouhan & Singh, 2011). A variety of phytoconstituents have also been isolated from different species of *Leucas* which include flavanoids, lignans, steroids, coumarins, aliphatic long-chain compounds, fatty acids and terpenes. For instance, *Leucas aspera* was found to contain xanthoproteins, flavonoids, phenols, glycosides and alkaloids (Chew *et al.*, 2012). *Leucas cephalotes* was also found to contain the following compounds; carbohydrates, phytosterols, phenolic compounds and flavanoids which create a novel for the study of human carbonic anhydrase inhibitors (Antariksh *et al.,* 2010). Literature shows that *Leucas lavandulaefolia* contains Luteolin **(4)**, chrysoeriol **(3)**, Salicylic acid **(2)**, Caffeic acid **(1)**, linifoliside, linifoliol, alkaloids triterpenoids, steroids, lupeol and fatty alcohols in methanol extracts (Makhija *et al*., 2011). Some of these compounds are shown in figure 2. In addition, a number of compounds have also been isolated from *Leucas inflata* which include chromone, coumarsabin, 8-methoxycoumarsabin, siderin and coumarleucasin (Chouhan & Singh, 2011).

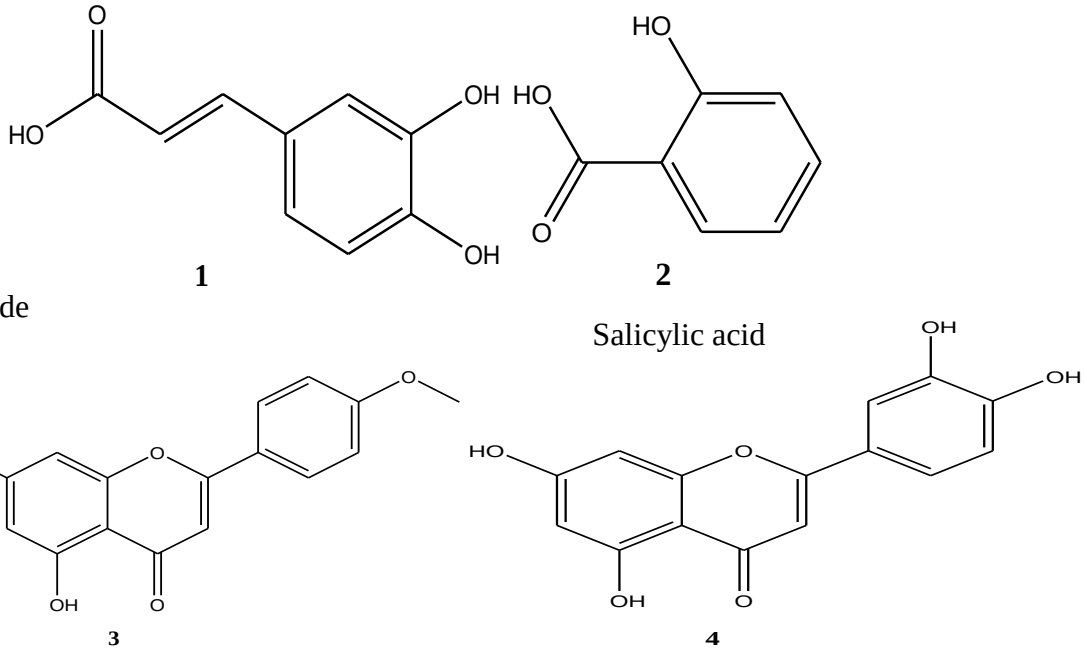


Figure 2: Secondary metabolites isolated from *Leucas lavandulaefolia*. (1) Linifoliside, (2) Salicyclic acid, (3) Chrysoeriol, (4) Luteolin

2.4 Endophytes

Endophytes are bacteria or fungi that inhabit inside plant tissues and do not cause any diseases (Porras-Alfaro & Bayman, 2011). Endophytes that reside in plants can either be localized at the point of entry or spread their entire plant. These endophytes may either be obligate or facultative since they are associated with lower and higher plants (Clay & Schardl, 2002). The associations of endophytes with their host are complex which are normally a cryptic phenomenon in nature (Rosenblueth & Martínez-Romero, 2006). Endophytes often contribute to the normal health and development of their hosts in exchange for a privileged niche (Zhang *et al.*, 2006). They increase plant biomass, confer drought tolerance and produce bioactive molecules which enables them to survive in adverse environmental conditions (Santoyo *et al.*, 2016). Furthermore, endophytes have been used in phytoremediation; assist plants to remove xenobiotics and heavy metals from the soil (Gao *et al.*, 2010). For instance, a research done by Babu *et al.* (2015) reported the potential use of *Pseudomonas koreensis* in association with *Miscanthus sinensis* to remediate heavy metals in soils of mining site. Some endophytes are able to infect a wide range of hosts

while others are specific or limited to one or a few hosts. They have shown resistance to plant pathogens especially nematodes by producing secondary metabolites having antagonistic activity (Yu *et al.*, 2010). Endophytes are a potential sources for new drugs or lead molecules since they have the capacity to synthesize organic compounds having diverse structural features.

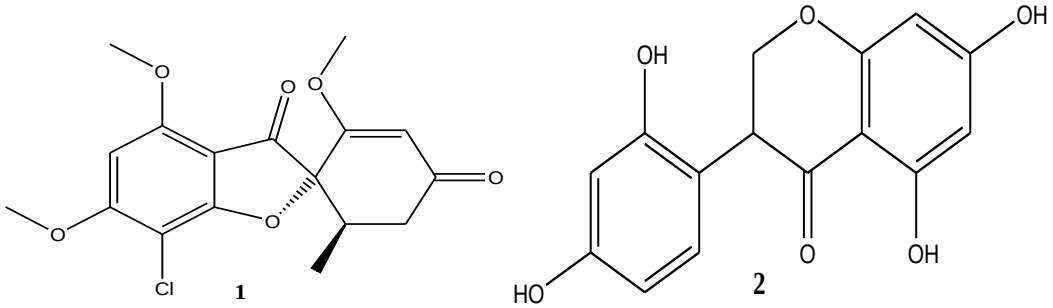
2.4.1 Fungal Endophytes

Approximately 1.5 million fungal species exist in the world and only 100,000 have been discovered (Arnold & Lutzoni, 2007). Fungal endophytes protect their host against herbivores; insect attacks which shows a mutualistic, parasitic and commensalistic relationship (Rodriguez *et al.*, 2009). They inhabit many different tissues of the plants which include stems, roots, branches, leaves, flowers, bark, seeds as well as petioles. In an individual organ or tissues of the same plant, fungal endophyte profiles can be completely different from those of other organs and tissues of the same plant. Fungal endophytes produce antimicrobial substances which have been shown to increase survival of their host to a variety of abiotic and biotic stresses (Rani *et al.*, 2017). Endophytic fungi are able to overcome host resistance by secretion of metabolites toxic to their host that modulate host phytohormones and detoxification of defence metabolites (Kusari *et al.*, 2012). Symbiotic relationship of the fungal endophytes with their host results in increased production of reactive oxygen species which are important in maintaining the mutualistic plant /fungal interaction. For instance, *Neoptyphodium* species members of *Clavicipitaceae* fungi are able to infect grasses and have been studied extensively because of their impact on agriculture (Tanaka *et al.*, 2012). This fungal species produces alkaloids which are toxic to livestock and limits the utilization of these grasses in forage and pasture. On the other hand, the alkaloids and metabolites produced by these fungal endophytes benefit their host by increasing resistance to insects and nematodes as well as increasing drought tolerance (Saikkonen *et al.*, 2016). Additionally, the endophyte *Fusarium culmorum* isolated from dune grass which grows in coastal habitats is necessary for salt tolerance of this plant (Martin & Dombrowski, 2015). According to Vega *et al.* (2008), every plant species studied harbors fungal endophytes within its aerial tissues.

2.4.2 Bioactive Metabolites from Endophytic Fungi

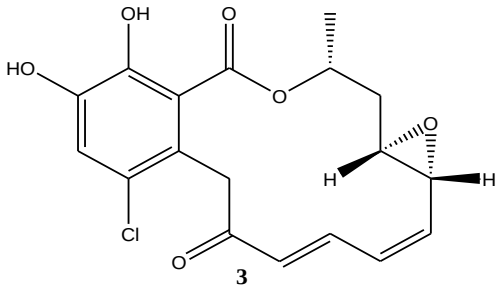
Endophytic fungi produce several bioactive compounds that are used as anticancer, antimicrobial, immunosuppressive, antioxidants and antiviral agents (Stierle & Stierle, 2015). For instance, the discovery of Taxol from Pacific yew tree, *Taxus brevifolia* increased the importance

of endophytes that shifted to the research of endophytic fungi. Taxol is the world's first billion-dollar anticancer drug that is used for the treatment of ovarian and breast cancers (Schulz *et al.*, 2002). According to Patil *et al.* (2016), Griseofulvin (**1**) is an effective antifungal agent used for the treatment of fungal infections of skin, hair and nails and it was isolated from *Penicillium griseofulvum.* Oocydin A is also an antifungal agent was isolated from *Serratia marcescens* (Higginbotham *et al.*, 2013). Furthermore, another important bioactive metabolite Cyclosporine was also isolated from *Tolypocladium inflatum* in 1971 and *Cylindrocarpon lucidum* fungal endophytes. This drug is an effective immunosuppressive agent that prevents rejection after organ or tissue transplant (Bhardwaj & Agrawal, 2014). Research has shown that several pharmacologically important compounds have also been isolated from fungal endophytes. They include antimycotics steroid; 22-triene-3 β-ol, anticancer cajanol (**2**) (Kumar *et al.*, 2014), anti-inflammatory ergoflavin, immunosuppressive Syndoxanthane A, B as well as cytotoxic Radicicol (**3**) (Song *et al.*, 2013).



Griseofulvin

Cajanol



Radicicol

Figure 3: Isolated bioactive metabolites from endophytic fungi (1) Griseofulvin, (2) Cajanol, (3) Radicicol

2.5 Antibiotics

The future of medicine depends on the effectiveness of antibiotics (Gelband *et al.*, 2015). Antibiotics are antimicrobial drugs that fight infections caused by bacteria and fungi, however they are not effective against viral infections such as common cold, most sore throat and flu. They exert their therapeutic effect by inhibiting the growth of bacteria (Fischbach & Walsh, 2009). The first antibiotic was discovered by Alexander Fleming in 1928 which was a breakthrough in the health sector (Gould, 2016). Between 1930 and 1962 more than 20 novel classes of antibiotics were produced. Since then, only two classes have been marketed (Demain & Sanchez, 2009). According to Butler and Paterson (2020) since 2000 the situation has changed with thirty eight more new classes of antibiotics launched worldwide which include (16 natural product derived and 20 synthetic derived, two natural product), three monoclonal antibodies and four new Beta-lactam combinations. There are several classes of antibiotics which include β-lactams, aminoglycosides, sulfonamides, macrolides and tetracyclines among others (Walsh & Wencewicz, 2016). However, some antibiotics are bactericidal which involves killing the bacteria while others are bacteriostatic meaning that they stop bacteria from multiplying (Wong *et al.*, 2012). For instance, the β-lactam antibiotics bind and inhibit enzymes required for bacterial cell wall synthesis. They include penicillin, cephalosporins, cephamycin among others (Moir *et al.*, 2012). The aminoglycosides inhibit protein synthesis in bacteria; they include gentamycin, amikacin, tobramycin, streptomycin among others (Riviere *et al.*, 2018). Current uses of antibiotics are unsustainable owing to the spread of antibiotic-resistant pathogens (Dyar *et al.*, 2016).

2.5.1 Plant-based Antibiotics

Plant-based antimicrobials have a potential to combat bacterial, fungal, protozoal and viral diseases without any side effects (Chandra *et al.*, 2017). For instance, griseofulvin (antifungal) and chloramphenicol (antibacterial) were isolated from the treated tissues of broad bean plant and are currently present in the market (Demain, 2009). Artemisinin was isolated from *Artemisia annua* and is used to alleviate chills and fever (Stringham *et al.*, 2018). Furthermore, a very effective and nonspecific antimicrobial drug pyrithione synthesized from the Chinese medicinal plant *Polyalthea nemoralis* is active against bacteria and fungi (Lewis & Ausubel, 2006). The isoquinoline alkaloid emetine obtained from part of *Cephaelis ipecacuanha* and related species has been used as an amoebicidal drug as well as for the treatment of liver abscesses. Additionally,

quinine was isolated from *Cinchona* bark though there has been controversies over its use while antileukaemic alkaloids vinblastine and vincristine were obtained from Madagascan periwinkle (Kumar & Pandey, 2013).

2.6 Antimicrobial Drug Resistance

The discovery of penicillin in 1929 enabled the effective control of infections caused by Gram-positive pathogens such as *Streptococcus* and *Staphylococcus aureus* while in 1943 a new aminoglycoside streptomycin was discovered and was used in the treatment of *Mycobacterium tuberculosis* (Brown & Wright, 2016). Since then, modern medicine has been transformed by the use of antibiotics and millions of lives have been saved (Marston *et al.*, 2016). In the 1940s, antibiotics were first prescribed to treat infections and penicillin was used to control bacterial infections among soldiers in the world war 11 (Wright, 2016). However, despite this breakthrough the spread of penicillin resistance was documented by 1942, when *S. aureus* was found to resist penicillin in hospitalized patients (Kapoor *et al.*, 2017). By the late 1960s, more than 80% of hospital and community acquired strains of *S. aureus* were resistant to penicillin (Lobanovska & Pilla, 2017). Since then, many advances have been made through the discovery of beta-lactam antibiotics to restore the confidence (Shaikh *et al.*, 2015). Later in 1960s, an aminoglycoside called gentamycin was introduced and was used to treat *P. aeruginosa* infections (Bush, 2015). The first incidence of Methicillin resistant *Staphylococcus aureus* was identified during the same decade: in the United Kingdom in 1962 and the United States in 1968 (Olsen, 2015). Vancomycin was later introduced into clinical practice in 1972 for the treatment of methicillin resistance in both *S. aureus* and coagulase-negative *staphylococci* (McGuinness *et al.*, 2017). Surprisingly, it was difficult to induce vancomycin resistance that it was believed unlikely to occur in a clinical setting. Unfortunately, cases of vancomycin resistance were reported in coagulase-negative *staphylococci* in 1979 and 1983 (Friães *et al.*, 2015). From the late 1960s through the early 1980s, the pharmaceutical industry introduced many new antibiotics to solve the resistance problem, but after that the antibiotic pipeline began to dry up and fewer new drugs were introduced (Deak *et al.*, 2016).

Bacteria have an extraordinary genetic plasticity that allows them to respond to a wide array of environmental threats, including the presence of antibiotic molecules that may jeopardize their existence (Blair *et al.*, 2015). These bacteria uses two major genetic strategies to adapt to the antibiotic attack. These include mutations in genes which are often associated with the mechanism

of action of the compound and acquisition of foreign DNA coding for resistance through horizontal gene transfer (HGT) (Munita & Arias, 2016). In mutational resistance, a subset of bacterial cells derived from a susceptible population develops mutations in genes that affect the activity of the drug, this results in preserved cell survival in the presence of the antimicrobial molecule (Lin *et al.*, 2015). Once a resistant mutant emerges, the antibiotic eliminates the susceptible population and the resistant bacteria predominates. Additionally, mutations resulting in antimicrobial resistance alter the antibiotic action via the following mechanisms; modifications of the antimicrobial target, decrease in drug uptake, activation of efflux mechanisms to extrude the harmful molecule and changes in metabolic pathways via modulation of regulatory networks (Holmes *et al.*, 2016). In horizontal gene transfer, bacteria acquire external genetic material through three main strategies; transformation (incorporation of naked DNA), transduction (phage mediated) and conjugation (Baym *et al.*, 2016). However, recent advances made in the discovery of new bioactive compounds from endophytes and medicinal plants could facilitate the return of susceptible microbes (Nisa *et al.*, 2015).

2.7 Bacteria and Human Diseases

The drug-resistant bacteria are either Gram-positive or Gram-negative depending on the composition of their cell walls. The Gram-positive bacteria have a thin peptidoglycan cell wall sandwiched between an inner cytoplasmic cell membrane and bacterial outer membrane. An example of Gram-positive bacteria that is used in this study is *Staphylococcus aureus.* The Gram-positive bacteria do not retain the crystal violet stain used in gram staining method hence they stain red. The Gram-negative bacteria include *Klebsiella pneumoniae*, *Escherichia coli, Salmonella typhi* and *Proteus vulgaris*; they stain blue-purple since they have larger peptidoglycan layer hence the iodine and crystal violet precipitate in the thickened cell wall are not eluted by alcohol (Costa *et al.*, 2015).

2.7.1 *Staphylococcus aureus*

The introduction of penicillin in the early 1940s dramatically improved the prognosis of patients with staphylococcal infection. However, as early as 1942, penicillin-resistant *Staphylococci* were recognized, first in hospitals and subsequently in the community. By the late 1960s, more than 80% of both community- and hospital-acquired staphylococcal isolates were resistant to penicillin (Foster, 2017). This pattern of resistance, first emerging in hospitals and then

spreading to the community, is now a well-established pattern that recurs with each new wave of antimicrobial resistance (Schito, 2006). *Staphylococcus aureus* is divided into 2 groups; methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive (MSSA). The therapeutic outcome of infections that result from MRSA is worse than the outcome of those that result from methicillin-sensitive strains (Cosgrove *et al.*, 2003). Methicillin-Resistant *S. aureus* is the most common cause of community-acquired skin infections. Hospital and community-acquired *Staphylococcus aureus* infections pose a substantial burden in terms of morbidity, mortality and healthcare costs. The introduction of new antibiotics to counter this pathogen has frequently been followed by the emergence of resistant strains. Most significantly, *S. aureus* isolates resistant to β-lactams have become common, and many of these are also resistant to β-lactamase-resistant penicillins (Foster, 2017) . Diagnosis can be through PCR or culture of the organism from the involved site. Dalbavancin, ceftaroline, tedizolid, oritavancin and ceftobiprole are the most recent antibiotic used for the treatment of MRSA (David *et al.*, 2017).

2.7.2 *Proteus vulgaris*

The genus *Proteus* currently consists of five named species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. myxofaciens* and *P. hauseri.* However, *P. mirabilis* is by far the most common species identified in clinical specimens (Hamilton *et al.*, 2018). *P. vulgaris*, previously considered bio-group 2, has been reported to cause UTIs, wound infections, burn infections, bloodstream infections, and respiratory tract infections (Jacobsen *et al.*, 2008). *P. vulgaris*, is an opportunistic pathogen and was firstly described by Margit Luise Hauser in 1885 (Mandal *et al.*, 2015). It is a rod-shaped chemoheterotrophic bacterium that possesses peritrichous flagella which makes it motile (Kothari & Sagar, 2008). *P. vulgaris* produces a chromosomally encoded beta-lactamase, referred to as the cefuroxime-hydrolyzing beta-lactamase (cefuroximase or CumA), which hydrolyzes cephalosporins. The enzyme can be induced by ampicillin, amoxicillin and first generation cephalosporins, weakly induced by carboxypenicillins, ureidopenicillins, cefotaxime and ceftriaxone, and inhibited by clavulanate (Baron *et al.*, 2018). Strains of *P. vulgaris* that have a mutation in the regulatory genes of this beta-lactamase produce high levels of the enzyme and are resistant to penicillins, cefuroxime, ceftriaxone and cefotaxime. However, these isolates are generally susceptible to ceftazidime, aztreonam, cephamycins, carbapenems and beta-lactam/beta-lactamase inhibitor combinations (Benmansour *et al.*, 2016). Samples containing *P. vulgaris* are first incubated on nutrient agar to form colonies. Gram stains and oxidase tests are performed to

test the gram-negative and oxidase-negative characteristics. The colonies of interest are then inoculated onto a selective culture medium MacConkey. This medium contains lactose which *Proteus* organisms do not ferment. (Feglo *et al.*, 2010).

2.7.3 *Salmonella typhi*

Salmonella the causative agent of salmonellosis is a rod-shaped gram-negative facultative anaerobe bacterium which belongs to the family Enterobacteriaceae (Parkhill *et al.,* 2001). *Salmonella typhi* is a strain of bacteria that lives only in humans and causes a bacterial infection of the intestinal tract and occasionally the bloodstream which is called typhoid fever which can be treated by antibiotic therapy (El-Sharkawy *et al.*, 2017). The bacteria are shed in the urine or stool of infected persons, including a chronic carrier, eating or drinking contaminated food or water or by contact with stool from infected persons (Schwarz & Johnson, 2016). The first line treatment for *S.typhi* was chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole. However, strains that were resistant to these three antibiotics were considered MDR and such isolates were first observed in late 1970s to early 1980s (Shaikh, 2015). Resistance to the second line antibiotics; fluoroquinolones have also been frequently reported since these became the preferred treatment in regions with MDR infections. Ceftriaxone, a third-generation cephalosporin and azithromycin, a macrolide are now used to treat *S.typhi* (Kariuki, 2015). However, sporadic cases of ceftriaxone or azithromycin resistance S. *typhi* have been reported. The bacteria diagnosis can be through culture technique or Widal test for antibody detection (Zhang *et al.*, 2008).

2.7.4 *Escherichia coli*

Escherichia coli was originally called Bacterium coli commune and was first isolated from the faeces of a child in 1885 by the Austrian paediatrician (Oundo *et al.,* 2008). This bacterium is part of a normal gut microflora hence plays a vital role in the synthesis of vitamin K and the metabolism of bile acids and other sterols (Zinnah *et al.,* 2007). Many strains are harmless and are non-spore forming rod however within the species there are 4 strains that cause diarrheal illness (de Muinck *et al.*, 2013). These species of *E. coli* can be subdivided into the following; intestinal non-pathogenic (commensal isolates), Intestinal pathogenic isolates and extra-intestinal pathogenic *E. coli* or ExPEC isolates (Lautenbach *et al.,* 2001). Management of infections caused by ExPEC has been complicated by the emergence of antimicrobial resistance, especially since the late 1990s. Until the late 1990s ExPEC were relatively susceptible to first line antibiotics, however

several surveillance studies during the 2000s across Europe, North and South America have shown that between 20% and 45% of ExPEC are resistant to first line antibiotics including the cephalosporins, fluoroquinolones, and trimethoprim–sulfamethoxazole (Pitout, 2012). The β-Lactam antibiotics, especially the third generation cephalosporins, are a major drug class used to treat serious community-onset or hospital-acquired infections caused by *E. coli* (Von Baum, 2005).

2.7.5 *Klebsiella pneumoniae*

Klebsiella. pneumoniae was first isolated in the late 19th century and was initially known as Friedlander's bacterium (Paczosa & Mecsas, 2016). Over the last few decades, there has been a rise in the acquisition of resistance to a wide range of antibiotics by strains derived from *K. pneumoniae*. As a consequence of this antibiotic resistance, simple infections such as urinary tract infections (UTIs) have become recalcitrant to treatment, and more serious infections such as pneumonia and bacteremia have become increasingly life-threatening (Pitout *et al.*, 2015). Two major types of antibiotic resistance have been commonly observed in *K. pneumoniae*. One mechanism involves the expression of extended spectrum-lactamases (ESBLs), which renders bacteria resistant to cephalosporins and monobactams. The other mechanism of resistance, which is even more troubling, is the expression of carbapenemases by *K. pneumoniae*, which renders bacteria resistant to almost all available lactams, including the carbapenems (Kidd *et al.*, 2017). *K. pneumoniae* is one of the most important causative agents of nosocomial infections. The presence of a capsule is important for the virulence of this organism (Holt *et al.*, 2015). This bacterium is rod-shaped lactose fermenting bacillus with a prominent capsule. It is an opportunistic pathogen and is found in the mouth, skin and intestines as well as hospital settings (Bialek-Davenet *et al.*, 2014). This bacterium is the cause of severe pneumonia in alcoholics in Africa and Asia (Vuotto *et al.*, 2014). *K.pneumoniae* is now recognized as an urgent threat to human health because of the emergence of MDR strains associated with hospital outbreaks and hyper virulent strains associated with severe community-acquired infections (Zowawi *et al.*, 2015). It is ubiquitous in the environment and can colonize and infect both plants and animals. However, little is known about the population structure of *K. pneumoniae*, so it is difficult to recognize or understand the emergence of clinically important clones within this highly genetically diverse species (Holt *et al.*, 2015).

2.8 Economic Importance of Fungi and Bacteria

Multiple resistant microbial infections have rendered therapy difficult and costly (Baym *et al.*, 2016). People have succumbed to Multiple Drug Resistant (MDR) infections because the available drugs have failed especially in the developing world (Micek *et al.*, 2015). Hospital and community MDR strains like *Mycobacterium tuberculosis, Enterococcus faecium, Enterobacter cloacae, Klebsiella pneumoniae, Staphylococcus aureus, Acinetobacter baumanii* as well as *Pseudomonas aeruginosa* have become difficult to treat (van Duin & Paterson, 2016). MDR enteric disease agents in developing countries such as *Salmonella enteritidis, Shigella flexneri* and *Vibrio cholera* among others have threatened public health (Levy & Marshall, 2004). High rates of MRSA imply that treatment for suspected *S. aureus* infections such as wound infections must rely on second-line drugs in many countries (Bal *et al.*, 2016).

In the early 1950s and 1960s, fungal infection was extremely very rare even in immunocompromised cancer patients. Surprisingly in the past 2 decades, fungal infections have increased frequency due to the growing number of immunosuppressed patients which has brought about high mortality rates (Khan *et al.*, 2010). The use of grafts and antineoplastic agents has also led to the development of more complicated infections. Patients with HIV and neutropenia (abnormally low count of neutrophils) are also exposed to these fungal infections. The National Nosocomial Infections Surveillance System has also reported *Candida* species as the fourth common bloodstream isolates in nosocomial infections in the USA. More than 90% of the reported fungal related deaths result from species that belong to one of the four genera; *Cryptococcus, Candida, Aspergillus* as well as *Pneumocystis* (Brown *et al.*, 2012).

2.9 Techniques used in Isolation of Fungal Endophytes

Traditionally, fungal endophytes are isolated using the cultivation-dependent method and direct observation (Götz *et al.*, 2006). The cultivation-dependent method involves washing of plant tissue under running tap water, surface sterilization of the plant tissue which applies different procedures on different tissue types (Li *et al.*, 2011). In direct observation, the fungal structures are directly examined under the light microscope or electron microscope, which shows all endophyte microbiota inside the plant tissue that cannot be cultured on standard media (Deckert *et al.*, 2001). However, most fungal endophytes have hyphal structures that cannot be identified in any taxonomic key due to lack of spore-producing structures (Su *et al.*, 2010). In addition,

endophytes cannot be obtained as microbial resources for further use with the direct observation method (Sun & Guo, 2012). Therefore, it is important to isolate fungal endophytes for detailed studies based on population dynamics, species diversity and characterization to improve screening for biologically active secondary metabolites (Lucero *et al.*, 2011).

2.10 Identification of Fungal Endophytes

2.10.1 Morphological Identification

According to Guo *et al.* (2000) fungal endophytes can be identified on the basis of morphological characteristics. Morphological identification should be done according to the standard taxonomic key which includes diameter, texture, colour and dimensions (Singh *et al.*, 2017). Different types of fungi produce different looking colonies; some colonies may be coloured, some circular in shape and some irregular (Lu *et al.*, 2012). According to Mane *et al.* (2018) microscopic examination and staining techniques are basic on differentiating hyphae which vary from simple single stain to complex multi-stain procedures. Caution should be taken because morphologically similar fungal endophytes are medium based and cultural conditions can affect the sexual and vegetative reproduction (Boddington & Dearnaley, 2008). Fungal endophytes that fail to sporulate in the culture are grouped as mycelia sterilia and conventional methods cannot be used. However, a large number of fungal endophytes do not sporulate in the culture (Boddington & Dearnaley, 2008)

2.10.2 Molecular Identification

Molecular techniques exhibit high specificity and sensitivity; they can be used for classifying fungal strains into diverse hierarchical taxonomic levels (Yoo & Eom, 2012). Molecular techniques are used in the confirmation of the results from morphological characterization (Leme *et al.*, 2013). The most commonly used region for identification purposes is the ITS region since it can be used in the analysis of the lower taxonomic levels (Sarsaiya *et al.*, 2020). This region contains highly conserved areas adequate for designing primers that allow discrimination over a wide range of taxonomic levels (Lutfia *et al.*, 2020). Other regions and genes that can also be used in the identification of fungi include; intergenic spacer sequence (IGS) regions placed between 28S and 18S rRNA genes. It is usually used where there are not enough difference available across the ITS (dos Santos Vieira *et al.*, 2020; Schena *et al.*, 2017), β-tubulin (Kulik *et al.*, 2020), Translation elongation factor 1 alpha (*TEF* 1α) (Geiser *et al.*, 2004),

calmodulin (Mulè *et al.*, 2004), virulence genes (Lievens *et al.*, 2009), and mitochondrial genes such as the multicopy *cox* I and *cox* II and their intergenic region (Martin & Tooley, 2003) are also used in the fungal identification. The protein coding markers such as RPB 1(largest subunit of RNA polymerase II), RPB 2(second largest subunit of RNA polymerase II) and MCM 7(gene encoding for a mini chromosomal maintenance protein) can also be used. These regions and markers have a high species resolving power but they are also prone to PCR failures (Schoch *et al.*, 2012).

The nuclear ribosomal ITS region is used as a universal DNA barcode marker for fungi. Ribosomal DNA (rDNA) ITS sequence analysis is widely used for the identification of fungal endophytes (González-Teuber *et al.*, 2017). ITS region is situated between the ribosomal RNA large sub-unit and small sub-unit genes on the fungal chromosomes, or its corresponding transcribed region on the polycistronic ribosomal RNA precursor transcript. This DNA barcoding marker is used for lower species level phylogeny determination because it is known to evolve very fast and are relatively conserved nucleotide sequence among the fungi (Iwen *et al.*, 2002). Among the regions of ribosomal cistrons, the ITS has the highest probability of successful identification for fungi, with the most clearly defined barcode gap between inter and intraspecific variation (Anderson *et al.*, 2003). ITS ribosomal DNA has been proven to be an important tool for distinguishing phylogenetic relationships among species or genera (Schoch *et al.*, 2012). Most importantly, ITS sequence analysis has been used in the identification of non-sporulating fungal endophytes since it has reduced biased judgment impact and Large Subunit (LSU) (Satari *et al.*, 2018). The eukaryotic rRNA cistron consists of the 18S, 5.8S, and 28S rRNA genes transcribed as a unit by RNA polymerase I. Posttranscriptional processes split the cistron, removing two internal transcribed spacers. These two spacers, including the 5.8S gene, are usually referred to as the ITS region. The 18S nuclear ribosomal small subunit rRNA gene (SSU) is commonly used in phylogenetics, and although its homolog (16S) is often used as a species diagnostic for bacteria, it has fewer hypervariable domains in fungi. The 28S nuclear ribosomal large subunit rRNA gene (LSU) sometimes discriminates species on its own or combined with ITS (Stackebrandt & GOEBEL, 1994). Ribosomal DNA ITS analysis was confirmed for the first time in the isolation of *Pleurostoma, Chaetomium, Xylaria, Coniochaeta, Daldinia, Nodulisporium, Cazia* and *Phellinus* as endophytes from *Huperzia serrata* (Nair & Padmavathy, 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of Plant Material

Fresh leaves from 20 *L. martinicensis* plants were collected from Mount Elgon National forest in Kenya prior to the onset of the long rains in March 2018. The forest stretches from N 01° 01.995' to E 034° 46.815' at an altitude of 2080 m. Sampled *L. martinicensis* plants were randomly selected in the forest, in sites that had less human activities. The specimens were collected and identification was done with the help of a taxonomist prior to depositing at Egerton University Biotechnology laboratory. The young leaves were transported to the laboratory within 24 hours in a cool box and processed.

3.2 Endophytic Fungi Isolation

Endophytic fungi were isolated from fresh *L. martinicensis* leaves using the procedure described by Marcellano *et al.* (2017) with slight modification. Briefly, sampled leaves were washed under running tap water to remove soil debris and blot-dried using filter papers. Thereafter, they were surface-sterilized for 3 minutes using 70% ethanol followed by 1% sodium hypochlorite. Sterilized leaves were rinsed in three changes of sterile dH_2O after sterilization with each of the disinfectant. The surface-sterilized leaves were cut into thin sections of approximately 1×4 mm and inoculated in petri-dishes containing Sabourand Dextrose Agar (SDA) media amended with 2 mg/l streptomycin to prevent bacterial growth. The petri-dishes were incubated at 28 ± 2°C for 1-3 weeks and with frequent monitoring to check for growth of fungal mycelia. Hyphal tips from the developing fungal colonies were sub-cultured on to fresh SDA petri-dishes until axenic cultures were obtained, herein referred to as fungal endophytes.

3.3 Morphological Characterization

The morphology of the axenic cultures was categorized using macroscopic and microscopic features. Macroscopic characteristics included shape and colour of mycelia, while microscopic characteristics included the presence of septate or aseptate hyphae: this was applicable to those fungal species that do not produce spores. The cellotape technique was used, that involved using a clear 2 cm wide cellotape and a wooden applicator stick. Using a sterile technique, the sticky

end of the cellotape was gently pressed onto the surface of the culture. The cellotape was then removed and a drop of 95% ethanol was applied to the cellotape to dissolve the glue. A drop of Lactophenol Cotton Blue was placed on a clean glass side and then the cellotape was gently placed on the slide, excess stain was removed using an absorbent paper. The slide was then placed on a light microscope and observed under x40 objective and with the help of a mycological handbook (Campbell & Johnson, 2013) and assistance of a mycologist the fungi were phenotypically identified to the genus level using the following keys: The colonies of *Nigrospora* isolates grow rapidly and produces white woolly colonies initially that turn to gray and finally black from both back and front and has aseptate hypha, *Diaporthe* isolates have sparse margins, radially white cottony mycelium on both sides and tanned concentric ring with dense aseptate hyphae while *Epicoccum* isolates have cottony to woolly colonies which are yellow to orange in color, the color is always the same when observed from back and front but it is more intense in the front. It turns the color of the media to orange, brown, red or yellow due to diffusible pigments. The hyphae is septate.

3.4 Molecular Characterization of Endophytes

3.4.1 DNA Extraction

Genomic DNA was extracted using BIO BASIC EZ -10 Spin column miniprep kit according to manufacturer's instructions (Bio Basic Inc.). Approximately 60 mg of the 3-5 day old fungal mycelia were placed into screw cap microfuge tube. Thereafter, 5-10 of 1.4 mm Precellys ceramic beads were added to the tube and the sample was covered with 200 µl of the plant cell lysis buffer (PCB) (Sodium propionate, sodium cacodylate and BIS-TRIS propane in a ratio of 2:1:2). The mixture was homogenized in a Precellys 24 homogenizer and incubated on a heating block set at 65°C for 30 minutes. After incubation, 30 µl of protein precipitation solution was added to the sample and incubated for 20 minutes on ice. The sample was centrifuged at 12 000 rpm at 4° C for 2 minutes. A clear lysate that formed as a result of centrifugation was transferred onto an EZ-10 spin Column using a micropipette. Phosphate Buffer Solution (250 µl) was added to the lysate and the samples were incubated at room temperature for 4 minutes with occasional mixing. The mixture was centrifuged at 12 000 rpm at 4°C for 30 seconds and thereafter the flow through was discarded. A wash solution (400 µl) was added to the sample and centrifuged at 12 000 rpm at room temperature for 30 seconds and the flow through discarded. This step was repeated twice. The samples were then centrifuged at 12 000 rpm at room temperature for 1 minute.

The collection tubes of the EZ -10 Spin columns were replaced with clean 1.5 ml reaction tubes. About 50 µl 65°C TE elution buffer was added to the filtrate and incubated at room temperature for 2-3 minutes, after which final centrifugation at 10 000 rpm was done for 2 minutes. The eluted DNA was stored at -4°C.

3.4.2 Polymerase Chain Reaction (PCR) Amplification

The Polymerase Chain Reaction (PCR) was conducted by amplifying the ITS region of the ribosomal DNA. PCR amplification was done in a final volume of 25 µl consisting of 2 µl (0.5 µg) of the genomic DNA, 0.5 µl of the forward primer ITSI (CTTGGTCATTTAGAGGAAGTAA), 0.5 µl of the reverse primer ITS4 (TCCTCCGCTTATTGATATGC) (Singh *et al.*, 2020), 12.5 µl of the jump start ready mix (20 mM Tris-HCl, pH 8.3, 100 mM KCL, 4 mM $MgCl_2$, 0.002% gelatin, 0.4 mM dNTPs, inert dye, stabilizers, 0.03 unit/ml Taq DNA polymerase, JumpStart ™ Taq Ready Mix™) and 9.5 µl of distilled water. The mixture was vortexed for 30 seconds and amplification conducted using the following program cycle; initial denaturation of 5 minutes at 94 °C, followed by 34 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 52 °C and elongation for 2 minutes at 72 °C. To confirm the quality of the PCR products, 3 µl of aliquots of PCR products were mixed with 5 µl of Midori green loading dye and resolved on 1.2% agarose gel. Gel electrophoresis was conducted using 1× TAE buffer for 20 minutes at 100 volts and visualization was done in a UV trans-illuminator (Nippon Genetics Europe GMbH). The PCR amplified products were purified according to BIO BASIC EZ-10 spin column (BIO BASIC INC.) following the manufacturer's instructions. The PCR reaction mixture was placed in 1.5 ml microfuge tubes and 5 ml of buffer B3 added. The mixture was then transferred to EZ-10 spin columns and left to stand for 2 minutes at room temperature and centrifuged at 10 000 rpm for 1 minute. The supernatant was removed and about 750 µl of wash solution was added to the column and further centrifuged at 10 000 rpm for 1 minute. The washing step was repeated twice. The columns were transferred into a clean 1.5 ml microfuge tubes and about 20 µl of TE elution buffer was added. This was incubated at room temperature for 2 minutes and then centrifuged at 10 000 rpm for 1 minute to elute the DNA and stored at -20°C.

3.4.3 Sequencing and Phylogenetic Analysis

The purified PCR amplified ITS rDNA fragments (18-30ng in 12 µl TE elution buffer) were submitted for sequencing using ITS1F and ITS4. Sequencing was conducted using Applied

Biosystems 3730x1 DNA Analyzer at Earth and Life Institute, Berlin. The sequence reads obtained were assembled using Geneious R7 software. The assembled sequences were used to search for related sequences deposited in GenBank database using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blst). NCBI BLASTN hits that matched the ITS query, with a coverage of ≥ 80% and sequence similarity of ≥ 96-100 % were considered a minimum threshold for species identification. Multiple Sequence Alignment of the ITS sequences was then done using Clustal Omega version 2.0. Search against UNITE fungi databases was also done to ascertained the similarities of the sequences. A phylogenetic analysis was done using distance based method in Molecular Evolutionary Genetics Analysis X (MEGA X) and Neighbor joining (NJ) tree was constructed using Tamura-Nei distance. Support for specific nodes on NJ was estimated by bootstrapping 1000 replications. All characters were equally weighted and unordered. Gaps and missing data were treated as complete deletion.

3.5 Pre-Screening of Fungal Endophytes

3.5.1 Fermentation and Antimicrobial Activity

Pre-screening for antimicrobial activity was conducted using dual culture assay following Srivastava and Anandrao (2015) protocol with slight modifications. Briefly, the pathogenic bacteria *Staphylococcus aureus, Salmonella typhi, Escherichia coli, Proteus vulgaris,* and *Klebsiella pneumoniae* were inoculated separately in 100 ml conical flasks containing Nutrient Broth (NB) and incubated for 24 h at 30°C. Then 100 µl of suspended bacteria cultures were transferred into different petri-dishes containing Mueller Hinton agar, spread using a sterile glass spreader and allowed to air dry under sterile conditions. Using a cork borer of 7 mm, agar plugs were cut from seven-day old mycelia. Three agar plugs were placed per dish facing downwards in contact with media containing the inoculated bacteria. Standard chloramphenicol disc (30 µg/disc) was used as positive control. Three independent replicate experiments were carried out with each fungal treatment consisting of three replicate plates for each bacterial pathogen. The petri-dishes were incubated at 32°C for 24 h followed by measuring the zone of inhibition using a ruler in millimeters and images taken.

3.5.2 Extraction of Secondary Metabolites from Endophytes

Fungal endophytes that possessed antimicrobial activity were cultivated on rice media following the procedure by Nascimento *et al.* (2012). Solid state fermentation was carried out in

twenty 500 ml Erlenmeyer flask containing 90 g of rice in 90 ml distilled water per flask, previously autoclaved twice at 121°C for 15 minutes. Five agar plugs were cut using 7 mm cork borer from 7-day old axenic endophyte cultures and inoculated on parboiled rice media. One flask, without inoculum, was used as a negative control. The Flasks contents were incubated in a Biological Oxygen Demand at 28°C for 21 days. After the incubation period, the rice media and endophyte biomass were fragmented into pieces with a spatula followed by addition of 150 ml methanol to each flask and allowed to stand overnight. The flasks were then placed in an ultrasonic cleaner (SB-120 DTN) to allow extraction of secondary metabolites. The mixture was filtered using Whatman filter paper No. 1 and repeated extraction with methanol till exhaustion. The filtrate was evaporated in a BUCHI rotavapor (R-205) at reduced pressure to yield methanol extracts. The extract was suspended in water to dissolve sugars and then subjected to liquid-liquid partitioning with hexane to remove fats and ethyl acetate to dissolve polar compounds. The resulting organic layers were evaporated under reduced pressure to produce hexane and ethyl acetate extracts. The ethyl acetate extract was subjected to further fractionation by column chromatography and purification by preparative HPLC.

3.5.3 Extraction of Secondary Metabolites from *L. martinicensis* Leaves

L. martinicensis leaves were dried under shade to constant weight for 2 weeks to avoid decomposition of secondary metabolites and ground into fine powder using a mill. Approximately 500 g of ground material was soaked in 1 L of methanol and allowed to stand overnight for extraction of secondary metabolites. The methanol extract was filtered using Whatman filter paper No. 1 and then concentrated using a BUCHI rotary evaporator under reduced pressure of 760 torr to obtain 3.5 g of methanol crude extract. The extract was suspended in distilled water to remove sugars, placed in a separating funnel and then 50 ml hexane was added to remove fatty acids. The hexane mixture was shaken while releasing pressure from the separating funnel and allowed to stand for 10 minutes to achieve equilibrium. The resulting hexane fraction was discarded while the aqueous fraction retained. Approximately 50 ml of distilled ethyl acetate solvent was added to the aqueous fraction and placed back in the separating funnel. The ethyl acetate procedure was repeated to allow maximum extraction of secondary metabolites and the extract placed in a 500 ml conical flask. The ethyl acetate crude extract was concentrated in a BUCHI rotary evaporator under reduced pressure of 760 torr. The ethyl acetate extract was fractionated using column chromatography.

3.6 Thin Layer Chromatography

Thin layer chromatography was performed using TLC plates containing silica GF 254 nm and 0.25 mm thickness. The dry ethyl acetate extracts were re-dissolved in ethyl acetate and mixed in an ultrasonic cleaner. Preliminary analysis was done to determine the optimum solvent systems for the mobile phase. The solvent mixture that gave optimum separation was (7:3) ethyl acetate-hexane (E: H) for the plant extract and (6:4) ethyl acetate-hexane for the endophytes. The samples were spotted separately on 1 cm x 5 cm TLC plates using a spotter. The TLC plates were then placed in 50 ml beakers containing 5 ml of the above solvent systems covered with aluminium foil. They were then allowed to develop up to 4 cm and the developed chromatograms visualized under a UV lamp (Uvitec-LF-204.LS) at 365 nm and 254 nm.

3.7 Column Chromatography

The optimum solvent systems acquired after an intensive TLC analysis of ethyl acetate crude extract from *Nigrospora osmanthi*, *Diaporthe* sp. and the leaf material, was used in column chromatography. The dry extracts were re-dissolved in 1 ml amount of ethyl acetate and loaded on evenly packed silica gel column, by dripping on the column wall, cautioning the disturbance of the silica gel layer. The silica gel used in the columns was 60 0.06-0.2 mm (70-230 mesh ASTM) supplied by Scharlau Lab supplies Limited. The column was eluted gradually at a flow rate of 15 ml/5 min. Ethyl acetate fractions of equal volumes were collected in test tubes and TLC for each fraction performed. Fractions with similar TLC pattern were pooled together. This yielded 4 fractions labelled F1-F4 from ethyl acetate extracts of the fungal endophytes and the plant material which were spotted again on TLC plates and then each fraction was dried and subjected to antimicrobial assay. From the TLC analysis, fraction F2 and F3 obtained from the *N. osmanthi* contained the same compound because they had similar TLC patterns. Purification by preparative HPLC was done on fraction F3 for *N. osmanthi* and F2 for *Diaporthe* sp. while fraction F2 for the plant.

3.8 Purification of the Fungal and Plant Fractions

Preparative HPLC system (Shimadzu-UFLC prominence), fitted with an auto-sampler (Model-SIL20ACHT) and a visible detector (Model-SPDA 20A) was used in the separation of compounds. Dried fractions F2 from *N. osmanthi*, F3 from *Diaporthe* and F2 from the leaves 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 the centrifuged samples were then loaded onto an auto-sampler. The separation was performed on Kromasil reverse phase ODS C18 5 µm column (4.6x250 mm). Gradient separation was performed using mobile phase A (100% Millipore water) and mobile phase B (100% HPLC grade methanol). The detection was carried out at 254 nm wavelength. The collected fractions were later concentrated under reduced pressure to yield 3 pure compounds. The pure compounds were then weighed and then analyzed using NMR and mass spectroscopy.

3.9 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 spectrometer at the Braunschweig Helmholtz Center for Infectious Diseases, Germany. The compounds were dissolved in Deuterated chloroform and others in DMSO. Tetramethylsilane was used as an internal standard and chemical shifts were given as δ (ppm). The results were analyzed by 1D and 2D spectroscopic techniques.

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 HSQC spectrum (Heteronuclear single quantum Coherence) was used to determine the connectivity of hydrogen to their respective carbons atoms.

3.10 Antimicrobial Assay for the Fungal and Plant Fractions

3.10.1 Disc Diffusion Assay

Susceptibility of bacterial pathogens to the plant and fungal endophytes extracts was determined following the procedure described by Srivastava and Anandrao (2015). Approximately 50 mg of the fractions were weighed using analytical balance and a dissolved in 10 ml of 0.1% of DMSO. A concentration of 5 mg/ml was made using the formula $C_1V_1 = C_2V_2$ where C stands for the concentration and V volume. A 100 µl of overnight grown pathogenic bacterial culture suspensions adjusted to 1.5 x 10^8 CFU/ml were spread on MHA petri-dishes using sterile glass rod and left to air dry in a laminar hood. Blank sterile discs of 6 mm in diameter were impregnated in 50 µl of different extracts and placed on MHA media containing test organisms. A standard chloramphenicol disc (30µg/disc) (6 mm) was used as a positive control, whereas sterile Whatman filter paper No.1 discs soaked in DMSO were used as negative control. Three independent replicate

experiments were conducted with each extract treatment consisted of three petri-dishes. Petri-dishes were placed in a refrigerator (4^oC) for 2 hours to ensure diffusion of the extracts into the media. Thereafter, they were transferred to an incubator for 24 hours at 32°C. The diameter of the zones of inhibition was determined in millimeters using a ruler.

3.10.2 Minimum Inhibitory Concentration (MIC)

The plant and endophyte semi pure fractions that possessed antimicrobial activity were subjected to MIC. The MIC of both extracts were quantified using agar disc diffusion assay Scorzoni *et al.*, 2007. The inhibitory effects were assessed based on two-fold dilution according to Zuo *et al.* (2008) with slight modifications. Briefly, a stock solution was prepared by dissolving 50 mg of the extracts in 10 ml of 0.1% DMSO. The stock solution (5 mg/ml) was further diluted in two-fold dilution to obtain the following concentrations (2.5, 1.25, 0.625 mg/ml). Approximately 100 µl of freshly grown cultures (24 h) containing bacterial population of $1.5x10^8$ CFU/ml was spread on MHA media using a sterile swab and allowed to dry. Thereafter Whatman No. 1 filter paper discs (6 mm) were soaked in 50 µl of different concentrations of the extracts. Using sterile forceps, four discs containing different concentrations of the extracts were placed on MHA petri dishes. The positive control was standard chloramphenicol discs (30µg/disc) (6 mm) while the negative control was sterile Whatman No.1 filter paper discs soaked in DMSO. The experiment was carried out in triplicate and the plates incubated at 32°C for 24 h. The zones of inhibition were determined in millimeters using a ruler.

3.11 Mass Spectrometry

The compounds mass spectra were recorded on FinniganTripple Stage Quadrupol Spectrometer (TSQ-70) with electron spray ionization method in the analysis. Thermo XcaliburQual computer software was used in the analysis of the mass chromatograms.

3.12 Statistical Analysis

The mean inhibition zones were calculated and the data was analyzed using SPSS software version 20. The homogeneity of variance was determined by One-way ANOVA using levene statistic. Mean comparison was done using Turkeys test (Honestly Significant Difference) with a significance level of $p<0.05$.

CHAPTER FOUR

RESULTS

4.1 Morphological Identification of Endophytes

A total of three axenic endophytes were isolated from leaves of *L. martinicensis* plants on SDA media. The fully grown axenic cultures displayed varied forms namely circular, irregular and filamentous, while colony colour was either red or white (Fig. 1A-C). The three endophytes were observed under ×400 magnification indicated that the three endophytes poses either aseptate or septate hyphae (Fig. 1 D-F).

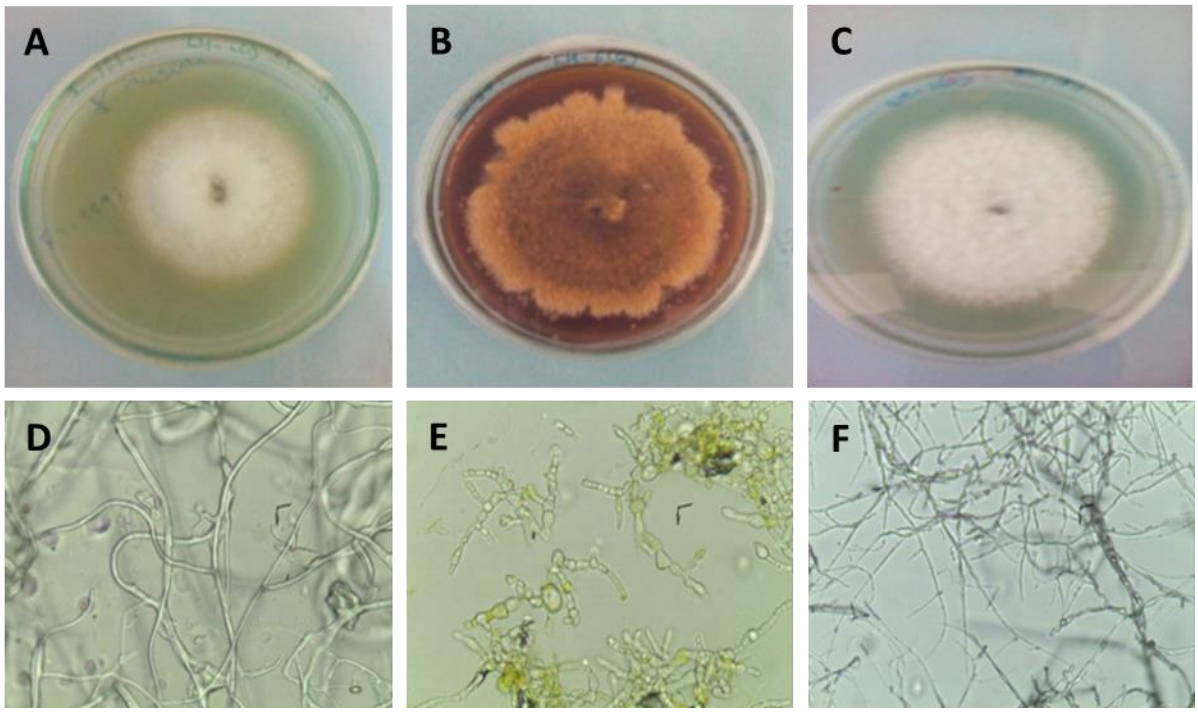


Figure 4: Morphological characteristics of fungal endophytes isolated from *L. martinicensis* leaves collected from Mt Elgon in Kenya. (**A)** Endophyte with circular colony form putatively identified as a member of Genus *Diaporthe*; (**B)** Endophyte with irregular colony form identified as a member of Genus *Epicoccum* **C.** Endophyte with filamentous colony form identified as a member of Genus *Nigrospora*; (**D-F)** Corresponding hyphae characteristic septate for isolates of Genus *Diaporthe* and *Nigrospora*, while aseptate for Genus *Epicoccum.*

The growth rate ranged from 4 to 10 days for the isolate to attain full growth (Genus *Diaporthe* taking 4 days, *Epicoccum* 10 days and *Nigrospora* 6 days). Morphological characteristics namely form, elevation, margins and colour of the colonies varied across the three isolates (Table 1).

Table 1: Morphological characteristics of axenic cultures of endophytic fungi isolated from *L. martinicensis* leaves

Fungal endophyte	**Genus**	**Phenotypic characteristics**
AD-L(1)	*Diaporthe*	Form: circular
		Elevation: crateriform
		Margin: entire
		Colour: white
		Hyphae: aseptate
LM-S(6)	*Nigrospora*	Form: irregular
		Elevation: Nmbonate
		Margin: undulate
		Colour: red
		Hyphae: septate
LM-L(1)	*Epicoccum*	Form: failamentous
		Elevation: crateriform
		Margin: filiform
		Colour: white
		Hyphae: aseptate

4.2 Molecular Characterization of the Endophytes Isolates

4.2.1 ITS Amplification and Sequencing

PCR products resolved on 1.2% agarose gel revealed that the amplicons obtained ranged between 500-700 bp. However, after sequencing the actual size of the amplicons were 564bp, 533bp and 528bp for isolate LM-L(1), AD-L(1) and LM-S(6), respectively. Alignment of the three

sequences using Clustal Omega also revealed variation between the ITS sequence nucleotide residues **(**Appendix 1**).** NCBI BLASTN-Targeted loci search using ITS nucleotide sequences of the 3 endophytes revealed that they belong to phylum Ascomycota. In addition, determination of the genus and species based on sequence identity of 100%, e-values of 0 and query coverage ≥ 90% the BLASTN-Targeted loci search returns indicated that ITS sequences for isolate LM-L(1), AD-L(1) and LM-S(6), were similar to those of *Nigrospora osmanthi* (NR_153474.1), *Diaporthe novem* (NR_111855.1) and *Epicoccum italicum* (NR_158264.1), respectively. In contrast to BLASTN-Targeted loci hits, search against UNITE fungi identification databases revealed that ITS sequences for LM-L(1) AD-L(1) and LM-S(6) isolates share sequence similarity with *Nigrospora sphaerica* (GenBank: MH645137), *Diaporthe pseudolongicolla* (GenBank: KU672724) and *Epicoccum nigrum* (GenBank: MG719634), respectively. Alignment of *Nigrospora* and *Epicoccum* sequences revealed truncation of nucleotides in the 5′and 3′ end for sequence from BLASTN-Targeted loci database compared to the UNITE database, whereas for *Diaporthe* the sequences from the two database were identical except for a single nucleotide substitution at position 111.

4.2.2 Phylogenetic Analysis of the Isolated Fungi

The evolutionary relationship of the *Diaporthe* sp., *Nigrospora osmanthi* and *Epicoccum italicum* with the top 10 BLASTN-Targeted loci hits and UNITE sequences inferred using Maximum likelihood method (Neighbor Joining approach), identified AD-L (1) isolate up to genus *Diaporthe* level. Whereas LM-L(1) clustered with two species from GenBank (*N. osmanthi* and *N. lactocolonia*) and one from UNITE (*N. sphaerica*) databases. On the other hand, LM-S(6) clustered with *E. italicum* and *E. nigrum* from GenBank and UNITE databases, respectively (Fig. 2).

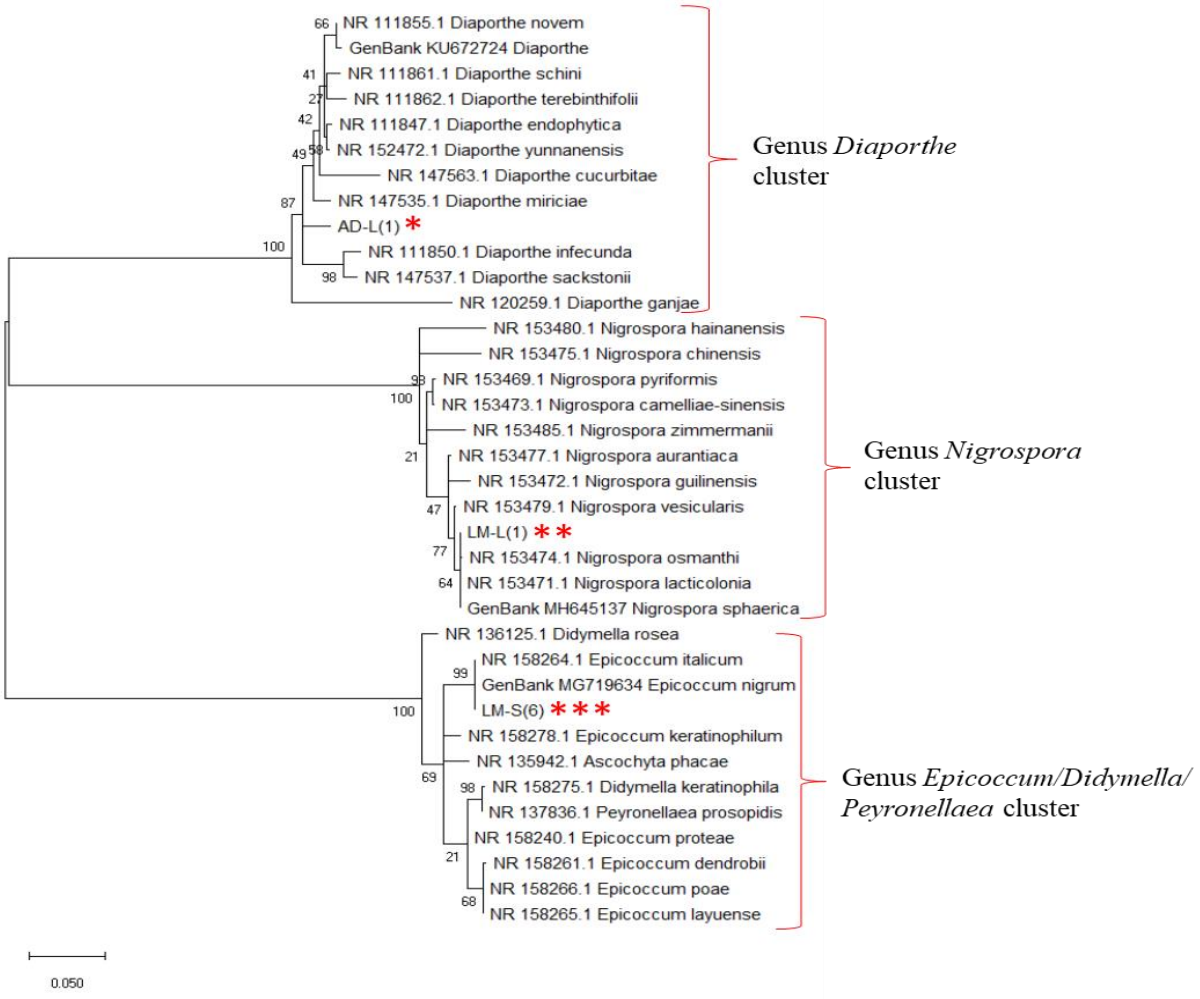


Figure 5: Evolutionary phylogenetic tree obtained from analysis of rDNA ITS sequences of fungal endophytes *Nigrospora osmanthi*, *Diaporthe* sp. and *Epicoccum italicum* isolated from *L. martinicensis* leaves and their closest relatives obtained from GenBank and UNITE fungal identification database. The tree was constructed with MEGA-X using Maximum likelihood method and 1000 bootstraps. The isolates AD-L (1)*, LM-L (1) ** and LM-S (6) *** clustered with fungi of the genus *Nigrospora*, *Diaporthe* and *Epicoccum*, respectively.

4.3 Antibacterial Activity of Endophytes

4.3.1 Pre-screening of Endophytes

In the dual culture assay, all endophyte isolates inhibited growth of test bacteria strains (Fig. 6).

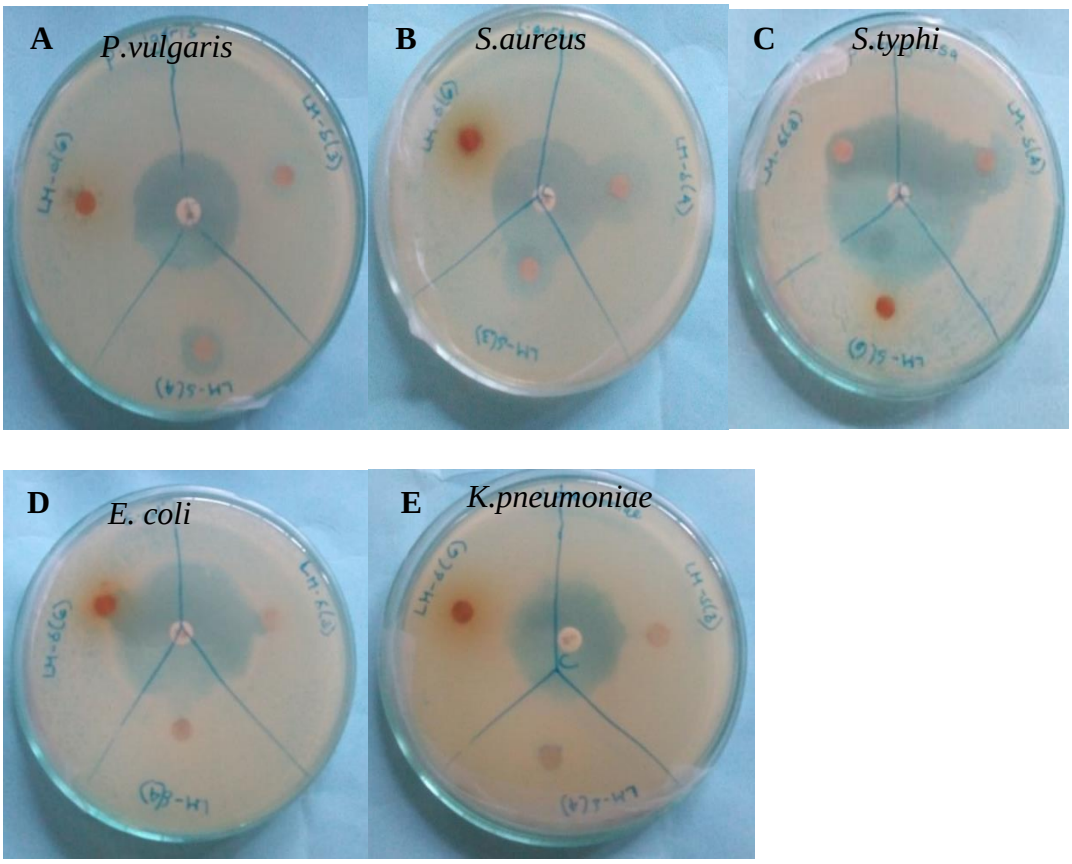


Figure 6: Dual culture assay of the isolated fungal endophytes against the test bacteria. Inhibition zones of the agar plugs of; AD-L(1) (*Diaporthe* sp.), LM-L(1) (*Nigrospora osmanthi*) and LM-S(6) (*Epicoccum italicum*). Positive control consists of discs impregnated with chloramphenicol (30 µg/disc).

Irrespective of the test bacteria, higher activity was displayed by isolate LM-L(1) whereas LM-S(6) isolate displayed the lowest activity though not significantly ($p<0.05$) different from AD-L(1) isolate (Table 2). The zones of inhibition produced by the endophytes against *E. coli* and *P. vulgaris* were not significantly ($p<0.05$) different. On the other hand, LM-L(1) produced significantly ($p<0.05$) higher inhibition zone against *S. aureus*, *K. pneumoniae* and *S. typhi* compared to LM-S (6) isolate (Table 2). Although the endophytes inhibited growth of test bacteria,

chloramphenicol at 30 µg/disc displayed significantly ($p<0.05$) higher activity of 2-3 fold higher than those displayed by the endophyte plugs (Table 2).

Table 2: Prescreening of antimicrobial activities for the isolated fungal endophytes against the test organisms using Dual Culture assay

Isolate	**Genus**	**Test Bacteria (Diameter in mm, n=4)**				
		E.coli	***P.vulgaris***	***S.aureus***	***K.pneumoniae***	***S.typhi***
AD-L(1)	*Diaporthe*	8*±0.00$^{b\#}$	8.3±0.58^{b}	9.7±1.15^{bc}	8.3±0.58^{bc}	9±1.00^{b}
LM-L(1)	*Nigrospora*	10±1.73^{b}	10±1.00^{b}	10.7±0.58^{b}	9±1.00^{b}	9.3±0.58^{b}
LM-S(6)	*Epicoccum*	8±0.00^{b}	7±0.00^{b}	7±0.00^{c}	7±0.00^{c}	7±0.00^{c}
Chlora.[1]		22±2.00^{a}	20±2.00^{a}	32±2.00^{a}	32±1.00^{a}	22±1.00^{a}

*Values are the mean diameter (mm) of 3 replicate experiments followed by ± S.E of the mean. #Values in a column with same letter are not significantly ($p<0.05$) different based on Turkey HSD test. [1]Chloramphenicol (30 µg/disc).

4.3.2. Antibacterial Activity of Endophytes Ethyl Acetate Extracts

Ethyl acetate fractions obtained from AD-L(1) isolate displayed antibacterial activity against all test bacteria except fraction F2, F3 and F4 when tested against *K. pneumoniae*. Generally, antibacterial activities of AD-L(1) fractions were not significantly ($p<0.05$) different when tested against *E. coli*, *P. vulgaris* and *S. typhi*. On the other hand, when tested against *K. pneumoniae* and *S. aureus* significant ($p<0.05$) activity was obtained with fraction F1 and F4, respectively (Table 3). Although ethyl acetate fractions showed activity against test bacterial strains, the activity of chloramphenicol was 2-3 fold higher (Table 3).

Table 3: The activity of ethyl acetate fractions extracted from *Diaporthe* against the test bacteria

Fractions	**Test Bacterial Strains (Diameter in mm, n=6)**				
	E.coli	***P.vulgaris***	***K.pneumoniae***	***S.typhi***	***S.aureus***
F1	8*±0.58$^{b\#}$	9±0.58^{b}	10±0.58^{b}	9.7±0.88^{b}	8±0.58^{c}
F2	9±0.58^{b}	10.3±0.88^{b}	0±0.00^{c}	8±0.58^{b}	8±0.58^{c}
F3	9±0.58^{b}	8.7±1.20^{b}	0±0.00^{c}	11±0.58^{b}	9.3±0.33^{c}
F4	9.3±0.88^{b}	8±0.58^{b}	0±0.00^{c}	10.3±0.88^{b}	13.7±0.88^{b}
Chloramphenicol	21±2.00^{a}	21±1.76^{a}	32±0.58^{a}	22±0.58^{a}	32±1.15^{a}
DMSO	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}	0±0.00^{d}

*Values are mean diameter (mm) of 3 replicate experiments followed by ± S.E of the mean. #Values in a column with same letter are not significantly ($p<0.05$) different based on Turkey HSD test.

For endophyte LM-L(1), antibacterial activity of fractions F1 to F4 against *P. vulgaris*, *E.* coli and *K. pneumonia* bacteria were not significantly ($p<0.05$) different, while only discs impregnated with fraction F1 showed activity against *S. typhi* (9.7±0.33). The activity of fraction F3 and F4 compared to F1 and F2 were significantly ($p<0.05$) different when tested against *S. aureus* (Table 4). However, activity of chloramphenicol was significantly ($p<0.05$) higher than those obtained on discs impregnated with LM-L(1) fractions at 5 mg/ml which was approximately 2-3 fold higher (Table 4).

Table 4: The activity of ethyl acetate fractions obtained from LM-L(1) isolate against the test bacteria

Fractions	**Test Bacteria (Diameter in mm, n=6)**				
	E.coli	***P. vulgaris***	***K. pneumoniae***	***S .typhi***	***S. aureus***
F1	8*±1.00$^{b\#}$	10±1.15^{b}	8.3±0.67^{b}	9.7±0.33^{b}	8.7±0.67^{c}
F2	8.7±1.20^{b}	11.3±2.03^{b}	8.7±0.88^{b}	0±0.00^{c}	10.7±0.67^{c}
F3	8±0.58^{b}	10.7±2.19^{b}	7±0.00^{b}	0±0.00^{c}	15±0.58^{b}
F4	8.3±1.33^{b}	12±2.08^{b}	8±0.58^{b}	0±0.00^{c}	15±1.53^{b}
Chloramphenicol	21±1.15^{a}	20±1.15^{a}	33±0.58^{a}	22±0.58^{a}	32±1.15^{a}
DMSO	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}	0±0.00^{d}

*Values are mean diameter (mm) of 3 replicate experiments followed by ± S.E of the mean. $^{\#}$Values in a column with same letter are not significantly ($p<0.05$) different based on Turkey HSD test.

4.3.3. MIC Assay for Endophyte Extracts

For MIC assay, increasing the concentration of AD-L(1) fractions from 0.625 to 5 mg/ml led to increased antibacterial activity for fractions F2 and F3, with highest activity obtained on 5 mg/ml. Antibacterial activity at 5 mg/ml for fraction F2 and F3 against *E. coli* and *S. typhi* was significantly ($p<0.05$) higher unlike for *P. vulgaris*, *S. aureus* and *K. pneumoniae*, where 5 mg/ml concentration was significantly different from activity of 0.625 mg/ml (Table 5).

Table 5: Minimum inhibitory concentrations of ethyl acetate fractions obtained from AD-L(1) isolate against test bacteria

Fraction	Concentration (mg/ml)	Test Bacterial Strains (Diameter in mm, n=10)				
		E.coli	***P.vulgaris***	***S.aureus***	***S.typhi***	***K.pneumoniae***
F2	5.0	14.7 ± 0.67^{b}	14 ± 1.15^{b}	14.3 ± 0.88^{b}	13.3 ± 0.67^{bc}	11.3 ± 0.67^{cd}
F2	2.5	12.7 ± 0.67^{bc}	12 ± 1.15^{bc}	12.7 ± 0.67^{bc}	10.7 ± 0.67^{cd}	9.7 ± 0.33^{de}
F2	1.25	10.7 ± 0.67^{cd}	10.3 ± 0.88^{bc}	10.7 ± 0.67^{bcd}	10 ± 0.58^{d}	8.3 ± 0.33^{de}
F2	0.625	8.3 ± 0.33^{de}	8 ± 0.58^{c}	8 ± 0.58^{d}	8 ± 0.58^{d}	7.3 ± 0.33^{e}
F3	5.0	13.3 ± 0.67^{bc}	12 ± 1.15^{bc}	14 ± 1.15^{b}	15 ± 0.58^{b}	16 ± 1.15^{b}
F3	2.5	10.7 ± 0.67^{cd}	10.3 ± 0.88^{bc}	12 ± 1.15^{bc}	13 ± 0.58^{bc}	14 ± 1.15^{bc}
F3	1.25	9 ± 0.58^{de}	9 ± 0.58^{c}	10.3 ± 0.88^{bcd}	10.7 ± 0.67^{cd}	12 ± 1.15^{cd}
F3	0.625	7.3±0.33e	7.7 ± 0.33^{c}	8.3 ± 0.88^{cd}	8.3 ± 0.33^{d}	9 ± 0.58^{de}
Chloramphenicol		22 ± 1.15^{a}	20 ± 1.15^{a}	32 ± 1.15^{a}	23 ± 0.58^{a}	33 ± 0.58^{a}
DMSO		0 ± 0.00^{f}	0 ± 0.00^{d}	0 ± 0.00^{e}	0 ± 0.00^{e}	0 ± 0.00^{f}

*Values are the mean diameter (mm) of 3 replicate experiments followed by ± S.E of the mean. #Values in a column with same letter are not significantly ($p<0.05$) different based on Turkey HSD test.

For LM-L(1) MIC assay, all the fractions concentrations tested showed activity against all test bacteria. Similar to AD-L(1) increasing fractions concentration from 0.625 mg/ml to 5 mg/ml resulted to increased antibacterial activity against test bacteria (Table 6). Furthermore, in all fractions the discs impregnated with 5.0 mg/ml generally had significantly ($p<0.05$) higher

activity compared to those impregnated with 0.625 mg/ml and 1.25 mg/ml. This trend was mainly observed against *E. coli*, *P. vulgaris* and *K. pneumoniae*, while for *S. aureus* and *S. typhi* the trend was observed for fractions F3 and F4 respectively. Unlike in the disc diffusion assay data presented in table 4, fraction F2-F4 produced activity against *S. typhi* (Table 6). Highest activity was obtained at concentration of 5 mg/ml against *P. vulgaris* produced by discs impregnated with all fractions (F2 (15.3±0.6), F3 (19±0.58 and F4 (16.3±1.33). However, its only discs impregnated with F3 at 5.0 mg/ml that produced activity that was not significantly ($p<0.05$) different from positive control chloramphenicol antibiotic (Table 6).

Table 6: Minimum inhibitory concentrations (mg/ml) of fraction obtained from LM-L(1) isolate against the test bacteria

Fraction	Concentration (mg/ml)	Test Bacterial Strain (Diameter in mm, n=14)				
		E.coli	***P.vulgaris***	***S.aureus***	***S.typhi***	***K.pneumoniae***
F2	5.0	13.3*±0.67$^{b\#}$	15.3±0.67^{cd}	11±0.58^{cde}	12±1.15^{cd}	11.7±0.33^{cd}
F2	2.5	11±0.58^{bcd}	13±0.58^{cde}	9.7±0.33^{defg}	10.3±0.88^{cde}	10.3±0.33^{cde}
F2	1.25	9.3±0.67^{cd}	9.7±0.33^{efg}	8.7±0.33^{efg}	8.7±0.33^{de}	8.7±0.33^{ef}
F2	0.625	8±0.58^{d}	7.7±0.33^{g}	7.3±0.33^{g}	7±0.00^{e}	7±0.00^{f}
F3	5.0	13±0.58^{b}	**19±0.58^{ab}**	14±0.58^{b}	11.3±0.67^{cd}	12.7±0.67^{bc}
F3	2.5	11±0.58^{bcd}	15.7±0.33^{bc}	12.7±0.67^{bc}	9.7±0.33^{de}	10.3±0.33^{cde}
F3	1.25	10±0.58^{bcd}	13±0.58^{cde}	10.7±0.67^{cdef}	8.7±0.33^{de}	9.3±0.33^{def}
F3	0.625	8.3±0.33^{cd}	9.3±0.33^{efg}	9±0.58^{efg}	7.3±0.33^{e}	7.7±0.33^{f}
F4	5.0	11.7±0.88^{bc}	16.3±1.33^{bc}	12±0.58^{bcd}	16±1.15^{b}	14.7±0.67^{b}
F4	2.5	10.3±0.88^{bcd}	14.3±0.88^{cd}	10.7±0.67^{cdef}	14±1.15^{bc}	12.7±0.67^{bc}
F4	1.25	9±0.58^{cd}	12±1.15^{def}	9.3±0.33^{defg}	10.7±0.67^{cde}	11±0.58^{cd}
F4	0.625	7.7±0.67^{d}	8.7±0.33^{fg}	8±0.58^{fg}	8.7±0.33^{de}	9±0.58^{ef}
Chloramphenicol		22±1.15^{a}	20±1.15^{a}	32±0.88^{a}	23±1.15^{a}	33±0.58^{a}
DMSO		0±0.00^{e}	0±0.00^{h}	0±0.00^{h}	0±0.00^{f}	0±0.00^{g}

*Values are mean diameter (mm) of 3 replicate experiment followed by ± S.E of the mean. $^{\#}$Values in a column with same letter are not significantly ($p<0.05$) different based on Turkey HSD test.

4.4 Antibacterial Activity of Secondary Metabolites from the Leaves

4.4.1 Disc Diffusion Assay of the Secondary Metabolites from the Leaf Extracts

Ethyl acetate extract of the leaf material exhibited highest activity against *S. aureus* (10±0.58) and *S.typhi* (10.3±1.20) and very little activity against *K. pneumoniae* (7.7±0.67) and *E.coli* (7.3±0.33). Similarly, methanol extract from the leaf material showed little activity against *E.coli* (7±0.00), *P.vulgaris* (7.3±0.33) and *K.pneumoniae* (7.7±0.33) (Table 7). After fractionation of the ethyl acetate extract, four fractions were obtained. Fractions F2, F3 and F4 displayed activity against *P.vulgaris, S.typhi, K. pneumoniae* and *S.aureus* while there was no activity against *E.coli*. Interestingly, F1 exhibited activity against all the test pathogens. However, there was no significant differences ($p< 0.05$) in activity of fractions F1-F4 against *P.vulgaris, E coli* and *K.pneumoniae* (Table 7).

Table 7: Inhibition zones of *L. martinicensis* fractions, methanol crude and ethyl acetate extracts

	Test organism (diameter in mm, n=8)				
Fractions	***E.coli***	***P.vulgaris***	***K.pneumoniae***	***S.typhi***	***S.aureus***
F1	8.7*±1.67^{b}	9.7±1.45^{b}	8.3±0.67^{b}	7±0.00^{c}	8.7±0.33^{b}
F2	0±0.00^{c}	7.3±0.33^{b}	7±0.00^{b}	7±0.00^{c}	7.7±0.33^{b}
F3	0±0.00^{c}	7±0.00^{b}	8±0.58^{b}	7.7±0.33^{c}	7.3±0.33^{b}
F4	0±0.00^{c}	8.3±0.33^{b}	7±0.00^{b}	8.3±0.33^{bc}	9±0.58^{b}
Methanol crude	7±0.00^{b}	7.3±0.33^{b}	7.7±0.33^{b}	9.3±0.33^{bc}	8.7±0.33^{b}
Ethyl acetate extract	7.3±0.33^{b}	8.7±0.33^{b}	7.7±0.67^{b}	10.3±1.20^{b}	10±0.58^{b}
Chloramphenicol	22±1.15^{a}	20±1.15^{a}	33±0.58^{a}	23±0.58^{a}	32±1.15^{a}
DMSO	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}

*The values represent the mean of the three experiments ± S.E of the mean. Within the column, inhibition zones of the plant fractions, methanol crude and ethyl acetate extracts sharing the same letter (s) are not significantly different while those sharing different letters are significantly different (α = 0.05, Turkey HSD).

4.4.2 Minimum Inhibitory Concentration Assay

Increasing the concentration of fractions from 0.625 mg/ml to 5.0 mg/ml lead to an increased activity against all the test bacteria, however their activities were significantly (*p>0.05*) different (Table 8). At highest concentration of 5.0 mg/ml F3 demonstrated highest inhibitory activities against *S.typhi* (15±0.78), *S. aureus* (14±1.22) and *K.pneumoniae* (16±1.04). Similarly, F3 also exhibited high inhibitory activity against *S.typhi* (13±0.78) , *S. aureus* (12±1.22) and *K.pneumoniae* (14±1.04) at an MIC of 2.5 mg/ml. On the other hand, at 5.0 mg/ml the activity of F2 against *E. coli*

(14.7±0.67), *P.vulgaris* (14±0.15)s and *S. aureus* (14.3±0.88) was not statistically different. However, the activity of chloramphenicol standard was 2-4 fold higher compared to F2 and F3 (Table 8).

Table 8: Minimum Inhibitory Concentration assay of *L. martinicensis* fractions against test bacteria

		Test organisms (Diameter in mm) n=10				
Fraction	**Concentration**	***E.coli***	***P.vulgaris***	***S.aureus***	***S.typhi***	***K.pneumoniae***
F2	0.625	8.3 ± 0.33^{de}	8 ± 0.58^{c}	8 ± 0.58^{d}	8 ± 0.58^{d}	7.3 ± 0.33^{e}
F2	1.25	10.7 ± 0.67^{cd}	10.3 ± 0.88^{bc}	10.7 ± 0.67^{bcd}	10 ± 0.58^{d}	8.3 ± 0.33^{de}
F2	2.5	12.7 ± 0.67^{bc}	12 ± 1.15^{bc}	12.7 ± 0.67^{bc}	10.7 ± 0.67^{cd}	9.7 ± 0.33^{de}
F2	5	14.7 ± 0.67^{b}	14 ± 0.15^{b}	14.3 ± 0.88^{b}	13.3 ± 0.67^{bc}	11.3 ± 0.67^{cd}
F3	0.625	7.3 ± 0.33^{e}	7.7 ± 0.33^{c}	8.3 ± 0.88^{cd}	8.3 ± 0.33^{d}	9 ± 0.58^{de}
F3	1.25	9 ± 0.58^{de}	9 ± 0.58^{c}	10.3 ± 0.88^{bcd}	10.7 ± 0.67^{cd}	12 ± 1.15^{cd}
F3	2.5	10.7 ± 0.90^{cd}	10.3 ± 1.23^{bc}	12 ± 1.22^{bc}	13 ± 0.78^{bc}	14 ± 1.04^{bc}
F3	5	13.3 ± 0.90^{bc}	12 ± 1.23^{bc}	14 ± 1.22^{b}	15 ± 0.78^{b}	16 ± 1.04^{b}
Chloramphenicol		22 ± 1.15^{a}	20 ± 1.15^{a}	32 ± 1.15^{a}	23 ± 0.58^{a}	33 ± 0.58^{a}
DMSO		0 ± 0.00^{f}	0 ± 0.00^{d}	0 ± 0.00^{e}	0 ± 0.00^{e}	0 ± 0.00^{f}

. * The values are the mean of the three experiments ± S.E of the mean. Within the column, the inhibition zones of the fractions sharing the same letter (s) are not significantly different while those with different letters are significantly different (α=0.05 Turkey HSD test).

4.5 Characterization of the Secondary Metabolites

In this study, a total of three pure compounds were isolated from the leaves (one) and fungal endophytes (two). The pure compounds were analyzed by mass spectrometry and spectroscopic techniques which included 1D and 2D NMR.

4.5.1 Structural Elucidation of Compound 5

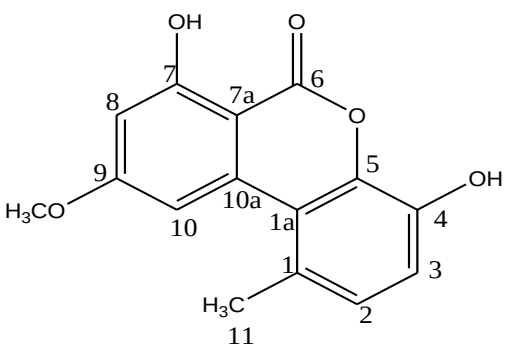
Compound **5** was obtained from F2 of *Nigrospora osmanthi* endophyte with a mass of 4.3 mg. The High Resolution Mass Spectroscopy (HRMS) of this compound showed ion peaks at m/z 273.0757 that corresponds to $[C_{15}H_{12}O_6 + H]^+$. This compound absorbs at a maximum wavelength of 256 nm as shown in figure 3. The number of carbon atoms was confirmed by ^{13}C NMR spectra (Appendix 2) which had 15 peaks. The analysis of DEPT spectra (Appendix 3) showed the presence of 4 methine carbons δ 99.1 (C8), δ103.3 (C10), δ101.7 (C2) and δ 117.8 (C3) while the combination of ^{13}C NMR and HMBC spectra showed presence of a methoxy δ 55.8 (C9), 2 oxygenated aromatic carbons δ152.7 (C4), δ164.2 (C7), a methyl group δ25.0 (C11) and nine quaternary carbons resonating at δc 138.3, 108.5, 152.7, 159.3, 164.7, 164.2, 137.9, 166.2, 98.4.

The protons directly attached to carbon atoms were assigned using HSQC spectrum (Appendix 4). This spectrum showed a correlation between protons resonating at δ_H 6.63, 6.72, 6.62, 7.22, 2.74 and 3.91 with carbon atoms absorbing at δ_c 101.7, 117.8, 99.1, 103.3, 25.0 and 55.8 respectively.

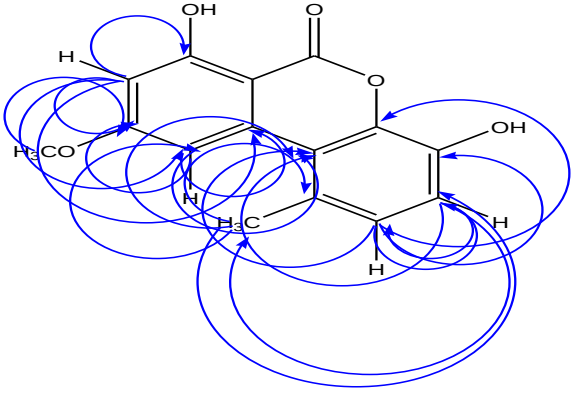
The HMBC spectrum (Appendix 5) showed a correlation between protons and carbons two or three bonds away from each other. From this spectrum, proton H-2 (δ 101.7) correlates with C-1a, C-3, C-4 and C-5, proton H-3 (δ 117.8) correlates with C-11, C-2 and C-1a, while proton H-8 (δ 99.1) correlates with C-10a, C-10, C-7, and C-9. The methyl proton C-11 correlates with C-10, C-1a, C-3 and C-1. Methoxy proton correlates with C-9 while proton H-10 (δ 103.3) correlates with C-10a, C-1a, and C-9. Based on literature (Darsih *et al.*, 2017) and spectra information from table 9 this compound is referred to as 4, 7- dihydroxy-9-methoxy-1-methylchromen-6-one .

Table 9: The NMR data of compound **5**

No.	^{13}C	$^{1}HNMR$	TYPE	HMBC
1	138.3	-	Cq	-
1a	108.5	-	Cq	-
2	101.7	6.63	CH	1a, 3, 4, 5
3	117.8	6.72	CH	11, 2, 1a,
4	152.7	-	Cq	-
5	159.3	-	Cq	-
6	164.7	-	Cq	-
7	164.2	-	Cq	-
7a	137.9	-	Cq	-
8	99.1	6.62	CH	10a, 10, 7, 9
9	166.2	-	Cq	-
10	103.3	7.22	CH	10a, 1a, 9
10a	98.4	-	Cq	-
11	25.0	2.74	CH_3	10, 1a, 3, 1
-OCH_3	55.8	3.91	CH	9



compound 5



4, 7- dihydroxy-9-methoxy-1-methylchromen-6-one → HMBC correlation

Figure 7: Structure of compound 5 and HMBC correlations

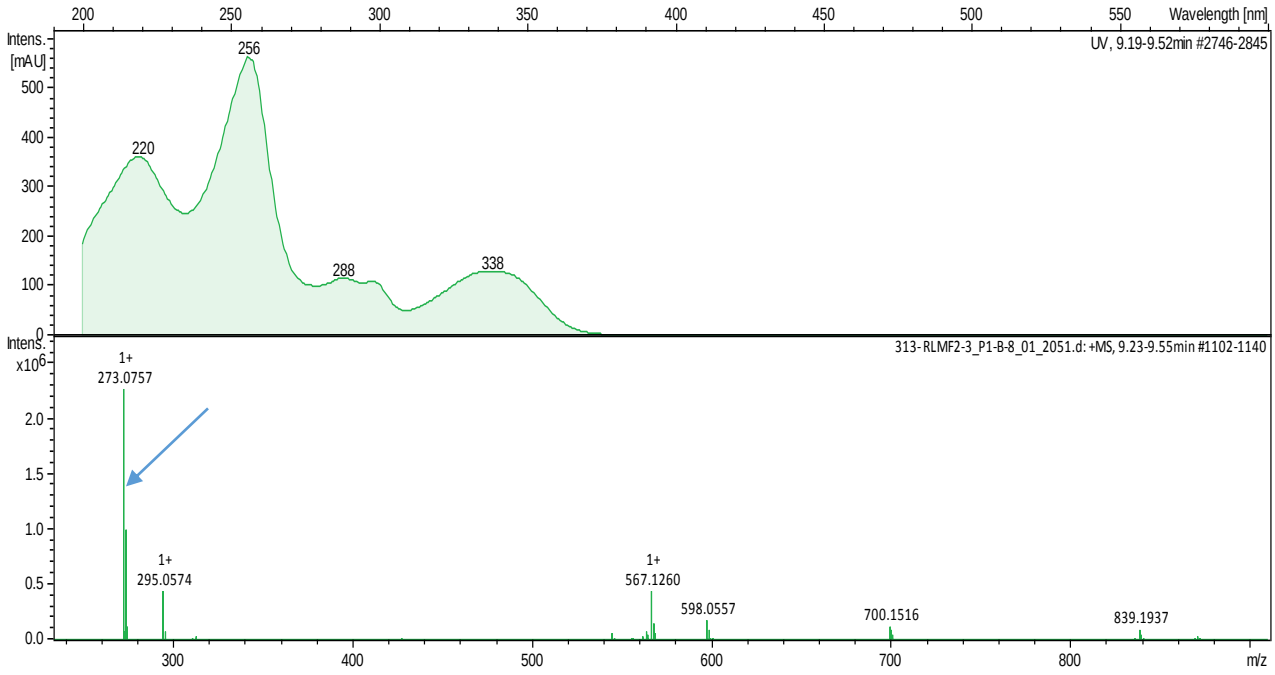


Figure 8: Mass spectrum of compound 5

4.5.2 Structure Elucidation of Compound 6

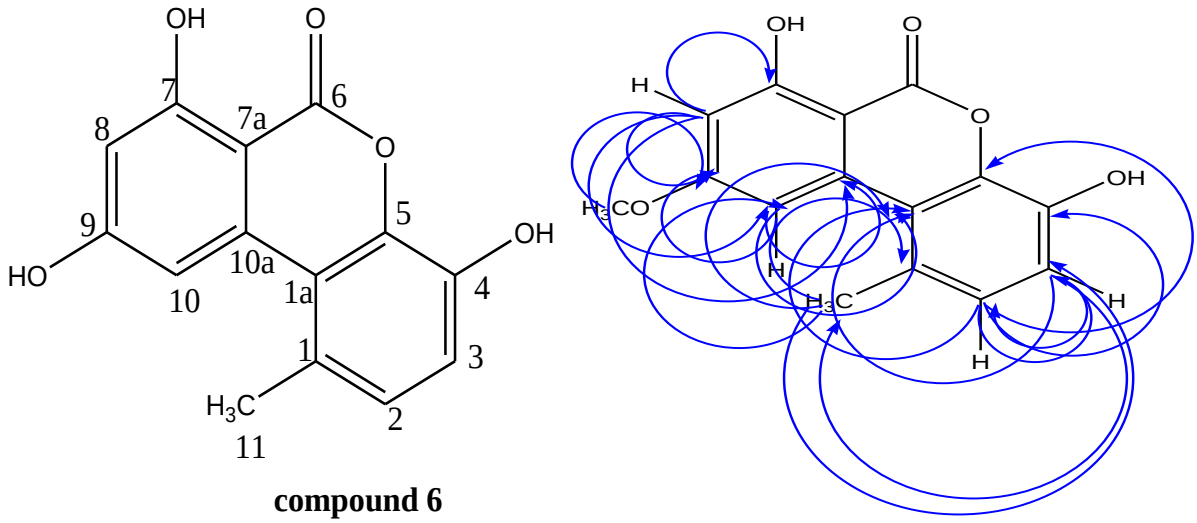
Compound **6** was obtained from F3 extract of *Diaporthe* sp. endophyte with a mass of 8.2 mg. The High Resolution Mass Spectroscopy showed ion peaks at m/z 259.0598 that corresponds to $[C_{14}H_{10}O_5+H]^+$ (Figure 5). The UV spectrum showed that the compound absorbs at a wavelength of 256 nm (Figure 5). The ^{13}C NMR (Appendix 6) showed the presence of 14 carbon atoms with a methyl group at C-1 while nine quaternary carbons were obtained from the HSQC spectra (Appendix 7). The quaternary carbons were resonating at δ_C 138.2, 108.9, 158.4, 152.6, 164.0, 164.7, 101.0, 137.9 and 96.6.

The 2 to 3 bond connectivity's was confirmed by the HMBC spectra (Appendix 8). Proton H-2 (δ 6.71) correlates with the C-11, C-3 and C-1a. Proton H-3(δ 6.62) correlates with C-1a, C-2, C-5, and C-4. The methyl proton H-1 (δ 2.68) correlates with C-10, C-1a, C-2 and C-1. Proton H-10 (δ 7.21) correlates with C-10a, C-7a, C-1a and C-7. This spectrum showed a correlation between protons resonating at δ_H 6.71, 6.62, 5.32, 7.21 and 2.68 with carbon atoms absorbing at

δc 117.5, 101.5, 129.6, 104.8 and 25.1. Based on spectra information from table 10, this compound is referred to as 4, 7, 9- trihydroxy-1-methylchromen-6-one.

Table 10: The assignment of ^{13}CNMR, DEPT, ^{1}HNMR and HMBC for Compound 6

No.	**^{13}C**	**^{1}HNMR**	**TYPE**	**HMBC**
1	138.2	-	C	-
1a	108.9	-	C	-
2	117.5	6.71	CH	11, 3, 1a
3	101.5	6.62	CH	1a, 2, 5, 4
4	158.4	-	C	-
5	152.6	-	C	-
6	164.0	-	C	-
7	164.7	-	C	-
7a	101.0	-	C	-
8	129.6	5.32	CH	11
9	137.9	-	C	-
10	104.8	7.21	CH	10a, 7a, 1a, 7
10a	96.6	-	C	-
11	25.1	2.68	CH_3	10, 1a, 2, 1



compound 6

4, 7, 9- trihydroxy-1-methylchromen-6-one → HMBC correlations

Figure 9: The structure of compound 6 and HMBC correlations

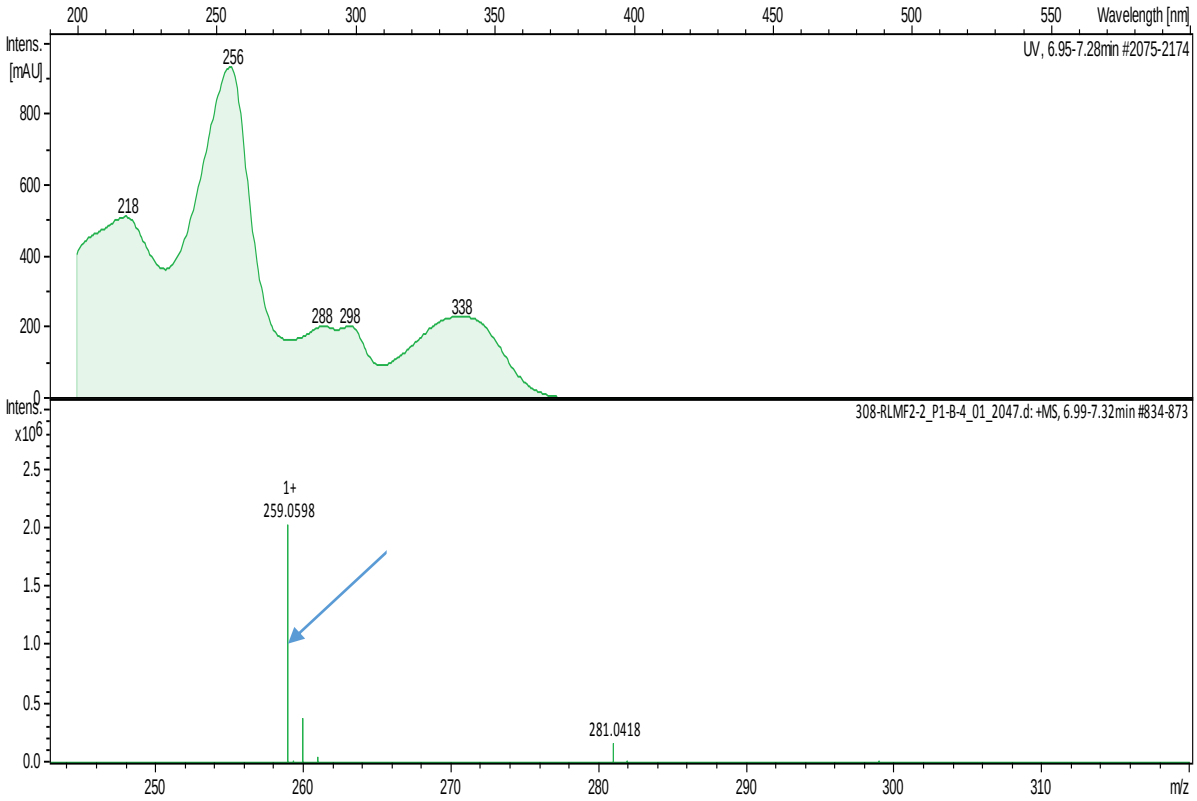


Figure 10: Mass spectrum of compound 6

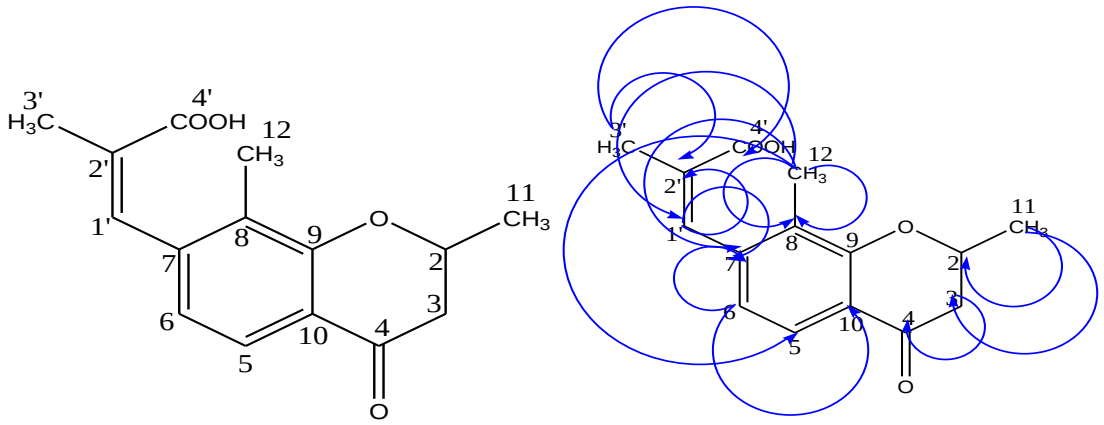
4.5.3 Structure Elucidation of Compound 7

Compound **7** was obtained from F2 extract of the plant. The mass spectrum showed a molecular mass of 259.0598 which corresponds to a molecular formula of $C_{15}H_{16}O_4$. The ^{13}CNMR spectra showed the presence of two methyl carbons at position δ 13.7 (C-11) and δ 25.1(C-12), the presence of methine carbons was confirmed by DEPT spectra (Appendix 11) at positions δ 21.8 (C-2), δ 101.0 (C-1′), δ 116.5 (C-5), δ 102.9 (C-6) and six quaternary carbons at 206.5 (C-4), 157.8 (C-2′), 164.0 (C-4'), 109.4 (C-7), 137.5 (C-8), 152.4 (C-9), 88.4 (C-10).

The protons directly attached the carbon atoms were assigned using the HSQC spectrum (Appendix 9). The spectra showed the presence of protons resonating at δ_H 1.23, 6.43, 6.52 and 5.55 which were attached to carbon atoms absorbing at δ 21.8 (C-2), 101.0 (C-1'), 116.6 (C-5) and 102.9 (C-6). The HMBC spectra (Appendix 10) was used to assign the 2-3 bond connectivities. From this spectrum, proton resonating at δ 2.08 shows a correlation with C-4 while proton resonating at δ 6.43 shows a correlation with C-7, C-5, C-9, and C-2'. Protons resonating at δ 2.05 shows a correlation with C-2′and C-4' while a proton resonating at 6.52 shows a correlation at C-12, C-1', C-7 and C-2'. The rest of HMBC correlations are shown in table 11. Based on the spectra information (Table 11) and literature (Al Yousuf *et al.*, 1999), this compound was known as 2, 8-dimethyl (2-methlyethenyl) benzo-4- acrylic acid.

Table 11: The assignment of ^{13}C NMR, DEPT, ^{1}HNMR and HMBC of compound **7**

Carbon	**^{13}CNMR**	**^{1}HNMR**	**TYPE**	**HMBC**
2	21.8	1.23	CH	-
3	31.0	2.08	CH_2	4
4	206.5	-	Cq	-
1'	101.0	6.43	CH	7, 5, 9, 2′
2'	157.8	-	Cq	-
3′	24.9	2.05	CH_3	2′, 4'
4′	164.0	-	Cq	-
5	116.5	6.52	CH	12, 1′, 7, 2′
6	102.9	5.55	CH	10, 7, 4'
7	109.4	-	Cq	-
8	137.5	-	Cq	-
9	152.4	-	Cq	-
10	88.4	-	Cq	-
11	13.7	0.85	CH_3	2, 3
12	25.1	2.05	CH_3	1′ , 7, 5, 8, 9



2, 8-dimethyl (2-methlyethenyl) benzo-4- acrylic acid ⟶ HMBC correlations

Figure 11: Structure of compound 7 and HMBC correlations

CHAPTER FIVE

DISCUSSION

5.1 Isolation and Identification of the Fungal Endophytes

Fungal endophytes are known to produce bioactive compounds with pharmaceutical, agricultural and industrial applications. However, isolation and characterization of fungi producing these bioactive compounds is crucial for large-scale production. In this study, isolation of fungal endophytes from *L. martinicensis* focused on leaf tissues. This choice was informed by reports indicating that distribution of fungal endophytes are organ specific with the highest numbers and diversity found in leaf tissues (Banhos *et al*., 2014; Suryanarayanan *et al*., 2012; Suryanarayanan *et al*., 2009) Despite this, in this study three endophytes were isolated from leaves obtained from different *L. martinicensis* plants. . This is in line with a study that was conducted by Rai *et al.* (2021) which indicates that young leaves are colonized by few endophytes as compare to older ones. This number is relatively low compared to a study by Pádua *et al.* (2018) where a total of 187 fungal endophytes were isolated from *Myracrodruon urundeuva* leaves. However, the population of endophytes in a plant is dependent on factors such as the stage of a developmental host, density of the inoculum, host species as well as environmental conditions (Dudeja & Giri, 2014). Although the number of endophytes was low, the endophytes represented three fungi genera obtained from *L. martinicensis* leaves collected from undisturbed tropical rain forest.

Endophytic fungi isolated from *L. martinicensis* leaves were identified by morphological characteristics when cultured on SDA media. However, many fungal endophytes fail to present all morphological structures in artificial cultures making their identification and classification difficult (García *et al.*, 2012). This study is in line with a research that was conducted by Yu *et al.* (2018) which shows that fungal endophytes lacked reproductive structures. Morphological identification is therefore somewhat challenging and hence difficult to objectively characterize endophytes to species level. This challenge can be answered by use of DNA barcodes such as ITS, β-tubulin among others which are relatively sensitive (González-Teuber *et al.*, 2017).

Further characterization of the endophytes was therefore undertaken using ITS barcoding to ascertain the morphological based identification. ITS ribosomal DNA sequences are all

important for distinguishing phylogenetic relationships among species or genera (Schoch *et al.*, 2012). Using Neighbor Joining approach to determine phylogenetic relationship between the three endophyte isolates with hits from NCBI and UNITE database confirmed that indeed the three endophyte fungi belong to phylum Ascomycota but from three different genera namely, *Diaporthe*, *Nigrospora* and *Epicoccum*. These results correlate with those obtained by Gonzaga *et al.* (2015) which shows that most of the isolated fungal endophytes belong to the phylum Ascomycota although some have been isolated from other phyla such as Basidiomycota, Oomycota, and Zygomycota. However, it was difficult to determine species of the isolates. The best hits obtained from BLAST search using the ITS sequences in NCBI and UNITE database suggested that two isolates *Nigrospora osmanthi* and *Epicoccum italicum* belong to two different species, while only genera for isolate *Diaporthe* could be determined. This clearly indicates that the use of BLAST search tool could have exhibited drawbacks due to inaccurately identified sequences present in the fungal databases (Raja *et al.*, 2017).

Endophytes from genera *Diaporthe* associate with a large number of host and are encountered as endophytes, saprobes as well as pathogens of forest trees, crops and ornamentals (Guarnaccia *et al*., 2018). Similar genera of fungal endophytes have been previously reported occurring as endophytes in medicinal plant such as *Luehea divaricata* (Specian *et al.*, 2012), *Hydnocarpus anthelminthicus* (Prachya *et al.*, 2007), *Annona squamosa* (Lin *et al.*, 2010), *Garcinia mangostona* and *Garcinia parvifolia* (Sim *et al.*, 2010). According to Gomes *et al.* (2013) and Santos *et al.* (2011) species of the genus *Diaporthe* have been targets for secondary metabolites research due to their ability to produce a huge number of unique low and high molecular weight metabolites and polyketides with varied activities as well as biological control of fungal pathogens. In this study, the fungi belonging to genera *Diaporthe* was not identified to species level which indicates that the use of ITS sequences have shortcomings in that they might not achieve a perfect alignment at higher taxonomic levels such as family, order and class (Lindhal *et al*., 2013) due to high ITS variability. According to Gomes *et al.* (2013), endophytes that belong to the genus *Diaporthe* are normally not always reliably identified to species-level because of variability in changing environmental conditions.

In contrast to the findings obtained from the current study, some species belonging to *Epicoccum* have been described as pathogens such as *Epicoccum sorghinum* isolated from *Saccharum officinarum, Nicotiana tabacum* as well as *Sorghum bicolor* (Lin *et al.* (2015). Despite

these, *Epicoccum* spp. has been isolated as an endophyte in various tissues of plants such as *Epicoccum nigrum* P16 from sugarcane (de Lima Favaro *et al.*, 2012). In comparison to this study *Epicoccum purpurascens* was isolated as an endophyte in a marine jellyfish *Aurelia aurita* (Wright *et al.*, 2003). In line with this study, genus *Nigrospora* has also been isolated as a fungal endophyte from the stems and leaves of various plants, leaf litter, dead larvae or from detritus as saprobes (Wu *et al.*, 2014).

5.2 Antibacterial Assay

All endophytes isolated from *L. martinicensis* leaves generally inhibited growth of gram negative *E. coli, P. vulgaris, K. pneumoniae* and *S. typhi*, and gram positive *S. aureus* bacteria. This clearly demonstrates that these endophytes possess antibacterial compounds. This is in line with reports indicating that fungi of *Nigrospora* sp., *Diaporthe* sp. and *Epicoccum* genera are known to produce different metabolites with antimicrobial activity against both gram positive and negative bacteria (Wu *et al.*, 2019). Antibacterial activity of metabolites extracted from *Epicoccum* showed relatively low activity. In contrast to this study, species of genus *Epicoccum* have been reported to produce metabolites with high activity against gram-positive and gram-negative bacteria (Dzoyem *et al*., 2017; Perveen *et al*., 2017)).

Ethyl acetate fractions obtained from *Nigrospora* isolate were active against *E.coli, P.vulgaris, K.pneumoniae* and *S.aureus* but not *S.typhi.* Their zones of inhibition were statistically ($p< 0.05$) different as compared to chloramphenicol. These results are in line with those obtained by Santos *et al.* (2015) who showed that ethyl acetate extracts were also active against both Gram-positive and Gram-negative bacteria. The ethyl acetate fractions obtained from *Diaporthe* sp. were also active against *E.coli, P. vulgaris, S.typhi,* and *S.aureus* but not active against *K.pneumoniae.* In line with these results Desale and Bodhankar (2013) showed that ethyl acetate fractions have significant antibacterial activity against *E.coli, S.aureus* and also *K.pneumoniae*.

Methanol and ethyl acetate extracts from *L. martinicensis* exhibited low activity against all the test bacteria. The low activities of these extracts might be attributed to low concentration of bioactive compounds in the metabolic extracts (Table 7). In contrast to this study, Das *et al.* (2012), showed that secondary metabolites extracted from the genus *Leucas* possess high antibacterial activity. A research done by Madhukiran *et al.* (2002) also demonstrated that methanol extract from leaves of *Leucas cephalotes* showed high activity against *S.aureus, E.coli, P.vulgaris* and *K.*

pneumoniae while the ethyl acetate extract also showed high activity against *E.coli* and *S.aureus.* Fractions obtained from *L. martinicensis* showed antibacterial activity against some test bacteria. For instance, F1 showed antibacterial activity against all the test pathogens while F2-F4 did not show any activity against *E.coli*. The differences in activity may be attributed to different composition and concentration of secondary metabolites. In contrast to this study, extracts from *Leucas aspera* have been shown to have antibacterial activity against some gram-positive and gram-negative bacteria (Chew *et al.*, 2012).

When subjected to Minimum Inhibitory Concentration assay, fractions obtained from *Nigrospora* isolate at concentration of 5.0 and 2.5 mg/ml generally showed high activity against the test bacterial strains (Table 4). The most notable activity was obtained with fraction F3 at 5.0 mg/ml against *P. vulgaris*, which was comparable to chloramphenicol antibiotic (30 µg/disc). The response obtained with *Nigrospora* fraction F3 suggested that the semi pure compound had capacity to substitute chloramphenicol in controlling *P. vulgaris*. Presence of compound **5** in fraction F3 is a clear indication that this could be promising in the management of bacterial infections. This result obtained from *Nigrospora* isolate fraction are in line with reports in literature and according to Chen *et al.* (2016), members of *Nigrospora* genus are sources of natural products for pharmaceutical uses. Increasing the concentration of ethyl acetate fractions obtained from *L. martinicensis* from 0.625 mg/m to 5.0 mg/ml increases activity against all the test bacteria. The most notable activity was obtained from fraction F3 at 5.0 mg/ml against *S.typhi* and *K.pneumoniae*. In contrast to this study, ethyl acetate extracts from the medicinal plant *Leucas indica* can inhibit the growth of *S.aureus* at a concentration of 6.25, 3.12, 1.56 mg/ml while 3.12 mg/ml and 1.56 mg/ml can inhibit *S.typhi* (Samanta *et al*., 2013). Despite fractions of the endophytes and the plant secondary metabolites showing activity against all the test bacterial strains, there was no activity observed for pure compound prepared from these fractions obtained. Lack of antibacterial activity for the pure compound suggests that the antibacterial activity observed in the fractions could be due to the interaction of two or more compounds. Purification of the fractions could lead to loss in activity. Furthermore, structural similarity of the pure compound from *Nigrospora* and *Diaporthe* with compounds designated as djalonensone and alternariol suggests that the pure compound could have antifungal activity.

5.3 Characterization of Secondary Metabolites

Fractionation of *Nigrospora osmanthi* led to the isolation of compound **5.** This compound was isolated for the first time from this endophyte however, it has the same skeleton with Alternariol and Alternariol 9-O-methyl ether compounds from the endophytic fungi *Alternaria* sp. isolated from *Catharanthus roseus* (Madagascar periwinkle) (Dasari *et al.*, 2012), the only difference occurring at position C-4 (-OH). Compound **5** has also the same skeleton with a new polyketide isolated from the endophytic fungus *Penicillium chermesinum* (Darsih *et al.*, 2017) the only difference occurring at position C-2 (CH) and C-3 (CH). In contrast to this study, compound **5** was isolated as a mycotoxin with mild antimicrobial activity (Dasari *et al.*, 2012). Compound **6** was isolated from *Diaporthe* sp. This compound has the same skeleton as compound **5** above the only difference occurring at position C-9. Fractionation of ethyl acetate extract of *L. martinicensis* led to the isolation of compound **7**. This compound was isolated for the first time in *L. martinicensis* but it has the same skeleton as Leucasone which was also isolated as a novel compound from *Leucas inflata.* (Al Yousuf *et al.*, 1999).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. From this study it is evident that Ascomycetes fungi inhabit the medicinal plant; *L. martinicensis*. ITS primers used were able to identify two endophytes to species level while only one was identified to the genus level.

ii. Isolated fungal endophytes, extracts from the plant and endophytes possess antibacterial activity due to the accumulation of various secondary metabolites.

iii. The structures of compounds **5**, **6** and **7** were successfully determined using a combination of spectroscopic techniques that include 1 and 2D high field NMR spectroscopy and Electrospray ionization-Mass Spectrometry (ESI-MS).

6.2 Recommendations

i. Further studies on characterization of fungal endophytes should be done using different selective media and different methods.

ii. This study has shown that the extracts from the fungal endophytes and *L. martinicensis* are promising in the control of human bacterial pathogens and should be considered for further evaluation.

iii. Isolation and characterization of pure compounds from the stem, roots and flowers of *L. martinicensis* and associated bacterial and fungal endophytes should be explored.

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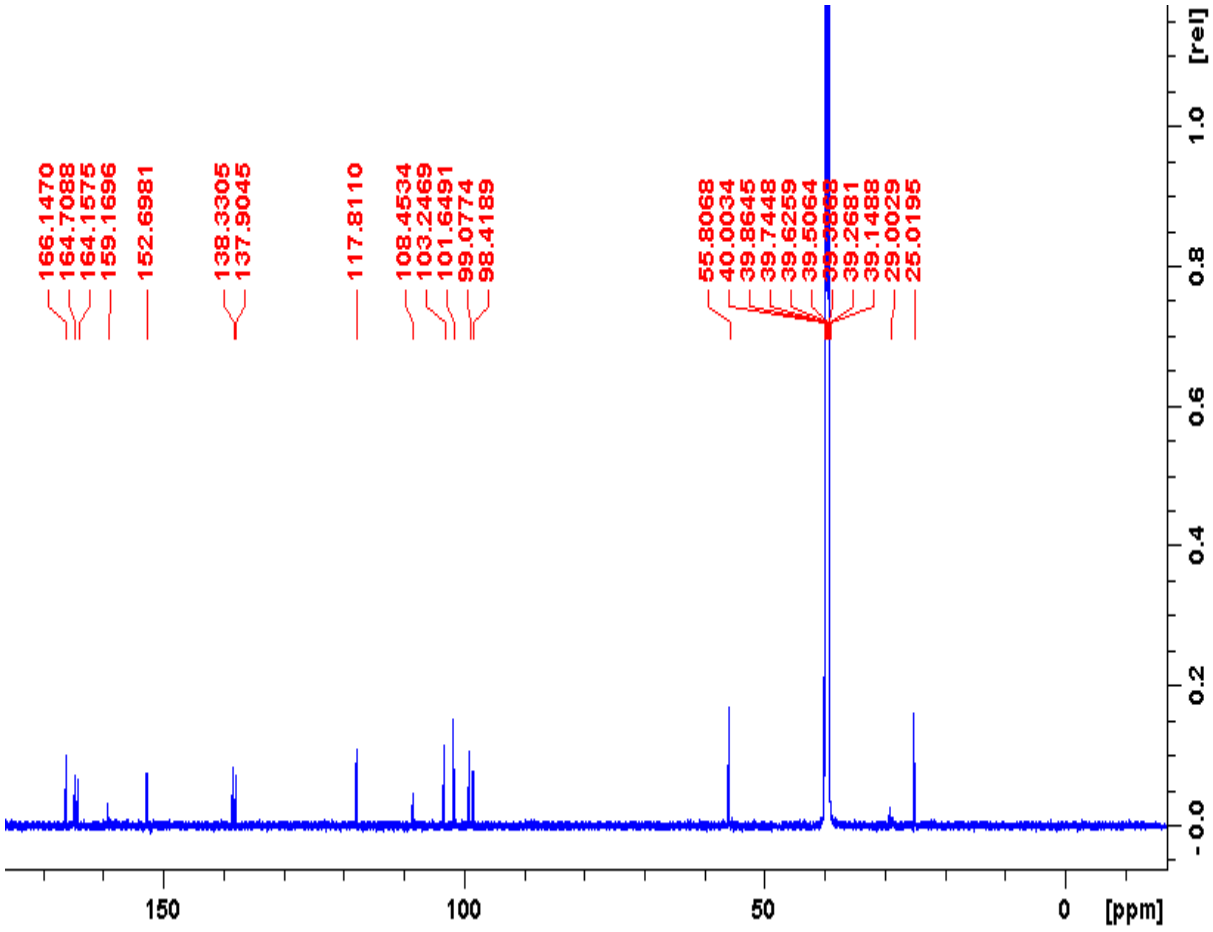
APPENDICES

Appendix A: ITS consensus sequences of endophytic fungal isolates

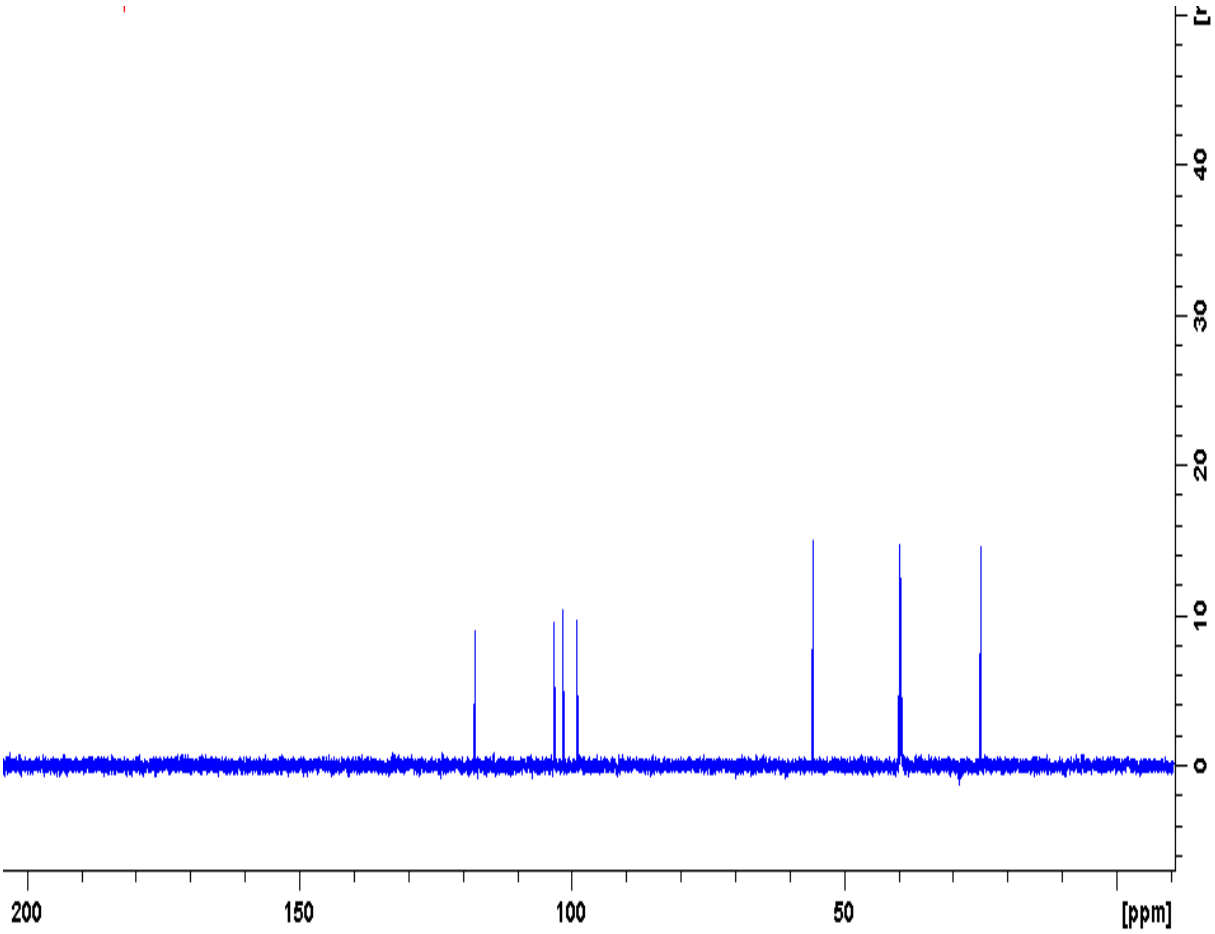
Endophyte Code	Sequences
AD-L(1)	TCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCTTCGGCGCAC CCAGAAACCCTTTGTGAACTTATACCTACTGTTGCCTCGGCGCAGGCCGGCT TCCTCACCGAAGCCCCCTGGAAACAGGGAGCAGCCCGCCGGCGGCCAACTA AACTCTGTTTCTATAGTGAATCTCTGAGTAAAAAACATAAATGAATCAAAA CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA CGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCATT TCAACCCTCAAGCCTGGCTTGGTGATGGGGCAGTGCCTTGGAGACAAGGCA CGCCCTGAAATCCAGTGGCGAGCTCGCCAGGACCCCGAGCGTAGTAGTTAT ATCTCGCTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTCT GAAAATTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATC
LM-L(1)	TCCGTTGGTGAACCAGCGGAGGGATCATTACAGAGTTATCCAACTCCCAAA CCCATGTGAACATATCTCTTTGTTGCCTCGGCGCAAGCTACCCGGGACCTCG CGCCCCGGGCGGCCCGCCGGCGGACAAACCAAACTCTGTTATCTTCGTTGA TTATCTGAGTGTCTTATTTAATAAGTCAAAACTTTCAACAACGGATCTCTTG GTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTAT TCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCCTAAGCACAGCTTAT TGTTGGGCGTCTACGTCTGTAGTGCCTCAAAGACATTGGCGGAGCGGCAGC AGTCCTCTGAGCGTAGTAATTCTTTATCTCGCTTCTGTTAGGCGCTGCCCCCC CGGCCGTAAAACCCCCAATTTTTTCTGGTTGACCTCGGATCAGGTAGGAATA CCCGCTGAACTTAAGC

LM-S(6)	TCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTTGTGGACTTCGGT CTGCTACCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTCCG CCCGCCGGTTGGACAACATTCAAACCCTTTGCAGTTGCAATCAGCGTCTGAA AAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA TGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGA ATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATG CCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTTG TCTCGCCTCCGCGCGCAGACTCGCCTTAAAACAATTGGCAGCCGGCGTATTG ATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAA AAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAAC TTAAGCA

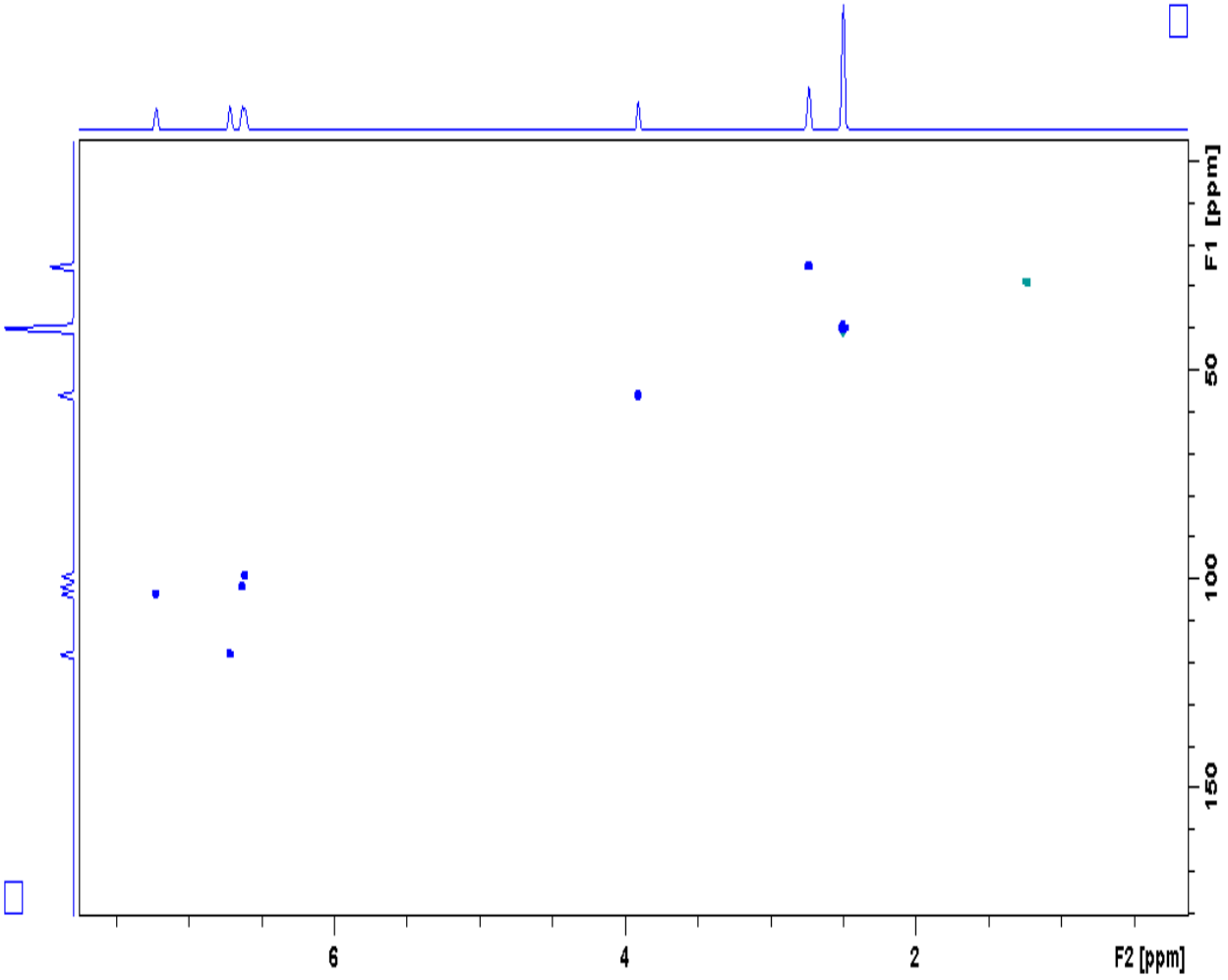
Appendix B: ^{13} CNMR of compound 5



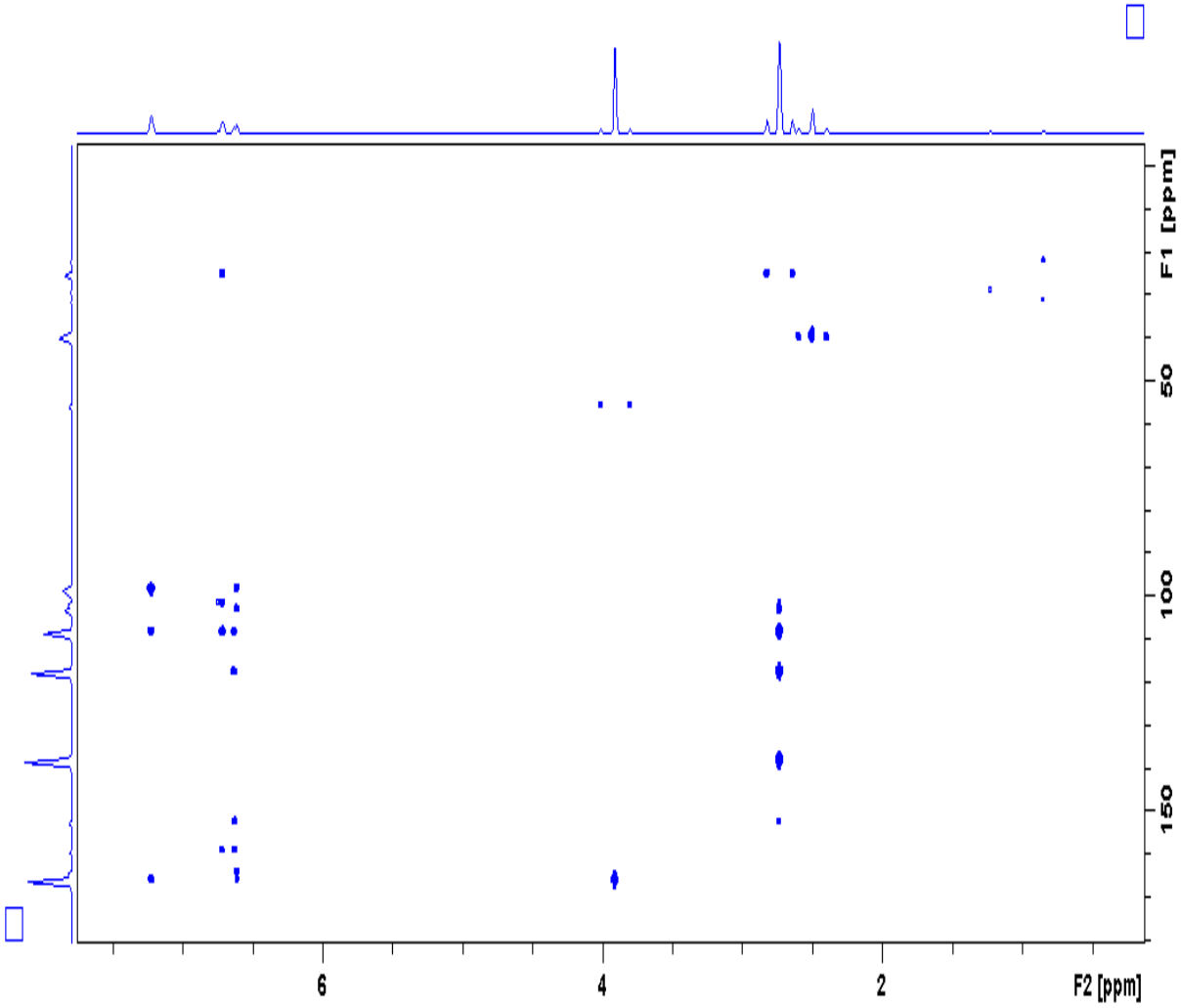
Appendix C: DEPT spectrum of compound 5



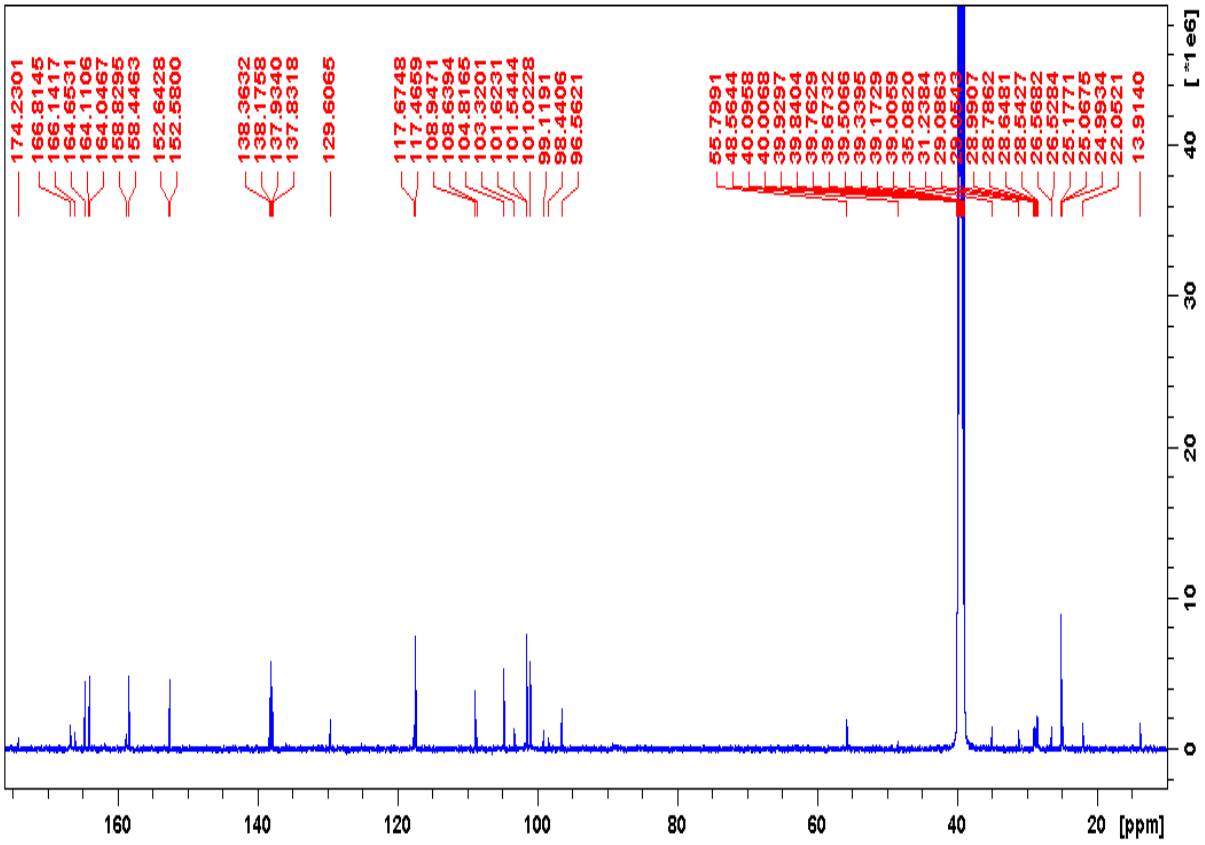
Appendix D: HSQC spectrum of compound 5



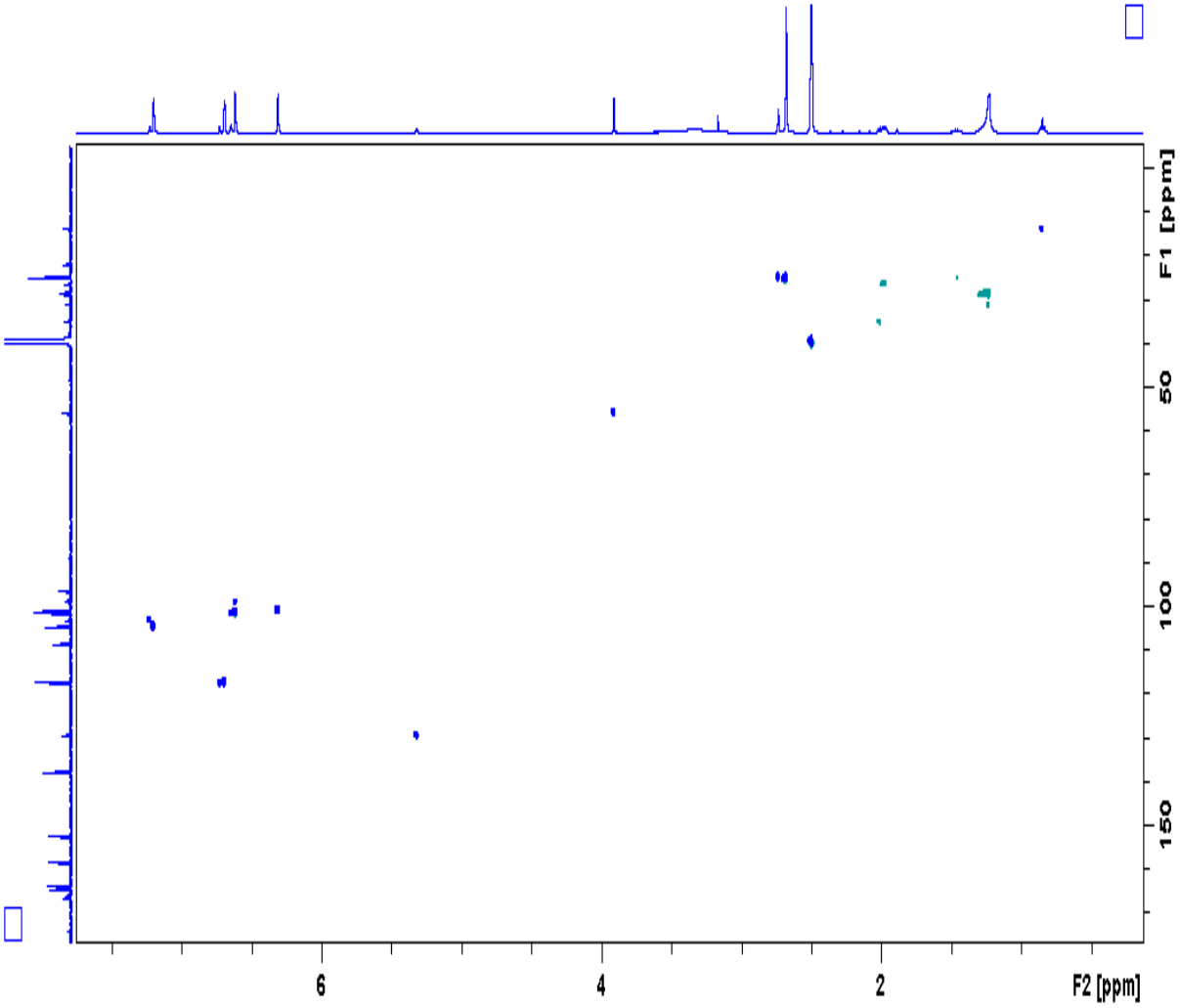
Appendix E: HMBC spectrum of compound 5



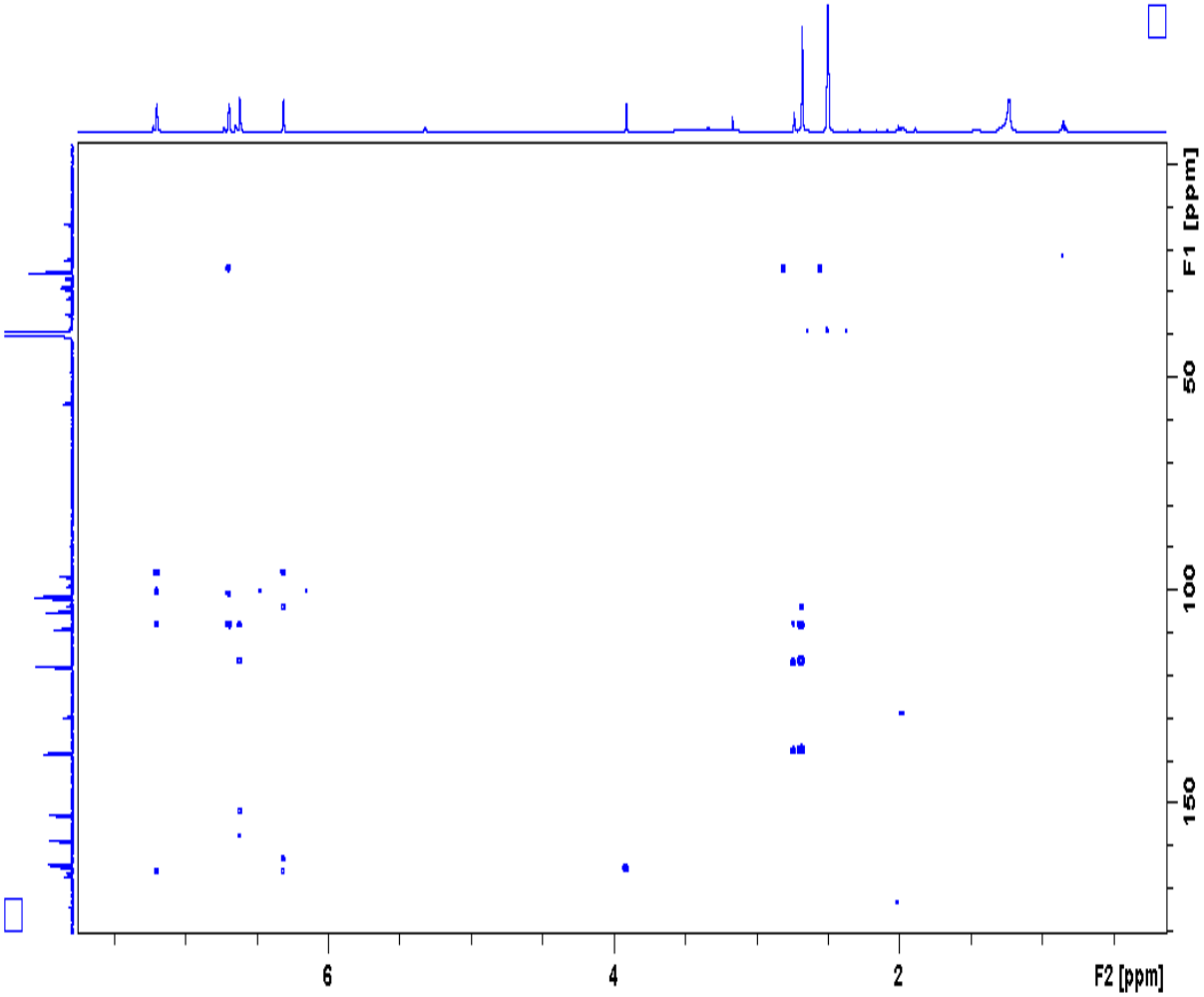
Appendix F: ^{13}CNMR for compound 6



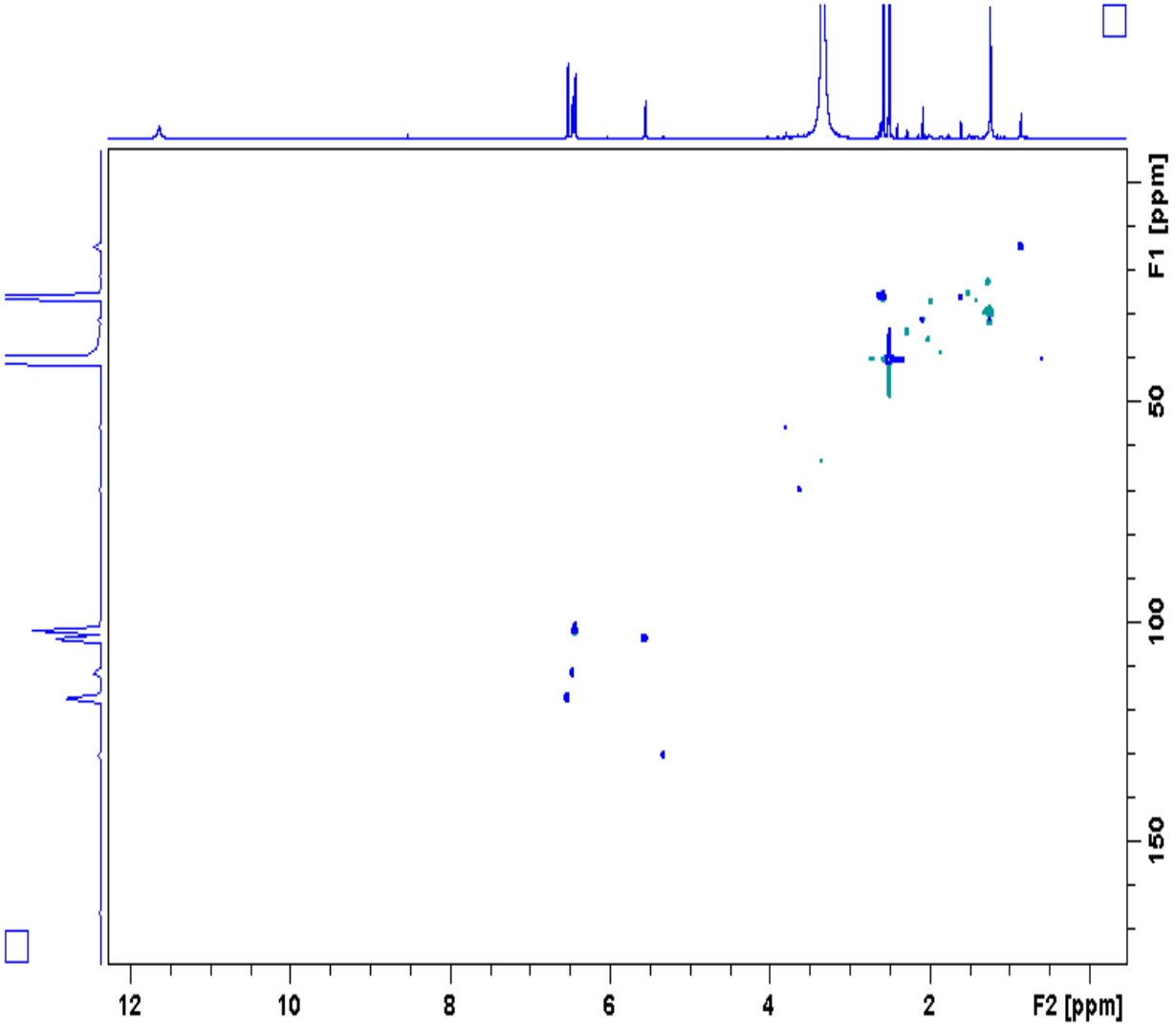
Appendix G: HSQC of compound 6



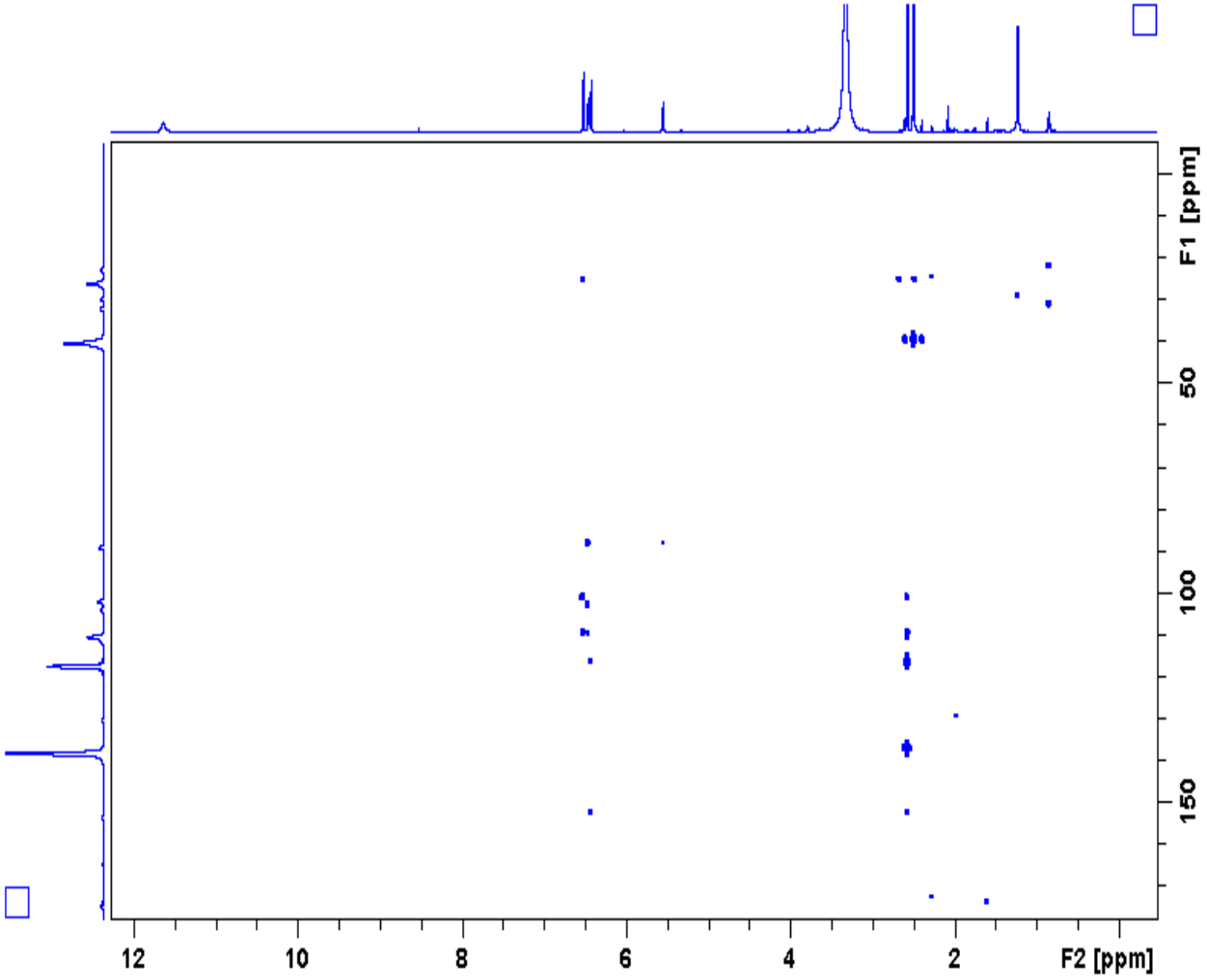
Appendix H: HMBC spectrum of compound 6



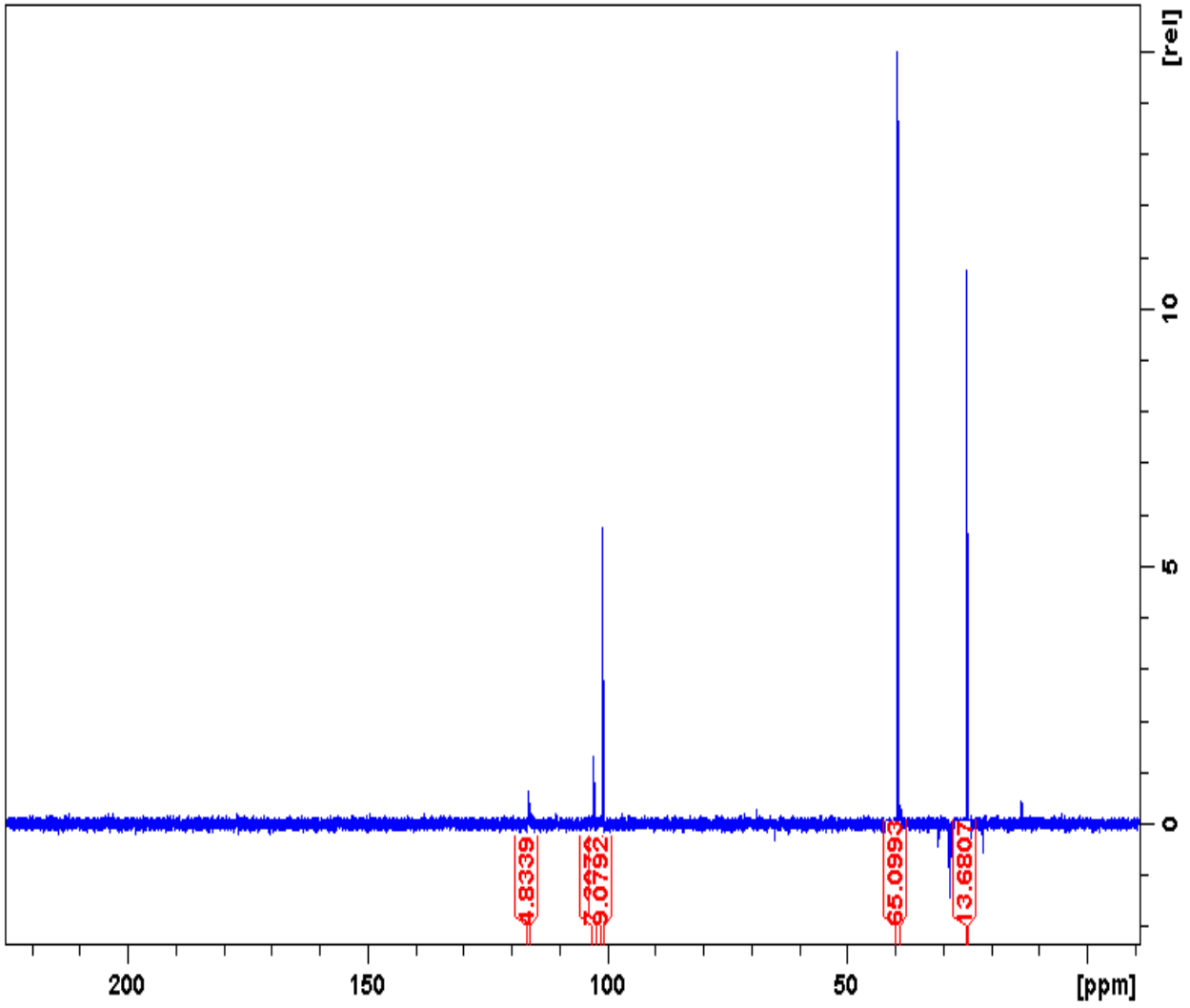
Appendix I: HSQC of compound 7



Appendix J: HMBC of compound 7



Appendix K: DEPT of compound 7



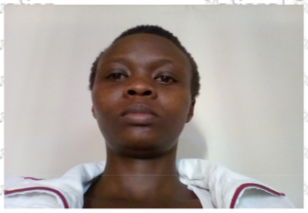
Appendix L: Research permit from NACOSTI

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **945645**

Date of Issue: **08/April/2021**



This is to Certify that Miss Ruth Moraa Obare of Egerton University, has been licensed to conduct research in Transnzoia on the topic: CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF SECONDARY METABOLITES EXTRACTED FROM ENDOPHYTIC FUNGI ISOLATED FROM *Leucas martinicensis* (Lamiaceae) for the period ending: 08/April/2022.

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945645

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Appendix M: Publication

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Full Length Research Paper

Antibacterial activity of endophytic fungi isolated from leaves of medicinal Plant *Leucas martinicensis* L. growing in a Kenyan tropical forest

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Infectious diseases are major threat to public health; a problem that has been exacerbated by emergence of multi-drug resistant (MDR) strains. Finding alternative antimicrobial compounds from natural sources such as fungal endophytes and medicinal plants is crucial for addressing antimicrobial resistance. Thus, in this study search for endophytes with antibacterial activities from leaves of medicinal plant *Leucas martinicensis* was undertaken. Three fungal endophytes were isolated from fresh leaves and characterized using ribosomal Internal Transcribed Spacer (ITS) DNA. Antibacterial activities against five bacterial pathogens were determined using dual cultures and, disc diffusion assay for ethyl acetate extracts and pure compounds. Fungal endophytes isolated were LM-L(1), AD-L(1) and LM-S(6) belonging to genera *Nigrospora*, *Diaporthe* and *Epicoccum*, respectively. Axenic cultures and ethyl acetate extracts displayed antagonistic activity against *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Salmonella typhi* unlike pure compounds. Irrespective of endophyte isolate, increasing the concentration of ethyl acetate fractions from 0.625 to 5.0 mg/ml during minimum inhibitory concentration (MIC) assay increased antibacterial activity; although 2 to 3 folds lower than chloramphenicol at 30 µg/disc. However, ethyl acetate fraction F3 at 5.0 mg/ml obtained from isolate LM-L(1) isolate belonging to genus *Nigrospora* produced activity that was not significantly ($p \geq 0.05$) different from chloramphenicol discs. Failure of pure compounds unlike ethyl acetate and axenic endophyte cultures suggests antibacterial activity observed was due to synergistic interactions of compounds. Nonetheless, the results demonstrate that fungal endophytes isolated from *L. martinicensis* possess antibacterial compounds which can be exploited further as lead compounds towards addressing antimicrobial drug resistance.

Key words: Fungal endophytes, antimicrobial activity, drug resistance, *Leucas martinicensis*.