DETERMINATION OF ANTIBACTERIAL ACTIVITY OF EXTRACTS FROM *Markhamia lutea*, *Zanthoxylum gilletii* AND THEIR ENDOPHYTES AGAINST COMMON BEAN BACTERIAL PATHOGENS

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EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

DECLARATION

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

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DEDICATION

To my family and friends for their love, care, encouragement and prayers in the development of this thesis.

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ABSTRACT

Common bean (*Phaseolus vulgaris* L.) is largely consumed among various communities in Kenya. However, its productivity is gradually declining due to infections such as common bacterial blight and halo blight. *Zanthoxylum gilletii* and *Markhamia lutea* have been used traditionally in the management of various human bacterial pathogens. In addition, plants are inhabited by fungal endophytes that produce bioactive secondary metabolites. This study therefore, was to determine the bioactivity of secondary metabolites from *Z. gilletii, M. lutea* and their fungal endophytes against *Xanthomonas axonopodis* pv. *phaseoli* and *Pseudomonas syringae* pv. *phaseolicola,* the causal agents of bean common bacterial blight and halo blight. The fungal endophytes were isolated from sterilized leaves, plated on Sabourand Dextrose Agar (SDA) media amended with streptomycin sulphate, incubated and subcultured. A total of 51 fungal endophytes were isolated and 50% identified. Fifteen (15) endophytes were active against the test organisms with *Fusarium solani* producing the best activity in the dual culture assay. The most active endophytes were fermented on rice media and extracted using methanol by ultrasonification. Methanol extract was partitioned between ethyl acetate and hexane to produce ethyl acetate and hexane extracts respectively. Purification of the ethylacetate extract produced 2-hydroxyphenylacetic acid (**14**), 4-hydroxyphenylacetic acid (**13**) and (*E*)-3,8-dimethyl-7-(4-methylhex-2-en-2-yl)-7,8-dihydro-5*H*pyrano[4,3-*b*]pyridine-2,4,8-triol(Lucinine)(**15**). Secondary metabolites from the dried leaves were extracted using methanol. Methanol extract was fractionated using methanol, ethyl acetate and hexane to obtain Skimmianine (**5**) from the methanol extract of *Z. gilletii*. The pure compounds were analyzed by a combination of mass spectrometry and spectroscopic techniques which included 1D and 2D NMR. All the extracts except methanol crude extract from *Z. gilletii* were active against *X. axonopodis* pv. *phaseoli* while only the *Fusarium* extracts were active against *P. syringae* pv. *phaseolicola*, in the disc agar diffusion assay. Qualitative and quantitative violacein assay were used to assess quorum quenching properties of these extracts. *Fusarium solani* extracts and *Z. gilletii* ethyl acetate extract showed a potential activity in the qualitative (overlays) and quantitative violacein assay. These results demonstrate the diversity of endophytic fungal genera inhabiting the two medicinal plants and the potential of these plants and their endophytic fungal extracts as sources of antibacterial and quorum quenching secondary metabolites. This study also provides leads for the control of the bacterial pathogens affecting common bean in Kenya.

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LIST OF ABBREVIATIONS AND ACRONYMS

ALS: Angular Leaf Spot

BCMV: Bean Common Mosaic Virus

BLAST: Basic Local Alignment Search Tool

CBB: Common Bacterial Blight

CFU: Colony Forming Unit

CIAT: International Center for Tropical Agriculture

COSY: Correlation Spectroscopy

DEPT: Distortion-less Enhancement by Polarization Transfer

DMSO: Dimethyl sulfoxide

DNA:Deoxyribonucleic Acid

dNTP: Deoxynucleotide Triphosphate

HMBC: Heteronuclear Multiple Bond Correlation

HPLC: High Performance Liquid Chromatography

HSQC: Heteronuclear Single Quantum Correlation

IGS: Intergenic Spacer Sequence

ITS: Internally Transcribed Region

LSU: Large Sub Unit

MEA: Malt Extract Agar

MEGA: Molecular Evolutionary Genetics Analysis

MH: Mueller Hinton

MIC: Minimum Inhibitory Concentration

MOA: Ministry of Agriculture

MOARD: Ministry of Agriculture and Rural Development

NCBI: National Center for Biotechnology Information

NJ: Neighbor Joining

NMR: Nuclear Magnetic Resonance

PCA: Potato Carrot Agar

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

Pv: Pathovar

QS: Quorum sensing

QQ: Quorum Quenching

RNA:Ribonucleic Acid

SAS: Statistical Analysis Software

SDA: Sabourand Dextrose Agar

TLC: Thin Layer Chromatography

WHO: World Health Organization

Xap: *Xanthomonas axonopodis* pv. *phaseoli*

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The common bean (*Phaseolus vulgaris* L.), also known as dry bean, is a leguminous food plant that belongs to the genus *Phaseolus*, family Leguminosae and sub- family Papilionoidae. This plant is a native of Central and South America which later spread to the tropical, sub- tropical and the temperate areas of America, Africa, Europe and Asia (Wortmann, 2006; Wortmann *et al.*, 2006). In Kenya, common bean is grown majorly along the slopes of Mt. Elgon, Western Kenya, the slopes of Mt. Kenya and the Aberdares in Central Kenya and in Eastern Kenya (Gray, 1990; Wortmann and Allen, 1994). Common bean is the major source of proteins, minerals (iron and zinc) and vitamins to both rural and urban households in Sub-Saharan Africa. As per Beebe *et al.* (2013), the annual per capita consumption of common beans is high among the low-income people who cannot afford to buy nutritious and expensive food such as meat and fish. Apart from its subsistence importance, the common bean is also an important source of income among various households in the rural areas of the sub Saharan Africa (Wortmann, 1998).

Despite its importance, the productivity of the common bean in the Sub-Saharan region is generally low and still declining further. This is illustrated in a report generated by Akibode and Maredia (2011) which shows that average yields of common beans in Uganda, Kenya, Angola, Malawi and Democratic Republic of Congo are 500, 490, 280, 490 and 540 kg ha^{-1}, respectively. These values are way below the expected output of 1500-3000 kg ha-^{1} in these areas where there are favorable conditions that support the growth of bean (Hillocks *et al.*, 2006). In Kenya for instance, there has been a decline in bean production per hectare from 600kg in the early 90's to 400 kg in 2004. The low productivity in these regions can be attributed to abiotic factors such as soils which lack nutrients such as nitrogen, phosphorous, potassium and nitrogen and also the increased variability of climatic changes (Namugwanya *et al.*, 2014). Other constraints include biotic factors such as fungi, bacteria and viruses which cause diseases that affect beans (Ferreira *et al.*, 2003a) with the bacterial pathogens being one of the reasons for the decline in productivity in Kenya and other parts of East Africa. Some of the fungal diseases that affect beans include angular leaf spot (ALS) (*Phaeoisariopsis griseola*), anthracnose (*Colletotrichum lindemuthianum*) and rust (*Uromyces appendiculatus*) which affect the leaves, stems and pods. The most common viral infection is the bean common mosaic caused by the bean common mosaic virus (BCMV) while the bacterial

infection includes common bacterial blight (*Xanthomonas axonopodis* pv. *phaseoli*), halo blight caused by *Pseudomonas syringae* pv. *phaseolicola* and bacterial brown spot caused by *Pseudomonas syringae* pv. *syringae.* The most common bacterial infection of common beans in Kenya is the common bacterial blight (CBB) (Njuguna *et al.*, 1980).

To control these pathogens and increase the productivity of common bean, the farmers have adopted the use of copper based foliar sprays, synthetic pesticides (Sartori *et al.*, 2004) for the treatment of infected plants and antibiotic such as streptomycin for treatment of infected seeds**.** However, these methods have proved to cause harm to the environment, the farmers and the consumers. There has been the occurrence of pesticide residues in the farm produce, emergence of pesticide resistant strains and pathogens. There has also been the occurrence of antibiotic resistance on the consumers which is an indirect effect of the use of antibiotics for seed treatments. Hence there has been an increasing interest in finding alternative ways of controlling these pathogens using extracts from natural sources such as medicinal plants, tropical and endophytic fungi.

Plants produce essential oils containing compounds such as neural, myrcene and geraniol which can inhibit microbial growth (Matasyoh *et al.*, 2007). They also produce secondary metabolites such as alkaloids, coumaric, anthracyonins among others which are important sources of microbicides, pharmaceuticals and pesticides (Bobbarala *et al.*, 2009) and hence the choice of these two medicinal plants; *Z. gilletii* and *M. lutea* for secondary metabolite extraction and fungal endophyte isolation. Newman and Cragg (2012), also emphasizes that the discovery of new bioactive natural products should focus on the microorganisms living in interaction with their host. Endophytes colonize healthy plant tissues intracellularly and/or extracellularly without causing any apparent symptoms of plant disease (Borges *et al.*, 2009). Endophytes are viewed as a source of bioactive natural products used in medicine and agriculture since they occupy many higher plants that grow in different environmental conditions (Strobel and Daisy, 2003). Studies have shown that natural products from fungal endophytes have a broad spectrum of biological activities such as antimicrobial, immunosuppressant, anticancer and may also act as bio-control agents (Gunatilaka, 2006; Borges *et al.*, 2009). Research shows that plant based pesticides and metabolites are considered one of the better alternatives since they have minimal detrimental environmental impact and danger to the farmers and consumers (Mohana *et al.*, 2008). Hence, this study was based on the investigation of the bactericidal properties of the endophytes and the secondary metabolites isolated from medicinal plants; *Z. gilletii* and *M.*

lutea against phytopathogens especially the bacterial pathogens of common bean; *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola.* The isolated endophytes were identified using molecular analysis of their ITS region. the primers used were ITS1F and ITS4. Quorum quenching/ anti-quorum sensing properties of the endophytes, their extracts and plants extracts were also assessed using *Chromobacterium violaceum* (CV12472).

1.2 Statement of the Problem

As the demand for common bean increases in Kenya, there has been a decline in its productivity since 2004. To meet this deficit, the country has to import almost 50% of beans from neighboring countries such as Uganda and Tanzania. The decline in productivity is partly attributed to bacterial infections such as common bacterial blight caused by *X. axonopodis* pv. *phaseoli* and halo blight caused by *P. syringae* pv. *phaseolicola* which affect the seeds and pods of the common beans. These bacterial infections can be prevented by use of copper based foliar bactericide sprays and antibiotics such as streptomycin which are also considered harmful to the environment, the consumer and other organisms in the ecosystem. Therefore, the need to develop an alternative control method using medicinal plants and their fungal endophytes against these bacterial pathogens.

1.3 Objectives

1.3.1 General Objective

To determine antibacterial activity of metabolites from *Z. gilletii, M. lutea* and their fungal endophytes against *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* that affect common bean.

1.3.2 Specific Objectives

1. To characterize fungal endophytes isolated from *Z. gilletii* and *M. lutea.*
2. To determine antibacterial activity and anti-quorum sensing activity of metabolites from *Z. gilletii*, *M. lutea* and their endophytes against *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola.*
3. To characterize secondary metabolites isolated from the medicinal plants and their endophytes.

1.4 Hypotheses

1. The endophytes from Z. *gilletii* and *M. lutea* have the same morphological and molecular characteristics.

2. The secondary metabolites from *Z. gilletii, M. lutea* and their endophytes have no biological activity against *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola.*
3. Metabolites from *Z. gilletii*, *M. lutea* and their endophytes have similar chemical characteristics.

1.5 Justification of the Study

Common bean is highly consumed for its protein, vitamin and iron among the low-income earners in the Sub-Saharan Africa who cannot afford the alternative sources of protein, iron and vitamins such as fish and meat. Therefore, this legume crop is important in the country's food security. However, its productivity is low and gradually declining. This is partly attributed to bacterial pathogens such as *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* which are the most common bacterial pathogens of common beans in Kenya. Currently, the methods used in the control of common bean bacterial pathogen are the use of copper based bactericide foliar sprays for the infected plants and the use of streptomycin for the infected seeds. These methods are considered expensive and unfriendly to the environment, the consumers and other organisms in the environment. *Zanthoxylum gilletii* and *M. lutea* are medicinal plants that have been used over the years in the management of human pathogens. Research has shown that these two medicinal plants contain secondary metabolites that have antibacterial activities against various human bacterial pathogens. However, limited work has been done to evaluate the antibacterial activity of metabolites and endophytes from *Z. gilletii* and *M. lutea* against these phytopathogens. This leaves a gap on the importance of these medicinal plants in the control of phytopathogens especially those that affect the common beans (*P. vulgaris* L.). Therefore, there was need for a study to be done on less harmful ways to manage these bacterial infections in common bean. This will help not only in increasing the productivity of the common beans in Kenya but also preventing environmental pollution from the chemical pesticides and reduce the occurrence of antibiotic resistance among the consumers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Common Bean (*Phaseolus vulgaris* L.)

Common bean (also known as string bean, field bean, flageolet bean, French bean, garden bean, green bean, haricot bean, pop bean or snap bean), is a major staple food in East Africa and the second most important source of dietary protein for human (Beebe *et al.*, 2012). This plant is always considered a short season crop with most of them growing between seasons. The common bean plants are of two types; erect herbaceous bushes which is the most predominant in Africa (Buruchara, 2007) and twinning climbing vines. The herbaceous bushes grow up to 20-60cm high while the twining climbing vines up to 2-5m long (Smoliak *et al.*, 1990). The root system of this plant is tap root with many adventitious roots. The herbaceous bushy type has stems that are slender with many branches while the twining type has a prostrate stem. The leaves are green or purple in color and are trifoliate (Wortmann *et al.*, 2006). The bean plant usually undergoes self- pollination; though cross pollination is also possible if the stigma meets pollen coated insects. Once pollinated, this plant gives rise to pods which are slender, green, yellow, black or purple in color. These pods are sometimes stripped and they can be cylindrical or flat, straight or curved 1-1.5cm wide and up to 20cm long in length (Wortmann *et al.*, 2006). The pods contain 4-12 seeds which are non-endospermic and kidney shaped. They vary in size and color depending on the variety from small black wild type to large white, brown, red, black or mottled seeds which are 7- 16mm long (Cobley and Steele, 1976).

In Kenya, common bean is grown on the slopes of Mt. Elgon, Western regions, slopes of Mt. Kenya, Aberdares in Central and Eastern parts of the country (Katungi *et al.*, 2009). Common bean crop is very important in its contribution to household`s income, diet and health (Buruchara *et al.*, 2011). Beans provide cheaper alternative source of protein and household food security to the low-income earners in towns and the rural poor population since animal protein sources are either very scarce or too expensive for majority to afford (Gichangi *et al.*, 2012). In Africa, the per capita consumption of beans is estimated at 31.4kg per year (Wortmann *et al.*, 1998) while in Kenya, consumption is estimated at 14kg per year but it can be as high as 66 kg in the Western part of the country (Buruchara, 2007). Common bean is an important source of protein, calcium, energy, folic acid, dietary fiber and carbohydrates (Buruchara, 2007; Katungi *et al.*, 2009). It also contains lysine, a nutrient that is relatively deficient in most staple diets which makes it a good complement to maize, rice,

vegetables, banana, cassava or potatoes to give a balanced diet (Keya and Mukunya, 1979; Beebe *et al.*, 2012). Health organizations promote the regular consumption of beans since it is believed that it reduces the risk of cancer, diabetes and coronary heart diseases due to its low-fat content and it is also cholesterol free. Young pods of certain varieties are used as green vegetables or canned as baked beans. Bean therefore, play an important role in alleviating malnutrition and health related issues (Katungi *et al.*, 2009; Beebe *et al.*, 2012).

In terms of productivity, Kenya is second after Uganda because there are relatively good and favorable environmental conditions that support the bean production in Uganda as compared to Kenya (Katungi *et al.*, 2009). As noted by Katungi *et al.* (2010), the productivity of common bean in Kenya has been declining all over the years. To meet this deficit the country has to import almost 50% of beans from neighboring countries such as Uganda and Tanzania (Karanja *et al.*, 2011). This decline can be attributed to biotic and abiotic constraints (Odendo *et al.*, 2004; Wagara and Mkimani, 2007). Some of the biotic constraints include fungi, bacteria and viruses which cause diseases that affect beans (Ferreira *et al.*, 2003b) with the bacterial pathogens being one of the reasons for the decline in productivity in Kenya and East Africa at large. The bacterial infections of common bean in Kenya include common bacterial blight (CBB) caused by *X. axonopodis* pv. *phaseoli*, halo blight caused by *P. syringae* pv. *phaseolicola* and bacterial brown spot caused by *P. syringae* pv. *syringae* (Nderitu *et al.*, 1997).

2.2 Bacterial Pathogens of Bean

In Kenya, the most common bacterial pathogens of common bean are *X. axonopodis* pv. *phaseoli* which causes common bacterial blight and *P. syringae* pv. *phaseolicola* which causes halo blight. These two bacterial pathogens frequently occur together (Wortmann *et al.*, 1998) and have almost similar symptoms.

2.2.1. Common Bacterial Blight

2.2.1.1 Description of Common Bacterial Blight

Common bacterial blight is caused by a gram-negative bacteria *X. axonopodis* pv. *phaseoli* and its fuscans variant *X. fuscans* subsp. *fuscans* (Xff) (Schaad *et al.*, 2005). This is the major bacterial disease that affect common bean worldwide (Tar'An *et al.*, 2001; Miklas *et al.*, 2003). In Africa, this disease has been reported in the Sub-Saharan African countries such as Kenya, Malawi, Uganda and Burundi (Njuguna *et al.*, 1980; Edje *et al.*, 1981; Opio *et al.*, 1993). According to Opio *et al.* (1996) and Birch *et al.* (1997) this disease is of major

economic importance since it causes about 10-40% yield reduction in the susceptible varieties in the whole world. In Kenya, it has been reported to cause 10% to 75% yield loss (Makini, 1995). The severity of the disease depends on the environment and the susceptibility of the cultivars of the common bean (Saettler, 1989).

2.2.1.2 Symptoms of Common Bacterial Blight

Xanthomonus axonopodis pv. *phaseoli* (*Xap*) infection is seed borne and it can survive in the seed if it is viable. This infection is severe in humid and warm condition and it affects the foliage and pods of the common bean. The infected crops present symptoms such as small water- soaked spots on the leaves which gradually enlarge and coalesce into irregular shapes which exhibit scalded appearance. These spots may become brown and surrounded by narrow, lemon- yellow colored margin due to the production of the bacterial toxins (Akhavan *et al.*, 2013). These spots normally occur along the leaf margin. The dead leaves may remain attached to the plant till maturity. The infected pods may exhibit circular slightly sunken water soaked areas which may produce yellow masses of bacterial ooze in humid condition. These areas usually dry up and become reddish brown lesions. The infected seed always present the symptoms of butter-yellow or brown spots that are distributed throughout the seed coat or they can be restricted to the hilum area. The infected seed may rot or shrivel or wrinkle if the infection occurs during pod development as shown in Plate 1. Such seeds show poor germination if sown (Jacques *et al.*, 2005; Darrasse *et al.*, 2007).

2.2.2 Halo Blight

Halo blight infection in common beans is caused by a single celled gram-negative bacterium known as *P. syringae* pv. *phaseolicola* (Audy *et al.*, 1996). In Africa, this pathogen is found in the great lakes region such as Malawi, Kenya and Zambia (Allen, 1983). The disease is favored by humid and cloudy conditions. According to Makini (1995), halo blight infection causes a crop loss of 23-40% in Kenya.

2.2.2.1 Symptoms of Halo Blight

Halo blight symptoms are mostly seen on the leaves. The first symptoms are water-soaked spots on the lower surface, little bigger than a pin-prick, scattered on the leaf blade.

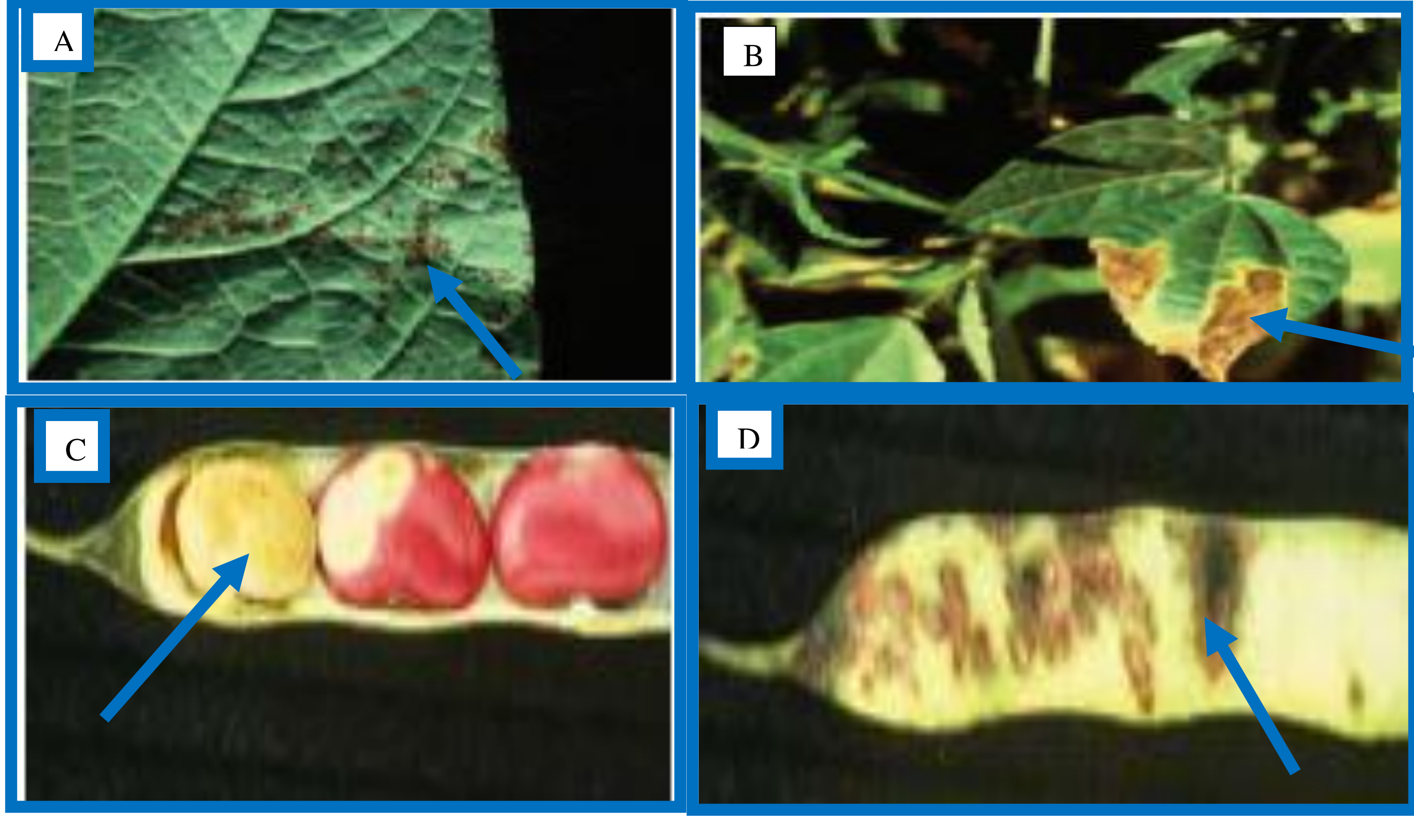


Plate 1: Bean leaves, seeds and pods infected by *X. axonopodis* pv. *phaseoli.*

A: Water soaked spots on the lower surface of the leaf; B: Necrotic lesions and yellowing on bean leaves; C: butter yellow spots on the seed coat of infected seeds; D: Water soaked spots on pods which later dry and develop a reddish brown narrow border.

The bacteria produce a toxic chemical which results in a yellow area (the 'halo') spreading outwards from the spots, which then go red, dry up and coalesce. Water-soaked areas or lesions also develop on pods, stems and leaf stalks, and sometimes produce a whitish ooze which contains bacteria. Seedlings that develop from diseased seed are systemically infected and lesions develop around the stem. The nodes rot and plants are stunted and distorted, with an overall lime-green color. The infected seeds may rot, wrinkle and dis-color (Plate 2) (Murillo *et al.*, 2010).

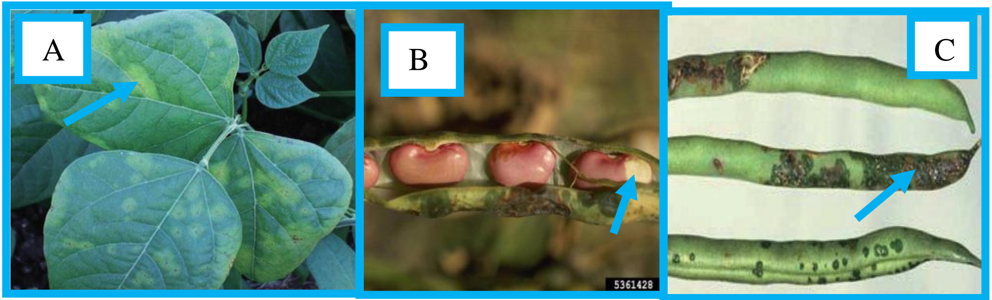


Plate 2: Disease symptoms induced by *P. syringae* pv. *phaseolicola* on leaves, pods and seeds.

A: Water soaked spots on the leaves leading to yellowing. B: yellowing of the infected seed; C: Water soaked spots on the infected pods (Murillo *et al*., 2010*).*

2.2.3 Pathogen Dissemination

Xanthomonus axonopodis pv. *phaseoli* and *P. syringae* pv. *phaseolicola* can be disseminated through splashing or wind-blown rain dispersal of bacteria from lesions and infected plant debris (Fininsa and Yuen, 2002). Overhead irrigation and splashing of rain water from infected plants are considered very effective in secondary spread of these bacteria. Surface irrigation water may also spread the bacteria (Akhavan *et al.*, 2013). They can also be disseminated through seed transmission since they are seed borne (Ito *et al.*, 2003; Belete and Bastas, 2017). Insect vectors such as white fly (Sabet *et al.*, 1969) and beetles also play a part in the transmission of *X. axonopodis* pv. *phaseoli* (Kaiser and Vakili, 1978). Plant to plant contact has also been considered as one of the ways of these pathogen dissemination (Arnold *et al.*, 2011).

2.2.4 Management Practices for Common Bacterial Blight and Halo Blight

As mentioned above, these two bacterial infections have a significant effect on the production of common bean in Kenya hence their management is necessary. There are several methods that have been used in the management of these pathogens as listed below.

2.2.4.1 Cultural Practices

Some of the cultural practices that have been used in the management of these two pathogens include elimination of weeds which could be a potential host of these pathogens, use of pathogen free seeds (Friesen *et al.*, 2014), deep ploughing to expose the debris to microorganisms which results to rapid degradation of the bacteria (Gilbertson *et al.*, 1990) destruction of affected plants, crop rotation (Abawi *et al.*, 2006) and intercropping the bean with other plants such as maize and sorghum (Moreno and Mora, 1984).

2.2.4.2 Chemical Control Methods

Currently, chemical control is the main method used in the management of these plant pathogens in the field. Some of the chemicals that have been applied in the control of these pathogens include copper sulphate, copper hydroxide and methyldithiocarbonate which are applied on the foliage. Research has shown that the use of copper based bactericides reduces the population of the bacteria in the infected plants (Shi *et al.*, 2011). Antibiotics such as streptomycin have also been used for seed treatments (Muthii, 2014). However, the intensive use of pesticides and chemical sprays has caused many negative effects to the environment such as water, soil and food contamination leading to poisoning of farmers and the consumers (Stangarlin *et al.*, 1999). In addition to that, the continued use of antibiotic treatment for the seeds leads to antibiotic resistance in the consumers. The use of copper based foliar sprays can also result to the selection of copper resistant strains of the bacteria (Mirik *et al.*, 2007). In addition, the unavailability as well as inflated cost of the chemicals is not so economical for the subsistence farmers. Hence there is need for a more economical way for the management of these pathogens.

2.2.4.3 Biological Control Methods

Biological control can be defined as the control of pest or pathogens by use of other organism or their extracts in the environment (Winston *et al.*, 2014). There have been some efforts made towards the use of this method to control *P. syringae* pv. *phaseolocola.* For instance, studies have shown that Lupine extracted from *Lupinus albus* and *L. luteus* can be used to control this pathogen (Múzquiz *et al.*, 2011). Another experiment done by Downes (1978), showed that *Erwinia herbicola* applied to seed inhibited growth of *P. syringae* pv. *phaseolicola* from infected seed onto agar. Common bacterial blight can also be biologically controlled by application of *Rhizobium leguminosarum* pv. *phaseoli* to common bean seeds both in field and greenhouse conditions (Osdaghi *et al.*, 2011). Bio-control experiment has also been performed on *X. axonopodis* pv. *phaseoli* using *Rahnella aquatilis* and *Pseudomonas* sp. which showed a reduction of this bacterial growth by 39%. This reduction was attributed to the production of phenolic compound by these biocontrol bacterial strains (Sallam, 2011).

All the above-mentioned methods have not been able to totally eradicate these bacterial pathogens (Sallam, 2011). Therefore, there is need to develop other means of controlling these pathogens such as the use of natural products which are biodegradable and hence reduce environmental pollution. This study mainly concentrated on the use of natural

products as antimicrobial agents that can either kill the bacteria or inhibit the growth of these bacteria. Apart from the bacteriostatic studies, this work also focused on the use of natural products to block the communication among the bacterial pathogens, also known as quorum sensing, that leads to their virulence in the host.

2.2.4.4 Anti-Quorum Sensing

During growth, bacteria produce small diffusible pheromone like chemical signals. The accumulation of these signals is directly proportional to the bacterial population. When it reaches a certain threshold, the bacteria activate gene expression for virulence. The relationship between the accumulation of these chemical signals and gene activation is known as Quorum Sensing (QS). Quorum sensing (QS) is a mode of bacterial communication which involves molecules known as auto-inducers (Pellegrini *et al.*, 2014). The autoinducers activate the receptors found on the surface of the bacteria which allow for the transcription of the battery gene (Raffa *et al.*, 2005). The expression of these genes controls different biochemical mechanisms that are involved in the bacterial survival and pathogenicity such as bioluminescence, biofilm formation, production of antibiotics, virulence factors and bacterial motility (Gram *et al.*, 2002; Schuster *et al.*, 2013). Quorum sensing is mediated through N-Acyl Homoserine Lactones (AHL) in gram negative bacteria. In gram positive bacteria QS is mediated through autoinducing peptides (AIPs) which are short peptides synthesized by the ribosome. With the current rise in the antibiotic resistance by several pathogenic gram negative bacteria, quorum sensing presents an alternative way for combating the drug resistance by the infectious bacteria (Damte *et al.*, 2013). Anti-virulence strategy that is directed towards the disruption of the quorum sensing system in a bacterial population is known as quorum quenching (QQ). Quorum quenching compounds do not cause cell death or growth rest and is assumed not to cause bacterial resistance (Otto, 2004) as it is known for the antibacterial compounds. Research has shown that plants such as cloves, cinnamon, pepper mint, lavender, rosemary, rose and geranium have anti QS activity (Szabó *et al.*, 2010; Stashenko, 2011). Some of the anti-QS compounds have been reported from penicillium (Stangarlin *et al.*, 1999; Rasmussen *et al.*, 2005) and Australian macro algae *Delissea pulchra* which produces halogenated furanones (Manefield *et al.*, 1999).

Quorum quenching is a novel technique that is considered to increase productivity of various crops to meet the global demand for food. De Kievit and Iglewski (2000), noted that various phyto-pathogens which cause a great loss in food production use quorum sensing systems to regulate the various process associated with their virulence. Hence quorum

quenching using auto-inducer molecules from natural products may prove a valuable way of controlling these diseases. This is also illustrated in a research done by Palmer *et al.* (2011) which shows that the virulence of *P. syringae* and *Pectobacterium carotovora* could be controlled using anti-QS molecules in the host plant.

Chromobacterium violaceum (CV) is a gram-negative soil and water bacterium which produces a water soluble purple pigment known as violacein as a phenotypic response regulated by QS mechanism. Hence this bacterium is used as a bioindicator to detect substances that block the QS mechanism (McLean *et al.*, 2004). *Chromobacterium violaceum* strain 12472 were used as bioindicator strains in this study.

2.3 Medicinal Plants

The term medicinal plants refer to the several types of plants used for herbal medicine. Some of these plants have a medicinal activity hence considered as a rich resource of ingredients which can be used in drug development and synthesis (Rasool Hassan, 2012). Study by the World Health Organization (WHO) estimates that 80% (approximately 3.3 million people) of the population living in rural areas use or depend on herbal medicine on regular basis for their health needs (WHO, 2002). For traditional medicine, the organs used as drugs include leaf, stem bark, root bark and whole plant. The sampling methods of these organs is very important to prevent plant extinction. Some of the methods used include; use of a hoe to sample the roots and the whole plant, machetes are used to sample stem and root barks while the leaves are usually hand-picked. Medicinal plants are also used as raw material in the extraction of active ingredients which are used in the synthesis of different drugs such as antibiotics. Research on medicinal plants to produce natural products has increased since these plants can produce many bioactive secondary metabolites that are used as chemical defense against predators. Plants also provide templates to design new drugs (Cragg and Newman, 2005; Kaufman *et al.*, 2006; Colegate and Molyneux, 2008). This study was based on the use of leaf and fungal endophytes extracts isolated and extracted from *Z. gilletii* and *M. lutea* to control two common bean bacterial pathogens. Both plants have been used in traditional herbal medicine in the management of various human pathogens but little work has been done on their importance in the control of phytopathogens.

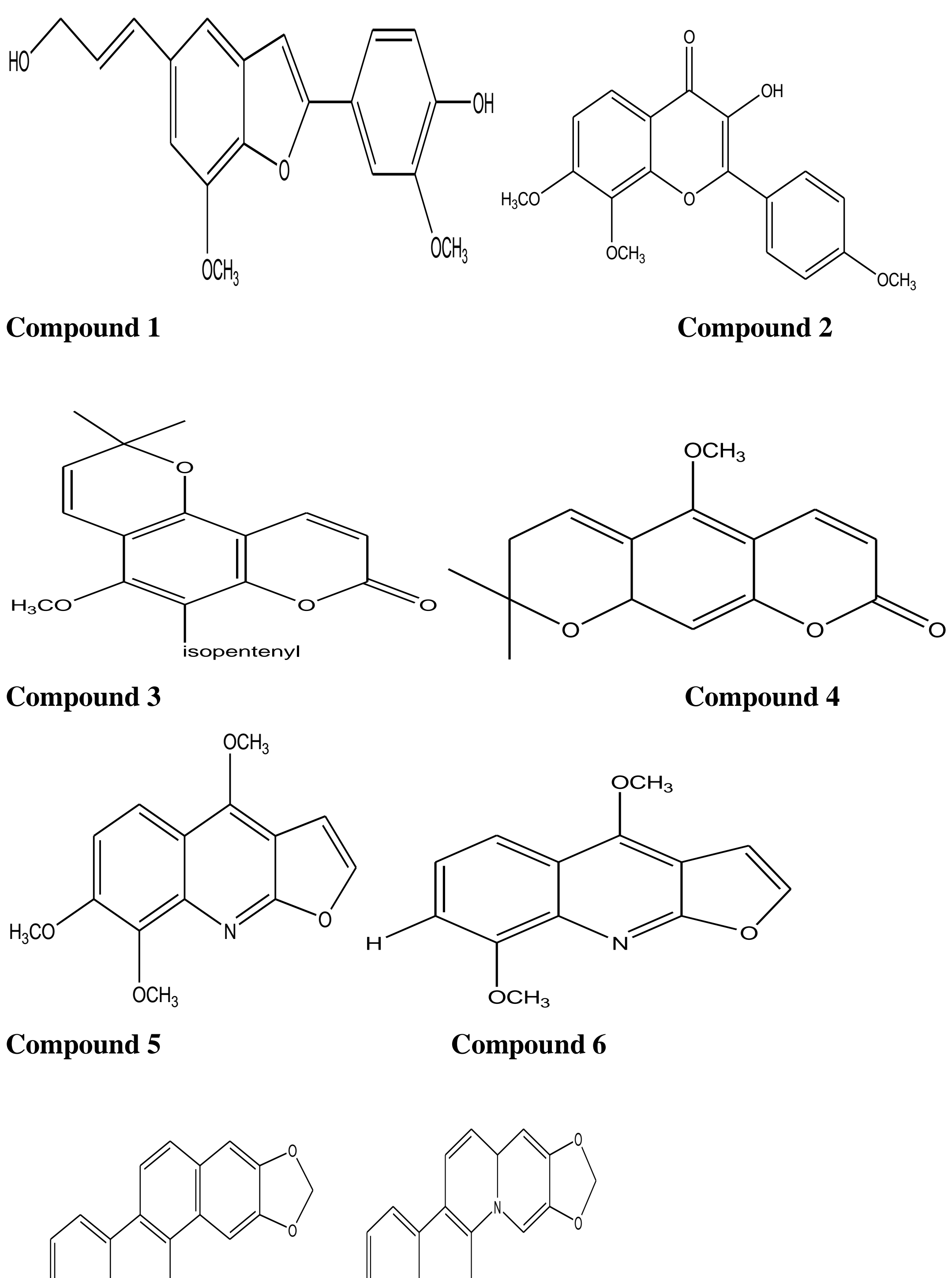
2.3.1 Genus *Zanthoxylum*

The plants in the genus *Zanthoxylum* are evergreen, aromatic deciduous shrubs or trees that belong to the family Rutaceae (Negi *et al*., 2011). *Zanthoxylum* comes from the word *Xanthoxylum* which is derived from Greek words Xanthon xylon that means “yellow

wood". They are found in the warm temperate and subtropical regions of the world. The plants in this genus are rich in various chemicals such as alkaloids, flavonoid, amides, ligans, sterols, coumarins and terpenes that have various medicinal values (Islam and Ahsan, 1997). This genus consists of about 250 species from small shrubs to large trees (Epifano *et al.*, 2011). Some of the African species include *Z. capense, Z. chalybeum, Z. davyi, Z. gilletii* among others. Some of the compounds with medicinal values that have been isolated from this genus include; neolignan ailanthoidol (AT) extracted from *Z. ailanthoides* (**1**) (Lippman and Hawk, 2009), Tambulin (3,5-dihydroxy-7,8,4'-trimethoxyflavone)**(2)** extracted from *Zanthoxylum alatum* (Epifano *et al.*, 2011), dipetalyne and xanthoxyletin **(3)** extracted from Z. *americanum* (Ju *et al.*, 2001), γ-fagarine**(4)** and Skimmianine **(5)** extracted from *Zanthoxylum integrifoliolum* (Chen *et al.*, 2005), Rutaceline **(6)** isolated from Z. *madagascariense* (Pachon *et al.*, 2007) and Berberine **(7)** isolated from *Zanthoxylum monophyllum* (Cordero *et al*., 2004) among others (Figure 1).

2.3.1.1 *Zanthoxylum gilletii*

Zanthoxylum gilletii (De Wild Waterman) locally known as East African satin wood. It is known as shikuma among the Luhya, sogowait among the Kipsigis and Kalenjin, sogomaitha among the Luo and muchagatha/mumondo among the Kikuyu. This plant is a native in Angola, Democratic Republic of Congo, Kenya, Nigeria, Sudan, Tanzania, Uganda, Zambia and Zimbabwe. It grows to a height of 10-35m high with a diameter of 30-90cm. The trunk, which is usually straight and branchless for several meters, is grey covered with thick corky thorns. The leaves are shinny and paripinnate, 3 to 4 pairs while the leaflets are aromatic and oblong with subacute base which is 5- 10cm long, 2-4cm broad. Its flowers are clustered and sessile. The fruits are reddish, sub globe 3.5-6mm in diameter, sessile or with a short stipe and persistent calyx. The seed is 2.5-3.5mm in diameter with a shiny black testa (Orwa *et al.*, 2009). The stem wood is bright yellow, hard sweet scented and termite resistant (Dharani, 2002) (Plate 3).



Compound 7 **Compound 8**

Figure 1: Bioactive secondary metabolites isolated from the genus *Zanthoxylum*

Phytochemical analysis of this medicinal plant has identified numerous compounds with medicinal and antioxidant potentials such as alkaloids, phenolic acids, saponins coumarins among others. Adesina and Reisch (1988) investigated the phytochemical

constituents of *Z. gilletii* in Nigeria which showed the presence of fluoroquinolone alkaloid, skimmianine, the cinnamic acid amide, fagaramide and many other alkaloids.

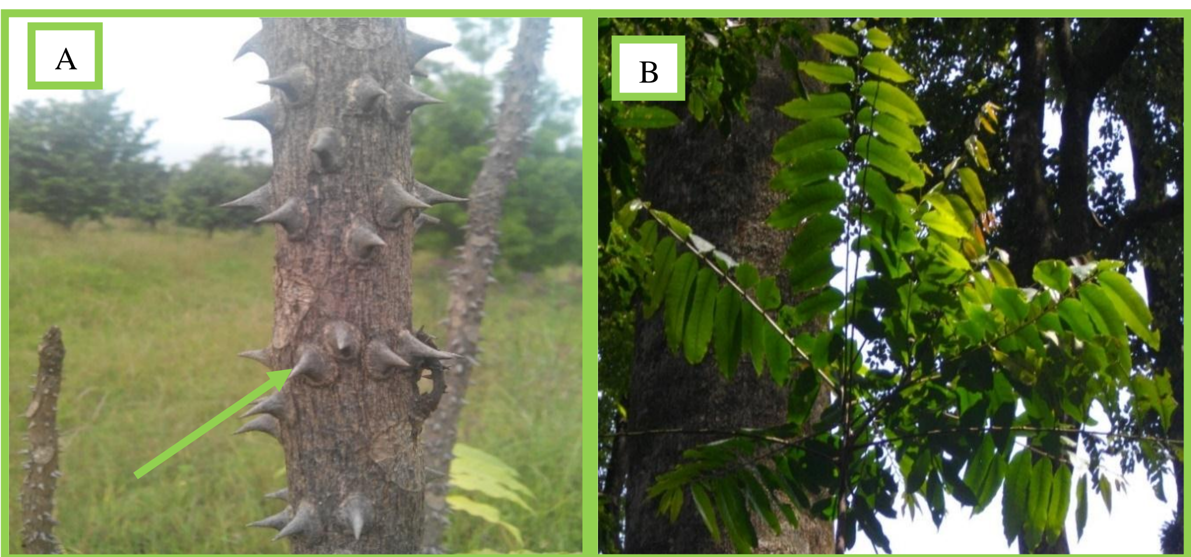


Plate 3: A: The stem showing the thick corky thorns, a key feature on the stem of *Z. gilletii;* B: Shiny paripinnate leaves of *Z. gilletii*.

Traditionally this plant has been used in various ways which include; the leaves and the seeds contain essential oils which are commonly used in food industries and perfumeries, a stem bark decoction is drunk for constipation, complicated gastrointestinal conditions, colds and fever, the bark is also chewed and the juice swallowed for the treatment of stomachache (Kokwaro, 1993). The root bark of *Z. gilletii* is used in traditional Tanzanian antimalarial preparations (Orwa *et al.*, 2009). The root bark decoction is also used as a tonic for both man and animals by the Zulu community in South Africa (Tarus *et al.*, 2006). The stem bark decoction is commonly used for back pain and externally for all urogenital infections. The root bark or fruit pulp is a liniment for rheumatism and all kinds of pain. Young leaves decoction eases cough and is said to be effective on gonorrhea and bilharzia (Gaya *et al.*, 2013). The Luhya community of Kenya uses the bark for antimalarial treatment (Nyunja *et al.*, 2009). Modern research has confirmed the presence of medically active compounds such as alkaloids and saponins which can lower blood pressure, combat cancer, relive pain and manage parasitic infections. The roots are usually used as chewing sticks to clean the teeth. It is believed that extracts from *Z. gilletii* has antifungal and anti-bacterial activity. This is illustrated by an *in vitro* experiment that showed that the extracts from this plant inhibited the growth of *Candida albicans, Cryptococcus neoformans* and other filamentous fungi (Addae Mensah, 1992). The essential oil extracts from this plant has shown larvicidal activity against mosquitoes (Japeth *et al.*, 2014). Due to the presence of various types of alkaloids whose

principle action is on the nervous system, this plant could act as a natural source of insecticide and fungicide (Pěnčíková *et al.*, 2011). Hence this makes an important plant for research on bean bacterial pathogens.

2.3.2 Genus *Markhamia*

Plants in the genus *Markhamia* are evergreen subtropical, flowering and they belong to the family Bignoniaceae. The genus was named by Berthold Seemann in honor of Sir Clements Robert Markham (1830–1916), who introduced quinine-yielding *Cinchona* into India. This genus contains five known species which include *M. lutea*, *M. obtusifolia*, *M. stipulata*, *M. tomentosa*, and *M. zanzibarica* (Ibrahim *et al.*, 2016). The plants in this genus are known to contain secondary metabolites such as saponins, tannins, flavonoids, quinones, alkaloids, anthracene derivatives, reducing sugars, glycosides, kaempferol, â-sitosterol, terpenes, steroids, coumarins and phenylpropanoid glycosides (PhGs) (Choudhury *et al.*, 2011).These compounds are known to contain antimicrobial properties such as antifungal, antiprotozoal, analgesic, anti-inflammatory and cytotoxic activities (Ibrahim *et al.*, 2016).

2.3.2.1 *Markhamia lutea*

Markhamia lutea (Nile tulip) commonly known as "Siala" in the Luo community of Kenya (Oloo *et al.*, 2013), is native in Kenya, Ethiopia, Tanzania and Uganda. It grows to a height of about 10-15m high. It has a light brown fissured bark and irregular crown. The leaves are compound, pinnate, often in bunches, thin and wavy while the leaflets are up-to 10cm long. The flowers are bright yellow and are trumpet shaped (Plate 4). Its fruit is long, thin with brown capsules up to 75cm in length. They hang in clusters and tending to spiral. These fruits often split on the tree to release many seed with transparent wings, 2.5cm long and yellow-whitish when mature (Orwa *et al.*, 2009). Some of the compounds which have antimicrobial activities that have been isolated from this plant include Luteoside A, Luteoside B, Luteoside C (Kernan *et al.*, 1998), Vabascoside (Gormann *et al.*, 2006) among others.

This plant is often used by carpenters since its wood is hard and known to be resistant to termites. This is illustrated by the work done by Syofuna *et al.* (2012) which demonstrated that the wood extracts from *M. lutea* played a major role in the protection of wood against termite and fungal attack. Hence in Uganda, its extract is used as a wood preservative. It is also used as ornamentals because of its large conspicuous yellow flowers (Schmidt and Mbora, 2008). The roots, bark and leaves are used in traditional medicine; leaves and bark are used to treat toothache, stomach-ache and headache, roots are administered to children to treat convulsions, and root and bark decoctions are taken against asthma, cough and

gonorrhea. Root decoctions are applied to treat earache and bark decoctions as aphrodisiac. Ground leaves and bark are applied externally to treat skin complaints and wounds. Leaves are used for the treatment of snakebites and young shoots to treat throat complaints, lumbago and diarrhea. Leaf extracts are taken to treat cough and malaria. In Uganda, the roots are a constituent of a complex herbal preparation used in the alleviation of AIDS symptoms (Lamorde *et al.*, 2010). This plant can also be used for control of the parasitic weed *Striga* in cereals by inducing germination in the absence of a host (Maroyi, 2012). Research has shown that petroleum-ether extract of the roots showed antibacterial activity while crude ethyl acetate leaf extracts exhibited *in-vitro* anti-parasitic activity against *Plasmodium falciparum*, *Trypanosoma brucei* and *Leishmania donovani*.

Plate 4: The bark leaves and flowers of *M. lutea*. A: Light brown fissured bark; B: Yellow trumpet shaped flowers together with the leaves

2.4 Endophytes

Clay (1990), defines endophytes as bacterial or fungal microorganisms that colonize healthy plant tissues intracellularly and/or extracellularly without causing any apparent symptoms of disease. The interaction between the plant and the endophyte is not clearly understood but some researchers believe that it can be symbiotic/ mutual (Sturz *et al.*, 1997; Kogel *et al.*, 2006). The endophytes can produce bioactive secondary metabolites and enzymes which assist the plant in chemical defense and adaptation of the plant to abiotic and biotic stress while the plant acts as the host to provide food and shelter (Schulz and Boyle, 2005; Arnold, 2007; Purahong and Hyde, 2011). As noted by Firáková *et al.* (2007), there is a dilemma whether the bioactive secondary metabolites of medicinal plants are produced by the plant itself or because of the mutual relationship with the endophytes. Some endophytes are not host specific since a single endophyte can survive in a wide range of hosts (Sandhu *et*

al., 2014) therefore they have the ability to colonize all plants while others are host specific. They have been isolated in almost all plants that have been studied to date. Some of these plants include large trees, palms, sea grasses, lichens, algae, mosses, ferns, conifers, and angiosperms (Fróhlich *et al.*, 2000; Alva *et al.*, 2002; Gonthier *et al.*, 2006; Li *et al.*, 2007; Oses *et al.*, 2008; Rodriguez *et al.*, 2009), among many more. They reside entirely within the plant tissues such as leaves, roots and stems.

2.4.1 Role of Endophytes

Researchers currently view endophytes as a rich source of bioactive compounds (Weber *et al.*, 2007), since they produce a wide variety of secondary metabolites. The secondary metabolites produced by the endophytes depend on the ecological niche and the metabolic interaction between the plant and the fungi (Rakshith *et al.*, 2013). Hence endophytes have various applications as stated below;

2.4.1.1 Production of Pharmaceuticals

The metabolites produced by endophytes have desirable antimicrobial properties such as antibacterial, antifungal, antiviral, antioxidant, somatic fat reducing, blood pressure regulating, anti-inflammatory among others (Stinson *et al.*, 2003). Therefore, they form a good source of potential novel drugs (Wang *et al.*, 2005) and leads for compounds of pharmaceutical importance. A research done by Haque *et al.* (2005) shows that *Colletotrichum gloeosporioides,* a fungal endophyte, produces Taxol which was found to be cytotoxic against human cancer cells. The fungal extracts were also active against *Bacillus cereus* and *Staphylococcus aureus*. Another study by Liu *et al.* (2008), showed that endophytic fungi of *Xylaria* sp. isolated from *Ginkgo biloba* produces L.7-amino- 4-methylcoumarin which is active against various food borne and food spoilage microorganisms such as *Staphylococcus aureus, Escherichia coli, Salmonella typhi, S. typhimurium, S. enteritidis, Aeromonas hydrophila, Yersinia* sp., *Vibrio anguillarum, Shigella* sp., *Vibrio parahaemolyticus, Candida albicans* among others (Pongcharoen *et al.*, 2008) and hence this compound was suggested to be used as a natural food preservative. The discovery of novel antimicrobial metabolites from endophytes is therefore considered an alternative way to overcome the increasing levels of drug resistance by plants and human pathogens (Yu *et al.*, 2010). Fungal endophytes are not only useful in medicine but also important in agriculture. Sandhu *et al.* (2014) and Chepkirui *et al.* (2016) noted that the discovery of strobilurines which was isolated from *Strobilurus* sp. served as the lead compound for synthetic fungicides such as trifloxystrobin. Most of the research done on the activity of the

endophytic extracts is based on human pathogens but little work has been done on the activity against plant pathogens especially the bean bacterial pathogens hence the main aim of the study.

2.4.1.2 Bio- Control Agents

Fungal endophytes have also been used as Biological Control Agents (BCA) (Guo *et al.*, 2008). Examples include the use of *Trichoderma* spp. which is a fungus to control the *F. solani* that causes *Fusarium* root rot in common bean (Abd-El-Khair *et al.*, 2010). *Catenaria anguillulae* has also been used to control nematodes.

2.4.1.3 Phytoremediation

Endophytes have been applied in phytoremediation; plant assisted removal of xenobiotics and heavy metals from soil (Ma *et al.*, 2011). A research done by Van Aken *et al.* (2004) reported that *Methylobacterium populum* was involved in the degradation of energetic compounds such as 2,4,6-trinitrotoluene, hexa-hydro-1,3,5-trinitro-1,3,5-triazine, and hexa-hydro-1,3,5-trimtro-1,3,5-triazine. The work done by Tesar *et al.* (2002), also showed that bacterial endophytes from the rhizosphere can be used in the phytoremediation of diesel oil.

2.4.1.4 Enzyme Production

For an endophyte to colonize a plant tissue, they usually produce enzyme that enable them to penetrate the plant tissues (Schulz *et al.*, 2002). The work done by Leuchtmann *et al.* (1992) showed that most endophytes utilize xylan, pectin, show lipolytic activity and produce peroxidases and laccases. They also produce chitinase (Li *et al.*, 2004) and glucanase (Moy *et al.*, 2002). *Streptosporangium* sp., an endophytic actinomycete isolated from leaves of maize, also showed gluco-amylase production. This enzyme exhibited thermostable properties (Stamford *et al.* 2002). This led to the conclusion that endophytes can be used in the novel production of enzymes such as xylanase and hemicellulose.

2.4.2 Fungal Endophytes

The term endophytes consist of both fungal and bacterial strains. Fungal endophytes are fungi that colonize plant tissues. Research has proven that fungi is the most important group of eukaryotic organisms that are well known for producing many novel metabolites which are directly used as drugs or function as lead compounds for synthetic modification (Kock *et al.*, 2001; Bode *et al.*, 2002; Donadio *et al.*, 2002; Gunatilaka, 2006; Chin *et al.*, 2006; Mitchell *et al.*, 2008; Stadler and Keller, 2008). Hence this study basically

concentrated on the fungal endophytes. Fungal endophytes consist of three basic ecological groups; mycorrhizal fungi, balancious or 'grass endophytes' and non-balancious endophytes (Schulz and Boyle, 2005). Research has shown that millions of fungal endophytes that exist in nature are likely to be found in a tropical ecosystem (Dreyfuss and Chapela, 1994) and they are very diverse. This is because in the tropical ecosystem highly diverse population of angiosperms exist (Arnold *et al.*, 2000).

Most of the fungal endophytes isolated from medicinal plants are Ascomycetes such as *Fusarium* sp., *Colletotrichum* sp, *Xylaria* sp., *Alternaria* sp., *Aspergillus* sp., *Scopulariopsis* sp., *Chaetomium* sp. Apart from the Ascomycetes there are also Basidiomycetes that have been isolated as endophytes such as *Coprinellus domesticus*, *Polyporus arcularius*, *Trametes* spp. (Thomas *et al.*, 2008).

2.4.3 Techniques used in the Isolation and Study of Endophytes

Endophytic fungi can be studied using either direct observation or using cultivation methods. The direct observation method involves the visualization of the fungal structures using either a light microscope or an electron microscope (Sun and Guo, 2012). This method can show all the endophytic microbiota within the plant tissues particularly the biotrophic fungi that cannot be cultured on standard growth media (Lucero *et al.*, 2011). Most endophytic fungi cannot be identified to any taxonomic level using their morphological characteristics since they lack the spore producing structures. Therefore, this method is not commonly used in the identification of fungal endophytes (Deckert and Struhl, 2001).

Cultivation methods have always been used in the isolation and study of endophytic fungi (Vieira *et al.*, 2011). Hyde and Soytong (2008) noted that the technique used in endophyte isolation influences the type of endophyte isolated. The isolation methods of fungal endophytes may vary in the surface sterilization of the host plant tissues such as leaves, stems, roots, barks, flowers and fruits. This sterilization process can influence the detection of endophytic fungi. It is worth noting that different host and plant organs require different sterilization times. For example, thicker leaves require longer sterilization time than thin leaves. Hence the strength of the free chlorine and the sterillant should be established to make sure all the epiphytic fungi are removed from the plant (Hyde and Soytong, 2008). The plant tissues can be disinfected with strong antioxidants or disinfectants for a specific period. The most commonly used agents include 1- 4% detergent, 3% H_2O_2, 2-10% NaOCl, or 70-95% ethanol. To determine the effectiveness of the surface sterilization method used, Schulz *et al.* (1998) suggested a method of achieving this, which involves making a leaf or stalk

imprint on agar surface. If fungi does not grow, then the sterilization protocol can be deemed effective. Márquez *et al.* (2007) also, noted that this protocol for testing isolation of endophyte is excellent and should be applied in all endophytes.

Another important parameter to be considered in the isolation of fungal endophytes is the culture media since different fungal strains behave differently on different media. Some of the commonly used media is Potato Dextrose Agar (PDA), Sabourand Dextrose Agar (SDA), yeast malt agar (YMA), malt extract agar (MEA), Potato Carrot Agar (PCA) and Water agar (Huang *et al*., 2008). These culture media are usually supplemented with antibacterial agents such as chloramphenicol, penicillin, ampicillin, tetracycline, ciprofloxacin and streptomycin among others to prevent bacterial growth (Luiz *et al*., 2011).

2.4.4 Identification of Fungal Endophytes

Endophytes from different classes produce different or the same types of secondary metabolites that are of immense importance to pharmaceutical industry hence the need for their identification once they are isolated. There are two main methods that have been used in the identification of the isolated fungal endophytes. These include; morphological and molecular techniques.

2.4.4.1 Morphological Identification

Most of the isolated fungal endophytes are mycelia sterillia; those species that do not produce spores (Guo *et al.*, 2001) hence their morphological identification involves grouping them into morpho species using their morphological characteristics such as the colony shape, growth rate, colony texture, diffuse pigmentation, and topography of the back of the colony (Arnold *et al.*, 2000; Fróhlich *et al.*, 2000). The sporulating ones can be identified by their microscopic reproductive structures such as the hyphae and the spores, using the microculture technique and comparing the obtained results with taxonomic keys (Barnett and Hunter, 1972). However, this method does not reflect the species phylogeny. In addition, it cannot be used to identify the mycelia sterile species; hence the need for more advanced identification method.

2.4.4.2 Molecular Identification

Molecular characterization can be used in the confirmation of results obtained from morphological characterization for the sporulating fungi (Huang *et al.*, 2009) while for the non-sporulating fungi, it is considered the best option for identification. The conserved known genes with enough variation are usually selected for designing PCR assays and

performing phylogenetic analysis. In fungal genome, the genes encoding ribosomal RNA are organized in arrays which contain repetitive transcriptional units involving 16-18S, 5.5S and 23-28S rRNA, two intergenic spacers ITS1 and ITS2 and two external spacer sequences (5′ and 3′ ETS) that are transcribed by RNA polymerase1. Posttranscriptional process splits the cistron where ITS1 and ITS2 are excised and three types of rRNAs produced; 5.8S gene (is highly conserved and used for the phylogenetic analysis of higher taxonomic levels), 18S nuclear ribosomal small subunit rRNA gene (SSU) used for phylogenetic analysis due to its few hypervariable domains in fungi and the 28S nuclear ribosomal large subunit (LSU) which is sometimes used to discriminate species on its own or combined with ITS (Schoch *et al.*, 2012). 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 (Guo *et al.*, 2001). This region contains highly conserved areas adequate for designing primers that allow discrimination over a wide range of taxonomic levels (White *et al.*, 1990). 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 (Schena *et al.*, 2004), β-tubulin (Mostert *et al.*, 2006; Aroca *et al.*, 2008). 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 and 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 minichromosomal 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 DNA is extracted and the target region amplified using a thermocycler, which allows the amplification of specific copies of specific DNA sequences by repeated cycles of denaturation, annealing, elongation and termination at different temperatures using specific primers, deoxyribonucleotide triphosphates (dNTPs) and thermostable *Taq* DNA polymerase in adequate buffer (Mullis and Faloona, 1987). The amplified DNA fragment is visualized by agarose gel electrophoresis with Ethidium bromide (EtBr), SYBR Green, colorimetric methods (Mutasa *et al.*, 1996) or fluorometric assays (Fraaije *et al.*, 1999). The presence of a band of the expected size in the gel indicates the presence of the target organism. The PCR technique is highly sensitive, specific and very reliable. The PCR bands are then purified and

sequenced to determine the exact order of nucleotide in the DNA. The sequence results are then compared to the available databases such as FUNgal phYlogenetic dataBASE (FUNYBASE) (Marthey *et al.*, 2008) and National Center for Biotechnology Information (NCBI). However, Kang *et al.,* (2010) noted that the use of sequence database to identify organisms based on the similarity on the DNA may have some disadvantages such as erroneous or incomplete sequences, sequences associated with misidentified organisms, inability to easily change or update data and problems associated with defining species boundaries.

2.4.5 Fermentation Techniques and Crude Extract Production

To extract the secondary metabolites that are produced, the endophytic fungal species are always subjected to fermentation procedure. Fermentation technique involves the biological conversion of complex substrate into simple compounds by a bacteria or fungi. The products of fermentation include carbon dioxide and alcohol. In addition to that, they also produce secondary metabolites which are bioactive since they possess biological activity against pathogens (Subramaniyam and Vimala, 2012). The secondary metabolites are produced during the stationary phase of growth when key nutrient source, such as carbon, nitrogen or phosphate, is depleted in the growth media. The metabolites range from antibiotics, peptides enzymes and growth factors (Robinson *et al.*, 2001; Machado *et al.*, 2004) among others. The production of secondary metabolites can be affected qualitatively and quantitatively by factors such as temperature, pH, medium composition, culture duration and aeration degree which can be modified to improve the production of the significant compounds (Barrios-González and Mejía, 1996). Fermentation outcome varies for each substrate used because microorganisms react differently on each substrate, the rate of substrate utilization also differs and so does the productivity.

There are two main types of fermentation based on the substrate used; solid state fermentation (SSF) and liquid fermentation/ submerged fermentation.

a) Solid State Fermentation

In solid state fermentation, the microorganism grows on solid material without the presence of free liquid (Steinkraus, 1984). This has been one of the oldest fermentation methods used by man. In this type of fermentation, the substrates are utilised very slowly and steadily which supports the controlled release of nutrients. It is considered one of the best methods in the fermentation of fungi. This is because in nature, fungi grow on solid substrates such as pieces of wood, seeds, stems, roots and dried parts of animal such as skins,

bones and faecal matters that have low moisture contents absorbed in the solid media (Hesseltine, 1977). This method cannot be used in the fermentation of bacteria since it requires a high-water activity (Babu and Satyanarayana, 1996). Some of the common substrates used include wheat bran, rice and rice straws, hay, fruits and vegetable waste, paper pulp, bagasse, synthetic media among others (Pandey *et al.*, 1999).

b) Liquid Fermentation/ Submerged Fermentation

This type of fermentation utilizes free flowing liquid substrate such as molasses and broth. The secondary metabolites are secreted into the fermentation broths and the substrate is utilized quite rapidly. Liquid fermentation is best suited for microorganism such as bacteria that require a high moisture content. The common substrates used include molasses, soluble sugars, liquid media, fruit and vegetable juices, sewage and waste water. The advantage of this method is that the purification of the products is easier as compared to the solid-state fermentation (Subramaniyam and Vimala, 2012).

2.4.6 Secondary Metabolites Isolated from Endophytic Fungi

Some of the secondary metabolites produced from the fermentation of fungal endophytes include; sesquiterpenes such as 2α-Hydroxydimenino (**9**) extracted from *Pestalotiopsis* species, diterpenes such as Guanacastepene (**10**), podophyllotixins (Stierle and Stierle, 2015), alkaloids such as peramine 1 (**11**) extracted from *Neotyphodium coenophialum,* phenolic acids such as 2-Hydroxy-6-methylbenzoic acid extracted from *Phoma* sp. (**12**), *p*-hydroxyphenyl acetic acid (**13**) isolated from *Epichloë typhina* (Tan and Zou, 2001). These compounds are shown in Figure 2.

Compound 9

Compound 10

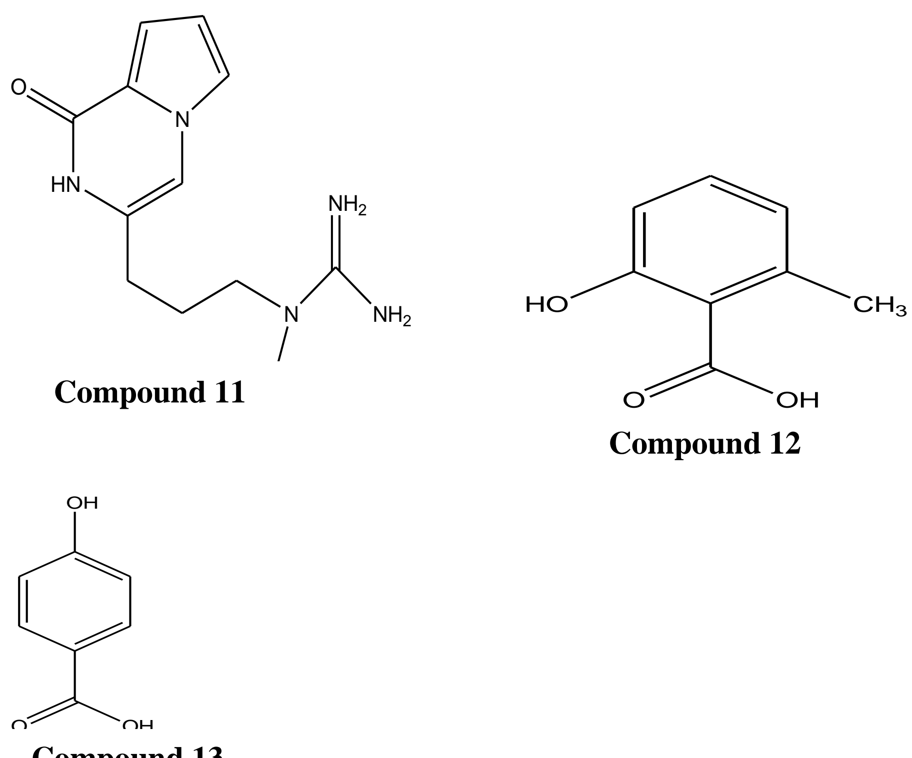


Figure 2: Compounds isolated from various endophytic fungi

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

Fresh leaves of *Z. gilletii* and *M. lutea* were collected from Kakamega Tropical Rainforest which stretches from 0° 10′ to 0° 21′N and longitude 34° 44′ to 34° 58′E and an altitude of 1524m above sea level. The leaves were identified with the help of a taxonomist and were deposited at the Biological Sciences Department, Egerton University.

3.2 Endophytic Fungi Isolation and Identification

This procedure was carried out at the place of stay after collection by improvising a clean bench.

3.2.1 Media Preparation

The media used for endophytic fungi isolation was Sabouraud Dextrose Agar (SDA). A portion (65g) of SDA was dissolved in 1000ml of distilled water in a conical flask and autoclaved at 120°C for 15 minutes. The media was then plated in petri dishes in a clean laminar flow by pouring 25ml of the media to each petri-dish and allowed to set.

3.2.2 Isolation of the Fungal Endophytes

Endophytic fungi were isolated from the leaves of *Z. gilletii* and *M. lutea* within 8 hours of collection as described by Zinniel *et al.* (2002) with slight modifications. In this method, the leaves were washed under running tap water to remove any soil or other foreign materials and blotted dry using filter papers. Thereafter, they were sterilized for 2 minutes using 70% ethanol followed by 1% sodium hypochlorite for 3 minutes and rinsed three times with sterile distilled water to remove any traces of the disinfectant. The sterilized leaves were cut aseptically into sections approximately 1mm by 4mm and inoculated in petri-dishes containing SDA amended with streptomycin sulphate antibiotic (2g/liter) to prevent bacterial growth. The plates were incubated at 25 ± 2°C for 1-4 weeks. Frequent monitoring was done to check for the growth of the endophytic fungi. After 3-4 days of incubation, the first fungal hyphae were visible from the edge of the leaf sample. Hyphal tips of the developing fungal colonies were transferred to fresh SDA plates to prepare pure cultures of the endophytes. This step was repeated several times until pure endophytic fungal strain with uniform colony was achieved (Plate 5). The cultures were then identified using molecular techniques.

3.2.3 Molecular Identification of the Isolated Fungi

Pure cultures of the endophytes were grown in 30ml of Yeast Malt broth (Malt extract- 10g/l source of additional carbon, D-glucose- 4g/l source of carbon, carbohydrate and energy, Yeast extract-4g/l provides vitamin B complex nutrient and growth factors). The final pH of the media was 6.3. The cultures were incubated at 28°C on an orbital shaker for 3-4 days to allow the fungal mycelia to grow for DNA extraction. The molecular identification was done at the Helmholtz Zentrum Für Infektionforschung (HZI) in Braunschweig.

3.2.3.1 DNA Extraction

The DNA extraction was performed using the BIO BASIC EZ-10 Genomic DNA kit following manufactures instruction. Approximately 6-10, 1.4mm Precellys Ceramic Beads were added to a 1.5ml screw cap reaction tube. Then approximately 60mg of the fungal hyphae obtained from a 3-4day old culture were added to the same tube. The sample was covered with 600µl Plant Cell lysis buffer (PCB) (Sodium propionate, sodium cacodylate and BIS-TRIS propane in a ratio of 2:1:2) and homogenized using a homogenizer (Precellys 24 lysis and homogenization, Peq lab, Bertin technologies). To aid in protein degradation 12µl of β mercaptoethanol was then added to the sample. The sample was vortexed (IKA MS3 Digital) to allow for the mixing of the components and then incubated for 25 minutes at 65°C in a metal block (MTB 250). Chloroform (600µl) was added to solubilize the proteins and polysaccharide from the DNA. The sample was centrifuged (5430 R) at 10,000 rpm for 2 minutes. The upper layer was transferred to a clean Eppendorf tube and the rest discarded. To allow for the binding of the DNA to the column, 200µl of BD (Binding buffer) buffer was added and the mixture vortexed, followed by addition of 200µl ethanol which was then later vortexed to mix. The mixture was transferred into EZ-10 column placed in a 2ml collection tube and centrifuged at 12,000 rpm for 1 minute. The flow through was discarded and then 500 µl of PW solution diluted with isopropanol was added to remove the extra protein and colored contaminants. The mixture was centrifuged at 12, 000 rpm and the flow through discarded then 500µl of Wash solution diluted with ethanol was added to remove the salts. The mixture was centrifuged at 12,000 rpm for 1 minute and the flow through discarded again. The column was again centrifuged at 12,000 rpm for 2 minutes to remove any remaining wash solution. The column was then transferred into an empty 1.5ml Eppendorf tube and 70µl of TE Buffer, pre- warmed to 60°C, added directly at the center of the EZ membrane to increase the elution efficiency. The sample was then incubated for 2 minutes at room temperature to increase the elution chances of the DNA from the membrane. The tube

was then centrifuged at 12,000 rpm for 2 minutes to elute the DNA. The DNA was stored at 4°C for further analysis.

3.2.3.2 Polymerase Chain Reaction (PCR) Amplification

The conserved ITS region on the fungal genome was amplified by conducting PCR. To a PCR tube 0.5µl of forward primer ITS1F (CTTGGTCATTTAGAGGAAGTAA) and 0.5µl of reverse primer ITS4 (TCCTCCGCTTATTGATATGC), were added, 12.5µL of the jump start ready mix that contained 20 mM Tris-HCl pH 8.3, 100 mM KCl, 3mM $MgCl_2$, 0.002% gelatin, 0.4mM dNTPs (dATP, dCTP, dGTP, dTTP), stabilizers, 0.1 unit/mL Taq DNA polymerase and JumpStart antibody was added. This was followed by 9.5µl of distilled water and 2µl of the template DNA to make a total volume of 25µl of the mixture per sample. For a negative control, 2 µl of distilled water was used instead of DNA template in the reaction mix. The amplification was done in a thermocycler (Eppendorf® Mastercycler® nexus Thermal Cycler) under the following conditions; initial denaturation of 5 minutes at 94°C, followed by 34 cycles of denaturation for 30 seconds at 94°C, annealing at 52°C for 30 seconds and elongation for 1 minute at 72°C. A final elongation of 10 minutes at 72°C. The PCR products were pre-stained with midori green dye and resolved in a 0.8% agarose gel. The visualization was done in a UV transilluminator (Nippon Genetics Europe GMbH) and photographs taken.

3.2.3.3 Purification of the Amplified PCR Products

The amplified PCR products were purified using BIO-BASIC EZ-10 spin column purification kit. To adjust the DNA to the binding conditions, 110µl of buffer 1 was added to the 22µl PCR product and mixed thoroughly. The mixed sample was then put on the EZ-10 spin column and incubated for 2 minutes at room temperature. Later, the sample was centrifuged at 10,000rpm for 30 seconds and the flow through discarded. The DNA was then washed by adding 500µl wash solution, centrifuged at 10,000rpm for 30 seconds and the flow through discarded. The same amount of wash solution was added to the sample and then centrifuged at 10,000rpm for 1 minute and the flow discarded. The amplified DNA was then eluted in a clean 1.5ml reaction tube by adding 20µl of elution buffer pre-warmed to 65°C. The sample was incubated at room temperature for 2 minutes and then centrifuged at 10,000 rpm for 1 minute. The EZ-10 spin column was discarded. The collected DNA were stored at -4°C ready for sequencing.

The amplified DNA was sequenced by Illumina genome analyzer sequencing machine (applied Bio systems 3730 xl DNA analyzer) at the Helmholtz Zentrum Für Infektionforschung (HZI) in Braunschweig, Germany. The forward and reverse primer sequences obtained from the sequencing were aligned by Genious R7 program to get the consensus sequences. The consensus sequences were deposited in NCBI GenBank and compared with those available in GenBank via BLAST searches. The accession numbers of the isolated fungal endophytes are shown in Appendix **28**. Phylogenetic analysis was conducted using the distance based neighbor joining methods in Molecular Evolutionary Genetics Analysis (MEGA) version 6.06. and the Neighbor joining (NJ) tree constructed using Tamura-Nei distance. All characters were equally weighted and unordered. Gaps and the missing data were treated as complete deletion. Support for the specific nodes on the NJ tree was estimated by bootstrapping 2000 replications. The substitution type was use of nucleotides and the pattern of lineage was homogeneous.

3.3 Bioassay of the Endophytic Fungi against the Plant Bacterial Pathogens

Bioassay of endophytic fungi against *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* was done at the biotechnology lab of Egerton university using dual culture assay as illustrated by Srivastava and Anandrao (2015) with slight modification. The media used for this assay were Muller Hinton Agar (38g/l) for the dual culture assay and Nutrient broth (21g/100ml of distilled water) for bacterial inoculation. Pure bacterial cultures of *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* were obtained from the Biological Science Department at Egerton University, main campus. The two bacteria were inoculated in the nutrient broth media in separate flasks under aseptic conditions, then incubated at 30°C for 24 hours. Using turbidity comparison, the bacterial suspension was prepared to match a 0.5 McFarland standard. At this concentration, the McFarland standard represents a bacterial cell density of approximately 1.5x 10^8 CFU/ml (1.0 x 108 – 2.0 x 108 CFU/ml).

From the respective nutrient broths subculture, 100µl of bacterial concentration of 5×10^5 CFU/mL was spread evenly on the MH media on petri-dishes using a sterile cotton swab and allowed to dry. Using a cork borer, a six-millimeter diameter of a 7day old mycelia plugs were placed on the MH media plate inoculated with the test bacteria. The plates were incubated at ± 32°C for 24 hours and the zone of inhibition was measured. Standard chloramphenicol antibiotic disc was used as a positive control while the negative control was a petri dish inoculated with the bacteria only. The experiment was carried out in triplicates for

each isolated endophyte. The most active endophytic fungi were subjected to solid state fermentation for secondary metabolites extraction.

3.4 Fermentation of the Endophytes and Extraction of Secondary Metabolites

Secondary metabolites were extracted from the endophytes that showed antagonistic activity against the bacteria in dual culture assay by fermentation. The fermentation was done at the biotechnology lab of Egerton university Njoro. Fermentation of the endophyte was carried out as described by Nascimento *et al.* (2012) with slight modification. The solid-state fermentation was carried out in 21, 500ml Erlenmeyer flasks containing 90g of rice in 90ml of distilled water per flask which were autoclaved twice at 120°C for 40 minutes. Agar plugs (3 pieces) of 2×2cm were cut from a 7-day old culture of the endophyte and then inoculated in the rice media. One flask without inoculum was kept as a control. The flasks were then incubated for 21 days at 30°C under static conditions. The flasks containing the cultures were checked periodically for contamination.

After the incubation period, the fermentation was ended with the addition of 150ml of methanol to each of the flask and left to stand overnight to enable the extraction of the secondary metabolites. The cultures were sliced with the aid of a spatula and the flasks placed in an ultrasonic cleaner (SB-120 DTN) to allow complete extraction of the secondary metabolites. The mixture was filtered using a Whatman filter paper no. 1 followed by repeated extraction with methanol until exhaustion. The filtrate was evaporated under reduced pressure using a rotavapor (BUCHI rotavapor R-205) to yield a methanol extract. The methanol extract was suspended in water to dissolve the glucose and glycoside that are not important, then subjected to a liquid- liquid partition between hexane to remove the fats and ethyl acetate which dissolved most polar compounds. The resulting organic layers were evaporated under reduced pressure using a rotavapor to yield hexane extract and the ethyl acetate extract. The extracts were subjected to antimicrobial assay and further fractionation and purification using preparative HPLC.

3.5 Extraction of Secondary Metabolites from Leaves of the Medicinal Plants and Fractionation

Extraction of the secondary metabolites from the leaves of *Z. gilletii* and *M. lutea* was done at the chemistry department of Egerton University using methanol solvent. The leaves were dried under a shade to avoid photo decomposition and destruction of the metabolites. The dried leaves were ground into a fine powder and 700g of each powder was soaked in

1.5L of methanol overnight and filtered through the Whatman filter paper no. 1. The filtrate was then evaporated using a rotor evaporator to remove the solvent and get methanol extract. The extract from *Z. gilletii* yielded 2.56g crude extract while that of *M. lutea* yielded 2.01 g. The crude methanol extracts were further purified using a solid phase extraction.

3.5.1 Reverse Phase-Solid Phase Extraction

Solid phase extraction was done using cartridges bound with C18 carbon which is less polar. The cartridge was inserted into a specially designed hole in a rubber stopper affixed to a glass tank to which vacuum was applied. The cartridge was first conditioned with 5ml of methanol. A portion (5ml) of the crude methanol extract was passed through the cartridge slowly into the flat-bottomed flask. This was followed by addition of 20ml methanol to absorb the polar compound. Then 20ml of ethyl acetate was added to remove the non-polar compounds that were trapped by the adsorbent material. The methanol and the ethyl acetate extract were later evaporated in a rotor vapor to obtain ethyl acetate and methanol extract. The vacuum was turned off after each liquid had passed to prevent the cartridge from drying up. The crude extract from *Z. gilletii* yielded 0.99g and 1.5g of ethyl acetate extract and methanol extracts respectively while the crude extract from *M. lutea* produced 0.99g methanol and 0.8g and ethyl acetate extracts. The methanol extracts were subjected to further purification using Thin Layer Chromatography (TLC).

3.5.2 Thin Layer Chromatography (TLC)

Thin layer chromatography was performed on silica GF 254nm, (Merck, Germany) 0.25mm thickness. The dry methanol extract was reconstituted in methanol and mixed thoroughly in an ultrasonic cleaner to ensure homogeneity. Preliminary analysis was performed to identify optimum solvent system for use as the mobile phase. The solvent mixture that gave optimum separation for the methanol extract of *Z. gilletii* were 6:4 ethyl acetate-hexane (E:H) mixture and for *M. lutea* was 3:7 ethyl acetate- methanol mixture. Samples were spotted on 2×5 aluminum backed TLC plates using a capillary tube. For each different plant, samples were spotted at about half a centimeter from the base on separate plates. The plates were then placed in 100ml beaker containing 10ml of the appropriate solvent system then covered with aluminum foil. It was then allowed to develop to up-to 4cm up the plate. The developed chromatogram was then visualized under UV lamp (Uvitec-LF-204.LS) at 254nm and 365nm. To get different fractions of the samples, the sample was again fractionated using column chromatography.

3.5.3 Column Chromatography

The sample was mixed with silica gel and methanol in a round bottomed flask. The solvent was then evaporated in a rotor vapor till dryness to allow the sample to adsorb into the silica gel. A column, 50cm length and 20mm diameter, was packed with silica gel 60 0.06-0.2mm (70-230 mesh ASTM). The adsorbed sample was placed above the layer of silica and the solvent system was added to the reservoir attached to the column. The column was eluted gradually and the flow rate maintained at approximately 15ml/5min. Fractions of each volume were collected and the TLC of each fraction performed. Fractions with similar TLC patterns were pooled.

Elution from the *Z. gilletii* extract was done on a gradient elution pattern in which the first solvent system to be used was 6:4 E:H mixture. This was followed by 9:1 E:H mixture, followed by 100% ethyl acetate and lastly 100% methanol. This yielded 7 fractions labelled F1-F7, with fraction 4 (0.010g) forming some characteristic yellow crystals (compound **5**) after standing for 3 weeks. Elution from *M. lutea* was done with only one solvent system, Methanol: Ethyl acetate (6:4) which did not show a good separation on TLC.

NMR analysis was performed on fraction 4 and other pure compounds from endophytic fungi to determine their mass and structure. All the fractions were subjected to antimicrobial assay.

3.5.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{1}H, ^{13}C, DEPT, HSQC, COSY and HMBC NMR spectra was recorded on the Bruker Advance 500 MHz NMR spectrometer at the Braunschweig Helmholtz Center for Infectious Diseases, Germany. Some of the readings were done in deuterated chloroform and others in DMSO (Dimethyl sulfoxide) and methanol, and chemical shift assigned by comparison with the residue proton and carbon resonance of the solvent. Tetra methylene (TMS) was used as an internal standard and the chemical shifts were given as δ (ppm). The results were analyzed using 1D and 2D spectrometric and spectroscopic techniques.

3.5.5 Two- Dimensional NMR Spectroscopy

The off- diagonal elements were used to identify the spin-spin coupling interactions in the ^{1}H-^{1}H (correlation spectroscopy). The proton-carbon connectivity, up to three bonds away was identified using ^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation) spectra. The ^{1}H-^{13}C HSQC spectra (Heteronuclear Single Quantum Coherence) were used to identify the

protons that were attached to the carbon atoms while the COSY spectrum were used to identify the protons attached to adjacent carbon atoms.

3.5.6 Mass Spectrometry

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

3.6 Antimicrobial Assays of the Extracts

3.6.1 Disc Agar Diffusion Assay

The antimicrobial assay of the plants and endophytic fungi extracts was performed using disc agar diffusion method (Kajaria *et al.*, 2012) at the biotechnology laboratory at Egerton university. The extracts were weighed to know the weight, then a 50mg/ml concentration of the extracts were made using the formula C=M/V. where C stands for concentration, M- mass of the extract and V is the volume of the DMSO to be added to the extract. Blank sterile discs of Whatman no.1 filter paper of 6mm in diameter were impregnated with the different extracts and plated against the test organisms. The media used in this section and the preparation of the test organisms was prepared as described in the section 3.3 above

Extracts to be tested were labelled M1 (Methanol crude extract), M2 (Methanol extract after SPE), E (Ethyl acetate extract after SPE), Y/ F4(the yellow crystalline compound) for Z. *gilletii* while for *M. lutea* were labelled M3(Methanol crude), M4(Methanol crude after SPE), E1(Ethyl acetate extracts after SPE), E2 (Ethyl acetate extract after portioning). These were prepared by dissolving them in DMSO. The sterile discs were impregnated by dipping into the extract and placed carefully on the inoculated petri dishes using a sterile-forceps and pressed gently to ensure contact with the agar medium. Chloramphenicol disc, was used as a positive control, while the negative control was a Whatmann filter paper no 1 soaked in DMSO. The plates were placed at 4°C for 30 minutes to ensure that the extracts diffused into the media. They were later incubated at ±32°C for 24 hours after which the diameter of the zone of inhibition, if any, was measured in millimeters using a ruler. The experiment was done in triplicates and the mean zone of inhibition calculated.

3.6.2 Anti-Quorum Sensing Assay

This assay was performed to assess the quorum quenching capability of some of the selected fungal endophytes together with their extract and the medicinal plant extracts. This assay was done at the university of Kwazulu Natal, South Africa.

3.6.2.1 Bacterial Strains, Media and Culture Conditions

The bacterial strains used in this study were *Chromobacterium violaceum* (CV) American Type Culture Collection (ATCC)12472. The strains were cultured in Luria- Bertani (LB) medium at 30°C for 24 hours. For the overlay and the quantitative assays, the inoculum was prepared by growing the bacteria in 3mL LB broth at 30°C in a shaking incubator at 130r/min.

3.6.2.2 Qualitative Violacein Inhibition

This assay was done using both the extracts from the medicinal plants and the selected fungal endophytes. The CV strains were grown under aerobic condition in LB Broth at 30°C for 24 hours with agitation. After 24 hours, 100µl of 1×10^7 CFU/ml of the 24-hour old culture was added to 5ml of LB soft Agar (Tryptone-10g/l, Yeast-5g/l, NaCl-10g/l, Agar-7.5g/l, pH- 7.5) and mixed gently by shaking. The soft Agar was then overlaid on LB Agar in a perti dish and spread evenly on the plate and left to dry. Agar well diffusion method was used in this assay for the extracts; Briefly, 6mm diameter holes were punched into the media using a cork borer and filled with of 20µl and 40µl of the extracts respectively. For the fungal strains, 1×1cm fungal plug was placed on-top of the over-laid LB plate. Furanone was used as the positive control. The plates were then incubated at 30°C for 24 hours after which they were examined for loss of the purple pigment and the presence of turbid halo-region. The active fungal strains were then subjected to liquid fermentation in yeast malt broth. The supernatant and the mycelia were both extracted using ethyl acetate. The extracts were then subjected to qualitative and quantitative violacein assay.

3.6.2.3 Quantitative Violacein Inhibition

Chromobacterium violaceum ATCC 12472 was pre-cultured in 3 ml LB broth at 30°C overnight. One hundred micro-liters of 1×10^7 CFU/ml culture was inoculated with the extracts of different concentrations; 100µg/ml, 250µg/ml, 500µg/ml, 750µg/ml, 1mg/ml and 2mg/m (6.2, 15.5, 31, 46.5, 62 and 124ml respectively) in 3 ml LB broth at 30°C overnight. The control was the culture without any treatment. Thereafter, 200µl of the culture was placed on a 96 well microtiter plate and the optical density was read in a microtiter plate

reader (GLOMAX multi detection system) at 600nm wavelength. One ml of an overnight culture of *C. violaceum* ATCC 12472 (with/without treatment) were transferred into a 1.5ml eppendorf tube and centrifuged at 13000rpm for 10min to precipitate the insoluble violacein. The culture supernatant was discarded and the pellet evenly re-suspended in 1 ml of DMSO. The solution was centrifuged again at 13000rpm for 10min to remove the cells. A portion (200µl) of Supernatant was again placed into a 98 well microtiter plate. All the experiments were done in triplicates. The violacein produced was quantified at $OD_{585\ nm}$ using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) and blanked with DMSO. The percentage of violacein inhibition was calculated by following the formula: percentage of violacein inhibition = (control $OD_{585\ nm}$– test $OD_{585\ nm}$ /control $OD_{585\ nm}$) × 100.

3.7 Data Analysis

The mean inhibition zones were calculated and data analysis was performed using SPSS version 24. The homogeneity of the variance was analyzed using one-way ANOVA using Levene`s test. The mean comparison was done using Turkey`s-Kramer (Honestly Significant Difference) with a significance level of 0.05.

CHAPTER FOUR

RESULTS

4.1 Isolation and Identification of the Fungal Endophytes

In this study, *Z. gilletii* and *M. lutea,* both important medicinal plants, were investigated for the presence of endophytic fungi in their leaf tissues. A total of 51 fungal endophytes were isolated from the leaf tissues of these two medicinal plants. Some of the pure cultures of the isolated endophytes are shown in Plate 5. After the DNA isolation, the optimal PCR products were obtained using primer pair ITS1F and ITS4 which varied in band sizes 500-700 base pairs (bps). The PCR products of 41 isolates (80%) were amplified in the ITS region (Plate 6). All the identified 24 fungal endophytes belonged to the phylum Ascomycota except *Trametes* aff. *maxima* which belonged to the phylum Basidiomycota. They were divided into the following groups; 63% *Fusarium* species, 4% *Fusarium solani*, 4% *Fusarium oxysporum*, 4% *Scopulariopsis flava*, 4% *Scopulariopsis brevacaulis*, 4% *Chaetomium* cf. *cochloides*, 4% *Chaetomium* spp and 4% *Trametes maxima* (Figure 3). The BLAST percentage was compared to sequences in NCBI from the previously identified fungi ranged from 95% to 100% (Appendix 28).

The evolutionary relationships of the isolated fungi were determined by generation of a distance based neighbor joining phylogenetic tree (Figure 4). The neighbor joining analysis placed the sequences into two groups; *Sordariomycetes* and *Agariomycetes*. The generated tree had two major clades that were divided into six sub clades of distinct species; sub-clade 1- *Fusarium* spp., sub-clade 2- *Scopulariopsis* sp., sub-clade 3- *Chaetomium* sp., sub-clade 4- *Trametes* sp., sub-clade 5- *Scopulariopsis* sp. and sub-clade 6- *Fusarium* spp. Approximately 75% of the isolated endophytes belonged to the genus Fusarium.

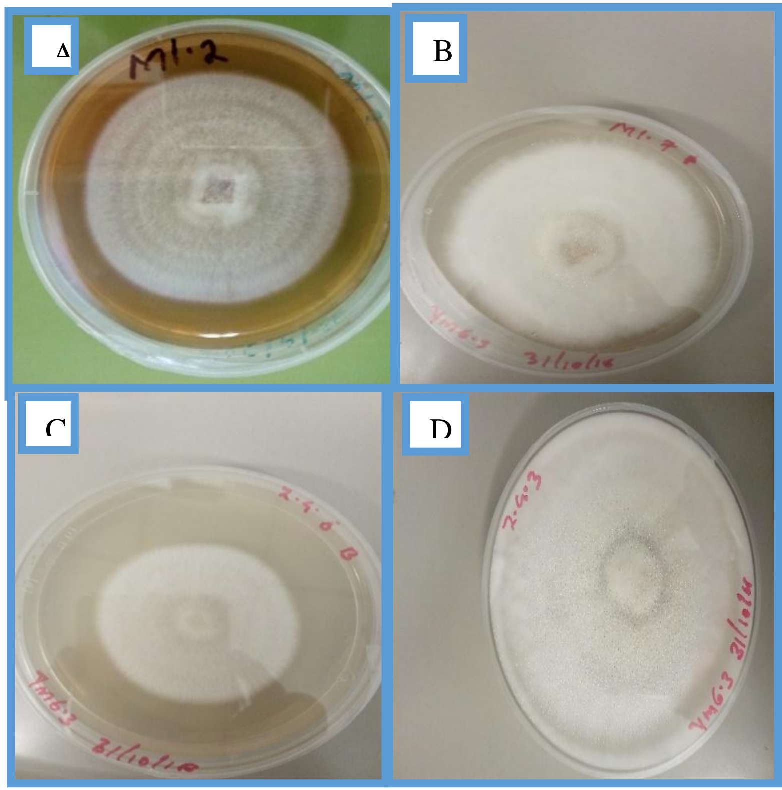


Plate 5: Pure cultures of fungal endophytes isolated from the two medicinal plants. A (*Fusarium solani*) and B (Fusarium sp): some of the pure cultures isolated from *M. lutea*; C (Fusarium sp) and D (Fusarium sp): some of the endophytes isolated from *Z. gilletii.*

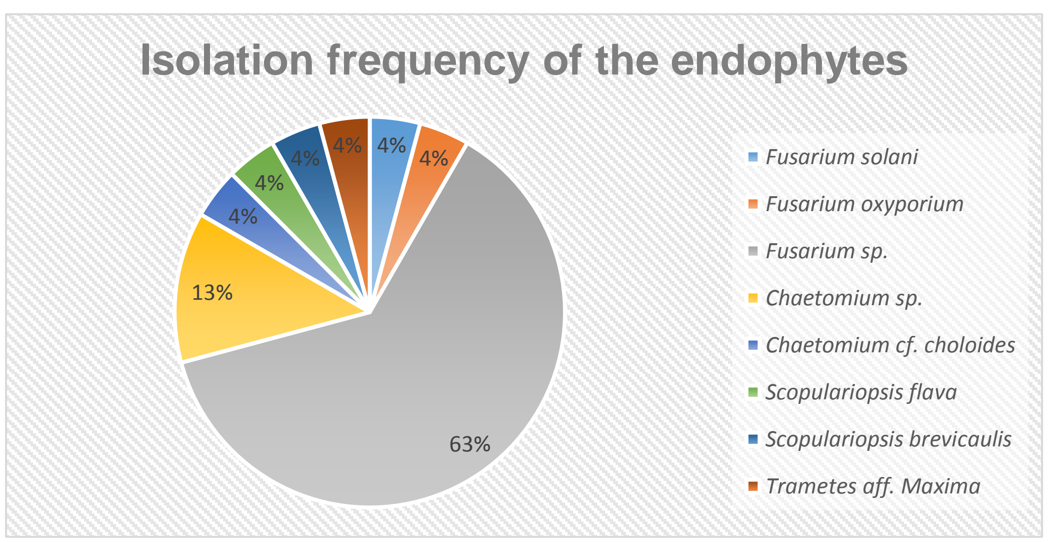


Figure 3: Isolation frequencies of the fungal endophytes from the two medicinal plants

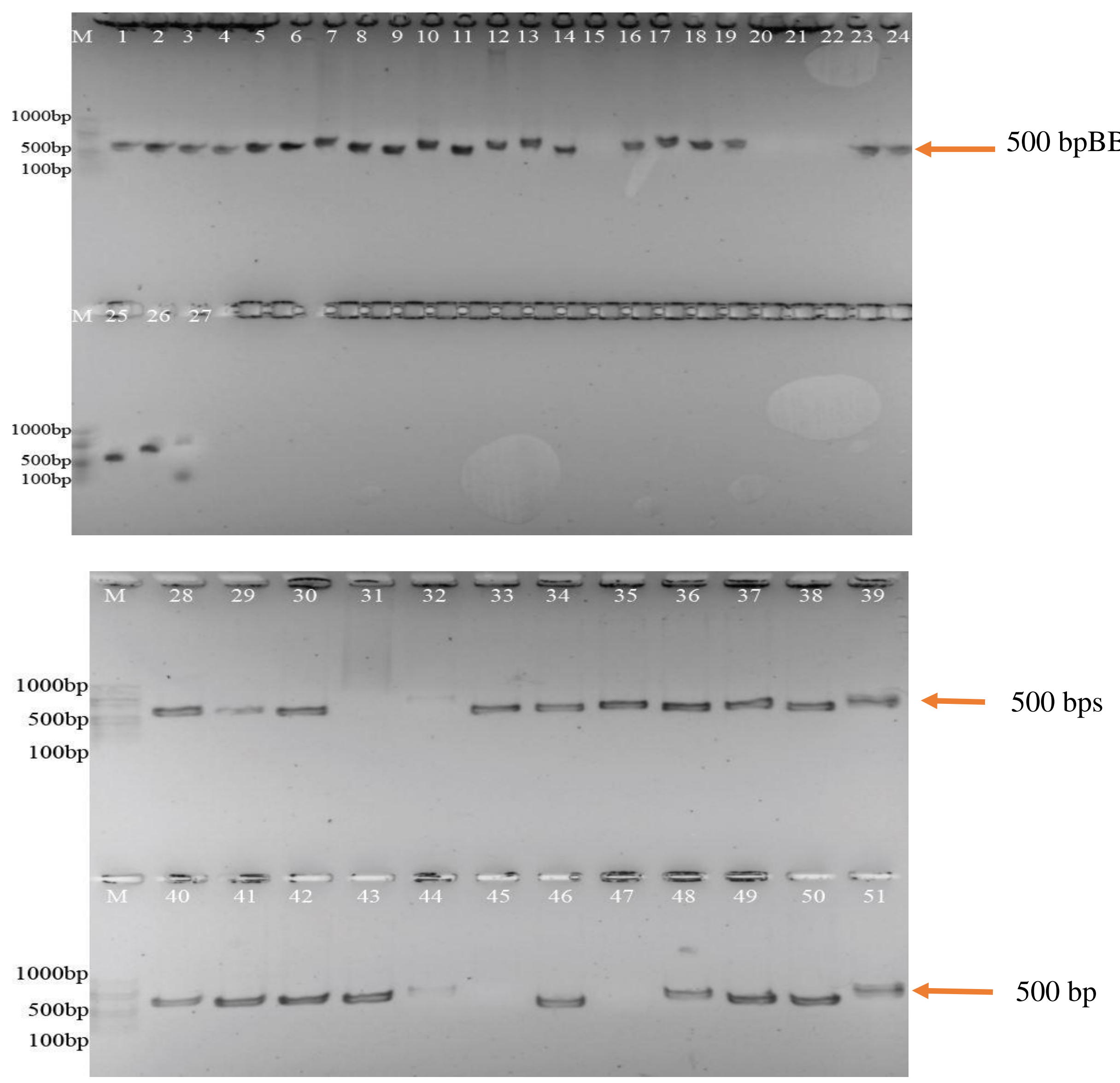


Plate 6: Agarose gel showing ITS PCR products of the isolated fungal endophytes. The molecular weight of the isolated DNA ranged from 500 bps-700bps.

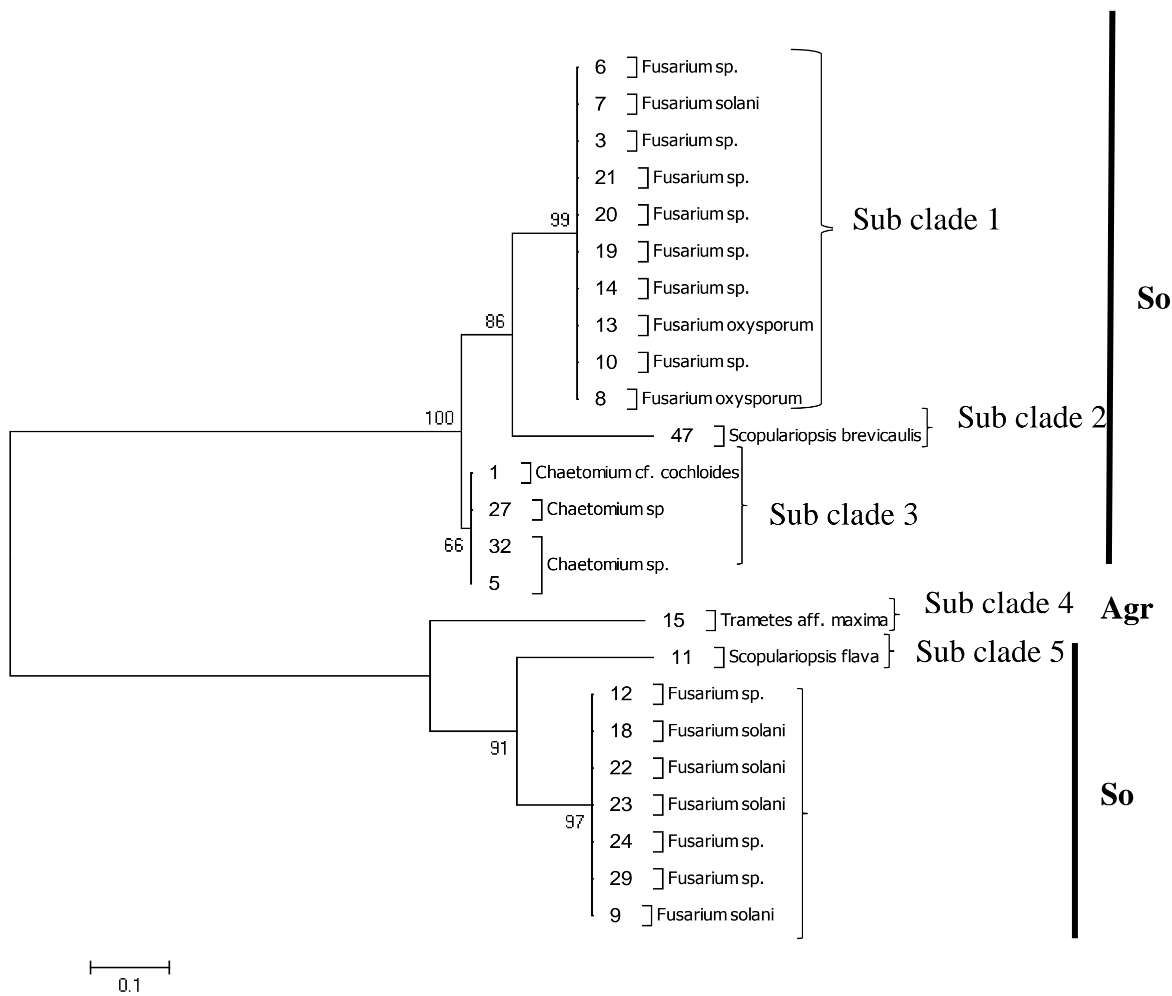


Figure 4: Phylogenetic Neighbour joining tree based on ITS analysis. (Maximum likelihood method; 2000 replicates bootstrap. SO- Sordariomycetes Agr- Agariomycetes).

4.2 Antibacterial Assay of Isolated Fungal Endophytes and Extracts from the Medicinal Plants

4.2.1 Dual culture assay of the isolated fungal endophytes

In this study, dual culture assay was used to assess the antagonistic effects of the isolated fungal endophytes against *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* (Appendix 26). As shown in Table 1, 15 out of 24 endophytes showed antagonistic activity against both *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola,* 13 of which belong to the genus *Fusarium*. Endophytic fungus, *F. solani* (Ml.2) had the largest inhibition zone of 20.3±1.5mm against *X. axonopodis* pv. *phaseoli* and 18.6±1.5mm against *P. syringae* pv. *phaseolicola*.

The one-way ANOVA- Leven's test showed a non- homogeneity of variance for the isolated endophytes with a p value of 0.001 (Appendix 30 and 31). The activity of fungal

endophyte Ml.2 against the test organism had no significant difference in activity when compared to chloramphenicol standard (Table 1).

Table 1: Inhibition zones (mm) of the isolated fungal endophytes against bean bacterial pathogens.

	Test organism (diameter of inhibition (mm)) n=3		
Endophytic isolate	**Endophytic identification**	*X. axonopodis* pv. *phaseoli*	*P. syringae* pv. *phaseolicola*
Ml.1	*Fusarium* sp.	10.6±1.15[cd]	10.3±1.15[de]
Ml.2	*Fusarium solani*	20.3±1.5[a]	18.6±1.15[a]
Ml.3A	*Chaetomium* cf. *cochloides*	0±0[e]	0±0[f]
Ml.4	*Fusarium* sp.	0±0[e]	0±0[f]
Ml.5	*Fusarium* sp.	14±2[bc]	15±1[bc]
Ml.6	*Scopulariopsis flava*	0±0[e]	0±0[f]
Ml. 6A	*Fusarium* sp	0±0[e]	0±0[f]
Ml.7	*Fusarium solani*	15±1[b]	17±1[ab]
Ml. 8	*Fusarium oxysporum*	15.3±1.15[b]	16.3±1.5[ab]
Ml.9	*Fusarium* sp.	12±2[bcd]	9.6±0.5[e]
Ml. 10	*Fusarium* sp.	15.3±1.15[b]	16±1.15[ab]
Ml.11	*Chaetomium* sp.	0±0[e]	0±0[f]
Ml.13	*Chaetomium* sp.	0±0[e]	0±0[f]
Ml.15	*Scopulariopsis brevicaulis*	0±0[e]	0±0[f]
Zg.1	*Fusarium* sp.	10.3±1.15[d]	11±1d[e]
Zg. 2	*Fusarium* sp.	12±2[bcd]	11±1[de]

Zg.3	*Fusarium* sp	11 ± 1^{cd}	11.3 ± 1.5^{de}
Zg.4	*Trametes aff. Maxima*	12 ± 2^{bcd}	12.6 ± 1.15^{cd}
Zg. 5	*Fusarium oxysporum*	9.6 ± 1.15^{d}	10.6 ± 0.5^{de}
Zg. 5A	*Fusarium* sp.	9.3 ± 0.5^{d}	11 ± 1^{de}
Zg.6	*Fusarium* sp.	0 ± 0^{e}	0 ± 0^{f}
Zg.7	*Fusarium* sp.	10 ± 1^{d}	9.7 ± 1.15^{e}
Zg.8	*Fusarium* sp.	11 ± 1.7^{cd}	10.6 ± 0.5^{de}
Zg.10	*Chaetomium* sp.	0 ± 0^{e}	0 ± 0^{f}
Chloramphenicol		20 ± 1^{a}	18.7 ± 1.15^{a}

Z.g- endophytes isolated from *Zanthoxylum gilletii* Ml- endophytes isolated from *Markhamia lutea*. *The values are the mean of three experiments ±S.E. of the mean. Within a column, fungal endophytes sharing the same letter(s) are not significantly different in antagonism against the two test organisms while those with different letters are significantly different (α =0.05, Tukey's test).

4.2.2 Disc agar diffusion assay of the plant and endophytic extracts

Fungal endophytes that produce the same bioactive compounds as the host plant have been reported (Kusari *et al.*, 2012). In this study, secondary metabolites were extracted from both the host plant and the isolated fungal endophytes. Extracts from both *M. lutea* and *Z. gilletii* showed some activity against *X. axonopodis* pv. *phaseoli* but this was not the case against *P. syringae* pv. *phaseolicola* (Table 2). The methanol crude extract from *Z. gilletii* showed no activity against both test organisms. This was also observed from the methanol extract after solid phase extraction (SPE). The ethyl acetate extract after the same procedure showed some activity against *X. axonopodis* pv. *phaseoli* and no activity against *P. syringae* pv. *phaseolicola*. The alkaloid skimmianine (**5**) isolated from the same plant produced a zone of inhibition of 12±2 mm against *X. axonopodis* pv. *phaseoli* while it showed no activity against *P. syringae* pv. *phaseolicola* (Table 2). All the extracts from *M. lutea* were active against *X. axonopodis* pv. *phaseoli* with the ethyl acetate extract after partitioning showing

the highest activity of 14±2 mm inhibition diameter against *X. axonopodis* pv. *phaseoli*. However, these extracts did not show any activity against *P. syringae* pv. *phasolicola* as similarly noticed in the extracts from *Z. gilletii.*

The extraction from endophytic fungi Ml.2 (*F. solani*), which was the most active as shown in the dual culture assay, yielded 0.4g hexane extract and 1.24g ethyl acetate extracts after partitioning while that of the second most active Ml.8 yielded 1.69g Ethyl acetate extract and 0.6g hexane extract. These extracts were then dissolved in DMSO to make a 50mg/ml stock solution for the antimicrobial assay. The dual culture results of the endophytes were in line with the results from the extracts of the fungal endophytes with the ethyl acetate extracts of Ml.2 giving the highest zone of inhibition of 15±0.5mm (Table 2). This was followed by the hexane extract that produced a zone of inhibition of 10±2mm (Table 2). The extracts from the second fungal endophyte (Ml.8) showed a low activity as compared to Ml.2 given that they both belong to the genus *Fusarium*.

The one-way ANOVA- Levenes test revealed non-homogeneity of variance by producing a p value of less than 0.005. Turkeys Honestly Significant Difference (HSD) test revealed that both the plant and endophytic extracts had significantly low activity when compared to the standard antibiotic chloramphenicol with the most active extract being Ml.2 ethyl acetate extract as shown in Table 2

4.2.3 Qualitative violacein inhibition assay

In this study, medicinal plants *M. lutea* and *Z. gilletii* and their selected endophytes were evaluated for quorum quenching activity. A total of ten fungal endophytes (chosen randomly irrespective of their antibacterial activity) and their extracts together with those from the plants were subjected to qualitative and quantitative violacein inhibition. The loss of the purple pigment in the *C. violaceum* and the presence of the turbid halos were indicatives of QS inhibition by either the fungi or the extracts while the presence of clear halos were indicative of antibacterial activity. *Fusarium solani* produced the best activity while other strains such as *Trametes* aff. *maxima*, *Chaetomium* sp., *Scopulariopsis* species and other *Fusarium* spp appeared as potential sources of anti-QS compounds. Some of the active strains are shown in plate 7. These potential strains were further subjected to liquid fermentation and the crude extracts analysed for qualitative violacein inhibition. The supernatant extract of *F. solani* produced a good activity against both the CV strains. This was followed by *Fusarium* species 1 which showed a little activity. The mycelial extracts however did not show any

activity against both strains (Plate 8). This could be due to the mode of extraction since the mycelia was extracted by ethyl acetate only which dissolves only slightly polar compounds as compared to acetone which is a very polar solvent. Extracts from the solid fermentation of both *Fusarium* sp.1 and *F. solani* did not show any activity.

Table 2: Inhibition zones (mm) of the plant and endophytic extracts against *X. axonopodis pv. phaseoli and P. syringae pv. Phaseolicola*

	Test organism (diameter in mm) n=3	
Extract code	***X. axonopodis* pv. *phaseoli***	***P. syringae* pv. *Phaseolicola***
Z.g MeOH crude	$0±0^{f}$	$0±0^{e}$
Z.g MeOH after SPE	$0±0^{f}$	$0±0^{e}$
Z.g EtA after SPE	$8.3±0.5^{e}$	$0±0^{e}$
Z.g Alkaloid	$12±2^{bcd}$	$0±0^{e}$
M.l MeOH crude	$7.6±0.5^{e}$	$0±0^{e}$
M.l MeOH after SPE	$8.6±1.5^{e}$	$0±0^{e}$
M.l EthA after SPE	$7.6±0.5^{e}$	$0±0^{e}$
M.l EthA after partion	$14±2^{bc}$	$0±0^{e}$
Ml.2 EtA	$14.6±0.5^{b}$	12.7^{b}
Ml.2 Hexane	$10.3±0.5^{de}$	$11±0.8^{cd}$
Ml.8 EtA	$11.6±0.5^{cd}$	$9.6±0.5^{d}$
Ml.8 Hexane	$9.6±0.5d^{e}$	$9.3±0.5^{c}$
Chloramphenicol	$20±0.5^{a}$	$18.7±1.15^{a}$
DMSO	$0±0a^{f}$	$0±0^{e}$

(Z.g- Extracts from the leaves of *Zanthoxylum giletii* Ml- Extracts from the leaves of *Markhamia lutea*.). *The values are the mean of three experiments ±S.E. of the mean. Within a column, the inhibition zones of extracts sharing the same letter(s) are not significantly different while those with different letters are significantly different (α =0.05, Tukey's test).

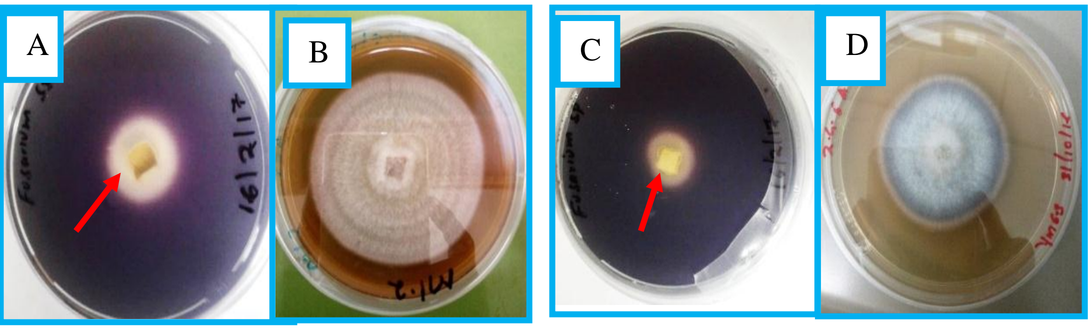


Plate 7: Qualitative violacein assay of *F. solani* and *Fusarium* sp.1 against CV 12472 .

A and C show the presence of the halo region indicating the activity of the endophytic fungi. ; B and D show the pure cultures of the endophytic fungi in a plate.

The crude and the fractionated extracts of the two medicinal plants were also subjected to the qualitative violacein assay. All the extracts did not show any potential activity except fraction 6 of *Z. gilletii* (S6) which showed some activity against the CV strain 12472 (Plate 8). The extracts from *F. solani*, *Fusarium* sp. 1 and Z.g fraction 6 (S6) that showed good activity were then subjected to quantitative violacein assay.

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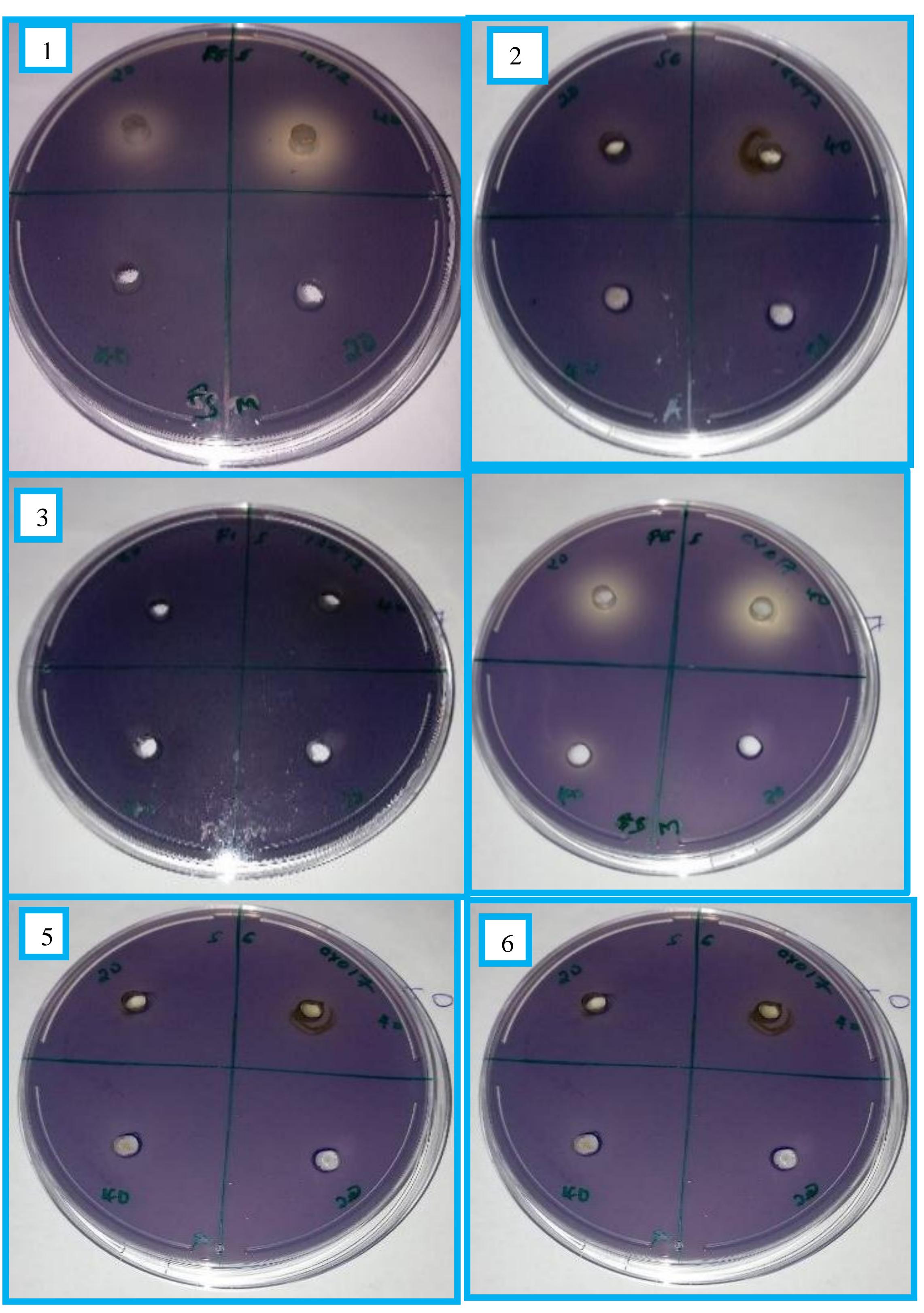

1
2
3
5
6

Plate 8: Qualitative violacein assay of the endophytic fungal and plant extracts against *Chromobacterium violaceum.* 1,2 and 3 shows the activity against CV12472 while 4,5 and 6 shows activity against CV017.

4.2.4 Quantitative violacein assay

The QS inhibitory activity of these extracts were further analysed by quantifying the amount of violacein produced. To determine whether the decrease in the violacein production was due to cell death or inhibition of the QS, cell viability was analysed after treatment. The qualitative assay was performed on the extracts that showed some potential activity on the qualitative screening, which included the supernatant extracts of *F. solani* and *Fusarium* sp. 1 and Zg fraction 1(S6).

The violacein production was inversely proportional to the cell viability and the increase in the extract concentration in some of the extracts. A concentration dependent inhibition of violacein production was observed in this experiment. Depending on the concentration, the extracts showed different levels of anti- QS activity as well as antibacterial activity. All the extracts showed a decrease in bacterial population as well as a violacein inhibition at different concentrations. The bacterial population decrease and the increase in the violacein inhibition was clearly seen in the extracts from *F. solani* (Figure 6) in which the bacterial count gradually decreased up to a concentration of 500µg/ml.

There was an increase in the bacterial count with increase in concentrations but then sharply decreased at 2000µg/ml with a 32% decrease in the violacein production. In contrast, the percentage violacein inhibition gradually increased with the concentration of the extracts. In such, it can be conclusive to note that the increase in the percentage violacein inhibition is both because of cell death as well as QS inhibition from the extracts of *F. solani.*

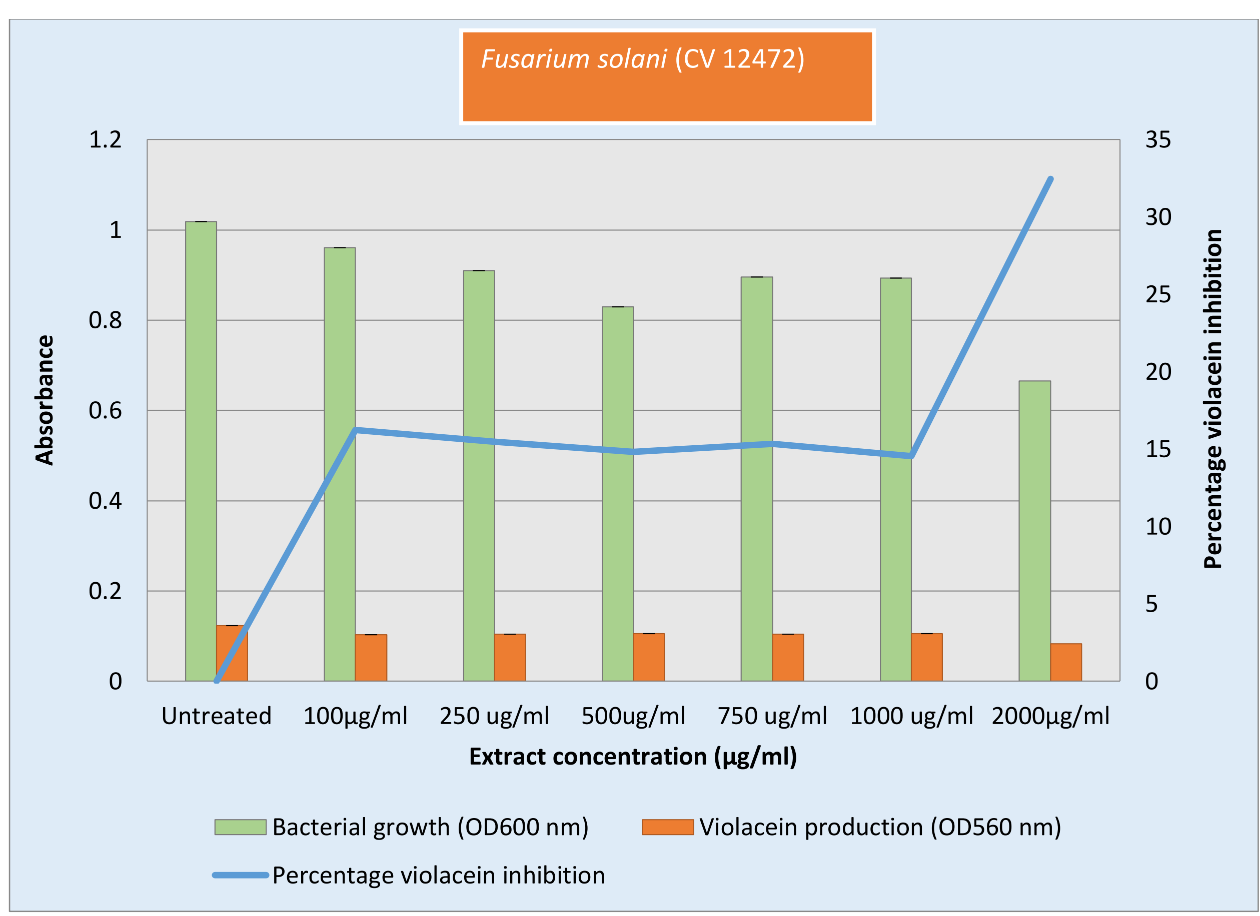


Figure 5: Effects of increasing the *F. solani* supernatant extract concentration on the bacterial growth and violacein production by *Chromobacterium violaceum* (Quantitative violacein assay).

The supernatant extracts from *Fusarium* sp.1 also induced both cell death and violacein production inhibition (Figure 7). However as compared to *F. solani*, there was a fluctuation in the production of the violacein, and hence the percentage violacein inhibition, with different concentration of the extracts. The bacterial growth was constant as compared to the control apart from the last 4 concentrations (250µg/ml, 750µg/ml, 1000µg/ml and 2000µg/ml) in which there was a slight decrease in the bacterial population. From this observation, the violacein inhibition could be majorly but not entirely due to QS inhibition since there was no major cell death observed.

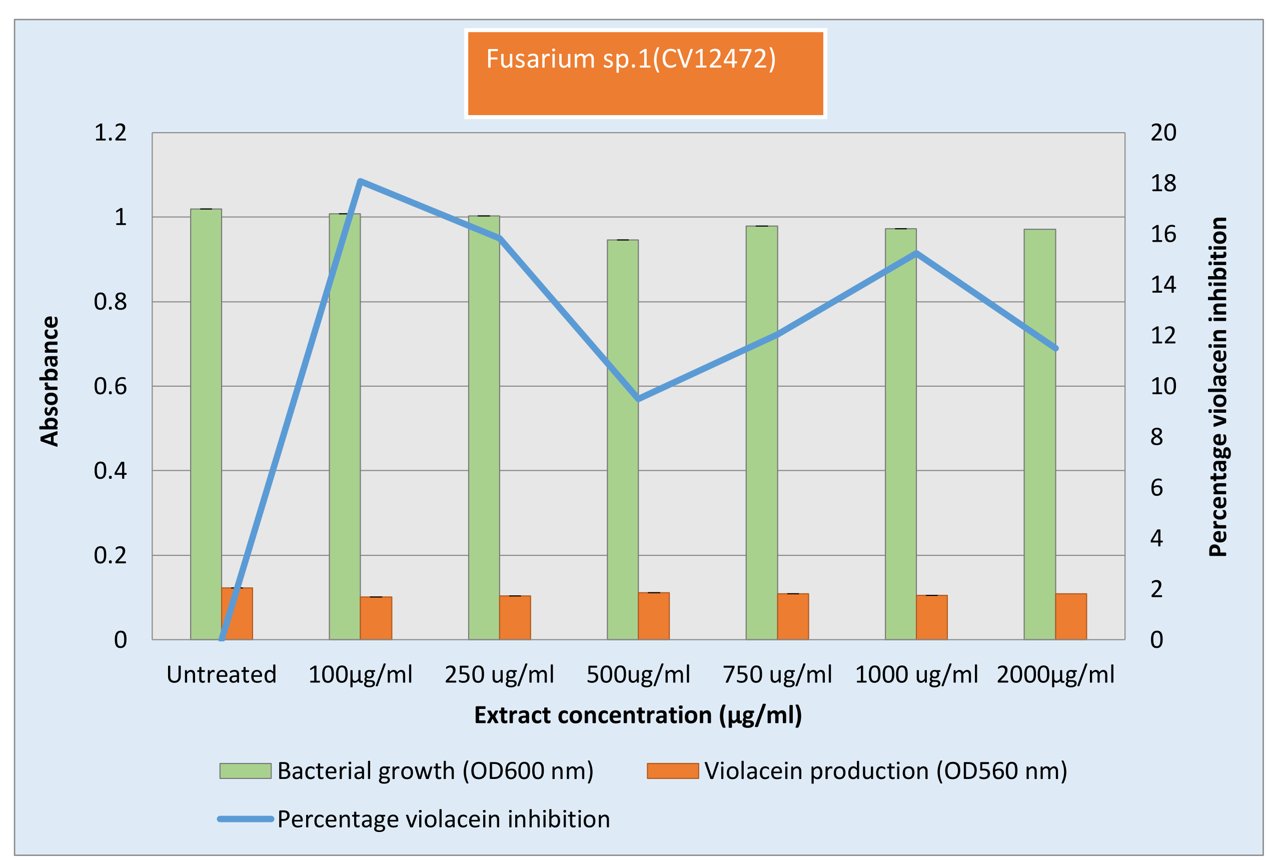


Figure 6: Effects of increasing the *Fusarium* sp.1 supernatant extract concentration on the growth and violacein production by *Chromobacterium violaceum* (Quantitative violacein assay)

The medicinal plant extracts from *Z. gilletii* (Zg. F6) also showed some potential quorum quenching activity with an increase or decrease in the percentage violacein inhibition as well as bacterial growth (Figure 8). The extract concentration of 2000 µg/ml showed the highest violacein inhibition of 29%. At this concentration, the bacterial population was also inhibited the most. Other concentrations tested also exhibited both violacein production inhibition as well as bacterial growth inhibition. This trend exhibited by this extract could be due to violacein inhibition by the extract as well as antibacterial activity of the extract.

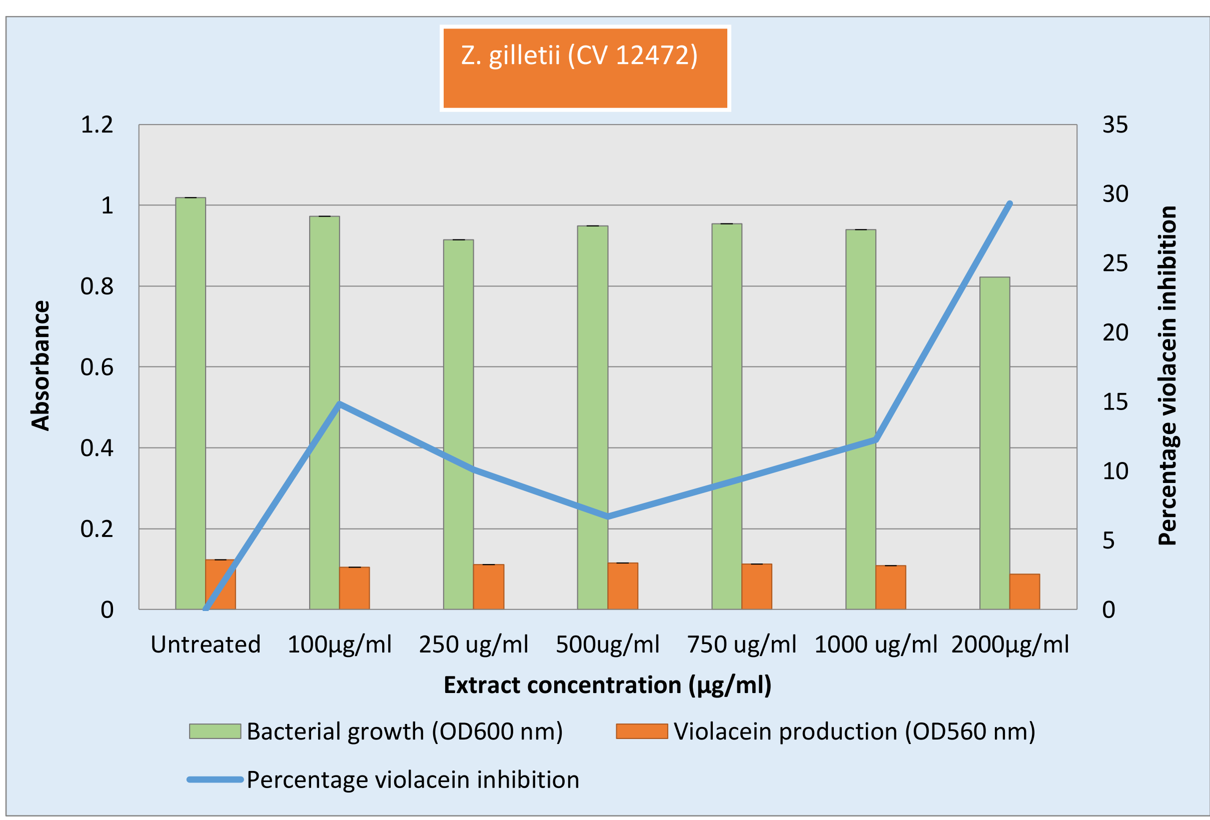


Figure 7: Effects of increasing the medicinal plant extract concentration on the growth and violacein production by *Chromobacterium violaceum* (Quantitative violacein assay)

4.3 CHARACTERIZATION OF THE SECONDARY METABOLITES

In this study, four pure compounds were extracted from both the leaves (compound **5**) and fungal endophyte (compound **13, 14** and **15**) isolated from the two medicinal plants. The pure compounds were analyzed by a combination of mass spectrometry and spectroscopic techniques which included 1D and 2D NMR.

4.3.1 Structural Elucidation of Compound 5

Compound **5** was obtained from the methanol extracts of *Z. gilletii* leaves. It appeared as yellow crystals that crystallized at room temperature. This compound showed a refractive index value of 0.66 in ethyl acetate: hexane (6:4) solvent system. The mass spectrum showed molecular ion at odd number 260 (*M+1*) *m/z* (Figure 10) which corresponds to the molecular formula of $C_{14}H_{13}NO_4$ (Sugeng *et al.*, 2001). This compound contains 14 carbon atoms which were shown by the ^{13}CNMR spectrum (appendix 1). The analysis of the DEPT spectra (appendix 3&4) showed the presence of 4 methine, 3 methoxy while the combination of ^{13}CNMR and HMBC spectra showed the presence of 7 quaternary carbon atoms. The

^{1}HNMR spectrum (appendix 2) further supported the presence of 13 protons in which 4 methine protons occurred as two sets of doublets while the other 9 protons represented 3 sharp methoxy singlets. A pair of coupled signals at 8.01 and 7.24 with a common coupling constant of *J*=9.5Hz were assigned to adjacent carbon atoms C-5 and C-6 respectively. Another pair of doublets occurred at 7.59 and 7.05 with a coupling constant of 2.80Hz. This can be due to the presence of two unsaturated methane protons in the furan ring. The rest of the protons appeared as 3 sharp singlets attributed to methoxy groups whose protons absorbed at 4.44, 4.04 and 4.12 and were assigned to C-4, C-7 and C-8 respectively. The assignment of the protons to their respective carbon atoms was done by HSQC (Appendix 7).

The HMBC spectrum (Appendix 5) showed the correlation between carbons and protons. This spectrum shows the correlation information between protons and carbon atoms which are two to three bonds away. From this spectrum, proton H1 resonating at 7.59 shows the correlation with C2, C3 and C3′. Proton H2 resonating at 7.05 also showed a correlation with C2, C2′ and C3. While proton H3 resonating at 4.44 showed a correlation with C3 and C4. The ^{1}H-^{1}H COSY (Appendix 6) correlation gives the information about the carbons attached to the adjacent carbons. From this spectrum, H2′ correlates with H3′ while H5 correlates with H6. The HMBC and COSY correlation is further illustrated in figure 5. The NMR data for this compound is summarized in Table 3.

Based on the spectra information (Table 3) and literature comparison, compound **5** was identified as 4, 7, 8-trimethoxyfuro [2,3-b] quinolone (Parhoodeh *et al*., 2012). This compound has previously been isolated from different plants of the family Rutaceae, some of which include; *Aegle marmelos* (Sugeng *et al.*, 2001), roots of *Glycosmis elongata* and *G. arborea* (Chakravarty *et al*., 1999; Rahmani *et al.,* 2010) and *Ruta graveolens* (Mancuso *et al.*, 2015).

Table 3: The assignment of ^{13}CNMR, ^{1}HNMR, COSY and HMBC of **compound 5**

NO	^{13}CNMR	TYPE/DEPT	^{1}HNMR	COSY	HMBC	^{1}HNMR (*J*)	^{1}HNMR LIT
2	164.4	Cq	-	-	-	-	-
2′	143	CH	7.59	3′	2,3,3′	2.8H_Z	7.58
3	102	Cq	-	-	-		

3′	104.6	CH	7.05	2′	2,2′,3	2.8Hz	7.03
4	157.2	Cq	-	-	-	-	
4′	59	CH_3	4.44	-	3,4		4.42
4a	114.9	Cq	-	-	-		
5	118.1	CH	8.02	6	4,6,7	9.5 Hz	8.01
5′	56.8	CH_3	4.04	-	-		4.03
6	112	CH	7.24	5	4a,5, 7,8	9.5 Hz	7.23
6′	61.6	CH_3	4.12	-	5′,8,8a		4.12
7	152.1	Cq	-	-	-	-	-
8	141.5	Cq	-	-	-	-	-
8a	142	Cq	-	-	-	-	-

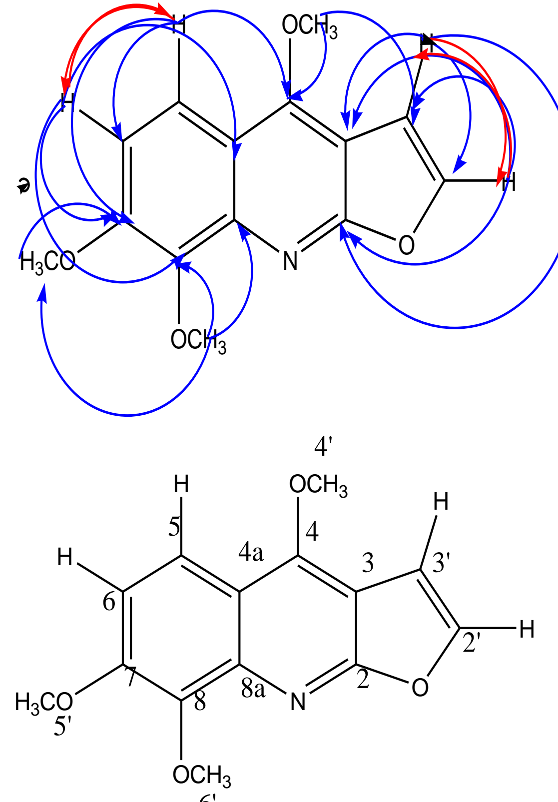


Figure 8: Structure and the HMBC- COSY correlation of compound 5

4.3.2 Structure Elucidation of Compound 15

Compound **15** was obtained from the ethyl acetate extract of *F. solani* (ML2) endophyte. The mass spectrum showed molecular ion at 307 *m/z* which corresponds to the molecular formula of $C_{17}H_{25}NO_4$. The number of the carbon atoms in this formula was confirmed by the ^{13}CNMR spectrum (appendix 8) which had 17 peaks. The ^{13}CNMR and DEPT (appendix 10) spectra showed the presence of two methylene carbon at δ 61.94 (C5) and δ 30.0 (C4′), three methine carbon δ 88.4 (C7), δ 137.5 (C2′) and δ33.7 (C3′), five methyl carbon δ 7.4 (C1″), δ12.2 (C1‴), δ19.4 (C1⁗), δ19.2 (C1″‴) and δ11.1 (C5′) and seven quaternary carbons at δ166.6 (C2), 99.2 (C3), 144.8 (C4), 109.1 (C4a), 68.9 (C8), 163.5 (C8a), 130.7 (C1′).

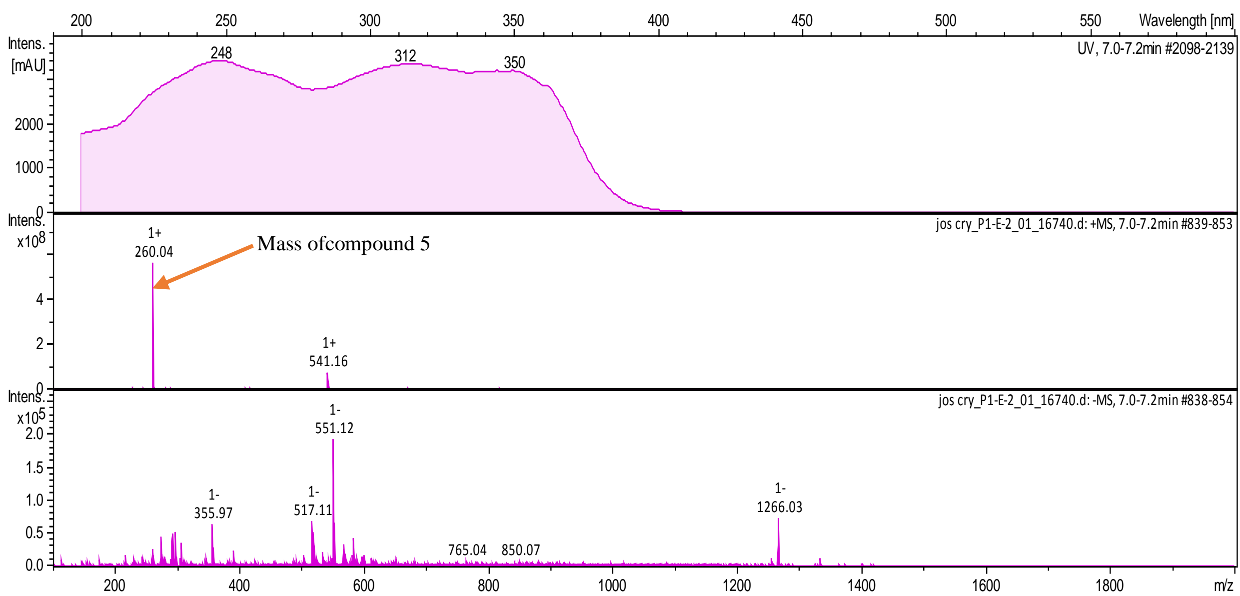


Figure 9: The Mass spectra of compound **5**

The protons attached directly to the carbon atoms were assigned using the HSQC spectrum (appendix 11) and supported using the ^{1}HNMR (appendix 9). These spectra showed the presence of protons with different multiplicities resonating at δ_H4.7 (H5) & 4.4 (H5) (dd *J*=15±0.1 Hz), 3.8 (H7), 1.9(H1″), 1.8 (H1‴) (d, *J*=1.07 Hz), 0.99 (H1⁗) (d, J=6.71), 1.4 (H1″‴), 5.2 (H2′) (d, J=9.47Hz), 2.4 (H3′), 1.4 (H4′), 0.93 (H5′) (d, J1=7.48, J2=7.32 Hz) which were attached to carbons absorbing at 61.94(C5), 88.4 (C7), 7.4 (C1″), 12.2 (C1‴) 19.4 (C1⁗), 19.2 (C1″‴), 137.5(C2′), 33.7(C3′), 30.0 (C4′) and 11.1(C5′) respectively. The

COSY (appendix 13) correlation was used to determine the protons attached to adjacent carbon atoms while the HMBC (appendix 12) was used to assign the 2-3 bond connectivity's in the structure. From this spectrum, proton H1 resonating at 4.72 & 4,42 shows correlation with C3, C4, C4a, C7, C8 and 8a while H2 resonating at 3.81 shows correlation with 1′,1‴,2′, 4a, 5 and 8. Proton H3 resonating at 1.94 shows correlation with C2, C3′, C4 and C4a while proton H4 resonating at 1.81 shows correlation with C1′, 1⁗′, 2′,3′ and 4′. The rest of the HMBC correlations are shown in table 4. The ^{1}H-^{1}H COSY (appendix 13) correlation gives the information about the carbons attached to adjacent carbons. The HMBC and COSY correlation is further illustrated in figure 5.

Data from these spectra was used in the structure elucidation of this compound which is shown in figure 10. Based on the spectra information (Table 4) and the IUPAC naming system, this compound was known as 3,8-dimethyl-7-(4-methylhex-2-en-2-yl)-7,8-dihydro-5*H*pyrano[4,3-*b*] pyridine-2,4,8-triol. This is the first isolation of this compound from *F. solani* and it was given it the trivial name Lucinine.

Table 4: The assignment of ^{13} CNMR, DEPT HSQC and COSY of compound **15**

NO	^{13}CNMR	DEPT	^{1}HNMR/ HSQC	COSY	HMBC	^{1}HNMR (*J*),
2	166.6	Cq	-	-	-	-
3	99.2	Cq	-	-	-	-
4	155.8	Cq	-	-	-	-
4a	109.1	Cq	-	-	-	-
5	61.9	CH_2	4.72, 4.42		3, 4, 4a, 7, 8, 8a	dd *J*=15 ±0.1 *Hz*
7	88.4	CH	3.805		1′, 1‴, 2′, 4a, 5, 8	
8	69	Cq	-	-	-	-
8a	163.5	Cq	-	-	-	-
1′	130.7	Cq	-	-	-	-
1″	7.4	CH_3	1.94		2, 3, 4, 4a,	
1‴	12.2	CH_3	1.81	3′	1′, 1⁗′, 2′, 3′, 4′	d, *J*= 1.07 *Hz*
1⁗	19.4	CH_3	0.99		2′, 3′, 4′	d, *J*= 6.71 *Hz*
1⁗′	19.2	CH_3	1.4		1′,2′, 7, 8, 8a	
2′	137.5	CH	5.2	3′	1‴, 1⁗, 3′,4′, 7	d, *J*= 9.47 *Hz*
3′	33.7	CH	2.43	1‴ 4′ 3′	1′, 1⁗, 2′, 4′, 5′	
4′	30	CH_2	1.43	5′ 3′	1⁗, 2′, 3′, 5′	

5′	11.1	CH_3	0.94	4′	3′, 4′	J_1=7.48, J_2=7.32 *Hz*

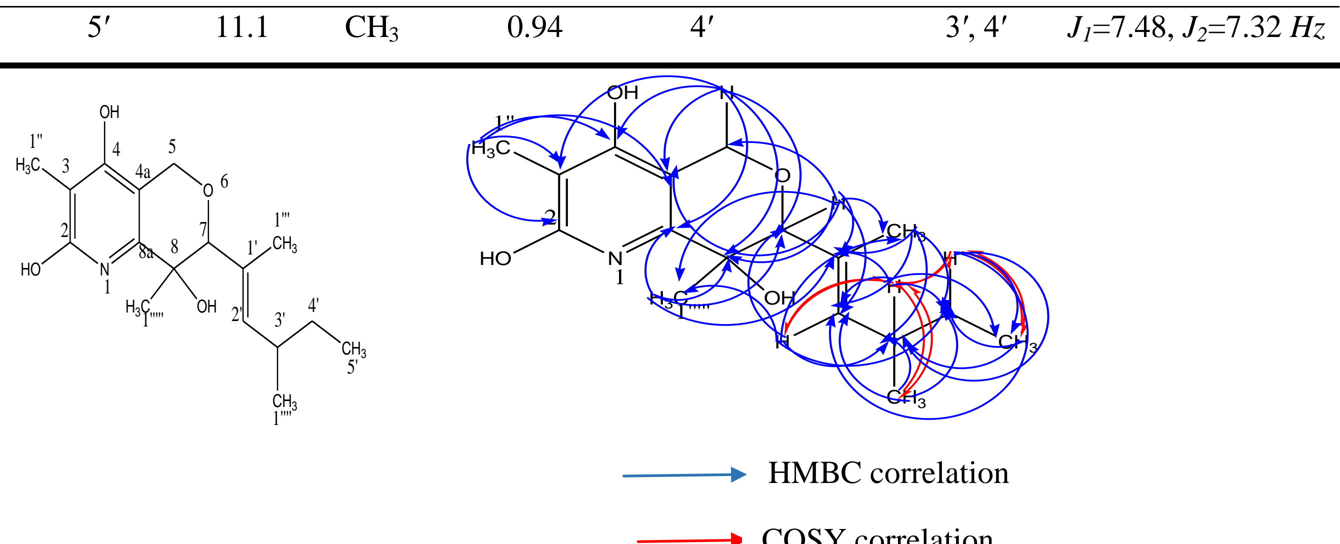


Figure 10: Structure and HMBC-COSY correlation of compound 15

4.3.3 Structure Elucidation of Compound 13

Compound **13** was obtained as a beige powder after drying from the ethyl acetate extract of *F. solani* (ML2) endophyte. The mass spectrum showed molecular ion at 152 *m/z* which corresponds to the molecular formula of $C_8H_8O_3$. The ^{1}HNMR spectrum analysis revealed the presence of a pair of doublets at δ 6.69 (H-3 and H-5 *J*= 8.55 Hz) and δ 7.04 (H-2 and H-6 *J*= 8.55 Hz) each integrating to two hydrogens. From the resonance of these protons, it suggests the presence of two aromatic hydrogen atoms in the *ortho* position. This spectrum also showed a pair of methylene hydrogens at δ 3.42 integrating as one hydrogen atom hence one peek. The HSQC spectra showed that these hydrogen atoms are attached to carbon δ130.7, δ 115.5 and δ 40.3) respectively. The ^{13}CNMR spectrum analysis disclosed the presence of 8 carbon while the DEPT spectrum showed the type of carbon atoms found in this compound. These carbon atoms included 6 aromatic carbon 156.5 (s, C-4′), 130.7 (d, C2′ and C6′), 115.5 (d, C3′ and C5′) and 125.6 (s, C1′), one methylene 40.3 (C2), one carbonyl 173.6 (C1) and 3 quaternary carbon atoms. The HMBC correlation was used to determine the 2-3 bond connectivity's while the COSY correlation was used to assign the Hydrogen attached to adjacent carbon atoms. The HMBC spectrum shows that proton H2 resonating at 3.42 shows correlation with C4′, C6′, 2′ and 1 while proton H2′and H6′ both shows correlation with C2′, C3′, C5′, C6′ and C1′. Protons H3′and 5′ shows correlation with C5′, C4′ and C1′. Further illustrations are shown in table 5.

These data presented in Table 2, shows the presence of a *para*-substituted aromatic ring. Based on the spectra information (Table 5) and literature comparison, compound **13** was identified as 4-hydroxyphenylacetic acid (*p*- hydroxyphenyl acetic acid) (Ohtani *et al.*, 2011).

Table 5: The assignment of ^{13}CNMR, DEPT, HMBC COSY of compound **13**

NO	^{13}CNMR	DEPT	^{1}HNMR/ HSQC	COSY	HMBC	^{1}HNMR (*J*)	LIT ^{13}CNMR	LIT ^{1}HNMR
1	173.6	Cq	-	-	-		173.2	
2	40.3	CH_2	3.42	-	4´6´, 2´, 1		40.4	3.49
1′	125.6	Cq	-	-	-		126.5	
2′ 6′	130.7	CH	7.04	3´, 6´	2´, 3´, 5´, 6´, 1´	d, *J*= 8.55)	131.2	7.14(J=8.7)
3′ 5′	115.5	CH	6.69	2´, 5´	5´, 4´, 1´	(d, *J*= 8.55)	151.9	6.77(J=8.7)
4′	156.5	Cq	-	-	-		157.1	

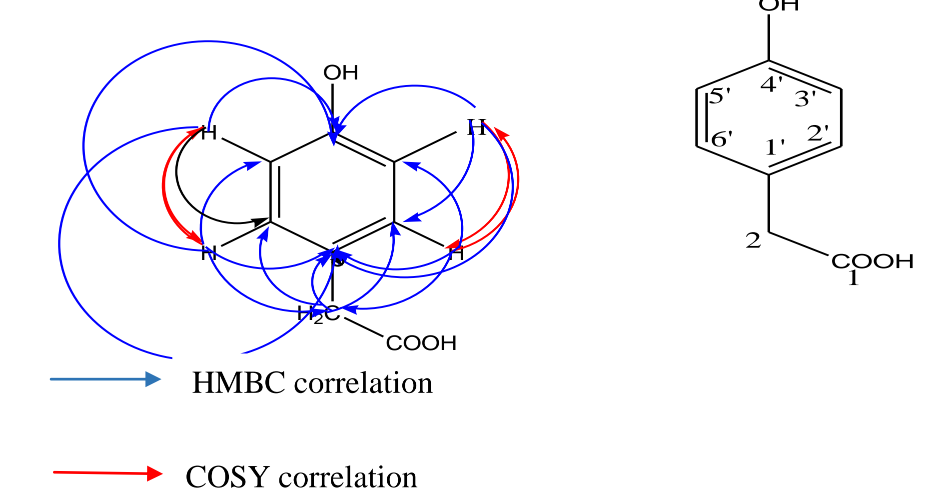


Figure 11: The HMBC-COSY correlation and the structure of compound **13**

This compound has been isolated from natural products such as *Bostrychiaradicans* (*Rhodomelaceae*) (Oliveira *et al.*, 2012) and *Undaria pinnatifida*, marine algae (Abe *et al.*, 1974). *p*-hydroxyphenylacetic acid has been identified as a phytotoxin produced by some plant pathogenic fungi (Aoki *et al.*, 1963). It has also been isolated as a secondary metabolite from fungi belonging to *Oidiodendron* spp. from which it was discovered that it has nematicidal activities against *P. penetrans* and *B. xylophilus* (Ohtani *et al.*, 2011). The

difference in the absorption of the ^{1}HNMR spectrum with the one in the literature could be due to the difference in the solvent used for the NMR. The solvent used in dissolving this compound was methanol while the one used in the literature was deuterated chloroform ($CDCl_3$).

4.3.4 Structure Elucidation of Compound 14

Compound **14** was obtained as a brown liquid from the column. Its chemical formula is $C_8H_8O_3$ with a molecular mass of 152*m/z*. The ^{13}CNMR (appendix 20) showed the presence of 8 carbon atoms absorbing at 172.3, 36.3, 122.5, 156, 119.1, 131.4, 115.3 and 128.8 ppm respectively. DEPT spectrum (appendix 21) showed that this compound has 3-quaternary carbons absorbing at 172.3(C1), 122.5(C1′),156.0(C2′),4-methoxyl,119.1(C3′),131.4 (C4′),115.3(C5′),128.8(C6′) and 1-methylene carbon, 40.3 (C2). From the ^{1}HNMR spectrum (appendix 22), this compound has 8 hydrogen atoms absorbing at 3.45, 6.72, 7.08, 6.78 and 7.05 respectively. The COSY (appendix 23) spectrum was used to determine the hydrogen atoms attached to adjacent carbon atom in space. From this spectrum, carbon 3´ is adjacent to carbon 4´ while carbon 5´ is adjacent to carbon 6´. The 2 to 3 bond connectivity's was confirmed by the HMBC spectra (appendix 25) proton H2 resonating at 3.45 shows correlation with C1, 1′, 2′ and 4′, proton 3′ resonating at 7.72 shows correlation with C1, C2′ and C3′, proton 4′ shows correlation with C1′, C5′ and C6′ while proton 5′ shows correlations with C2′ and C4′.

Based on the spectra information (Table 6) and literature comparison**,** compound **14** was identified as 2-hydroxyphenylacetic acid which an isomer of compound **13** (Yang and Fu, 2010; de Oliveira *et al.*, 2013). 2-hydroxyphenylacetic acid is used as an intermediate in the preparation of biologically active products such as anti-hypertensives in the pharmaceutical industries. Apart from being produced as a secondary metabolite, the compound can also be produced using enzymatic reaction by fungi and also through biotransformation of phenylacetonitrile (de Oliveira *et al.*, 2013).

Table 6: The assignment of ^{13} CNMR, DEPT HSQC and COSY of compound **14**

NO	^{13}CNMR	DEPT	^{1}HNMR/HSQC	COSY	HMBC
1	172.3	Cq	-	-	
2	36.3	CH_2	3.45	-	1, 1′, 2′, 4′
1′	122.5	Cq	-	-	-

2′	156	Cq	-	-	-
3′	119.1	CH	6.72	4′	1, 2′, 3′
4′	131.4	CH	7.08	3′	1′,5′, 6′
5′	115.3	CH	6.78	6′	2′,4′
6′	128.8	CH	7.05	5′	2, 2′,6′

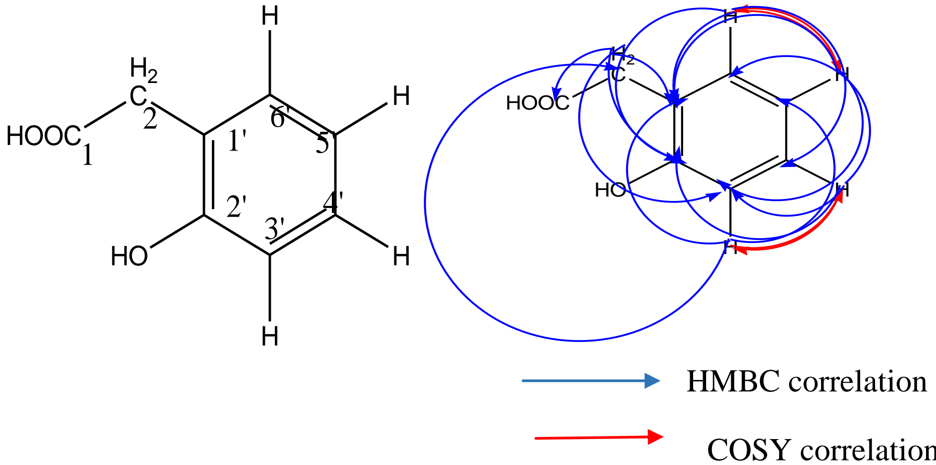


Figure 12**:** The structure and the HMBC-COSY correlation of compound 14

CHAPTER FIVE

DISCUSSION

5.1 Isolation and Identification of the Fungal Endophytes

In this study, fresh leaves of the medicinal plants (*Z. gilletii* and *M. lutea*) were used for the isolation of the endophytes in SDA media as well as extraction of secondary metabolite. As compared to this work, several reports have indicated leaf tissues as a source of endophytic fungi (Suryanarayanan *et al.*, 2009). This may relate to how the endophytes penetrate the plant tissues through the aerial interaction and it is also considered that the leaf tissues provide favorable conditions for fungus penetration (Banhos *et al.*, 2014). This make their identification using morphological characteristics especially the morpho species difficult (Liu *et al.*, 2009) hence making the molecular identification the most appropriate.

As per the results obtained from the gel electrophoresis, 41 out of 51 isolates (80%), could be amplified by PCR at the ITS region using ITS1F and ITS4 set of primers while the remaining 20% did not show any amplification bands. This could be due to the production of PCR inhibitory secondary metabolites such as humic acid, fluvic acid among others, during the growth period. The production of these secondary metabolites inhibit the amplification of the ITS region of the fungal genome (Paterson, 2004). Another reason could be due to primer mismatch bias which is also known to impede PCR amplification (Ihrmark, 2012). The DNA band sizes of 500-700 base pairs were obtained from the separation in the gel as expected from the size of the ITS region of the fungal genome. This kind of amplification was also noted by Bellemain *et al.*, 2010. The forward and the reverse sequence data showed that 31 sequences produced readable consensus sequences after alignment with Genious R7 program. The rest of the sequences could not be assembled to produce readable consensus sequence. This could be due to the errors that might have occurred during the DNA purification stage or the sequencing stage that is a very sensitive technique (Paterson, 2004).

The BLAST search of the sequences showed that 95% of the isolated fungi belonged to the phylum Ascomycota and 5% to Basidiomycota, with 75% of the fungal endophytes belonging to the genus Fusarium. Various research works show that endophytic fungi mostly consist of members of Ascomycota although some taxa of Basidiomycota, Zygomycota and Oomycote have also been isolated (Guo *et al.*, 2001).The results from this study correlate with the results obtained by (BaiWan *et al.*, 2009; Chen *et al.*, 2010; Xing *et al.*, 2011) which shows that fungal endophytes have been isolated from various medicinal plants and the dominant strains belong to the genus Fusarium. Chen *et al.* (2010) explains that fungal

endophyte diversity in a plant could be due to environmental factors, host species and the host genotype. The genus Fusarium is a common inhabitant in many plants with more than 120 species being identified (Michielse and Rep, 2009). Although Fusarium spp. are always considered as fungal pathogens on plants, they are often isolated as endophytes from various plants and they are also capable of producing various secondary metabolites with medicinal properties (Deng *et al.*, 2009; Tayung *et al.*, 2011). Bacon and Yates (2006) also notes that endophytic Fusarium are capable of inducing plant host resistance to pathogens and increase the plants environmental fitness. This adaptation enables them to produce various secondary metabolites that have medicinal properties such as antimicrobial and anticancer (Shiono *et al.*, 2007). It is worth noting that despite their biomedical importance, various Fusarium strains have not been identified to the species level and have not been phylogenetically characterized hence making their phylogenetic identification quite difficult (Hidayat *et al.*, 2016).

Another group of fungi that was isolated in this study was *Chaetomium* species from both *M. lutea* and *Z. gilletii*. *Chaetomium* belongs to the family Chaetomiceae which has more than 100 species. This genus has been reported to be a large source of secondary metabolites such as azaphilones, terpenoids, steroids, chaetoglobosins, xanthones, chromones, epipolythiodioxopiperazines, depsidones and anthraquinones (Fatima *et al.*, 2016). As compared to this study, this group of fungi have been isolated as endophytes from various plants such as *Ephedra fasciculate* (Bashyal *et al.*, 2005), *Ginkgo biloba* (Qin *et al.*, 2009), Marine red algae *Polysiphonia urceolata* (Wang *et al.*, 2006), *Populus tomentosa* and *Aspidosperma tomentosum* (Rodrigues *et al.*, 2005; Gao *et al.*, 2005), *Adenophora axilliflora* and *Imperata cylindrica* (Jiao *et al.*, 2006). In addition, it has also been isolated from *Aegle marmelos* (Gond *et al.*, 2007) which belongs to the same family Rutaceae as *Z. gilletii* among others. Studies have shown that the secondary metabolites from Chaetomium species can be used in medicine and can also be applied in agriculture (Abdullah and Azzo, 2015).

The other endophytic fungi that were found to be in association with these two medicinal plants were *Scopulariopsis flava* and *S. brevacaulis*. These species are widely distributed especially in the marine environment and they are also important as human pathogens (Dewey *et al.*, 1984; Cuenca-Estrella *et al.*, 2003). They have been isolated as fungal endophytes from lichens (Li *et al.*, 2007), from marine sponge *Tethya aurantium* (Wiese *et al.*, 2011) among others. Despite their wide distribution, little work has been done

in identification of the secondary metabolites in this genus (Yu *et al.*, 2008). Some of the secondary metabolites isolated from this genus are Scopularides A and B and an antifungal pyranol derivative (Cuenca-Estrella *et al.*, 2003; Yu *et al.*, 2008).

Apart from the ascomycetes that were isolated in this study, a basidiomycete *Trametes* aff. *maxima* was also isolated from *Z. gilletii*. This is a white rot fungus that belongs to the family Polyporales. As compared to this work, fungi of genus *Trametes* have been reported as endophytes from various plants such as *Theobroma giler*, *T. Cocoa*, *Podophyllum hexandrum* and *Taxus globose* (Crozier *et al.*, 2006; Puri *et al.*, 2006; Rivera-Orduña *et al.*, 2011). This genus has been reported to contain various medically important secondary metabolites such as the Krestin which is used as antitumor (Puri *et al.*, 2006). Apart from production of secondary metabolites, some species in this genus such as *T. vaseculour* has been used to transform chlorophenols which is an environmental pollutant. Hence fungi of this genus can be used in the isolation of novel antibacterial compounds. This study therefore revealed the presences of diverse species of endophytic fungi inhabiting these two medicinal plants.

5.2 Antibacterial Activity of Isolated Fungal Endophytes and Extracts from the Plants and the Endophytes

From the dual culture antagonistic assay against two test organisms *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola*, most (80%) of the active fungal endophytes belonged to the genus Fusarium with varied species displaying different antimicrobial activity. Endophytic fungus initially given the code Ml.2 and identified as *F. solani*, produced the largest zone of inhibition. Its activity was not statistically different from that of chloramphenicol by producing a zone of inhibition of 20.3mm while the other Fusarium species also showed some activity by different zones of inhibitions. This kind of trend was also observed by Gong and Guo (2009) and Vaz *et al.* (2009) in which different species of Fusarium such as *F. oxysporum* as well as other species displayed varying antimicrobial activity. Different sub species of *F. oxysporum* also exhibited different levels of activity against the test organisms. This kind of trend was also noted by Sette *et al.* (2006). Endophytic Chaetomium spp. did not show any activity against the test organisms. In contrast to this study, other species of endophytic Chaetomium such as *Chaetomium globosum* were found to be active against some gram negative human bacterial strains (Momesso *et al.*, 2008; Rani *et al.*, 2017). Other strains that exhibited some potential activity were *Trametes* aff. *maxima*. It produced a zone of inhibition of approximately 12mm against both strains which

was statistically different from that of the control. Fungal species from this genus have been shown to possess antibacterial activity against gram positive and gram-negative bacteria. For instance, in a research done by Waithaka *et al.* (2017), *Trametes gibbosa* showed potential activity against human gram negative bacteria as well as gram negative bacteria.

When subjected to solid fermentation in rice media and later fractionation *Fusarium solani* (Ml.2) produced two fractions; ethyl acetate extract and hexane extracts which were both active against the two-test organism and their inhibition zones were statistically different as compared to chloramphenicol standard. These results are in comparison with those obtained by Devaraju and Satish (2011) and Specian *et al.* (2012) in which different extracts from fungal endophytes isolated from various plants were active against *X. axonopodis* pv. *phaseoli.*

Fractionation of the crude extracts from the two medicinal plants led to the production of eight fractions which were subjected to antimicrobial analysis. The activity of these extracts against the test organisms were different depending on the level of fractionation. In the case of *Z. gilletii*, the methanol crude and the methanol extracts after SPE were not active against both test organisms while the ethyl acetate extract after SPE was active against *X. axonopodis* pv. *phaseoli*. This could be because the extracts did not contain the active secondary metabolites or the percentage composition of the active components in the crude extracts may be too low to show any activity (Tavares *et al.*, 2014). Skimmianine (compound **5**) obtained from the methanol extract after SPE also exhibited activity against *X. axonopodis* pv. *phaseoli*. These results are in comparison to those obtained by Tavares *et al.* (2014) in which the alkaloids isolated from *Zanthoxylum rhoifolium* showed activity against both the gram positive and the gram negative test organisms. As for the extracts from *M. lutea*, the most active were ethyl acetate extract after partition which produced an inhibition zone of 14±2mm. The crude methanol extract, the methanol extracts after SPE and ethyl acetate extract after SPE exhibited little activity against *X. axonopodis* pv. *phaseoli* while there was no activity against *P. syringae* pv. *phaseolicola*. All the medicinal plant extracts were not active against *P. syringae* pv. *phaseolicola.* As compared to this work, extracts from *Ginkgo biloba* also showed activity against *X. axonopodis* pv. *phaseoli* (Sati and Joshi, 2011). In contrast to these results, garlic extracts have been shown to inhibit the growth of *P. syringae* pv. *phaseolicola in vitro* (Hassan Eman and El-Meneisy Afaf, 2014). Different strains of *Pseudomonas* species of bacteria have been known for their antibacterial resistance against the available antibiotics. There are different modes of acquisition antibacterial resistance in these gram-negative bacteria some of which are horizontal gene transfer from other bacterial

in the ecosystem and the presence of the efflux pumps. E- flux pumps are intrinsic resistance mechanism which are known for substrate specificities as well as drug extrusion. This prevents the accumulation of antibacterial drug in the bacterial system and thus from reaching its target site (Sun *et al.*, 2014).

5.3 Anti-Quorum Sensing Activity of Metabolites from *Z. Gilletii*, *M. Lutea* and their Endophytes

This study also went further to study the anti-quorum sensing capabilities of the isolated fungal endophytes together with the secondary metabolites isolated from them. The quorum sensing system controlled phenotypic characteristic, producing a purple pigment, of *C. violaceum* was used to screen the potential of extracts from medicinal plants; *M. lutea* and *Z. gilletii* and their endophytes. The findings of this study indicate that medicinal plants *Z. gilletii* and endophytic *F. solani* and other *Fusarium* species are a potential source of quorum quenching compounds. The extracts from *M. lutea* and other fractions from *Z. gilletii* did not show any quorum quenching activity though they showed antibacterial activity. Similar observation was also noted by Zaki *et al.* (2012) in which ethanolic extracts from the leaves of *S. molle* did not show any quorum quenching activity while the rest of the extracts exhibited antibacterial as well as anti-QS activity. The ethyl acetate extract from *Z. gilletii* and the supernatant extracts of *F. solani* and *Fusarium* sp. 1 showed activity in the qualitative assay, which was concentration based. The extracts showed both antibacterial activity and quorum quenching activity which increased with extract concentration. These results correspond to the results obtained by Zahin *et al.* (2010), in which medicinal plants from India showed both antibacterial activity and quorum quenching activity at different concentrations. It was noted that the supernatant extracts from the liquid fermentation of the Fusarium strain showed significant activity while those from the solid rice media did not show any activity. As noted by (Jia *et al.*, 2016) the type of secondary metabolites produced are usually affected by the mode of fermentation as well as the media used.

The mechanism of action by the phytochemicals responsible for the QS interference of these crude extracts can be assumed to be direct or indirect (Zahin *et al.*, 2010). Some of these compounds may be responsible for inhibitions of biosynthesis of the signal molecule by the *luxI* encoded AHL synthase or targets the luxR signal receptor (Rasmussen *et al.*, 2005) while some can be responsible for the biodegradation of the QS molecules (Defoirdt *et al.*, 2004).

Research shows that plant extracts that contain high concentration of phenolic compounds such as garlic acids are known to possess quorum quenching activates (Bodini *et al.*, 2009) Therefore, the activity shown by *F. solani* and *Fusarium* species 1 could be partly attributed to the hydroxyphenyl acetic acids (Compound **13** and compound **14**) extracted from this species in this study

5.4 Characterization of the Secondary Metabolites

Four pure compounds were isolated from the leaves and the endophytic fungi analyzed in this study. The fractionation of the plants extracts led to the isolation of 4, 7, 8-trimethoxyfuro [2,3-b] quinolone, compound **5.** This alkaloid has been isolated from different medicinal plants in the family Rutacea such as *Glycosmis elongate, G. arborea, Zanthoxylum integrifoliolum* among others (Chen *et al.*, 2005; Mancuso *et al.*, 2015).

Further fractionation of the ethyl acetate extract of *F. solani* (ML.2) led to the extraction of two different hydroxyphenyl acetic acid (p- hydroxyphenyl acetic acid (Compound **13**) and 2- hydroxyphenyl acetic acid (compound **14**) and (*E*)-3,8-dimethyl-7-(4-methylhex-2-en-2-yl)-7,8-dihydro-5*H*pyrano[4,3-*b*]pyridine-2,4,8-triol(compound **15**). The hydroxyphenyl acetic acids (Compound **13** and **14**) have also been isolated as natural products from marine algae such as *Bostrychia radicans* (*Rhodomelaceae*) (Oliveira *et al.*, 2012) and *Undaria pinnatifida* (Abe *et al.*, 1974). *p*-hydroxyphenylacetic acid has been identified as a phytotoxin produced by some plant pathogenic fungi such as *Colletotrichum gloeosporioides* (Aoki *et al.*, 1963; Chapla *et al.*, 2014). P- hydroxyphenyl acetic acid has also been isolated as a secondary metabolite from fungi belonging to *Oidiodendron* species from which it was discovered that it has nematicidal activities against *Pratylenchus penetrans* and *Bursaphelenchus xylophilus* (Ohtani *et al.*, 2011). This is the first report on the isolation and structure elucidation of Compound **15** hence it was given the trivial name Lucinine.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

From this study, it is evident that diverse types of fungal endophytes, both the ascomycetes and the basidiomycetes, inhabit the two medicinal plants; *Z. gilletii* and *M. lutea* with the highest percentage being the ascomycetes. Fusarium spp. exhibited the highest isolation frequency from the leaves of both medicinal plants whereas Chaetomium sp, Scopulariopsis sp. and Trametes sp. exhibited the lowest isolation frequency. The ITS primers used in this study could identify the isolated fungi to the genus level with only a low percentage being identified to the species level.

From dual culture antagonistic assay of the isolated fungal endophytes, it is evident that *F. solani* and other Fusarium species, *T. aff. maxima* possess antibacterial activity due to a mixture of secondary metabolites that can be used to control *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola*. The endophytic fungi *F. solani* and Fusarium sp. 1 and the extract from *Z. gilletii* possess quorum quenching activity on *C. violaceum* bioindicator. These extracts showed a concentration based inhibition in which at different concentrations these extracts showed both quorum quenching activity and antibacterial activity.

Fractionation of the extracts from both the medicinal plants and endophytic *F. solani* led to the isolation of four pure compound namely Skimmianine (**5**), 4-hydroxyphenylacetic acid (compound **13**), 2-hydroxyphenylacetic acid (Compound **14**) and compound **15**. This the first report on the isolation of compound **15**.

6.2 RECOMMENDATIONS

1. Further studies on the isolation and identification of the endophytic fungi that inhabit both *M. lutea* and *Z. gilletii* should be done using different methods and selective media commercially available. In addition, alternative molecular markers such as β-tubulin gene, Elongation Factor-1 α gene should be used in the identification of the isolated fungi in addition to the commonly used ITS markers.
2. This study has shown that the extracts from these two medicinal plants are promising in the control of *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *phaseolicola* which should be considered for further investigations. It provides new leads towards the control of these two bacterial pathogens using extracts from *M. lutea* and *Z. gilletii* as

well as their endophytic fungi. There is need to popularize organic/biodegradable pesticides which are hoped to be of no harm to mankind and animals.

3. The active compounds isolated from the fungal endophytes and plants extracts can be extracted in large scale to make a biodegradable pesticide. Apart from that, further fractionation analysis of the extracts from *F. solani* and the medicinal plants is recommended.

REFERENCES

Abawi, G. S., Ludwig J. W. and Gugino B. K. (2006). Bean root rot evaluation protocols currently used in New York. *ANNUAL REPORT-BEAN IMPROVEMENT COOPERATIVE,* **49**: 83.

Abd-El-Khair, H., Khalifa, R. M. and Haggag, K. (2010). Effect of *Trichoderma* species on damping off diseases incidence, some plant enzymes activity and nutritional status of bean plants. *J American Sci,* **6**: 486-497.

Abdullah, S. K. and Azzo, N. M. (2015). Two New Records of *Chaetomium* Species Isolated From Soil under Grapevine Plantations and a Checklist of the Genus in Iraq. *Journal of Agricultural Technology,* **11**: 1515-1522.

Abe, H., Uchiyama, M. and Sato, R. (1974). Isolation of phenylacetic acid and its p-hydroxy derivative as auxin-like substances from *Undaria pinnatifida*. *Agricultural and Biological Chemistry,* **38**: 897-898.

Addae Mensah, I. (1992). Towards a rational scientific basis for herbal medicine, a phytochemists two-decade contribution. *Accra: Ghana Universities Press vii*.

Adesina, S. K. and Reisch, J. (1988). Arnottianamide and other constituents of *Zanthoxylum gillettii* root. *Journal of natural products,* **51**: 601-602.

Akhavan, A., Bahar, M., Askarian, H., Lak, M. R., Nazemi, A. and Zamani, Z. (2013). Bean common bacterial blight: pathogen epiphytic life and effect of irrigation practices. *SpringerPlus,* **2**: 41.

Akibode, S and Maredia, M. (2011). Global and regional trends in production, trade and consumption of food legume crops. Report Submitted to CGIAR Special Panel on Impact Assessment, 27 March 2011. 83 pp. *impact. cgiar. org/sites/default/files/images/Legumetrendsv2. pdf*.

Allen, D. J. (1983). *The pathology of tropical food legumes: disease resistance in crop improvement*: John Wiley and Sons.

Alva, P., McKenzie, E. H. C., Pointing, S. B., Pena-Muralla, R. and Hyde, K. D. (2002). Do sea grasses harbour endophytes? *Fungal Diversity Research Series*.

Aoki, H., Sassa, T. and Tamura, T. (1963). Phytotoxic metabolites of *Rhizoctonia solani*. *Nature,* **200**: 575-575.

Arnold, A. E. (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biology Reviews,* **21**: 51-66.

Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D. and Kursar, T. A. (2000). Are tropical fungal endophytes hyperdiverse? *Ecology Letters,* **3**: 267-274.

Arnold, D. L., Lovell, H. C., Jackson, R. W. and Mansfield, J. W. (2011). *Pseudomonas syringae* pv. *phaseolicola:* from 'has bean'to supermodel. *Molecular Plant Pathology,* **12**: 617-627.

Aroca, A., Raposo, R. and Lunello, P. (2008). A biomarker for the identification of four *Phaeoacremonium* species using the β-tubulin gene as the target sequence. *Applied Microbiology and Biotechnology,* **80**: 1131-1140.

Audy, P., Braat, C. E., Saindon, G., Huang, H C. and Laroche, A. (1996). A rapid and sensitive PCR-based assay for concurrent detection of bacteria causing common and halo blights in bean seed. *Phytopathology,* **86**: 361-366.

Babu, K. R. and Satyanarayana, T. (1996). Production of bacterial enzymes by solid state fermentation. *Journal of Scientific & Industrial Research,* **55**: 464-467.

Bacon, C. W. and Yates, I. E. (2006). Endophytic root colonization by *Fusarium* species: histology, plant interactions, and toxicity. In *Microbial Root Endophytes* (pp. 133-152): Springer.

BaiWan, D., KaiHui, L., WenQiang, C., XiaoWei, D. and XiuChao, X. (2009). *Fusarium solani*, Tax-3, a new endophytic taxol-producing fungus from *Taxus chinensis*. *World Journal of Microbiology & Biotechnology,* **25**: 139-143.

Banhos, E. F., Souza, A. Q. L, ., Andrade, J. C., Souza, A. D. L., Koolen, H. H. F. and Albuquerque, P. M. (2014). Endophytic fungi from *Myrcia guianensis* at the Brazilian Amazon: distribution and bioactivity. *Brazilian Journal of Microbiology,* **45**: 153-162.

Barnett, H. L. and Hunter, B. B. (1972). Illustrated genera of imperfect fungi: JSTOR.

Barrios-González, J. and Mejía, A. (1996). Production of secondary metabolites by solid-state fermentation. *Biotechnology Annual Review,* **2**: 85-121.

Bashyal, B. P., Wijeratne, E. M K., Faeth, S. H. and Gunatilaka, A. A. L. (2005). Globosumones A− C, Cytotoxic Orsellinic Acid Esters from the Sonoran Desert Endophytic Fungus *Chaetomium globosum* 1. *Journal of Natural Products,* **68**: 724-728.

Beebe, S. E., Rao, I. M., Blair, M. W. and Acosta-Gallegos, J. A. (2013). Phenotyping common beans for adaptation to drought. *Drought Phenotyping in Crops: From Theory to Practice,* **4**: 1-20.

Beebe, S. E., Rao, I. M., Mukankusi, C. and Buruchara, R. (2012). Improving resource use efficiency and reducing risk of common bean production in Africa, Latin America, and the Caribbean. In: Centro Internacional de Agricultura Tropical (CIAT).

Belete, T. and Bastas, K. K. (2017). Common Bacterial Blight (*Xanthomonas axonopodis* pv. *phaseoli*) of Beans with Special Focus on Ethiopian Condition. *J Plant Pathol Microbiol,* **8**: 2.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P. and Kauserud, H. (2010). ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC Microbiology,* **10**: 189.

Birch, P. R. J., Hyman, L. J., Taylor, R., Opio, A. F., Bragard, C. and Toth, I. K. (1997). RAPD PCR-based differentiation of *Xanthomonas campestris* pv. *phaseoli* and *Xanthomonas campestris* pv. *phaseoli* var. *fuscans*. *European Journal of Plant Pathology,* **103**: 809-814.

Bobbarala, V., Katikala, P. K., Naidu, K. C. and Penumajji, S. (2009). Antifungal activity of selected plant extracts against phytopathogenic fungi *Aspergillus niger* F2723. *Indian Journal of Science and Technology,* **2**: 87-90.

Bode, H. B., Bethe, B., Höfs, R. and Zeeck, A. (2002). Big effects from small changes: possible ways to explore nature's chemical diversity. *ChemBioChem,* **3**: 619-627.

Bodini, S. F., Manfredini, S., Epp, M., Valentini, S. and Santori, F. (2009). Quorum sensing inhibition activity of garlic extract and p- coumaric acid. *Letters in Applied Microbiology,* **49**: 551-555.

Borges, W. S., Borges, K. B., Bonato, P. S., Said, S. and Pupo, M. T. (2009). Endophytic fungi: natural products, enzymes and biotransformation reactions. *Current Organic Chemistry,* **13**: 1137-1163.

Buruchara, R. (2007). Background information on Common Beans (*Phaseolus vulgaris* L) in Biotechnology, Breeding & Seed Systems for African Crops. *The Rockefeller Foundation, Nairobi*.

Buruchara, R., Chirwa, R., Sperling, L., Mukankusi, C., Rubyogo, J. C., Mutonhi, R. and Abang, M. M. (2011). Development and delivery of bean varieties in Africa: the Pan-Africa Bean Research Alliance (PABRA) model. *African Crop Science Journal,* **19**: 227-245.

Chakravarty, A. K., Sarkar, T., Masuda, K. and Shiojima, K. (1999). Carbazole alkaloids from roots of *Glycosmis arborea*. *Phytochemistry,* **50**: 1263-1266.

Chapla, V. M., Zeraik, M. L., Leptokarydis, H., Silva, G. H., Bolzani, V. S., Young, M. C. M., Pfenning, L. H. and Araújo, A. R. (2014). Antifungal compounds produced by *Colletotrichum gloeosporioides*, an endophytic fungus from *Michelia champaca*. *Molecules,* **19**: 19243-19252.

Chen, J., Fang, H., Duh, C. and Chen, I. (2005). New indolopyridoquinazoline, benzo [c] phenanthridines and cytotoxic constituents from *Zanthoxylum integrifoliolum*. *Planta Medica,* **71**: 470-475.

Chen, X. M., Dong, H.L., Hu, K.X., Sun, Z.R., Chen, J. and Guo, S.X. (2010). Diversity and antimicrobial and plant-growth-promoting activities of endophytic fungi in *Dendrobium loddigesii* Rolfe. *Journal of Plant Growth Regulation,* **29**: 328-337.

Chepkirui, C., Richter, C., Matasyoh, J. C. and Stadler, M. (2016). Monochlorinated calocerins AD and 9-oxostrobilurin derivatives from the basidiomycete *Favolaschia calocera*. *Phytochemistry,* **132**: 95-101.

Chin, Y., Balunas, M. J., Chai, H. B. and Kinghorn, A. D. (2006). Drug Discovery from Natural Sources. *The AAPS Journal,* **8**: E239-E253.

Choudhury, S., Datta, S., Talukdar, A. D. and Choudhury, M. D. (2011). Phytochemistry of the Family Bignoniaceae-A review. *Assam University Journal of Science and Technology,* **7**: 145-150.

Clay, K. (1990). Fungal endophytes of grasses. *Annual Review of Ecology and Systematics,* **21**: 275-297.

Cobley, L. S. and Steele, W. M. (1976). An Introduction to the Botany of Tropical Crops Longman. *London,* **371**.

Colegate, S. M. and Molyneux, R. J. (2008). Bioactive Natural Products: Detection. *Isolation, and Structural Determination, New York, USA*.

Cordero, C. P., Gómez-González, S., León-Acosta, C. J., Morantes-Medina, S. J. and Aristizabal, F. A. (2004). Cytotoxic activity of five compounds isolated from Colombian plants. *Fitoterapia,* **75**: 225-227.

Cragg, G. M. and Newman, D. J. (2005). Biodiversity: A continuing source of novel drug leads. *Pure and Applied Chemistry,* **77**: 7-24.

Crozier, J., Thomas, S. E., Aime, M. C., Evans, H. C. and Holmes, K. A. (2006). Molecular characterization of fungal endophytic morphospecies isolated from stems and pods of *Theobroma cacao*. *Plant Pathology,* **55**: 783-791.

Cuenca-Estrella, M., Gomez-Lopez, A., Mellado, E., Buitrago, M. J., Monzón, A. and Rodriguez-Tudela, J. L. (2003). *Scopulariopsis brevicaulis*, a fungal pathogen

resistant to broad-spectrum antifungal agents. *Antimicrobial Agents and Chemotherapy,* **47**: 2339-2341.

Damte, D., Gebru, E., Lee, S. J., Suh, J. W. and Park, S. C. (2013). Evaluation of anti-quorum sensing activity of 97 indigenous plant extracts from Korea through bioreporter bacterial strains *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. *Journal of Microbiology Biochemistry and Technology,* **5**: 42-46.

Darrasse, A., Bureau, C., Samson, R., Morris, C. E. and Jacques, M. A. (2007). Contamination of bean seeds by *Xanthomonas axonopodis* pv. *phaseoli* associated with low bacterial densities in the phyllosphere under field and greenhouse conditions. *European Journal of Plant Pathology,* **119**: 203-215.

De Kievit, T. R. and Iglewski, B. H. (2000). Bacterial quorum sensing in pathogenic relationships. *Infection and Immunity,* **68**: 4839-4849.

de Oliveira, J. R., Mizuno, C. M., Seleghim, M. H. R., Javaroti, D. C. D., Rezende, M. O. O., Landgraf, M. D., Sette, L. D. and Porto, A. L. M. (2013). Biotransformation of phenylacetonitrile to 2-hydroxyphenylacetic acid by marine fungi. *Marine Biotechnology,* **15**: 97-103.

Deckert, J. and Struhl, K. (2001). Histone acetylation at promoters is differentially affected by specific activators and repressors. *Molecular and Cellular Biology,* **21**: 2726-2735.

Defoirdt, T., Boon, N., Bossier, P. and Verstraete, W. (2004). Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. *Aquaculture,* **240**: 69-88.

Deng, B. W., Liu, K. H., Chen, W. Q., Ding, X. W. and Xie, X. C. (2009). *Fusariumásolani,* Tax-3, a new endophytic taxol-producing fungus from *Taxusá chinensis*. *World Journal of Microbiology and Biotechnology,* **25**: 139.

Devaraju, R. and Satish, S. (2011). Endophytic Mycoflora of L. and Studies on Antimicrobial Activity of its Endophytic sp. *Society of Applied Sciences,* **2**: 75-79.

Dewey, F. M., Hunter-Blair, C. M., Banbury, G. H. and Donnelly, K. A. (1984). Isolation of *Scopulariopsis brevicaulis* from *Eucheuma striatum* and its ability to degrade seaweeds and their soluble products. *Transactions of the British Mycological Society,* **83**: 621-629.

Dharani, N. (2002). *Field guide to common trees & shrubs of East Africa*: Struik.

Donadio, S., Carrano, L., Brandi, L., Serina, S., Soffientini, A., Raimondi, E., Montanini, N., Sosio, M. and Gualerzi, C. O. (2002). Targets and assays for discovering novel antibacterial agents. *Journal of Biotechnology,* **99**: 175-185.

Downes, M. J. (1978). *Erwinia herbicola* for the control of Pyrenophora avenae on oat seedlings and of other organisms. Paper presented at the Proceedings of seminar on biological control.

Dreyfuss, M. M. and Chapela, I. H. (1994). Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. *Biotechnology (Reading, Mass.),* **26**: 49.

Edje, O. T., Mughogho, L. K., Rao, Y. P. and Msuku, W. A. B. (1981). Bean production in Malawi. Paper presented at the Potential for field beans in Eastern Africa: proceedings of a Regional Workshop held in Lilongwe, Malawi, 9-14 March 1980/sponsored by University of Malawi, Bunda College of Agriculture...[et al.].

Epifano, F., Curini, M., Carla Marcotullio, M. and Genovese, S. (2011). Searching for novel cancer chemopreventive plants and their products: the genus *Zanthoxylum*. *Current Drug Targets,* **12**: 1895-1902.

Fatima, N., Muhammad, S. A., Khan, I., Qazi, M. A., Shahzadi, I., Mumtaz, A., Hashmi, M. A., Khan, A. K. and Ismail, T. (2016). Chaetomium endophytes: a repository of pharmacologically active metabolites. *Acta Physiologiae Plantarum,* **38**: 1-18.

Ferreira, J. J., Rodríguez, C., Pañeda, A. and Giraldez, R. (2003a). Allelism test for resistance to race 38 of anthracnose in common bean differential cultivar,'widusa'. *Bean Imrovement Corperative*.

Ferreira, J. J., Rodríguez, C., Pañeda, A. and Giraldez, R. (2003b). Allelism test for resistance to race 38 of anthracnose in common bean differential cultivar,'widusa'. *BEAN IMPROVEMENT COOPERATIVE*.

Fininsa, C. and Yuen, J. (2002). Temporal progression of bean common bacterial blight (*Xanthomonas campestris* pv. *phaseoli*) in sole and intercropping systems. *European Journal of Plant Pathology,* **108**: 485-495.

Firáková, S., Šturdíková, M. and Múčková, M. (2007). Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia,* **62**: 251-257.

Fraaije, B. A., Lovell, D. J., Rohel, E. A. and Hollomon, D. W. (1999). Rapid detection and diagnosis of Septoria tritici epidemics in wheat using a polymerase chain reaction/PicoGreen assay. *Journal of Applied Microbiology,* **86**: 701-708.

Friesen, A. P., Conner, R. L., Robinson, D. E., Barton, W. R. and Gillard, C. L. (2014). Effect of microwave radiation on dry bean seed infected with *Xanthomonas axonopodis* pv. *phaseoli* with and without the use of chemical seed treatment. *Crop Protection,* **65**: 77-85.

Fróhlich, J., Hyde, K. D. and Petrini, O. (2000). Endophytic fungi associated with palms. *Mycological Research,* **104**: 1202-1212.

Gao, K., Liu, X., Kang, Z. and Mendgen, K. (2005). Mycoparasitism of Rhizoctonia solani by endophytic *Chaetomium spirale* ND35: ultrastructure and cytochemistry of the interaction. *Journal of Phytopathology,* **153**: 280-290.

Gaya, C. H., Kawaka, J. F., Muchugi, A., & Ngeranwa, J. J. (2013). Variation of alkaloids in the Kenyan Zanthoxylum gilletii (De Wild Waterman). *Afr. J. Plant Sci*, *7*(9), 438-444.

Geiser, D. M., del Mar Jiménez-Gasco, M., Kang S., Makalowska, I., Veeraraghavan, N., Ward, T. J., Zhang, N., Kuldau, G. A. and O'Donnell, K. (2004). FUSARIUM-ID v. 1.0: A DNA Sequence Database for Identifying Fusarium. In *Molecular Diversity and PCR-detection of Toxigenic Fusarium Species and Ochratoxigenic Fungi* (pp. 473-479): Springer.

Gichangi, A., Maobe, S. N., Karanja, D., Getabu, A., Macharia, C. N., Ogecha, J. O., Nyang'au M. K., Basweti, E. and Kitonga, L. (2012). Assessment of production and marketing of climbing beans by smallholder farmers in Nyanza region, Kenya. *World Journal of Agricultural Sciences,* **8**: 293-302.

Gilbertson, R. L., Rand, R. E. and Hagedorn, D. J. (1990). Survival of *Xanthomonas campestris* pv. *phaseoli* and pectolytic strains of *X. campestris* in bean debris. *Plant Disease,* **74**: 322-327.

Gond, S. K., Verma, V. C., Kumar, A., Kumar, V. and Kharwar, R. N. (2007). Study of endophytic fungal community from different parts of *Aegle marmelos* Correae (Rutaceae) from Varanasi (India). *World Journal of Microbiology and Biotechnology,* **23**: 1371-1375.

Gong, L. and Guo, S. (2009). Endophytic fungi from *Dracaena cambodiana* and *Aquilaria sinensis* and their antimicrobial activity. *African Journal of Biotechnology,* **8**.

Gonthier, P., Gennaro, M. and Nicolotti, G. (2006). Effects of water stress on the endophytic mycota of *Quercus robur*. *Fungal Divers,* **21**.

Gormann, R., Kaloga, M., Ferreira, D., Marais, J. P. J. and Kolodziej, H. (2006). Newbouldiosides A–C, phenylethanoid glycosides from the stem bark of *Newbouldia laevis*. *Phytochemistry,* **67**: 805-811.

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B. and Givskov, M. (2002). Food spoilage—interactions between food spoilage bacteria. *International Journal of Food Microbiology,* **78**: 79-97.

Gray, J. (1990). Bean (*Phaseolus vulgaris*) distribution in Africa. First approximation.

Gunatilaka, A. A. L. (2006). Natural Products from Plant-Associated Microorganisms: Distribution, Structural Diversity, Bioactivity, and Implications of Their Occurrence⊥. *Journal of Natural Products,* **69**: 509-526.

Guo, B., Wang, Y., Sun, X. and Tang, K. (2008). Bioactive natural products from endophytes: a review. *Applied Biochemistry and Microbiology,* **44**: 136-142.

Guo, L. D., Hyde, K. D. and Liew, E. C. Y. (2001). Detection and taxonomic placement of endophytic fungi within frond tissues of *Livistona chinensis* based on rDNA sequences. *Molecular Phylogenetics and Evolution,* **20**: 1-13.

Haque, M. A., Hossain, M. S., Rahman, M. Z., Rahman, M. R., Hossain, M. S., Mosihuzzaman, M., Nahar, N. and Khan, S. I. (2005). Isolation of bioactive secondary metabolites from the endophytic fungus of *Ocimum basilicum*. *J Pharm Sci,* **4**: 127-130.

Hassan Eman, O. and El-Meneisy Afaf, ZA. (2014). Biocontrol of halo blight of bean caused by *Pseudomonas phaseolicola*. *International Journal of Virology,* **10**: 235-242.

Hesseltine, C. W. (1977). SOLID-STATE FERMENTATION. 1. *Process Biochemistry,* **12**: 24-27.

Hidayat, I., Radiastuti, N., Rahayu, G., Achmadi, S. and Okane, I. (2016). Three Quinine and Cinchonidine producing *Fusarium* species from Indonesia. *Current Research in Environmental & Applied Mycology,* **6**: 20-34.

Hillocks, R. J., Madata, C. S., Chirwa, R., Minja, E. M and Msolla, S. (2006). Phaseolus bean improvement in Tanzania, 1959–2005. *Euphytica,* **150**: 215-231.

Huang, W. Y., Cai, Y. Z., Hyde, K. D., Corke, H., & Sun, M. (2008). Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal diversity*.Hyde, K. D. and Soytong, K. (2008). The fungal endophyte dilemma. *Fungal Divers,* **33**: e73.

Ibrahim, M. B., Kaushik, N., Sowemimo, A. A. and Odukoya, O. A. (2016). Review of the phytochemical and pharmacological studies of the Genus *Markhamia*. *Pharmacognosy Reviews,* **10**: 50.

Islam, S. N., & Ahsan, M. (1997). Biological activities of the secondary metabolites isolated from Zieria smithii and Zanthoxylum elephantiasis on microorganisms and brine shrimps. *Phytotherapy Research*, *11*(1), 64-66.

Ihrmark, K. (2012). B ödeker ITM, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, et al. New primers to amplify the fungal ITS2 region e evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiol Ecol,* **82**: e77.

Ito, M. F., Castro, J. L., Menten, J. O. M. and Moraes, M. H. D. (2003). Importância do uso de sementes sadias de feijão e tratamento químico. *O Agronômico,* **55**.

Jacques, M. A., Josi, K., Darrasse, A. and Samson, R. (2005). *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* is aggregated in stable biofilm population sizes in the phyllosphere of field-grown beans. *Applied and Environmental Microbiology,* **71**: 2008-2015.

Japeth, O. O., Josphat, M. C. and John, V. M. (2014). Chemical composition and larvicidal activity of *Zanthoxylum gilletii* essential oil against *Anopheles gambiae*. *African Journal of Biotechnology,* **13**.

Jia Min, Chen Ling, Xin Hai-Liang, Zheng Cheng-Jian, Rahman Khalid, Han Ting and Qin Lu-Ping. (2016). A friendly relationship between endophytic fungi and medicinal plants: a systematic review. *Frontiers in Microbiology,* **7**.

Jiao, R. H., Xu, S., Liu, J. Y., Ge, H. M., Ding, H., Xu, C., Zhu, H. L. and Tan, R. X. (2006). Chaetominine, a cytotoxic alkaloid produced by endophytic *Chaetomium* sp. IFB-E015. *Organic Letters,* **8**: 5709-5712.

Ju, Y., Still, C. C., Sacalis, J. N., Li, J. and Ho, C. T. (2001). Cytotoxic coumarins and lignans from extracts of the northern prickly ash (*Zanthoxylum americanum*). *Phytotherapy Research,* **15**: 441-443.

Kaiser, W. J. and Vakili, N. G. (1978). Insect transmission of pathogenic xanthomonads to bean and cowpea in Puerto Rico. *Phytopathology,* **68**: 1057-1063.

Kajaria, D., Gangwar, M., Kumar, D., Sharma, A. K., Nath, G., Tilak, R., Tripathi, Y. B., Tripathi, J. S. and Tiwari, S. K. (2012). Qualitative phytochemical characterization and antimicrobial evaluation of a polyherbal compound-Bharangyadi. *Journal of Pharmacy Research,* **5**: 416-419.

Kang, S., Mansfield, M. A., Park, B., Geiser, D. M., Ivors, K. L., Coffey, M. D., Grünwald, N. J., Martin, F. N., Lévesque, C. A. and Blair, J. E. (2010). The promise and pitfalls of sequence-based identification of plant-pathogenic fungi and oomycetes. *Phytopathology,* **100**: 732-737.

Karanja, D., Endire, S. G., Ruraduma, C., Kimani, P. M., Kweka, S. O. and Louis, B. (2011). Value added bean technologies for enhancing food security, nutrition, income and resilience to cope with climate change and variability challenges in Eastern Africa.

Katungi, E., Farrow, A., Chianu, J., Sperling, L. and Beebe, S. E. (2009). Common bean in Eastern and Southern Africa: a situation and outlook analysis. *International Centre for Tropical Agriculture,* **61**.

Katungi, E., Farrow, A., Mutuoki, T., Gebeyehu, S., Karanja, D., Alamayehu, F., Sperling, L., Beebe, S., Rubyogo, J. C. and Buruchara, R. (2010). Improving common bean productivity: An Analysis of socioeconomic factors in Ethiopia and Eastern Kenya. *Baseline Report Tropical Legumes II. Centro Internacional de Agricultura Tropical-CIAT. Cali, Colombia*.

Kaufman, P. B., Kirakosyan, A., McKenzie, M., Dayanandan, P., Hoyt, J. E., Li, C., Cseke, L., Kirakosyan, A., Kaufman, P. and Warber, S. (2006). The uses of plant natural products by humans and risks associated with their use. *Natural Products from Plants*: 441-473.

Kernan, M. R., Amarquaye, A., Chen, J. L., Chan, J., Sesin, D. F., Parkinson, N., ... & Sloan, B. (1998). Antiviral phenylpropanoid glycosides from the medicinal plant Markhamia lutea. *Journal of Natural Products*, *61*(5), 564-570.

Keya, S. O. and Mukunya, D. M. (1979). The influence of seed- bone anthracnose and halo blight inocula on yield and disease development in a Canadian wonder bean selection at Kabete.

Kock, J. L. F., Strauss, T., Pohl, C. H., Smith, D. P., Botes, P. J., Pretorius, E. E., Tepeny, T., Sebolai, O., Botha, A. and Nigam, S. (2001). Bioprospecting for novel oxylipins in fungi: the presence of 3-hydroxy oxylipins in Pilobolus. *Antonie van Leeuwenhoek,* **80**: 93-99.

Kogel, K. H., Franken, P. and Hückelhoven, R. (2006). Endophyte or parasite–what decides? *Current Opinion in Plant Biology,* **9**: 358-363.

Kokwaro, J. O. (1993). Medicinal plants of east Africa. *Nairobi: Kenya Literature Bureau 401p. ISBN,* **9966**: 190.

Kusari, S., Hertweck, C. and Spiteller, M. (2012). Chemical ecology of endophytic fungi: origins of secondary metabolites. *Chemistry & Biology,* **19**: 792-798.

Lamorde, M., Tabuti, J. R. S., Obua, C., Kukunda-Byobona, C., Lanyero, H., Byakika-Kibwika, P., Bbosa, G. S., Lubega, A., Ogwal-Okeng, J. and Ryan, M. (2010). Medicinal plants used by traditional medicine practitioners for the treatment of HIV/AIDS and related conditions in Uganda. *Journal of Ethnopharmacology,* **130**: 43-53.

Leuchtmann, A., Petrini, O., Petrini, L. E. and Carroll, G. C. (1992). Isozyme polymorphism in six endophytic *Phyllosticta* species. *Mycological Research,* **96**: 287-294.

Li, H. M., Sullivan, R., Moy, M., Kobayashi, D. Y. and Belanger, F. C. (2004). Expression of a novel chitinase by the fungal endophyte in Poa ampla. *Mycologia,* **96**: 526-536.

Li, W., Zhou, J., Guo, S. and Guo, L. (2007). Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. *Fungal Divers,* **25**: 69-80.

Lievens, B., Houterman, P. M. and Rep, M. (2009). Effector gene screening allows unambiguous identification of *Fusarium oxysporum* f. sp. *lycopersici* races and discrimination from other formae speciales. *FEMS Microbiology Letters,* **300**: 201-215.

Lippman, S. M. and Hawk, E. T. (2009). Cancer prevention: from 1727 to milestones of the past 100 years. *Cancer Research,* **69**: 5269-5284.

Liu, K., Ding, X., Deng, B. and Chen, W. (2009). Isolation and characterization of endophytic taxol-producing fungi from *Taxus chinensis*. *Journal of Industrial Microbiology & Biotechnology,* **36**: 1171.

Liu, X., Dong, M., Chen, X., Jiang, M., Lv, X. and Zhou, J. (2008). Antimicrobial activity of an endophytic *Xylaria* sp. YX-28 and identification of its antimicrobial compound 7-amino-4-methylcoumarin. *Applied Microbiology and Biotechnology,* **78**: 241-247.

Lucero, M. E., Unc, A., Cooke, P., Dowd, S. and Sun, S. (2011). Endophyte microbiome diversity in *micropropagated Atriplex canescens* and *Atriplex torreyi* var *griffithsii*. *PLoS One,* **6**: e17693.

Ma, Y., Prasad, M. N. V., Rajkumar, M. and Freitas, H. (2011). Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. *Biotechnology Advances,* **29**: 248-258.

Machado, C. M. M., Oishi, B. O., Pandey, A. and Soccol, C. R. (2004). Kinetics of *Gibberella fujikuroi* Growth and Gibberellic Acid Production by Solid- State Fermentation in a Packed- Bed Column Bioreactor. *Biotechnology Progress,* **20**: 1449-1453.

Makini F. W. (1995). Bean production and constraints in Kenya with emphasis on diseases. Paper presented at the Breeding for disease resistance with emphasis on durability. Proceedings of a regional workshop for eastern, central and southern Africa, held at Njoro, Kenya, October 2-6, 1994.

Mancuso, G., Borgonovo, G., Scaglioni, L. and Bassoli, A. (2015). Phytochemicals from *Ruta graveolens* activate TAS2R bitter taste receptors and TRP channels involved in gustation and nociception. *Molecules,* **20**: 18907-18922.

Manefield, M., de Nys, R., Naresh, K., Roger, R., Givskov, M., Peter, S. and Kjelleberg, S. (1999). Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology,* **145**: 283-291.

Maroyi, A. (2012) *Markhamia lutea* (Benth.) K. Schum. [Internet] Record from PROTA4U. In Lemmens, R. H. M. J., Louppe, D., & Oteng-Amoako, A. A. (Eds). PROTA (Plant Resources of Tropical Africa/Resources végétales de l'Afrique tropicale), Wageningen, Netherlands. http://www.prota4u.org/search.asp. Accessed 9 Oct 2015.

Márquez, S. S., Bills, G. F. and Zabalgogeazcoa, I. (2007). The endophytic mycobiota of the grass *Dactylis glomerata*. *Fungal Divers,* **27**: 171-195.

Marthey, S., Aguileta, G., Rodolphe, F., Gendrault, A., Giraud, T., Fournier, E., Lopez-Villavicencio, M., Gautier, A., Lebrun, M. H. and Chiapello, H. (2008). FUNYBASE: a FUNgal phYlogenomic dataBASE. *Bmc Bioinformatics,* **9**: 456.

Martin, F. .N and Tooley, P. W. (2003). Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia,* **95**: 269-284.

Matasyoh, L. G., Matasyoh, J. C., Wachira, F. N., Kinyua, M. G., Muigai, A. W. T. and Mukiama, T. K. (2007). Chemical composition and antimicrobial activity of the essential oil of *Ocimum gratissimum* L. growing in Eastern Kenya. *African Journal of Biotechnology,* **6**.

McLean, R. J. C., Pierson, L. S. and Fuqua, C. (2004). A simple screening protocol for the identification of quorum signal antagonists. *Journal of Microbiological Methods,* **58**: 351-360.

Michielse, C. B. and Rep, M. (2009). Pathogen profile update: *Fusarium oxysporum*. *Molecular Plant Pathology,* **10**: 311-324.

Miklas, P. N., Coyne, D. P., Grafton, K. F., Mutlu, N., Reiser, J., Lindgren, D. T. and Singh, S. P. (2003). A major QTL for common bacterial blight resistance derives from the common bean great northern landrace cultivar Montana No. 5. *Euphytica,* **131**: 137-146.

Mirik, M., Aysan, Y. and Cinar, O. (2007). Copper-resistant strains of *Xanthomonas axonopodis* pv. *vesicatoria* (Doidge) Dye in the eastern Mediterranean region of turkey. *Journal of Plant Pathology,* **89**: 153-154.

Mitchell, A. M., Strobel, G. A., Hess, W. M., Vargas, P. N. and Ezra, D. (2008). *Muscodor crispans*, a novel endophyte from *Ananas ananassoides* in the Bolivian Amazon. *Fungal Diversity,* **31**: 37-43.

Mohana, D. C., Raveesha, K. A. and Lokanath, R. K. M. (2008). Herbal remedies for the management of seed-borne fungal pathogens by an edible plant *Decalepis hamiltonii* (Wight & Arn). *Archives of Phytopathology and Plant Protection,* **41**: 38-49.

Momesso, L. S., Kawano, C. Y., Ribeiro, P. H., Nomizo, A., Goldman, G. H. and Pupo, M. T. (2008). Chaetoglobosins produced by *Chaetomium globosum*, endophytic fungus found in association with *Viguiera robusta* Gardn (Asteraceae). *Química Nova,* **31**: 1680-1685.

Moreno, R. A. and Mora, L. E. (1984). Cropping pattern and soil management influence on plant disease, 2: Bean rust epidemiology. *Turrialba (IICA).*

Mostert, L., Halleen, F., Fourie, P. and Crous, P. W. (2006). A review of *Phaeoacremonium* species involved in Petri disease and esca of grapevines. *Phytopathologia Mediterranea,* **45**: S12.

Moy, M., Li, H. M., Sullivan, R., White, J. F. and Belanger, F. C. (2002). Endophytic fungal β-1, 6-glucanase expression in the infected host grass. *Plant Physiology,* **130**: 1298-1308.

Mulè, G., Susca, A., Stea, G. and Moretti, A. (2004). A species-specific PCR assay based on the calmodulin partial gene for identification of *Fusarium verticillioides, F. proliferatum* and *F. subglutinans*. In *Molecular Diversity and PCR-detection of Toxigenic Fusarium Species and Ochratoxigenic Fungi* (pp. 495-502): Springer.

Mullis, K. B. and Faloona, F. A. (1987). [21] Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology,* **155**: 335-350.

Murillo, J., Bardaji, L. and Führer, M. E. (2010). La grasa de las judías, causada por la bacteria *pseudomonas syringea* pv. *phaseolicoda*. *Phytoma España: La revista profesional de sanidad vegetal*: 27-31.

Mutasa, E. S., Chwarszcynska, D. M. and Asher, MJC. (1996). Single-tube, nested PCR for the diagnosis of *Polymyxa betae* infection in sugar beet roots and colorimetric analysis of amplified products. *Phytopathology,* **86**: 493-497.

Muthii, K. T. (2014). Quality status of farm saved bean seed in Maragua sub-county and management of seed-borne diseases by seed treatment. University of Nairobi.

Múzquiz, M., Guillamon, E., Burbano, C., Pascual, H., Cabellos, B., Cuadrado, C. and Pedrosa, M. M. (2011). Chemical composition of a new Lupinus species found in Spain, *Lupinus mariae*-josephi H. Pascual (Fabaceae). *Spanish Journal of Agricultural Research,* **9**: 1233-1244.

Namugwanya, M., Tenywa, J. S., Otabbong, E., Mubiru, D. N. and Masamba, T. A. (2014). Development of common bean (*Phaseolus vulgaris* L.) production under low soil phosphorus and drought in Sub-Saharan Africa: A Review. *Journal of Sustainable Development,* **7**: 128.

Nascimento, A. M., Conti, R., Turatti, I. C. C., Cavalcanti, B. C., Costa-Lotufo, L. V., Pessoa, C., Moraes, M. O., Manfrim, V., Toledo, J. S. and Cruz, A. K. (2012). Bioactive extracts and chemical constituents of two endophytic strains of *Fusarium oxysporum*. *Revista Brasileira de Farmacognosia,* **22**: 1276-1281.

Nderitu, J. H., Buruchara, R. A. and Ampofo, J. K. O. (1997). Relationships between bean stem maggot, bean root rots and soil fertility: literature review with emphasis on research in eastern and central Africa. *AHI Technica Report Series (ICRAF). no. 4.*

Negi, J. S., Bisht, V. K., Bh, A. K., Singh, P. and Sundriyal, R. C. (2011). Chemical constituents and biological activities of the genus *Zanthoxylum*: a review. *African Journal of Pure and Applied Chemistry,* **5**: 412-416.

Newman, D. J. and Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Products,* **75**: 311-335.

Njuguna, S. K., Ndegwa, A. M. M., Van Rheenen, H. A and Mukunya, D. M. (1980). *Bean production in Kenya.* Paper presented at the Regional Workshop on potentials for field beans in Eastern Africa, Lilongwe, Malawi.

Nyunja, A. R. O., Onyango, J. C. and Erwin, B. (2009). The Kakamega forest medicinal plant resources and their utilization by the adjacent Luhya community. *International Journal of Tropical Medicine,* **4**: 82-90.

Odendo, M., David, S., Kalyebara, R., Ostyula, R. and Buruchara, R. (2004). The key role of beans in poverty alleviation: Lessons from the impact of Improved bean varieties in western Kenya. *Occasional Publication Series*.

Ohtani, K., Fujioka, S., Shimada, A. and Kimura, Y. (2011). Nematicidal activities of 4-hydroxyphenylacetic acid and oidiolactone D produced by the fungus *Oidiodendron* sp. *Zeitschrift für Naturforschung C,* **66**: 31-34.

Oliveira, A. L., Silva, D. B., Lopes, N. P., Debonsi, H. M. and Yokoya, N. S. (2012). Chemical constituents from red algae *Bostrychia radicans* (Rhodomelaceae): new amides and phenolic compounds. *Química Nova,* **35**: 2186-2188.

Oloo, J. O., Makenzi, P. M., Mwangi, J. G. and Abdulrazack, A. S. (2013). Dominant Tree Species for Increasing Ground Cover and their Distribution in Siaya County, Kenya. *International Journal of Agriculture Innovations and Research,* **2**: 373-377.

Opio, A. F., Allen, D. J. and Teri, J. M. (1993). Studies on seed transmission of *Xanthomonas campestris* pv *phaseoli* in common beans in Uganda [online].

Opio, A. F., Allen, D. J. and Teri, J. M. (1996). Pathogenic variation in *Xanthomonas campestris* pv. *phaseoli*, the causal agent of common bacterial blight in Phaseolus beans. *Plant Pathology,* **45**: 1126-1133.

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R. and Simons, A. (2009). Agroforestree database: a tree species reference and selection guide version 4.0. *World Agroforestry Centre ICRAF, Nairobi, KE*.

Osdaghi, E., Shams-Bakhsh, M., Alizadeh, A., Lak, M. R. and Maleki, H. H. (2011). Induction of resistance in common bean by *Rhizobium leguminosarum* bv. *phaseoli* and decrease of common bacterial blight. *Phytopathologia Mediterranea,* **50**: 45-54.

Oses, R., Valenzuela, S., Freer, J., Sanfuentes, E. and Rodriguez, J. (2008). Fungal endophytes in xylem of healthy Chilean trees and their possible role in early wood decay. *Fungal Divers,* **33**.

Otto, M. (2004). Quorum-sensing control in Staphylococci–a target for antimicrobial drug therapy? *FEMS Microbiology Letters,* **241**: 135-141.

Pachon, G., Rasoanaivo, H., Azqueta, A., Rakotozafy, J. C., Raharisololalao, A., DE CERAIN A., De Lapuente J., Borras M., Moukha S. and Centelles J. J. (2007). Anticancer effect of a new benzophenanthridine isolated from *Zanthoxylum madagascariense* (Rutaceline). *In Vivo,* **21**: 417-422.

Palmer, A. G., Streng, E. and Blackwell, H. E. (2011). Attenuation of virulence in pathogenic bacteria using synthetic quorum-sensing modulators under native conditions on plant hosts. *ACS Chemical Biology,* **6**: 1348-1356.

Pandey, A., Selvakumar, P., Soccol, C. R. and Nigam, P. (1999). Solid state fermentation for the production of industrial enzymes. *Current Science,* **77**: 149-162.

Parhoodeh, P., Rahmani, M., Hashim, N. M., Sukari, M. A. and Gwendoline, C. E. (2012). Alkaloid Constituents of *haplophyllum laeviusculum* (Rutaceae)(Kandungan alkaloid daripada *haplophyllum laeviusculum* (Rutaceae). *Sains Malaysiana,* **41**: 47-52.

Paterson, R. R. (2004). The isoepoxydon dehydrogenase gene of patulin biosynthesis in cultures and secondary metabolites as candidate PCR inhibitors. *Mycological Research,* **108**: 1431-1437.

Pellegrini, M. C., Alvarez, M. V., Ponce, A. G., Cugnata, N. M., De Piano, F. G. and Fuselli, S. R. (2014). Anti-quorum sensing and antimicrobial activity of aromatic species from South America. *Journal Of Essential Oil Research,* **26**: 458-465.

Pěnčíková, K., Urbanová, J., Musil, P., Táborská, E. and Gregorová, J. (2011). Seasonal variation of bioactive alkaloid contents in *Macleaya microcarpa* (Maxim.) Fedde. *Molecules,* **16**: 3391-3401.

Pongcharoen, W., Rukachaisirikul, V., Phongpaichit, S., Kühn, T., Pelzing, M., Sakayaroj, J. and Taylor, W. C. (2008). Metabolites from the endophytic fungus *Xylaria* sp. PSU-D14. *Phytochemistry,* **69**: 1900-1902.

Purahong, W. and Hyde, K. D. (2011). Effects of fungal endophytes on grass and non-grass litter decomposition rates. *Fungal Diversity,* **47**: 1-7.

Puri, S. C., Nazir, A., Chawla, R., Arora, R., Riyaz-ul-Hasan, S., Amna T., Ahmed, B., Verma, V., Singh, S. and Sagar, R. (2006). The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. *Journal of Biotechnology,* **122**: 494-510.

Qin, J. C., Zhang, Y. M., Gao, J. M., Bai, M. S., Yang, S. X., Laatsch, H. and Zhang, A. L. (2009). Bioactive metabolites produced by *Chaetomium globosum*, an endophytic fungus isolated from *Ginkgo biloba*. *Bioorganic & Medicinal Chemistry Letters,* **19**: 1572-1574.

Raffa, R. B., Iannuzzo, J. R., Levine, D. R., Saeid, K. K., Schwartz, R. C., Sucic, N. T., Terleckyj, O. D. and Young, J. M. (2005). Bacterial communication ("quorum sensing") via ligands and receptors: a novel pharmacologic target for the design of antibiotic drugs. *Journal Of Pharmacology and Experimental Therapeutics,* **312**: 417-423.

Rahmani, M., Serang, R. M., Hashim, N. M., Sukari, M. A., Ee, G. C. L., Ali, A. M. and Ismail, H. B. . (2010). Alkaloids and sulphur-containing amides from *Glycosmis citrifolia* and *Glycosmis elongata*. *Sains Malaysiana,* **39**: 445-451.

Rakshith, D., Santosh, P., & Satish, S. (2013). Isolation and characterization of antimicrobial metabolite producing endophytic Phomopsis sp. from Ficus pumila Linn.(Moraceae). *International Journal of Chemical and Analytical Science*, *4*(3), 156-160.

Rani, R., Sharma, D., Chaturvedi, M. and Yadav, JP. (2017). Antibacterial Activity of Twenty Different Endophytic Fungi Isolated from *Calotropis procera* and Time Kill Assay. *Clin Microbiol,* **6**: 2.

Rasmussen, T. B., Bjarnsholt, T., Skindersoe, M. E., Hentzer, M., Kristoffersen, P., Köte, M., Nielsen, J., Eberl, L. and Givskov, M. (2005). Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *Journal of Bacteriology,* **187**: 1799-1814.

Rasool Hassan, B. A. (2012). Medicinal plants (importance and uses). *Pharmaceut Anal Acta,* **3**: e139.

Rivera-Orduña, F. N., Suarez-Sanchez, R. A., Flores-Bustamante, Z. R., Gracida-Rodriguez, J. N. and Flores-Cotera, L. B. (2011). Diversity of endophytic fungi of *Taxus globosa* (Mexican yew). *Fungal Diversity,* **47**: 65-74.

Robinson, T., Singh, D. and Nigam, P. (2001). Solid-state fermentation: a promising microbial technology for secondary metabolite production. *Applied Microbiology and Biotechnology,* **55**: 284-289.

Rodrigues, K. F., Costa, G. L., Carvalho, M. P. and Epifanio, R. A. (2005). Evaluation of extracts produced by some tropical fungi as potential cholinesterase inhibitors. *World Journal of Microbiology and Biotechnology,* **21**: 1617-1621.

Rodriguez, R. J., White, Jr J. F., Arnold, A. E. and Redman, R. S. (2009). Fungal endophytes: diversity and functional roles. *New Phytologist,* **182**: 314-330.

Sabet, K. A., Ishag, F. and Khalil, O. (1969). Studies on the bacterial diseases of Sudan crops. *Annals of Applied Biology,* **63**: 357-369.

Saettler, A. W. (1989). Common bacterial blight. *Bean production problems in the tropics,* **2**: 261-283.

Sallam, N. M. (2011). Biological control of common blight of bean (*Phaseolus vulgaris*) caused by *Xanthomonas axonopodis* pv. *phaseoli* by using the bacterium *Rahnella aquatilis*. *Archives of Phytopathology And Plant Protection,* **44**: 1966-1975.

Sandhu, S. S., Kumar, S. and Aharwal, R. P. (2014). Isolation and Identification of endophytic fungi from *Ricinus communis* Linn. and their antibacterial activity. *Int J Res Pharm Chem, 2014d,* **4**: 611-618.

Sartori, A. F., Reis, E. M. and Casa, R. T. (2004). Quantificação da transmissão de Fusarium moniliforme de sementes para plântulas de milho. *Fitopatologia Brasileira,* **29**: 456-458.

Sati, S. C. and Joshi, S. (2011). Antibacterial activities of *Ginkgo biloba* L. leaf extracts. *The Scientific World Journal,* **11**: 2237-2242.

Schaad, N. W., Postnikova, E., Lacy, G. H., Sechler, A., Agarkova, I., Stromberg, P. E., Stromberg, V. K. and Vidaver, A. K. (2005). Reclassification of *Xanthomonas campestris* pv. citri (ex Hasse 1915) Dye 1978 forms A, B/C/D, and E as X. smithii subsp. citri (ex Hasse) sp. nov. nom. rev. comb. nov., X. fuscans subsp. aurantifolii (ex Gabriel 1989) sp. nov. nom. rev. comb. nov., and *X. alfalfae* subsp. citrumelo (ex Riker and Jones) Gabriel et al., 1989 sp. nov. nom. rev. comb. nov.; *X. campestris* pv *malvacearum* (ex Smith 1901) Dye 1978 as X. smithii subsp. smithii nov. comb. nov. nom. nov.; X. campestris pv. alfalfae (ex Riker and Jones, 1935) Dye 1978 as X. alfalfae subsp. alfalfae (ex Riker et al., 1935) sp. nov. nom. rev.; and "var. fuscans" of X. campestris pv. phaseoli (ex Smith, 1987) Dye 1978 as X. fuscans subsp. fuscans sp. nov. *Systematic and Applied Microbiology,* **28**: 494-518.

Schena, L., Nigro, F., Ippolito, A. and Gallitelli, D. (2004). Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology,* **110**: 893-908.

Schmidt, L. H. and Mbora, A. (2008). *Markhamia lutea* (Benth) K. Schum. *Seed Leaflet*.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Bolchacova, E., Voigt, K. and Crous, P. W. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences,* **109**: 6241-6246.

Schulz, B. and Boyle, C. (2005). The endophytic continuum. *Mycological Research,* **109**: 661-686.

Schulz, B., Boyle, C., Draeger, S., Römmert, A. K. and Krohn, K. (2002). Endophytic fungi: a source of novel biologically active secondary metabolites** Paper presented at the British Mycological Society symposium on Fungal Bioactive Compounds, held at the University of Wales Swansea on 22–27 April 2001. *Mycological Research,* **106**: 996-1004.

Schulz, B., Guske, S., Dammann, U. and Boyle, C. (1998). Endophyte-host interactions. II. Defining symbiosis of the endophyte-host interaction. *Symbiosis, Philadelphia, Pa.(USA)*.

Schuster, M., Joseph, S. D., Diggle, S. P. and Peter, G. E. (2013). Acyl-homoserine lactone quorum sensing: from evolution to application. *Annual Review of Microbiology,* **67**: 43-63.

Sette, LD, Passarini, MRZ, Delarmelina, C, Salati, F and Duarte, MCT. (2006). Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. *World Journal of Microbiology and Biotechnology,* **22**: 1185-1195.

Shi, C., Navabi, A. and Yu, K. (2011). Association mapping of common bacterial blight resistance QTL in Ontario bean breeding populations. *BMC Plant Biology,* **11**: 52.

Shiono, Y., Tsuchinari, M., Shimanuki, K., Miyajima, T., Murayama, T., Koseki, T., Laatsch, H., Funakoshi, T., Takanami, K. and Suzuki, K. (2007). Fusaristatins A and B, two new cyclic lipopeptides from an endophytic *Fusarium* sp. *Journal of Antibiotics,* **60**: 309.

Smoliak, S., Ditterline, R. L., Scheetz, J. D., Holzworth, L. K., Sims, J. R., Wiesner, J. R., Baldridge, D. E. and Tibke, G. L. (1990). Common bean. *Montana State University, Animal and Range Sciences Extension Service, Forage extension program, Bozeman, USA*.

Specian, V., Sarragiotto, M. H., Pamphile, J. A. and Clemente, E. (2012). Chemical characterization of bioactive compounds from the endophytic fungus Diaporthe helianthi isolated from *Luehea divaricata*. *Brazilian Journal of Microbiology,* **43**: 1174-1182.

Srivastava, A. and Anandrao, R. K. (2015). Antimicrobial potential of fungal endophytes isolated from leaves of *Prosopis juliflora* (DC). An important weed. *Int J Pharm Pharmacol Sci,* **7**: 128-136.

Stadler, M. and Keller, N. P. (2008). Paradigm shifts in fungal secondary metabolite research. *Mycological Research,* **112**: 127-130.

Stamford, T. L. M., Stamford, N. P., Coelho, L. C. B. B., & Araujo, J. M. (2002). Production and characterization of a thermostable glucoamylase from Streptosporangium sp. endophyte of maize leaves. *Bioresource technology*, *83*(2), 105-109.

Stangarlin, J. R., Schwan-Estrada, K. R. F., Cruz, M. E. S. and Nozaki, M. H. (1999). Plantas medicinais e controle alternativo de fitopatógenos. *Biotecnologia Ciência & Desenvolvimento,* **11**: 16-21.

Stashenko, E. (2011). Antiquorum sensing activity of essential oils isolated from different species of the genus Piper. *Vitae,* **18**: 77-82.

Steinkraus, K. H. (1984). Solid- state (solid- substrate) food/beverage fermentations involving fungi. *Engineering in Life Sciences,* **4**: 83-88.

Stierle, A. A. and Stierle, D. B. (2015). Bioactive secondary metabolites produced by the fungal endophytes of conifers. *Natural product communications,* **10**: 1671.

Stinson, M., Ezra, D., Hess, W. M., Sears, J. and Strobel, G. (2003). An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. *Plant Science,* **165**: 913-922.

Strobel, G. and Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews,* **67**: 491-502.

Sturz, A. V., Christie, B. R., Matheson, B. G. and Nowak, J. (1997). Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils,* **25**: 13-19.

Subramaniyam, R. and Vimala, R. (2012). Solid state and submerged fermentation for the production of bioactive substances: a comparative study. *Int J Sci Nat,* **3**: 480-486.

Sugeng, R. M., Sukari, A., Rahmani, M., Ee, G. C. L., Taufiq-Yap, Y. H., Aimi, N. and Kitajima, M. (2001). Alkaloids from *Aegle marmelos* (Rutaceae). *Malaysian Journal of Analytical Sciences,* **7**: 463-465.

Sun, J., Deng, Z. and Yan, A. (2014). Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. *Biochemical and Biophysical Research Communications,* **453**: 254-267.

Sun, X. and Guo, L. D. (2012). Endophytic fungal diversity: review of traditional and molecular techniques. *Mycology,* **3**: 65-76.

Suryanarayanan, T. S., Thirunavukkarasu, N., Govindarajulu, M. B., Sasse, F., Jansen, R. and Murali, T. S. (2009). Fungal endophytes and bioprospecting. *Fungal Biology Reviews,* **23**: 9-19.

Syofuna, A., Banana, A. Y. and Nakabonge, G. (2012). Efficiency of natural wood extractives as wood preservatives against termite attack. *Maderas. Ciencia y tecnología,* **14**: 155-163.

Szabó, M. Á., Varga, G. Z., Hohmann, J., Schelz, Z., Szegedi, E., Amaral, L. and Molnár, J. (2010). Inhibition of quorum- sensing signals by essential oils. *Phytotherapy Research,* **24**: 782-786.

Tan, R. X. and Zou, W. X. (2001). Endophytes: a rich source of functional metabolites. *Natural Product Reports,* **18**: 448-459.

Tar'An, B., Michaels, T. E. and Pauls, K. P. (2001). Mapping genetic factors affecting the reaction to *Xanthomonas axonopodis* pv. *phaseoli* in *Phaseolus vulgaris* L. under field conditions. *Genome,* **44**: 1046-1056.

Tarus, P. K., Coombes, P. H., Crouch, N. R. and Mulholland, D. A. (2006). Benzo [c] phenanthridine alkaloids from stem bark of the Forest Knobwood, *Zanthoxylum davyi* (Rutaceae). *South African Journal of Botany,* **72**: 555-558.

Tavares, L. C., Zanon, G., Weber, A. D., Neto, A. T., Mostardeiro, C. P., Da Cruz, I. B. M., Oliveira, R. M., Ilha, V., Dalcol, I. I. and Morel, A. F. (2014). Structure-activity relationship of benzophenanthridine alkaloids from *Zanthoxylum rhoifolium* having antimicrobial Activity. *PloS one,* **9**: e97000.

Tayung, K., Barik, B. P., Jha, D. K. and Deka, D. C. (2011). Identification and characterization of antimicrobial metabolite from an endophytic fungus, *Fusarium solani* isolated from bark of Himalayan yew. *Mycosphere,* **2**: 203-213.

Tesar, M., Reichenauer, T. G. and Sessitsch, A. (2002). Bacterial rhizosphere populations of black poplar and herbal plants to be used for phytoremediation of diesel fuel. *Soil Biology and Biochemistry,* **34**: 1883-1892.

Thomas, S. E., Crozier, J., Aime, M. C., Evans, H. C. and Holmes, K. A. (2008). Molecular characterisation of fungal endophytic morphospecies associated with the indigenous forest tree, *Theobroma gileri*, in Ecuador. *Mycological Research,* **112**: 852-860.

Van Aken, B., Yoon, J. M. and Schnoor, J. L. (2004). Biodegradation of nitro-substituted explosives 2, 4, 6-trinitrotoluene, hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine, and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (Populus deltoides× nigra DN34). *Applied and Environmental Microbiology,* **70**: 508-517.

Vaz Aline, BM, Mota Rubens, C, Bomfim Maria Rosa, Q, Vieira Mariana, LA, Zani Carlos, L, Rosa Carlos, A and Rosa Luiz, H. (2009). Antimicrobial activity of endophytic fungi associated with *Orchidaceae* in Brazil. *Canadian Journal of Microbiology,* **55**: 1381-1391.

Vieira, M. L. A., Hughes, A. F. S., Gil, V. B., Vaz, A. B. M., Alves, T. M. A., Zani, C. L., Rosa, C. A. and Rosa, L. H. (2011). Diversity and antimicrobial activities of the fungal endophyte community associated with the traditional Brazilian medicinal plant *Solanum cernuum* Vell.(Solanaceae). *Canadian Journal of Microbiology,* **58**: 54-66.

Wagara, I. N. and Mkimani, P. (2007). Resistance of nutrient-rich bean varieties to major biotic constraints in Kenya. Paper presented at the 8th African Crop Science Society Conference, El-Minia, Egypt, 27-31 October 2007.

Waithaka, Paul Njenga, Gathuru Eliud Mugu, Githaiga Benson Muriuki and Onkoba Keziah Moraa. (2017). Antimicrobial Activity of Mushroom (Agaricus Bisporus) and Fungal

(Trametes Gibbosa) Extracts from Mushrooms and Fungi of Egerton Main Campus, Njoro Kenya. *Journal of Biomedical Sciences,* **6**.

Wang, S., Li, X. M., Teuscher, F., Li, D. L., Diesel, A., Ebel, R., Proksch, P. and Wang, B. G. (2006). Chaetopyranin, a benzaldehyde derivative, and other related metabolites from *Chaetomium globosum*, an endophytic fungus derived from the marine red alga *Polysiphonia urceolata*. *Journal of Natural Products,* **69**: 1622-1625.

Wang, Y., Guo, L. D. and Hyde, K. D. (2005). Taxonomic placement of sterile morphotypes of endophytic fungi from *Pinus tabulaeformis* (Pinaceae) in northeast China based on rDNA sequences. *Fungal Diversity*.

Weber, R. W. S., Kappe, R., Paululat, T., Mösker, E. and Anke, H. (2007). Anti-Candida metabolites from endophytic fungi. *Phytochemistry,* **68**: 886-892.

White, T. J., Bruns, T., Lee, S. J. W. T. and Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications,* **18**: 315-322.

WHO World Health Organization. (2002). WHO traditional medicine strategy 2002-2005.

Wiese, J., Ohlendorf, B., Blümel, M., Schmaljohann, R. and Imhoff, J. F. (2011). Phylogenetic identification of fungi isolated from the marine sponge *Tethya aurantium* and identification of their secondary metabolites. *Marine Drugs,* **9**: 561-585.

Winston, RL, Schwarzländer Mark, Hinz Hariet, L, Day Michael, D, Cock Matthew, JW and Julien, MH. (2014). Biological control of weeds: a world catalogue of agents and their target weeds. *Biological control of weeds: a world catalogue of agents and their target weeds.*

Wortmann, C. S. (1998). *Atlas of common bean (Phaseolus vulgaris L.)* production in Afric*a*: CIAT.

Wortmann, C. S. (2006). *Phaseolus vulgaris* L.(common bean). *Record from PROTA4U. Bring M. and Belay, G.(Edirots). PROTA (Plant Resource of Tropical Africa). Wageningen, Netherlands*.

Wortmann, C. S., Brink, M. and Belay, G. (2006). *Phaseolus vulgaris* L.(common bean). Record from PROTA4U. *PROTA (Plant Resources of Tropical Africa/Ressources végétales de l'Afrique tropicale)*.

Wortmann, C. S., Kirkby, R. A., Eledu, C. A. and Allen, D. .J. (1998). Atlas of common beans (*Phaseolus vulgaris* L.). *Pan African Bean Research Alliance. CIAT*: 133.

Wortmann Charles, S and Allen David, J. (1994). *African bean production environments: their definition, characteristics and constraints*: CIAT.

Xing, Y. M., Chen, J., Cui, J. L., Chen, X. M. and Guo, S. X. (2011). Antimicrobial Activity and Biodiversity of Endophytic Fungi in *Dendrobium devonianum* and *Dendrobium thyrsiflorum* from Vietman. *Current Microbiology,* **62**: 1218-1224.

Yang, D. and Fu, H. (2010). A Simple and Practical Copper- Catalyzed Approach to Substituted Phenols from Aryl Halides by Using Water as the Solvent. *Chemistry–A European Journal,* **16**: 2366-2370.

Yu, H., Zhang, L., Li, L., Zheng, C., Guo, L., Li, W., Sun, P. and Qin, L. (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes. *Microbiological Research,* **165**: 437-449.

Yu, Z., Lang, G., Kajahn, I., Schmaljohann, R. and Imhoff, J. F. (2008). Scopularides A and B, cyclodepsipeptides from a marine sponge-derived fungus, *Scopulariopsis brevicaulis*. *Journal of Natural Products,* **71**: 1052-1054.

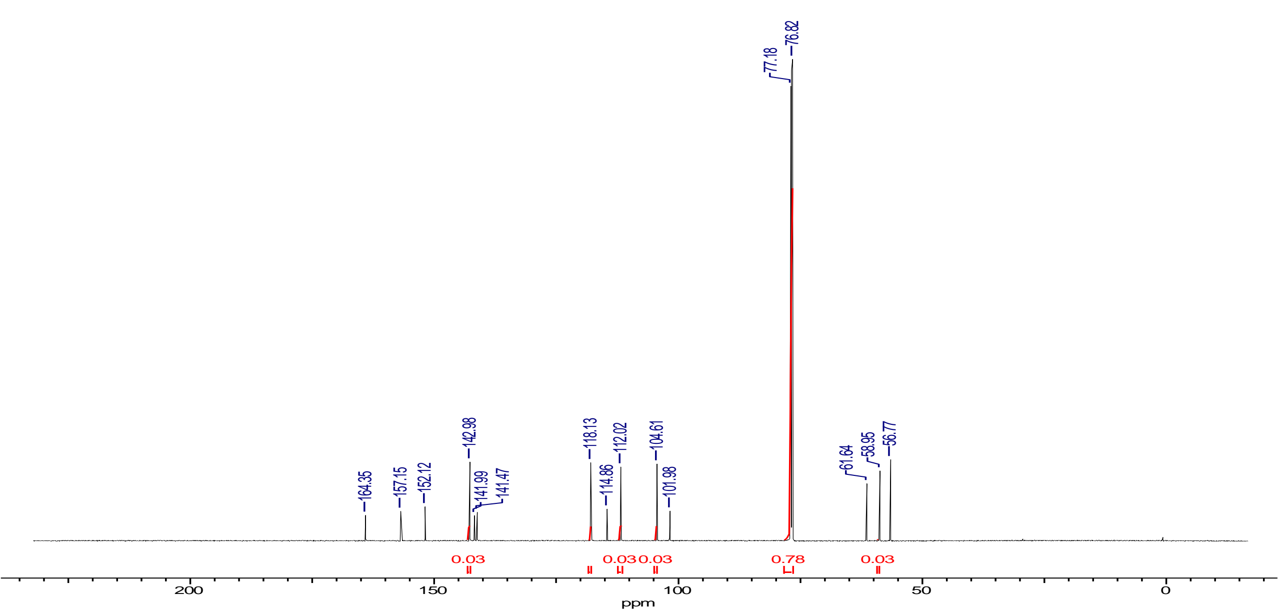
Zahin, M., Hasan, S., Aqil, F., Khan, M., Ahmad, S., Husain, F. M. and Ahmad, I. (2010). Screening of certain medicinal plants from India for their anti-quorum sensing activity.

Zaki, A. A., Shaaban, M. I., Hashish, N. E., Amer, M. A. and Lahloub, M. F. (2012). Assessment of anti-quorum sensing activity for some ornamental and medicinal plants native to Egypt. *Scientia Pharmaceutica,* **81**: 251-258.

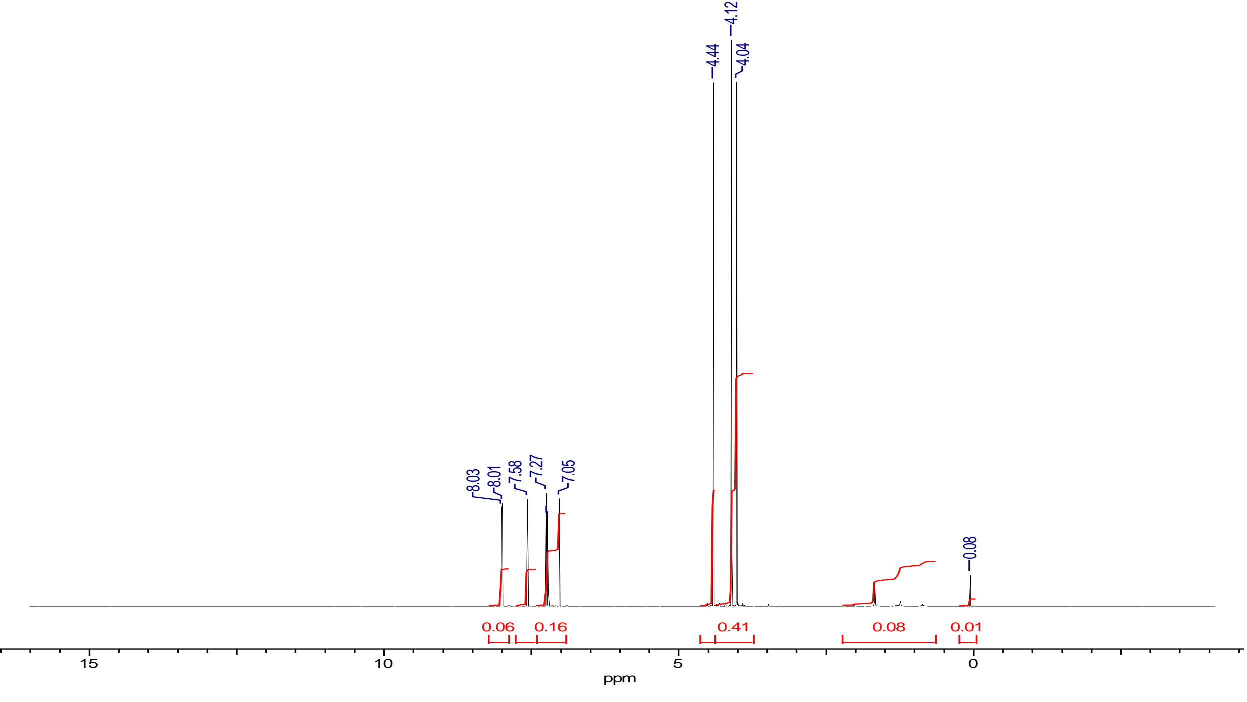
Zinniel, D. K., Lambrecht, P., Harris, N.B., Feng, Z., Kuczmarski, D., Higley, P., Ishimaru, C.A., Arunakumari, A., Barletta, R.G. and Vidaver, A. K. (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Applied and Environmental Microbiology,* **68**: 2198-2208.

APPENDICES

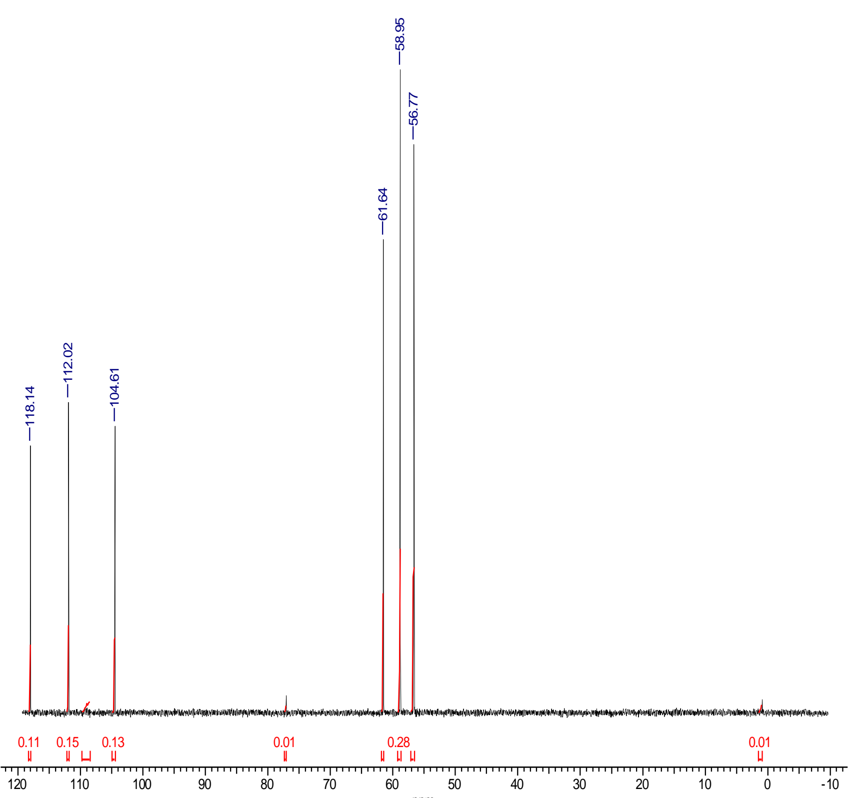
Appendix 1: ^{13}CNMR spectra of compound 5



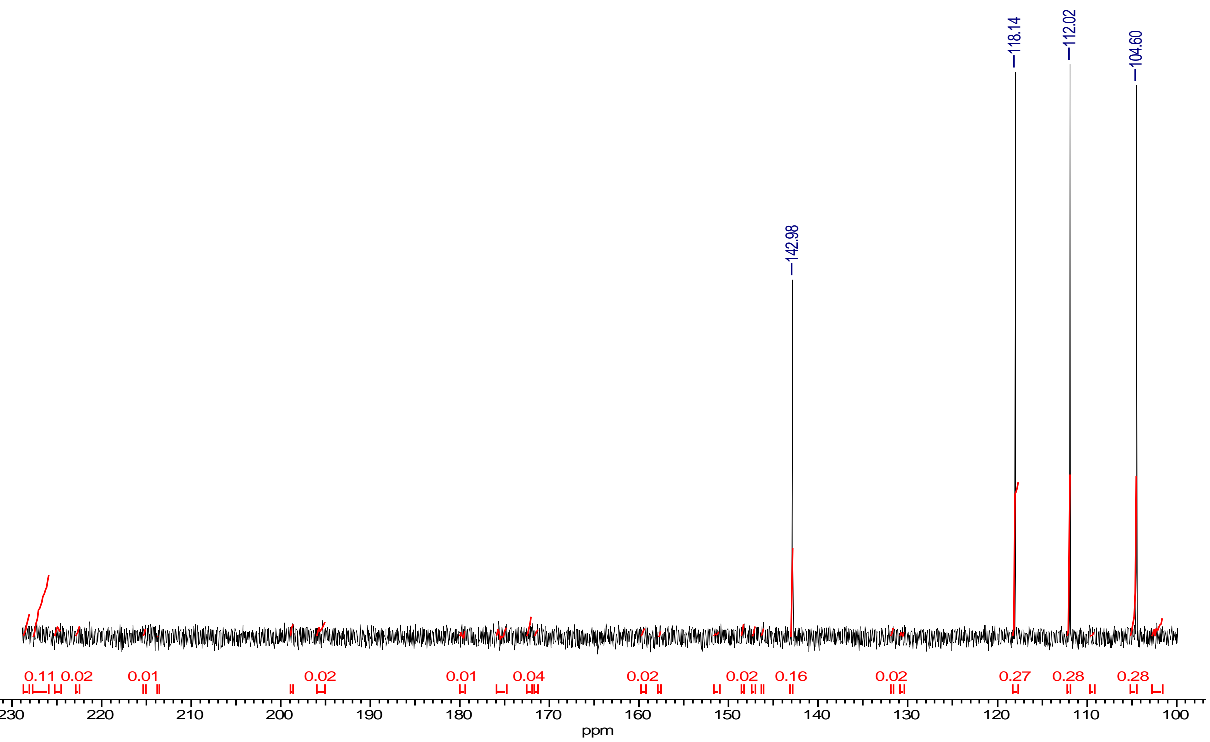
Appendix 2: ^{1}HNMR spectra of compound 5



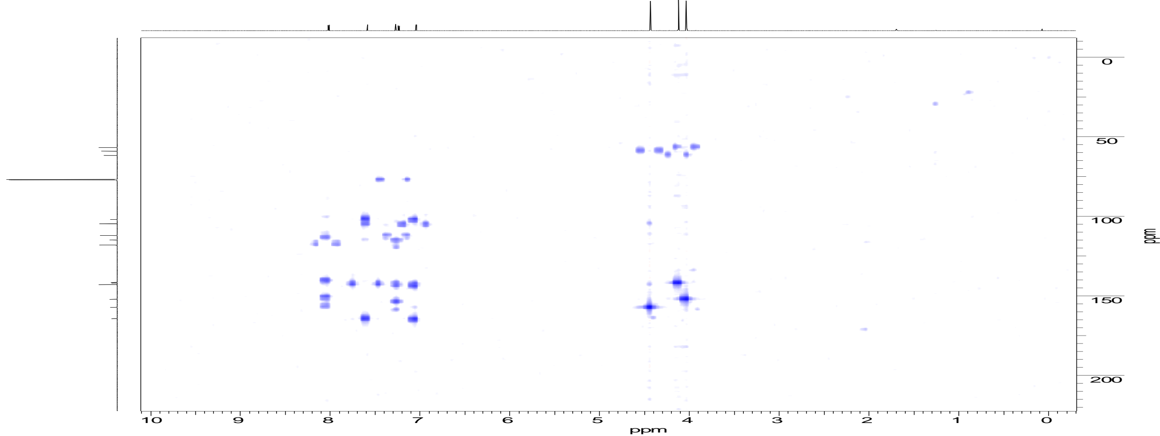
Appendix 3: DEPT spectra of compound 5



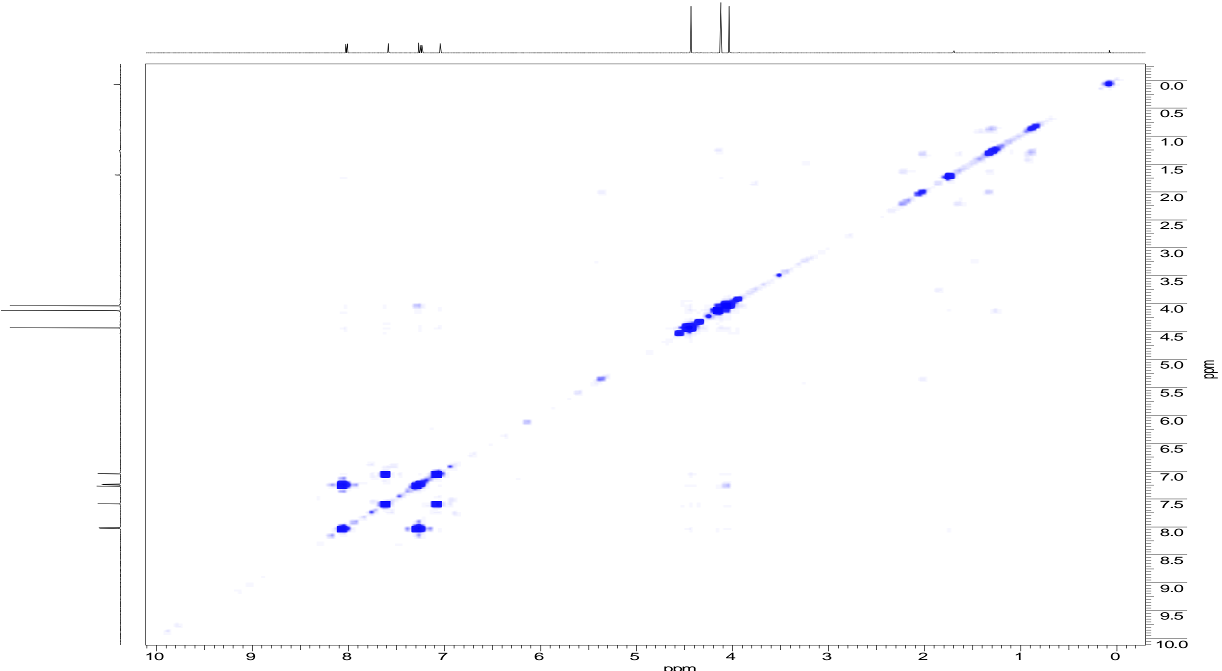
Appendix 4: DEPT of compound 5 continued



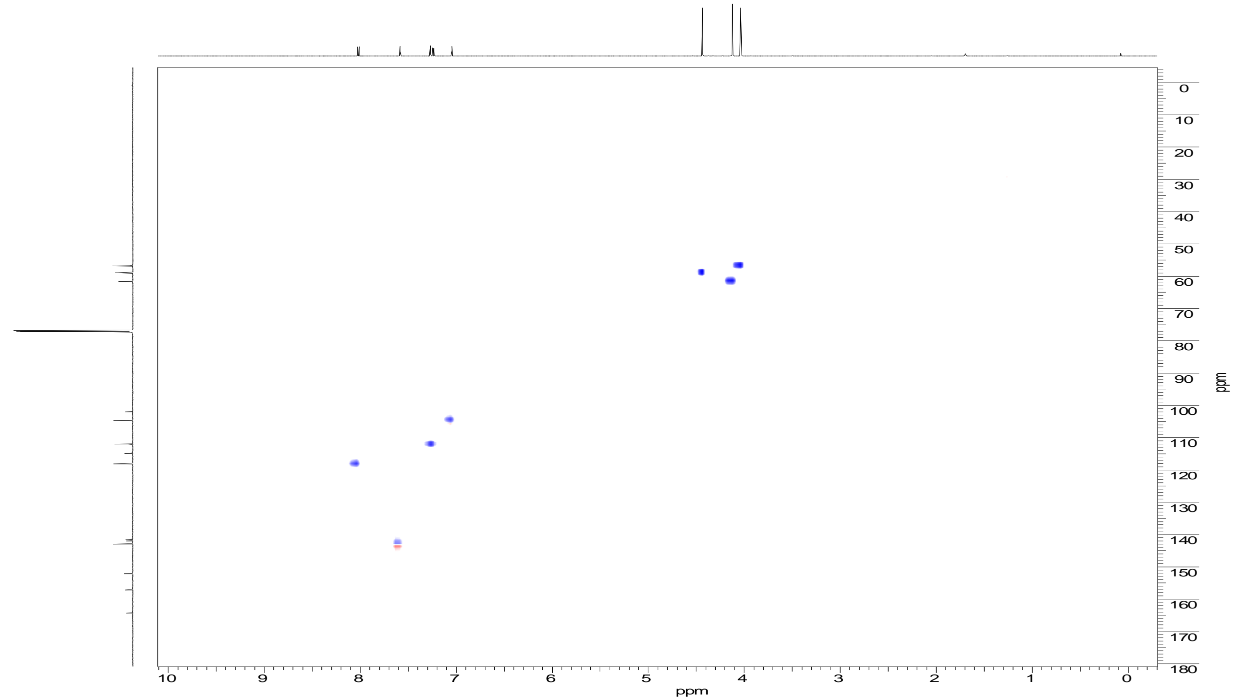
Appendix 5: HMBC spectra of compound 5



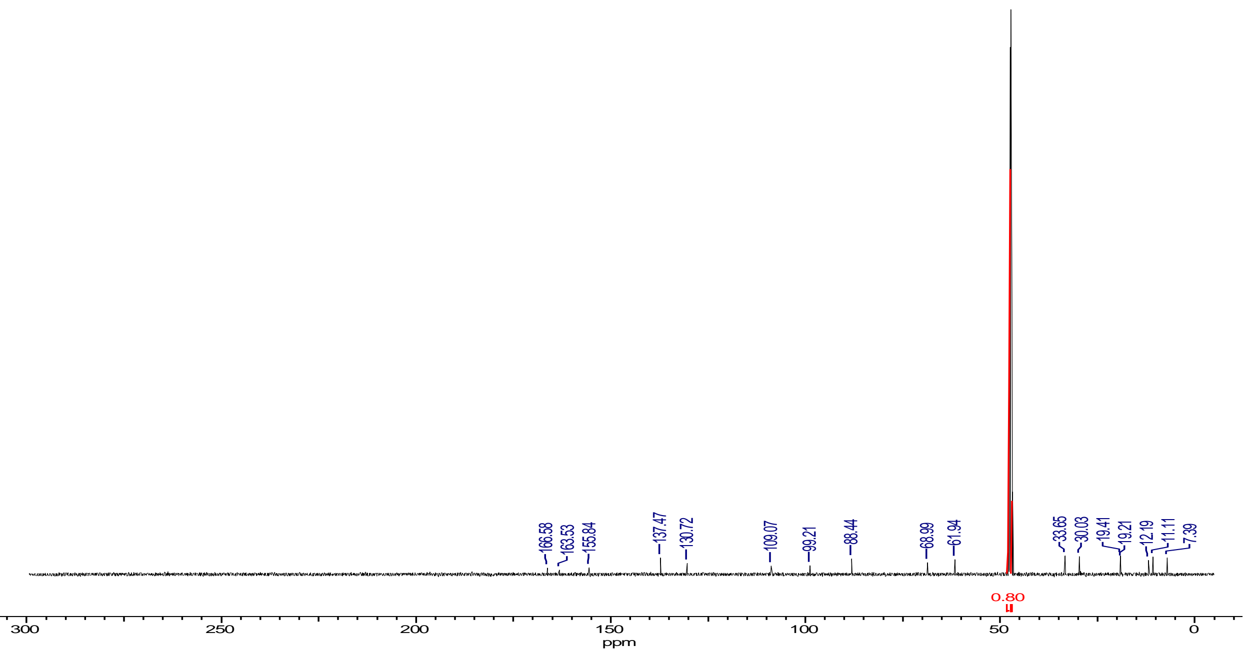
Appendix 6: COSY spectra of compound 5



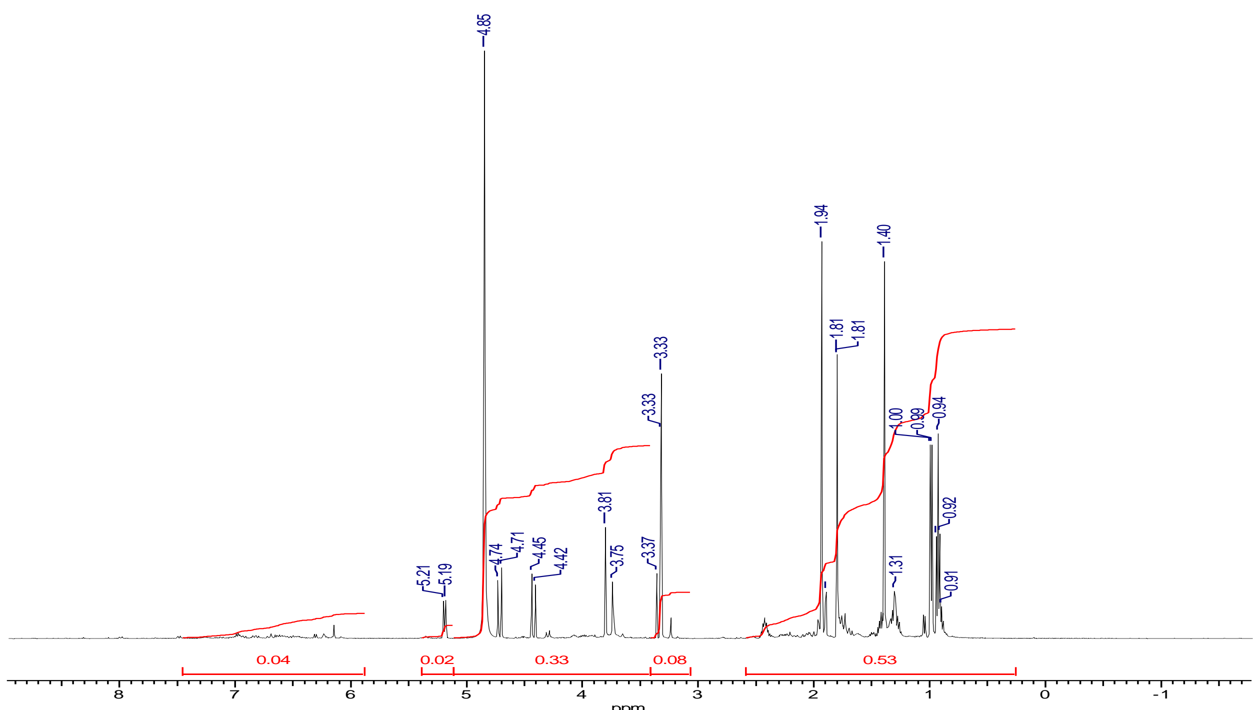
Appendix 7: HSQC spectra of compound 5



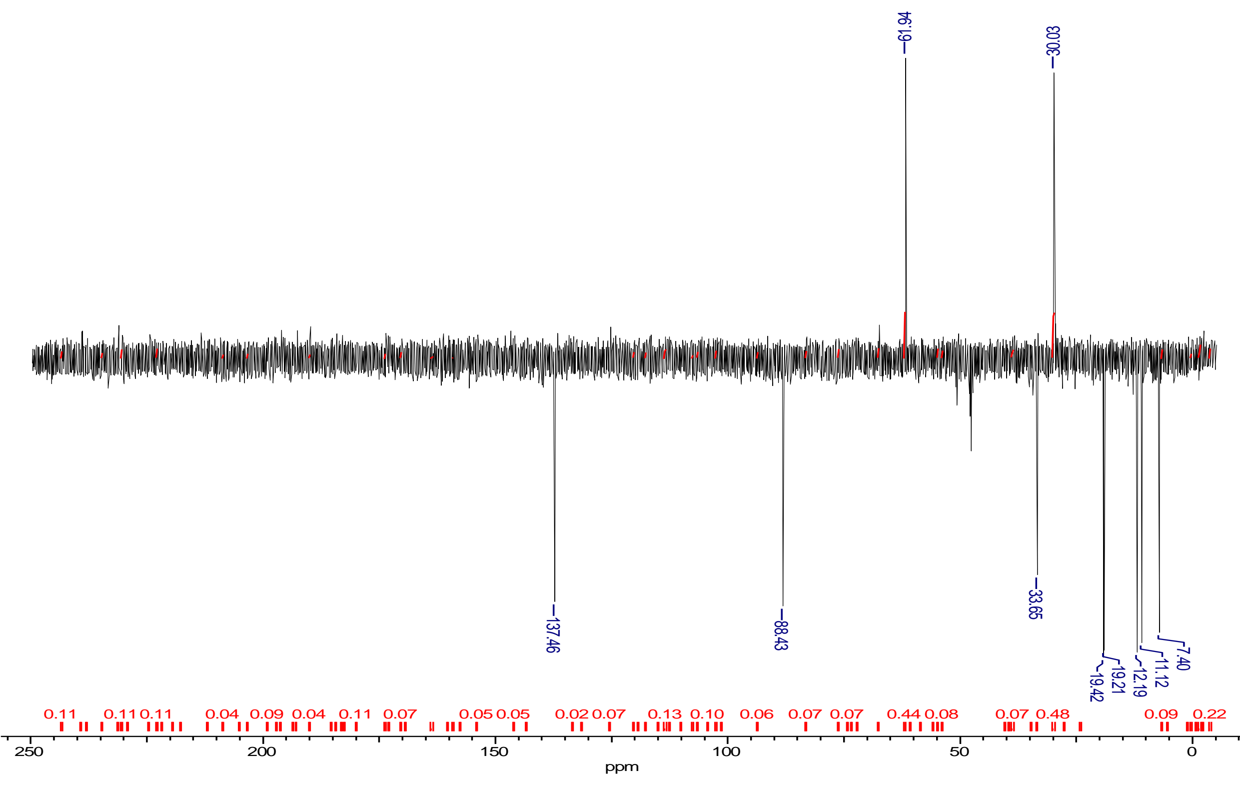
Appendix 8: ^{13}CMNR of compound 15



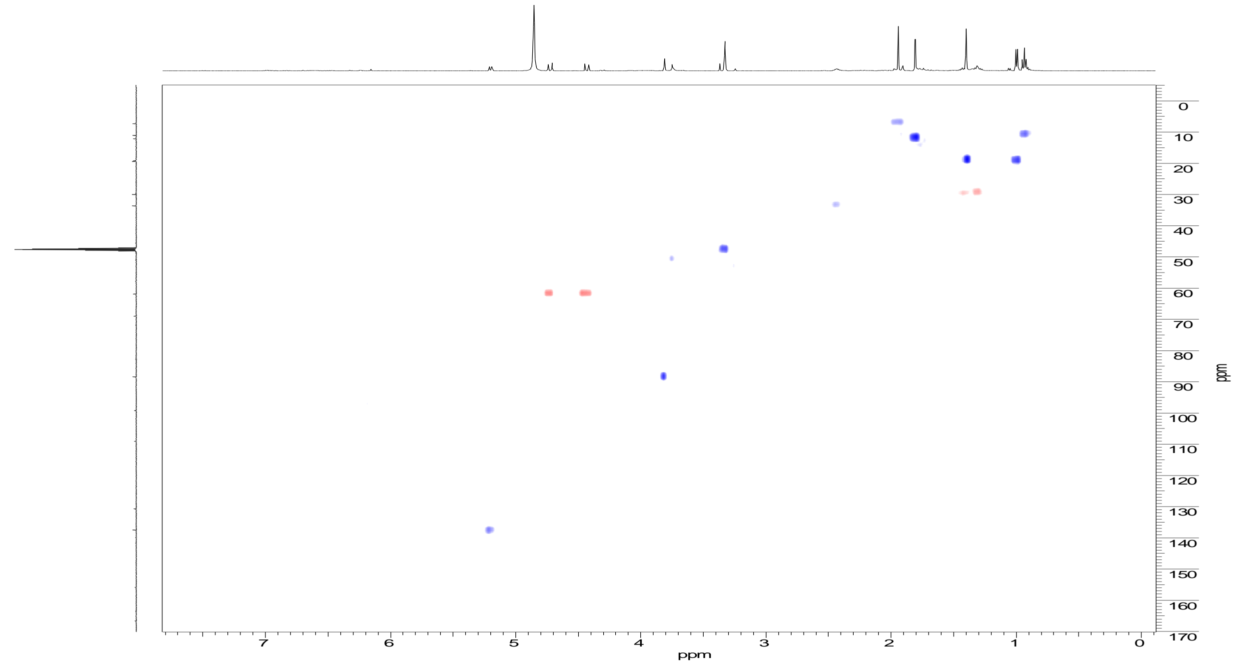
Appendix 9: ^{1}HNMR of compound 15



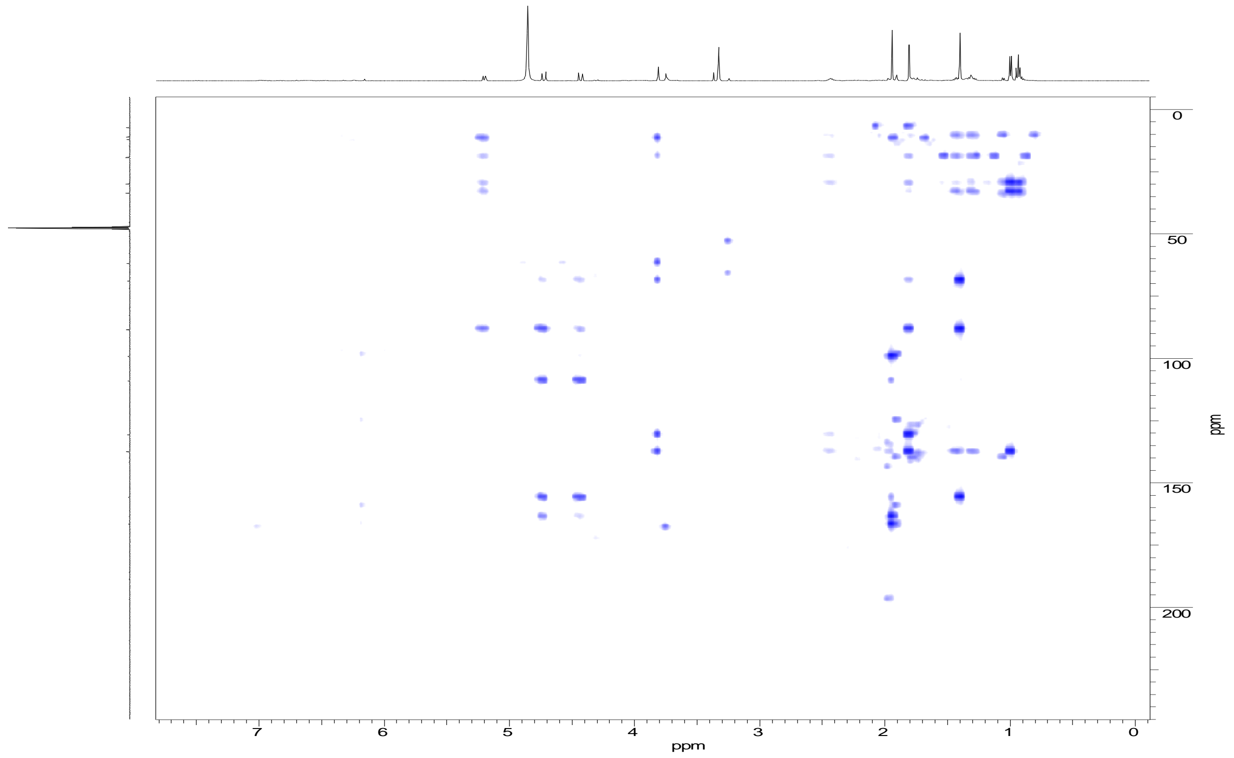
Appendix 10: DEPT spectrum of compound 15



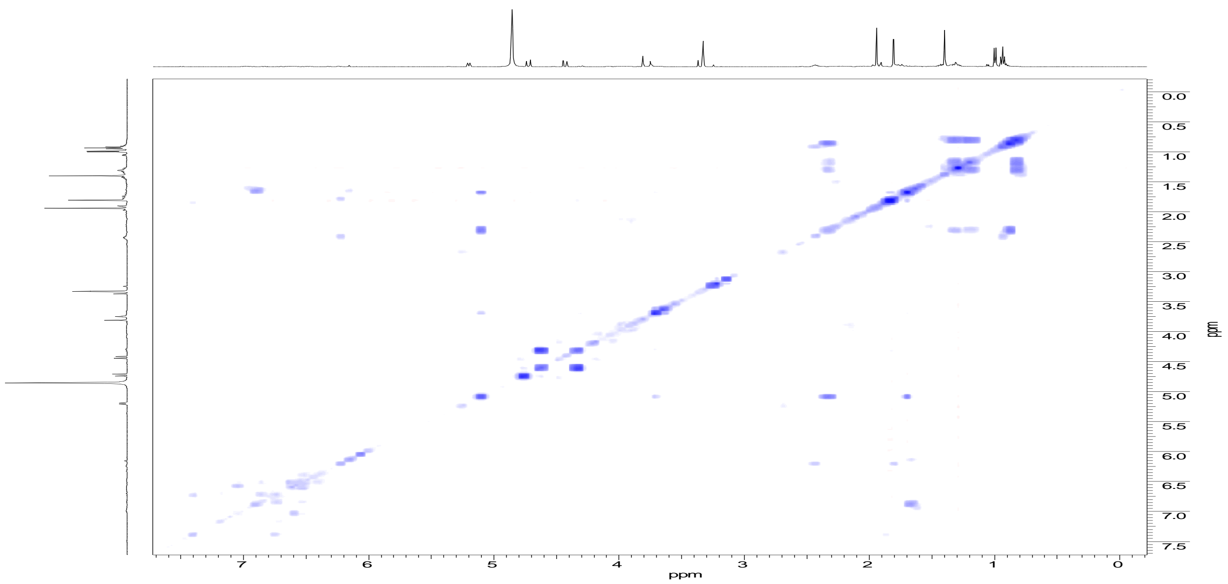
Appendix 11: HSQC spectrum of compound 15



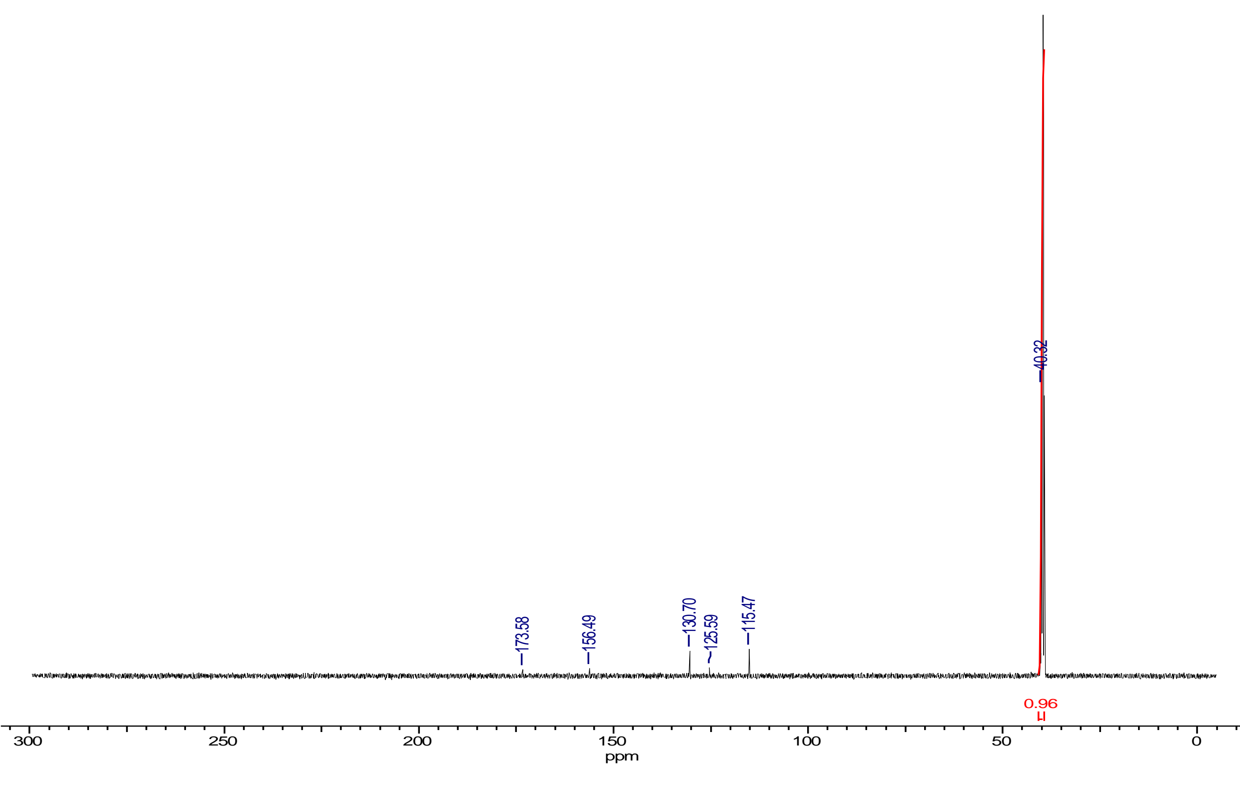
Appendix 12: HMBC spectrum of compound 15



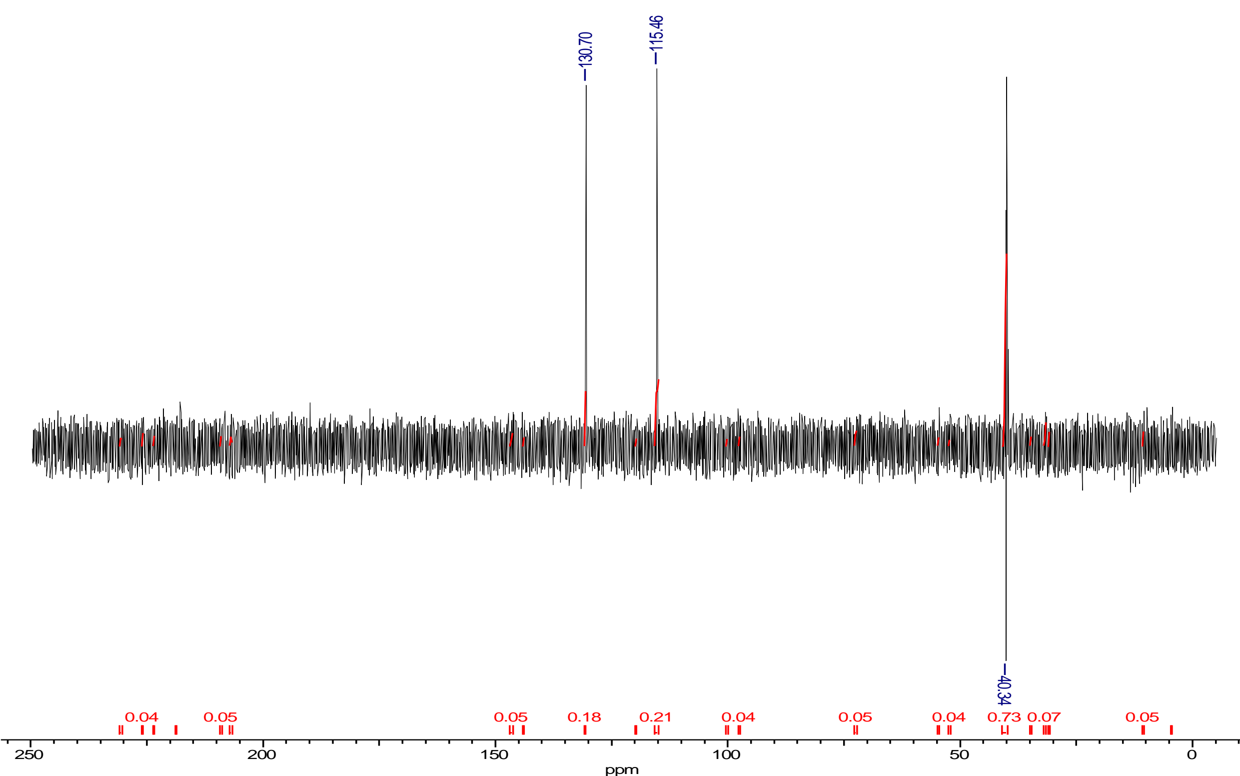
Appendix 13: COSY spectrum of compound 15



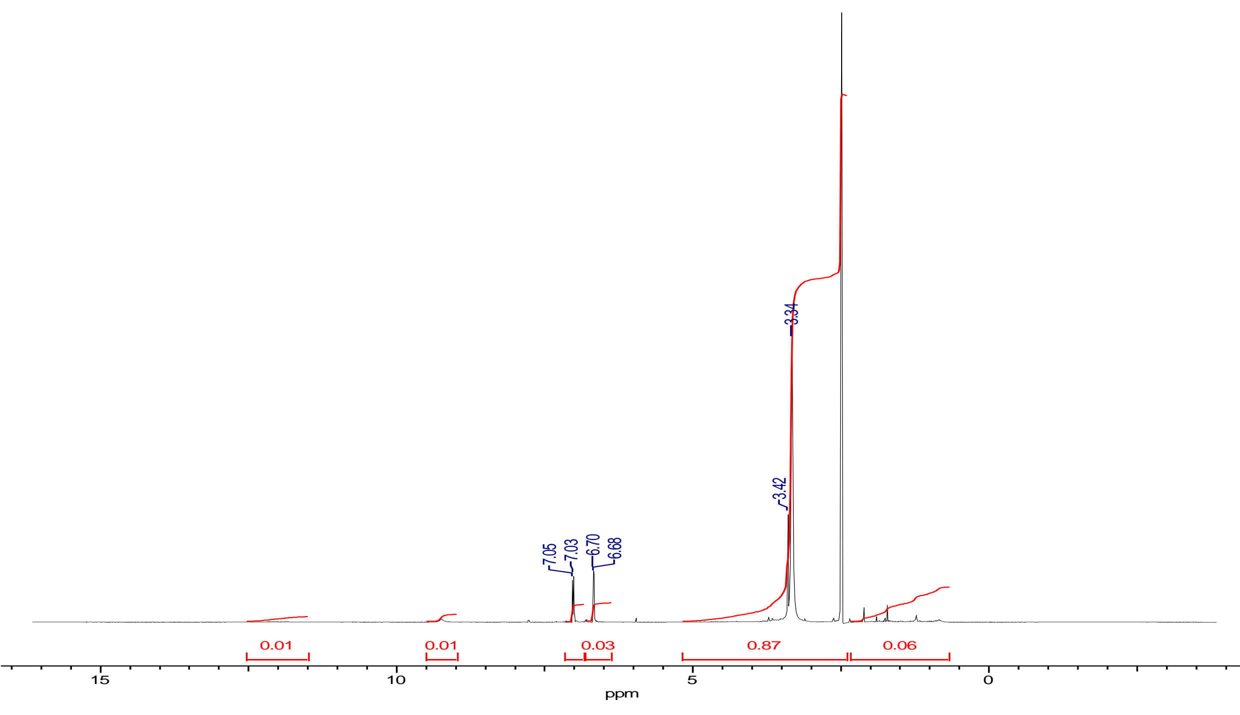
Appendix 14: ^{13}CNMR spectrum of compound 13



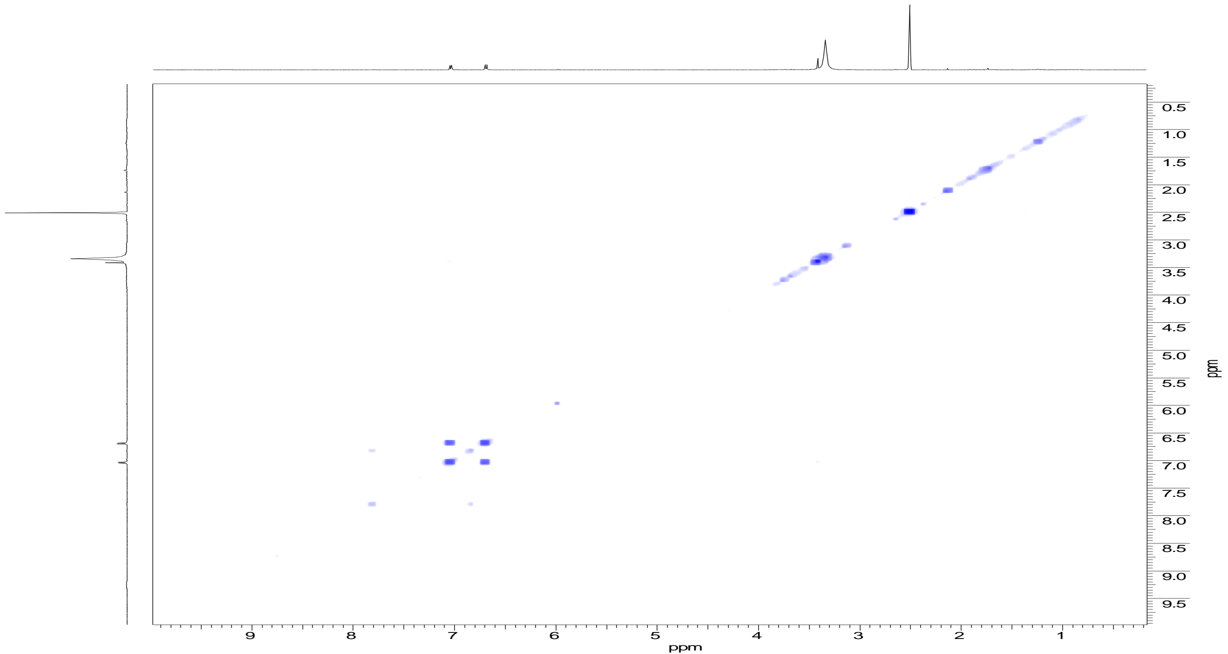
Appendix 15: DEPT spectrum of compound 13



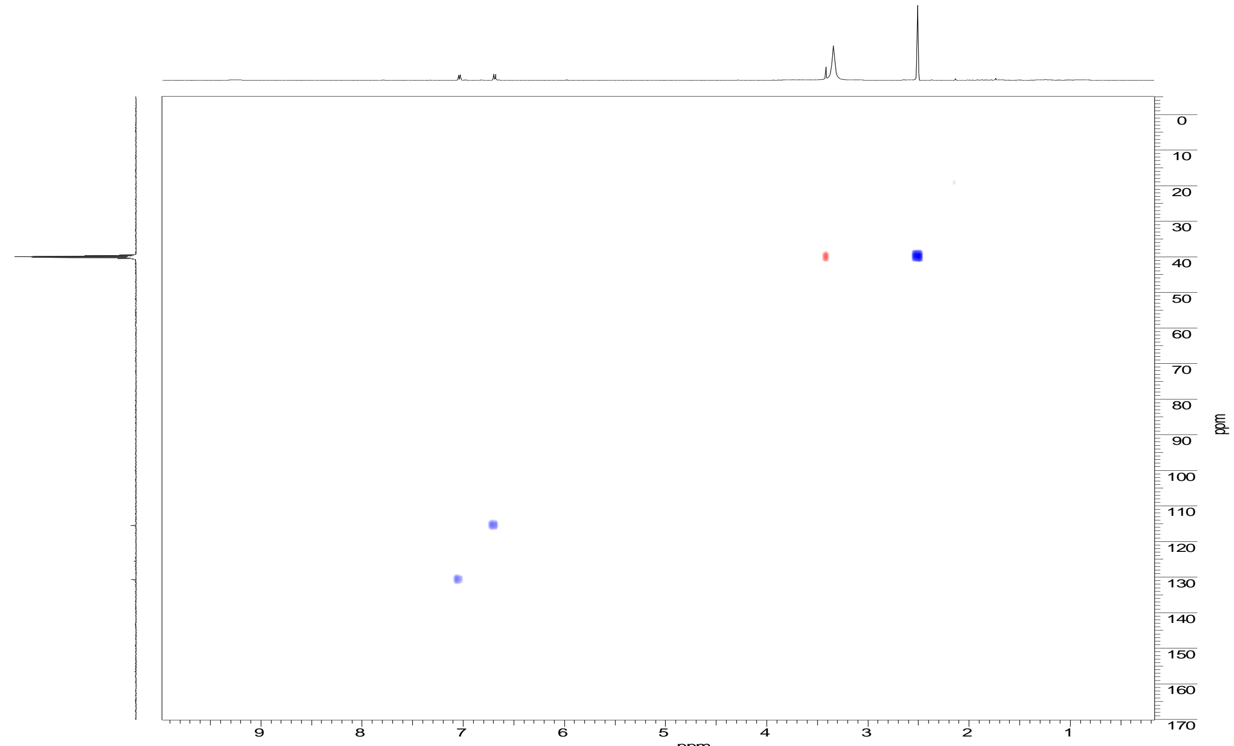
Appendix 16: ^{1}HNMR of compound 13



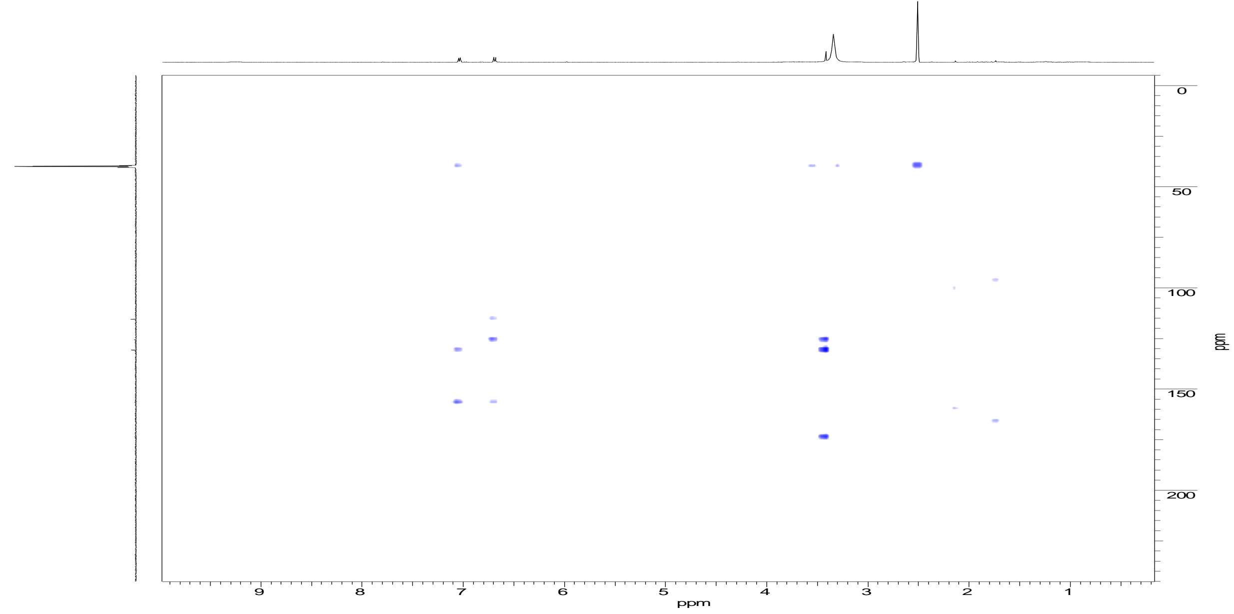
Appendix 17: COSY spectrum of compound 13



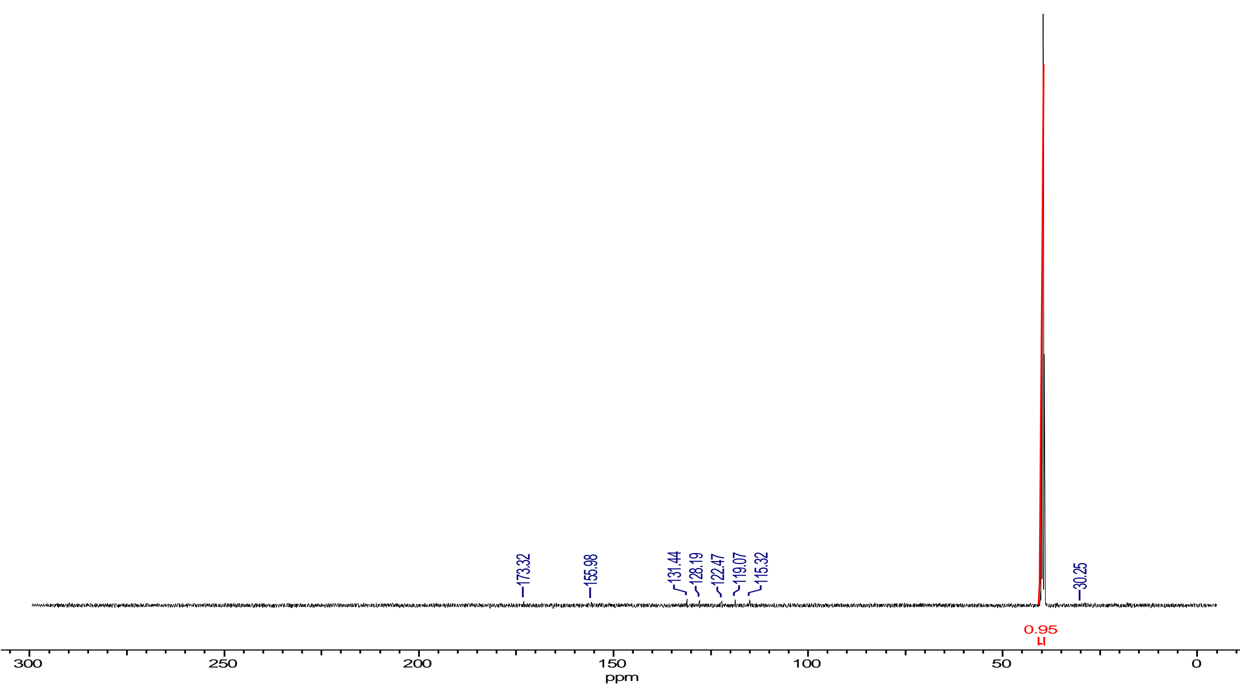
Appendix 18: HSQC spectrum of compound 13



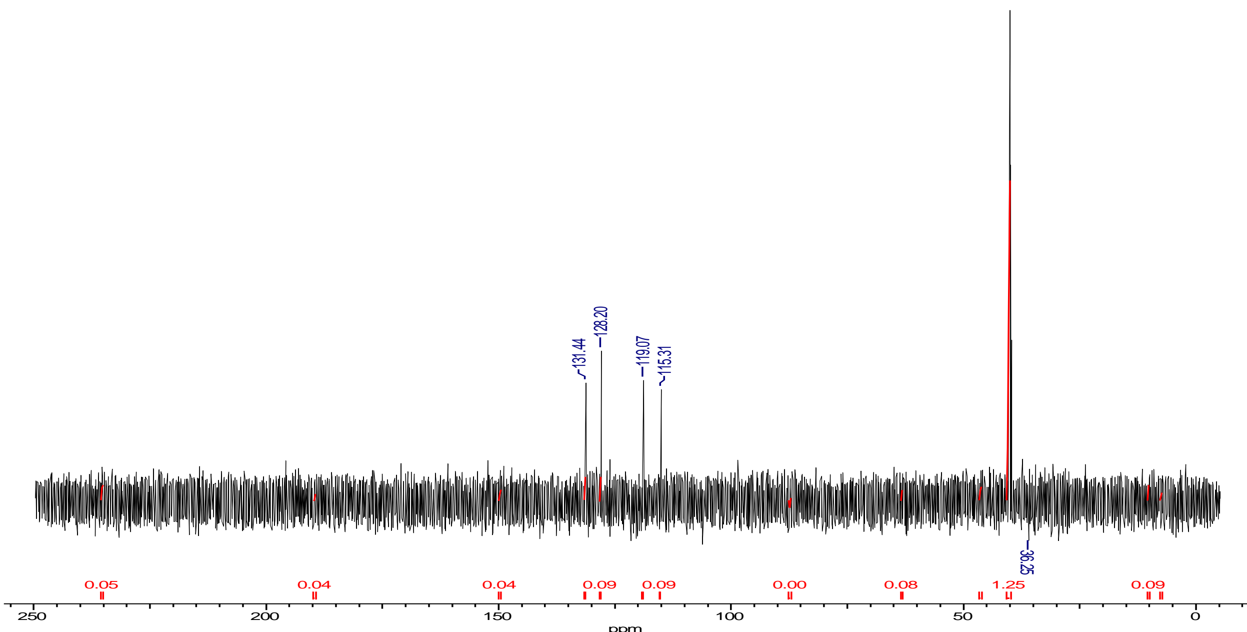
Appendix 19: HMBC spectrum of compound 13



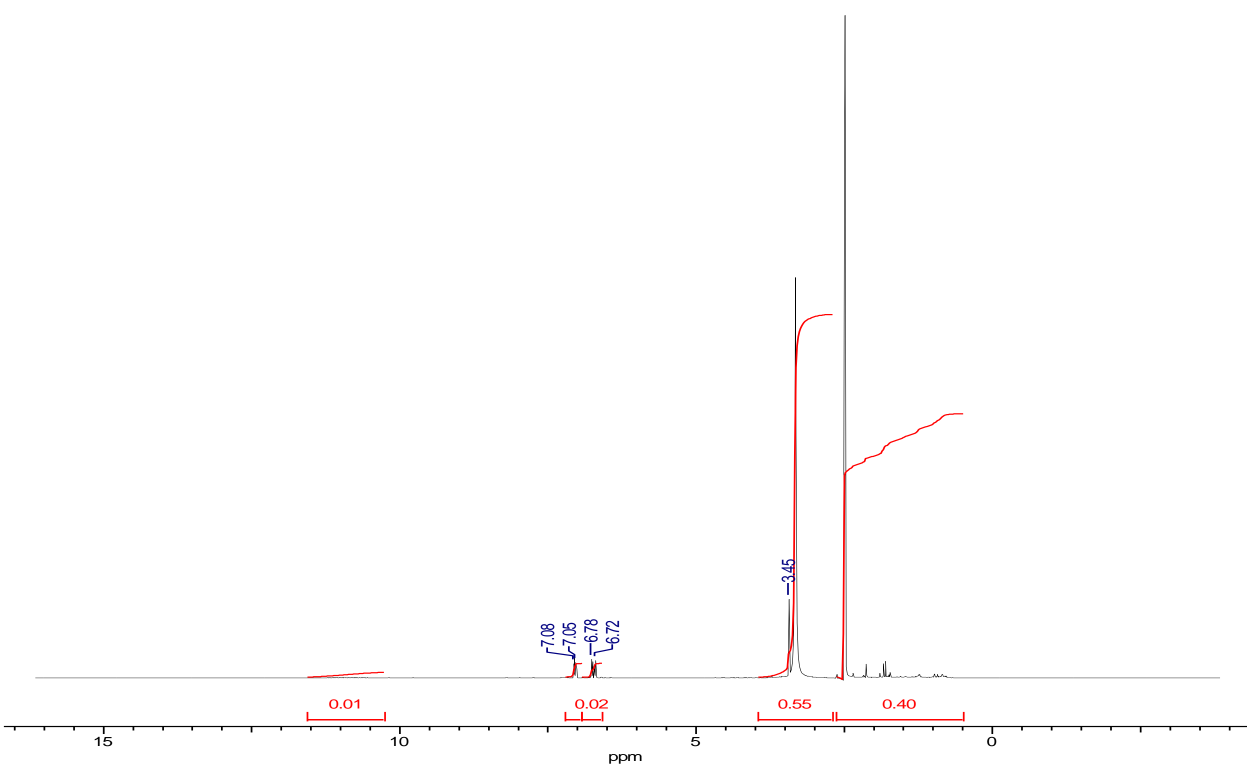
Appendix 20: ^{13}CNMR of compound 14



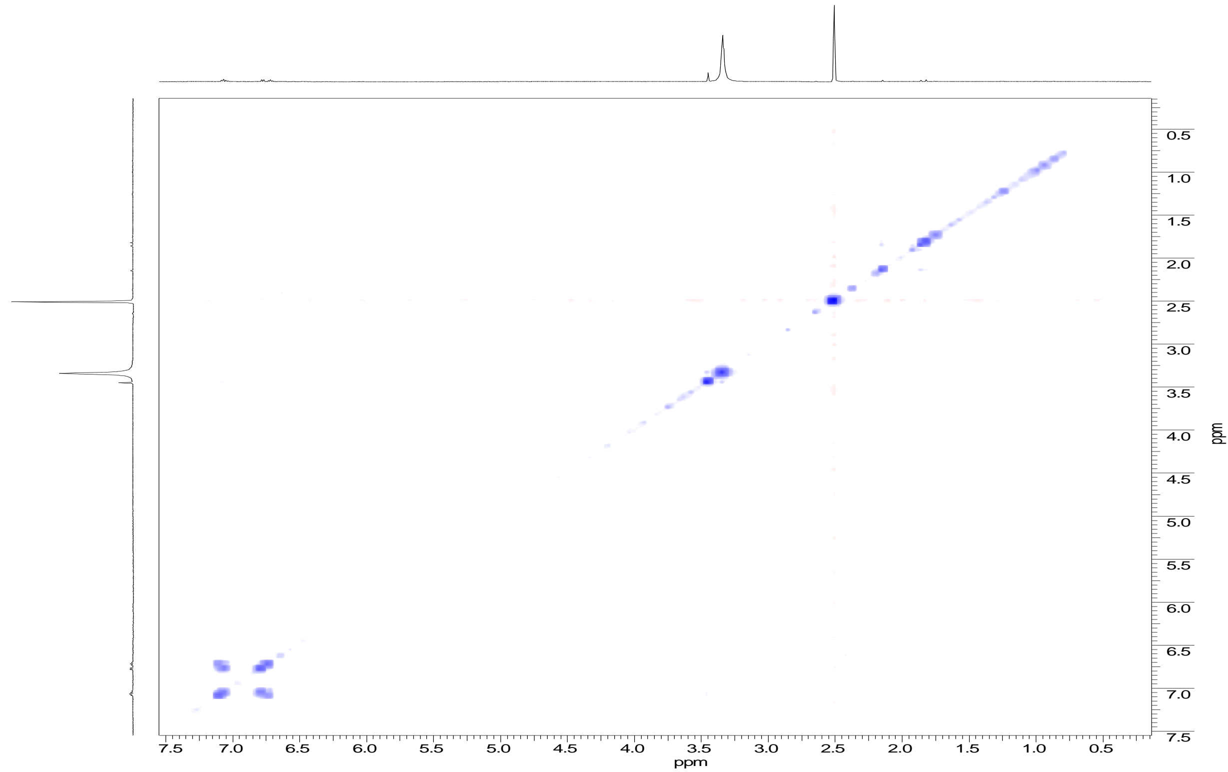
Appendix 21: DEPT spectrum of compound 14



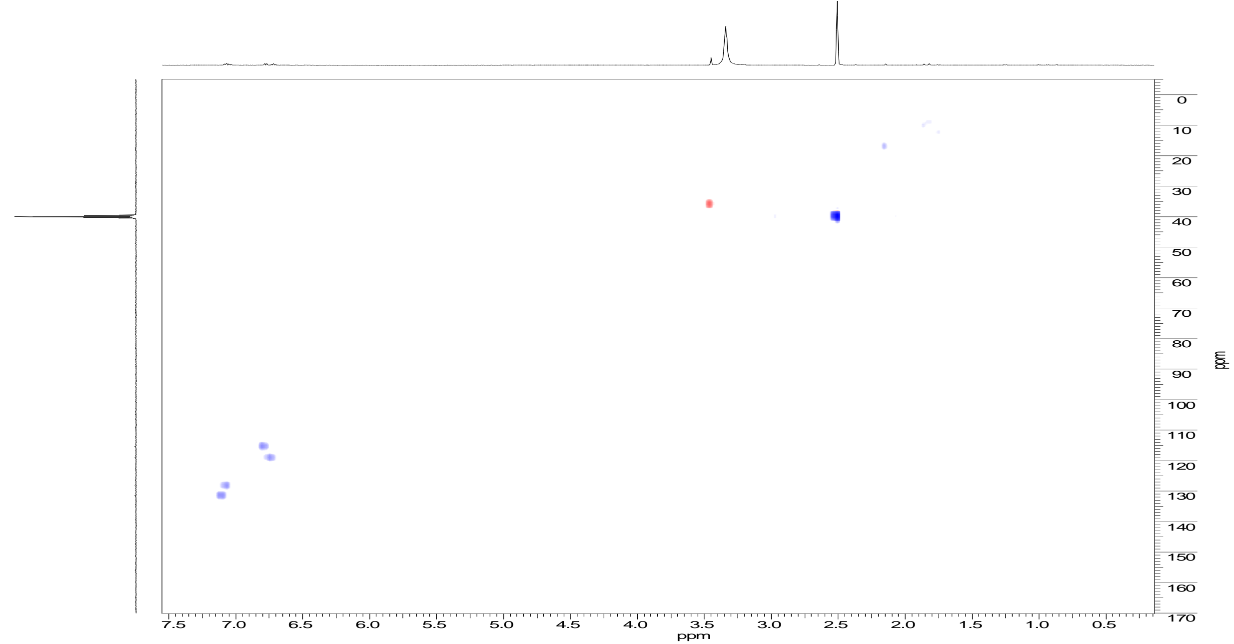
Appendix 22: ^{1}HNMR of compound 14



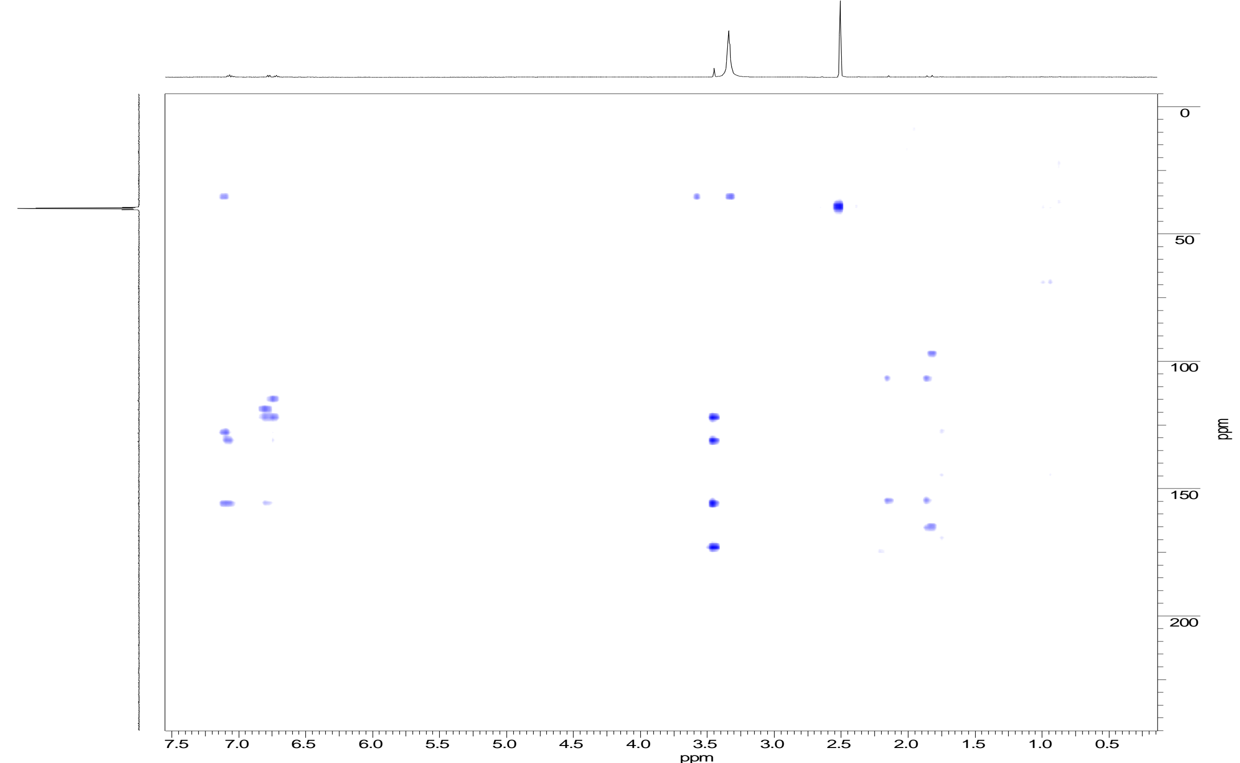
Appendix 23: COSY Spectrum of compound 14



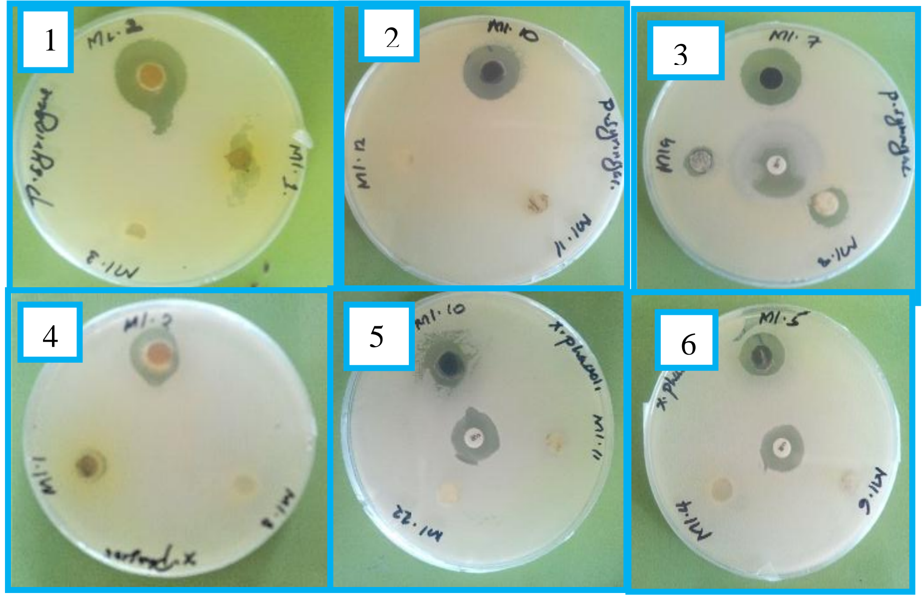
Appendix 24: HSQC spectrum of compound 14



Appendix 25: HMBC spectrum of compound 14

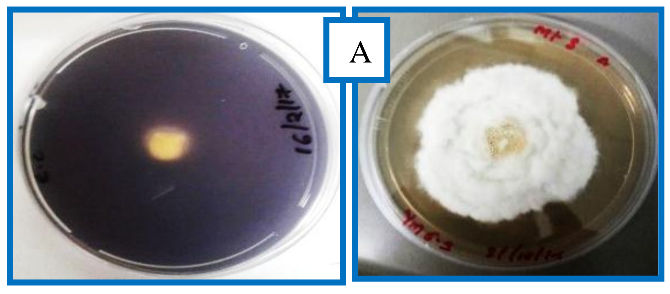


Appendix 26: Agar plug diffusion assay of the bioactive fungal endophytes against *X. axonopodis* pv. *Phaseoli* and *P. syringae* pv. *phaseolicola*

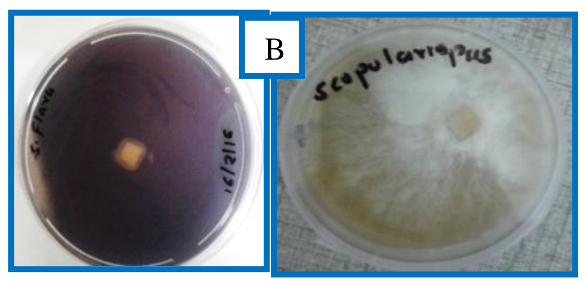


These plates show some of the active fungal endophytes that were isolated from both medicinal plants

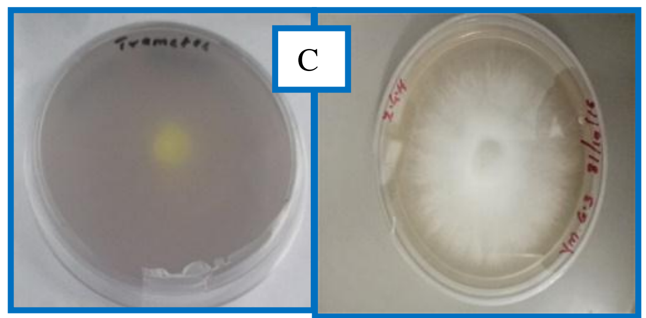
Appendix 27: Qualitative violacein assay of the selected fungal endophytes



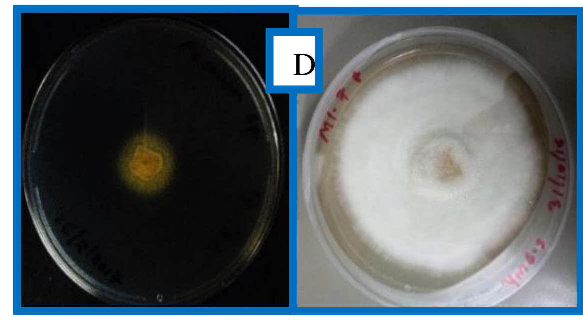
Chaetomium cochloides – **Potential activity**



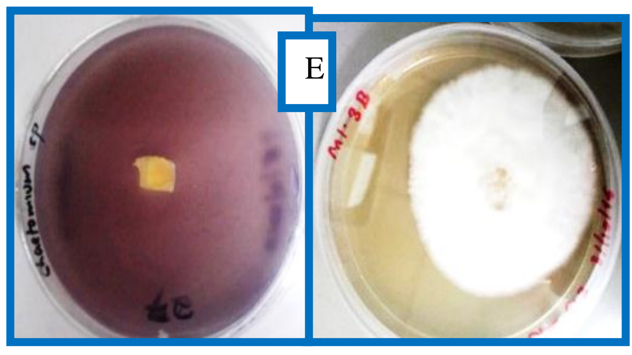
Scopulariopsis Flava– **Potential**



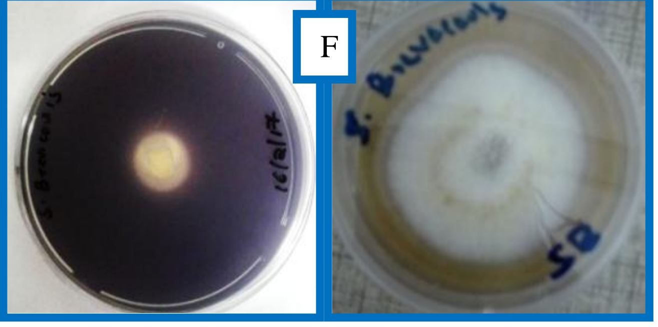
Trametes maxima species **potential activity**



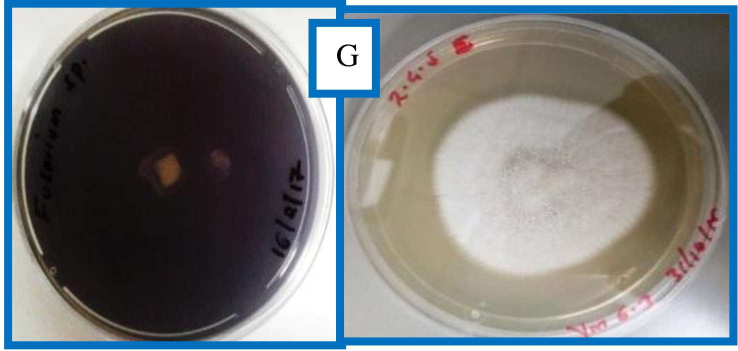
Fusarium species 2- **Positive**



Chaetomium sp. – **Negative**



Scopolariopsis brevicaulis– **antimicrobial**



Fusarium sp. **potential activity**

These plates show the activity of the selected fungal endophytes against *Chromobacterium violaceum.* The presence of the halo region in the plate indicate that the fungal strain has a potential as a quorum quenching source of secondary metabolites while the lack of it shows that the fungal strain is not active or the active secondary metabolites concentration is low.

Appendix 28: BLAST search hits of endophytic fungal isolates

NO:	Identity	Max. Score	Total score	Query cover	E-value	Identity	Accession number
1	*Chaetomium cf. cochloides*	1109	1109	99%	0	99%	KT895345.1
3	*Fusarium sp*	1077	1077	99%	0	100%	EU750687.1
5	*Chaetomium sp*	1120	1120	99%	0	99%	KM520350.1
6	*Fusarium sp.*	1083	1083	100%	0	100%	EU750687.1
7	*Fusarium solani Strain* GWG5(1)	1053	1053	100%	0	99%	KM268689.1
8	*Fusarium oxysporum*	780	780	100%	0	96%	KJ573079.1
9	*Fusarium solani Strain* GWG4(1)	1068	1068	100%	0	100%	KM268688.1
10	*Fusarium oxysporum (*isolate 850)	1040	1040	72%	0	99%	JN232136.1
11	*Scopulariopsis flava*	1153	1153	100%	0	99%	LN850790.1
12	*Fusarium sp.* 18014	1099	1099	94%	0	99%	EU750687.1
13	*Fusarium oxysporum*	1074	1074	100%	0	100%	KM889544.1
14	*Fusarium sp*	1064	1064	100%	0	100%	KM889541.1
15	*Tramets aff. Maxima*	1007	1007	99%	0	95%	JN164918.1
18	*Fusarium solani*	1064	1064	100%	0	100%	EU029589.1
19	*Fusarium solani*	1050	1050	89%	0	99%	KM268689.1
20	*Fusarium sp*	1044	1044	89%	0	95%	KT313630.1
21	*Fusarium sp.*	1083	1083	100%	0	100%	KM889544.1
22	*Fusarium solani*	1059	1059	100%	0	100%	KM268689.1
23	*Fusarium solani*	1053	1053	100%	0	100%	AB369907.1
24	*Fusarium sp.*	1092	1092	99%	0	99%	KM889544.1
27	*Chaetomium sp*	1070	1070	99%	0	99%	KM520346.1
29	*Fusarium sp*	1074	1074	100%	0	100%	EU750687.1
32	*Chaetomium sp.*	1016	1016	100%	0	100%	KR012907.1
47	*Scopulariopsis brevicaulis*	939	939	87%	0	99%	KP132728.1

Appendix 29: ITS consensus sequences of endophytic fungal isolates

S/N	Endophytic code		% Identity	Species Identity
1	Ml. 3A	TTCCTCTTATTGATATGCTTAAGTTCAGCGGGTCTTCC TACCTGATCCGAGGTCAACCTTGGGTTAAAAGGTGGT TTAACGGCCGGAACCCGCAGCACGCCCAGAGCGAGA TGTATGCTACTACGCTCGGTGTGACAGCGAGCCCGCC ACTGCTTTTCAGGGCCTGCGGCAGCCGCAGGTCCCCA ACACAAGCCGGGGGCTTGATGGTTGAAATGACGCTC GAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAA TGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGC AATTCACATTACTTATCGCATTTCGCTGCGTTCTTCAT CGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTT GACTTATTCAGTACAGAAGACTCAGAGAGGCCATAA ATTATCAAGAGTTTGGTGACCTCCGGCGGGCGCCCGC GGTGGGGCCCAGGGGCGCCCGGGGGGTAAACCCCGG GGCCGCCCGCCGAAGCAACGGTTTAGGTAACGTTCAC AATGGTTTAGGGAGTTTTGCAACTCTGTAATGATCCC TCCGCTGGTTCACCAACGGAGACCTTGTTACGACTTT TACTTCCTCTAAATGACCAAGA	99%	*Chaetomium cf. cochloides*
3	Ml.8	TATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTG ATTCGAGGTCAACATTCAGAAGTTGGGTGTTTTACGG CATGGCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCT ACTACGCAATGGAAGCTGCGGCGGGACCGCCACTGT ATTTGAGGGACGGCGTGTGCCCACAGGGGGCTTCCGC CGATCCCCAACGCCAGGCCCGGGGGCCTGAGGGTTG TAATGACGCTCGAACAGGCATGCCCGCCAGAATACT GGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCA CTGAATTCTGCAATTCACATTACTTATCGCATTTCGCT GCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTG TTGAAAGTTTTAATTTATTTGCTTGTTTACTCAGAAAA ACATTATAAAAACAGAGTTAGGGGTCCTCTGGCGGG GGCGGCCCGTTGTTACAGGGCCGTCTGTTCCCGCCGA AGCAACGTTTTAGGTATGTTCACAGGGTTGATGAGTT GTATAACTCGGTAATGATCCCTCCGCTGGTTCACCAA CGGAGACCTTGTTACGACTTTTACTTCCTCA	100%	*Fusarium* sp.
5	Ml.11	TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTCTT CCTACCTGATCCGAGGTCAACCTTGGGTTAAAAGGTG GTTTAACGGCCGGAACCCGCAGCACGCCCAGAGCGA GATGTATGCTACTACGCTCGGTGTGACAGCGAGCCCG CCACTGCTTTTCAGGGCCTGCGGCAGCCGCAGGTCCC CAACACAAGCCGGGGGCTTGATGGTTGAAATGACGC TCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGC AATGTGCGTTCAAAGATTCGATGATTCACTGAATTCT GCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTC ATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTT TTGACTTATTCAGTACAGAAGACTCAGAGAGGCCATA AATTATCAAGAGTTTGGTGACCTCCGGCGGGCGCCCG CGGTGGGGCCCAGGGGCGCCCGGGGGGTAAACCCCG GGGCCGCCCGCCGAAGCAACGGTTTAGGTAACGTTC	99%	*Chaetomium* sp.

		ACAATGGTTTAGGGAGTTTTGCAACTCTGTAATGATC CCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACT TTTACTTCCTCTAAATGACCAAGA		
6	Zg. 5	TTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTG ATTCGAGGTCAACATTCAGAAGTTGGGTGTTTTACGG CATGGCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCT ACTACGCAATGGAAGCTGCGGCGGGACCGCCACTGT ATTTGAGGGACGGCGTGTGCCCACAGGGGGCTTCCGC CGATCCCCAACGCCAGGCCCGGGGGCCTGAGGGTTG TAATGACGCTCGAACAGGCATGCCCGCCAGAATACT GGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCA CTGAATTCTGCAATTCACATTACTTATCGCATTTCGCT GCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTG TTGAAAGTTTTAATTTATTTGCTTGTTTACTCAGAAAA ACATTATAAAAACAGAGTTAGGGGTCCTCTGGCGGG GGCGGCCCGTTGTTACAGGGCCGTCTGTTCCCGCCGA AGCAACGTTTTAGGTATGTTCACAGGGTTGATGAGTT GTATAACTCGGTAATGATCCCTCCGCTGGTTCACCAA CGGAGACCTTGTTACGACTTTTACTTCCTCTA	100%	*Fusarium* sp.
7	Ml.7	GATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTC GAGGTCAACATTCAGAAGTTGGGTGTTTTACGGCATG GCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCTACTA CGCAATGGAAGCTGCGGCGGGACCGCCACTGTATTTG AGGGACGGCGTGTGCCCACAGGGGGCTTCCGCCGAT CCCCAACGCCAGGCCCGGGGGCCTGAGGGTTGTAAT GACGCTCGAACAGGCATGCCCGCCAGAATACTGGCG GGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGA ATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGT TCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGA AAGTTTTAATTTATTTGCTTGTTTACTCAGAAAAACAT TATAAAAACAGAGTTAGGGGTCCTCTGGCGGGGGCG GCCCGTTGTTACAGGGCCGTCTGTTCCCGCCGAAGCA ACGTTTTAGGTATGTTCACAGGGTTGATGAGTTGTAT AACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGA ACCTTGTTACACTTTTACTTCC	99%	*Fusarium solani Strain GWG5(1)*
8	Ml. 5	AGGTGTTAGCTACTACCAATGGAAGCTGCGGCGGGA CCGCCACTGTAAGGGACGGCGTGTGCCCACAGGGGG CTTCCGCCGATCCCCAACGCCAGGCCCGGGGGCCTGA GGGTTGTAATGACGCTCGAACAGGCATGCCCGCCGA ATACTGGCGGGCGCAATGTGCGTTCAAAGATTCATGA TTCACTGAATTCTGCAATTCACATTACTTATCCATTTC CTGCGTTCTTCATCGATGCCAAGCCAAGAGATCCGTT GTTGAAAGTTTTAATTTATTTGCTTGTTTACTCAAAAA ACATTATAAAAACAGATTAGGGGTCCTCTGGCGGGG GCGGCCCGTTGTTACAGGGCCGTCTGTTCCCGCCGAA GCAACGTTTTAGGATGTTCACGGGTTGATGATTGTAT AACTCGGTAATGATCCCTCCGCTGGTTCACCAACGAA CCTTGTTACACTTTTACTTCCTCTA	96%	*Fusarium oxysporum*
9	Ml.2	GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAA CCAGCGGAGGGATCATTACCGAGTTATACAACTCATC AACCCTGTGAACATACCTAAAACGTTGCTTCGGCGGG AACAGACGGCCCTGTAACAACGGGCCGCCCCCGCCA GAGGACCCCTAACTCTGTTTTTATAATGTTTTTCTGAG TAAACAAGCAAATAAATTAAAACTTTCAACAACGGA	100%	*Fusarium solani Strain GWG4(1)*

		TCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAA TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC ATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATT CTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCT CAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAG CCCCCTGTGGGCACACGCCGTCCCTCAAATACAGTGG CGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACA CCTCGCAACTGGAGAGCGGCGCGGCCATGCCGTAAA ACACCCAACTTCTGAATGTTGACCTCGAATCAGGTAG GAATACCCGCTGAACTTAAGCATATCA		
10	Zg.1	GATATGCTTAAGTTCAGCGGGTATTCCTACTGATTCG AGGTCAACATTCAGAAGTTGGGTGTTTTAGGCATGGC CGCGCCGCTCTCCAGTTGCGAGGTGTTAGCTACTACG CAATGGAAGCTGCGGCGGGACCGCCACTGTATTTGA GGGACGGCGTGTGCCCACAGGGGGCTTCCGCCGATC CCCAACGCCAGGCCCGGGGGCCTGAGGGTTGTAATG ACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGG GCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAA TTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTT CTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAA AGTTTTAATTTATTTGCTTGTTTACTCAGAAAAACATT ATAAAAACAGAGTTAGGGGTCCTCTGGCGGGGGCGG CCCGTTGTTACAGGGCCGTCTGTTCCCGCCGAAGCAA CGTTTTAGGTATGTTCACAGGGTTGATGAGTTGTATA ACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAA CCTTGTTACACTTTTACTTCCCCACAGGTAACCTTGAT ATGTGGGGGGACGGCAGAACCCACGCGCGCCCTGAG CGAGGAGTTTACGCACTCGGTACACACACCTGGATTT GCTGCGGACAGGGCCACAAGCCGGGGCTTGATGTTG AACGCTCGAACAGGCATGCCCGCCAATACTGGCCAA TGTGCGTTCAAAGATTCATGATTCACTGATCGCAATT CAATTAATCAATTT	99%	*Fusarium oxysporum (isolate 850)*
11	Ml.6	AAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCG GAGGGATCATTACCGAAGTTACTCTTCAAAACCCATT GTGAACCTTACCTCTTGCCGCGCGTTGCCTCGGCGGG GAGGCGGGGTCTGGGTCGGCGCGCCCCTCACCGGGC CGCCGTCCCCGTCCCCGTCCCCGCCGGCCGCGCCAAA CTCTAAATTTGAAAAAGCGTACTGCACGTTCTGATTC AAAACAAAAAACAAGTCAAAACTTTTAACAACGGAT CTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT GCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA TCGAATCTTTGAACGCACATTGCGCCCGGCAGCAATC TGCCGGGCATGCCTGTCCGAGCGTCATTTCTTCCCTC GAGCGCGGCTAGCCCTACGGGGCCTGCCGTCGCCCG GTGTTGGGGCTCTACGGGTGGGGCTCGTCCCCCCCGC AGTCCCCGAAATGTAGTGGCGGTCCAGCCGCGGCGC CCCCTGCGTAGTAGATCCTACATCTCGCATCGGGTCC CGGCGAAGGCCAGCCGTCGAACCTTTTATTTCATGGT TTGACCTCGGATCAGTAGGGTACCCGCTGAACTTAAG CATAT	99%	*Scopulariopsis flava*
12	Zg.8	CTTGGTCATTTAGAGGAAGTAAAAGTGTAACAAGGTC TCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTT ATACAACTCATCAACCCTGTGAACATACCTAAAACGT TGCTTCGGCGGGAACAGACGGCCCTGTAACAACGGG	99%	*Fusarium* sp. *18014*

		CCGCCCCCGCCAGAGGACCCCTAACTCTGTTTTTATA ATGTTTTTCTGAGTAAACAAGCAAATAAATTAAAACT TTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAG AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG AATTCAGTGAATCATCGAATCTTTGAACGCACATTGC GCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCG TCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGG GATCGGCGGAAGCCCCCTGTGGGCACACGCCGTCCCT CAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCG TAGTAGCTAACACCTCGCAACTGGAGAGCGGCGCGG CCATGCCGTAAAACACCCAACTTCTGAATGTTGACCT CGAATCAGGTAGGAATACCCGCTGAACTTAAGCATAT CAATAAGGGAGGAACTGGTTCACCAACGGAACCTTG TACGACTTTTACT		
13	Zg. 5	GATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTC GAGGTCAACATTCAGAAGTTGGGTGTTTTACGGCATG GCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCTACTA CGCAATGGAAGCTGCGGCGGGACCGCCACTGTATTTG AGGGACGGCGTGTGCCCACAGGGGGCTTCCGCCGAT CCCCAACGCCAGGCCCGGGGGCCTGAGGGTTGTAAT GACGCTCGAACAGGCATGCCCGCCAGAATACTGGCG GGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGA ATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGT TCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGA AAGTTTTAATTTATTTGCTTGTTTACTCAGAAAAACAT TATAAAAACAGAGTTAGGGGTCCTCTGGCGGGGGCG GCCCGTTGTTACAGGGCCGTCTGTTCCCGCCGAAGCA ACGTTTTAGGTATGTTCACAGGGTTGATGAGTTGTAT AACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGA GACCTTGTTACGACTTTTACTTCCTCTA	100%	*Fusarium oxysporum*
14	Ml.9	GATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTC GAGGTCAACATTCAGAAGTTGGGTGTTTTACGGCATG GCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCTACTA CGCAATGGAAGCTGCGGCGGGACCGCCACTGTATTTG AGGGACGGCGTGTGCCCACAGGGGGCTTCCGCCGAT CCCCAACGCCAGGCCCGGGGGCCTGAGGGTTGTAAT GACGCTCGAACAGGCATGCCCGCCAGAATACTGGCG GGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGA ATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGT TCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGA AAGTTTTAATTTATTTGCTTGTTTACTCAGAAAAACAT TATAAAAACAGAGTTAGGGGTCCTCTGGCGGGGGCG GCCCGTTGTTACAGGGCCGTCTGTTCCCGCCGAAGCA ACGTTTTAGGTATGTTCACAGGGTTGATGAGTTGTAT AACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGA GACCTTGTTACGACTTTTACTTC	100%	*Fusarium* sp
15	Zg.4	GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAA CCTGCGGAAGGATCATTAACGAGTTTTGAAACGGGTT GTAGCTGGCCCTCCGGGGCATGTGCACACTCTGCTCA TCCACTCTACACCTGTGCACTCATTGTAGGTTGGCGT GGGCTCCGAGCTTCCGGGCTCGGGGCATTCTGCCGGC CTATGTACACTACAAACACTTTAAAGTATCAGAATGT CAACGCGTGTAACGCACCTTTATACAACTTTCAGCAA CGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGC	95%	*Tramets aff. maxima*

		GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT GAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTG GTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGGAA TTCTCAACCAGTAAATCCTTGTGGTTTACGGGCTTGG ATTTGGGGGCTTGCCGGCTCCAGTAAAGTCGGCTCCT CTTGAATGCATTAGCTTGATTCCGTGCGGATCGGCTC TCAGTGTGATAATTGTCTACGCTGTGGCCGTGAAGCG TTTGGCAAGCTTCTAACCGTCCTTTTAGGACAACGAT CTGACATCTGACCTCAAATCAGGTAGGACTACCCGCT GAACTTAAGCATATCAT		
18	Ml.1	GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAA CCAGCGGAGGGATCATTACCGAGTTATACAACTCATC AACCCTGTGAACATACCTAAAACGTTGCTTCGGCGGG AACAGACGGCCCTGTAACAACGGGCCGCCCCCGCCA GAGGACCCCTAACTCTGTTTTTATAATGTTTTTCTGAG TAAACAAGCAAATAAATTAAAACTTTCAACAACGGA TCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAA TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC ATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATT CTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCT CAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAG CCCCCTGTGGGCACACGCCGTCCCTCAAATACAGTGG CGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACA CCTCGCAACTGGAGAGCGGCGCGGCCATGCCGTAAA ACACCCAACTTCTGAATGTTGACCTCGAATCAGGTAG GAATACCCGCTGAACTTAAGCATAT	100%	*Fusarium solani*
19	Ml. 10	TGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATK MGAGGTCAACATTCAGAAGTTGGGTGTTTTACGGCAT GGCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCTACT ACGCAATGGAAGCTGCGGCGGGACCGCCACTGTATTT GAGGGACGGCGTGTGCCCACAGGGGGCTTCCGCCGA TCCCCAACGCCAGGCCCGGGGGCCTGAGGGTTGTAAT GACGCTCGAACAGGCATGCCCGCCAGAATACTGGCG GGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGA ATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGT TCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGA AAGTTTTAATTTATTTGCTTGTTTACTCAGAAAAACAT TATAAAAACAGAGTTAGGGGTCCTCTGGCGGGGGCG GCCCGTTGTTACAGGGCCGTCTGTTCCCGCCGAAGCA ACGTTTTAGGTATGTTCACAGGGTTGATGAGTTGTAT AACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGA ACCTTGTTACACTTTTACTTCCAGAAAGATCTCCGCCT TTTACCATTACCCCCCTACTTTCTAAGTTTCGAAAGGC CTAGAAACCATGA	99%	*Fusarium solani*
20	Zg. 2	TTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTG ATTCGAGGTCAACATTCAGAAGTTGGGTGTTTTACGG CATGGCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCT ACTACGCAATGGAAGCTGCGGCGGGACCGCCACTGT ATTTGAGGGACGGCGTGTGCCCACAGGGGGCTTCCGC CGATCCCCAACGCCAGGCCCGGGGGCCTGAGGGTTG TAATGACGCTCGAACAGGCATGCCCGCCAGAATACT GGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCA CTGAATTCTGCAATTCACATTACTTATCGCATTTCGCT GCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTG	995%	*Fusarium* sp.

		TTGAAAGTTTTAATTTATTTGCTTGTTTACTCAGAAAA ACATTATAAAAACAGAGTTAGGGGTCCTCTGGCGGG GGCGGCCCGTTGTTACAGGGCCGTCTGTTCCCGCCGA AGCAACGTTTAGGTATGTTCACAGGGTTGATGAGTT GTATAACTCGGTAATGATCCCTCCGCTGGTTCACCAA CGGAGACCTTGTTACGACTTTACTTCCTCTAAATGA CCAAGA		
21	Zg.3	TTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTG ATTCGAGGTCAACATTCAGAAGTTGGGTGTTTACGG CATGGCCGCGCCGCTCTCCAGTTGCGAGGTGTTAGCT ACTACGCAATGGAAGCTGCGGCGGACCGCCACTGT ATTTGAGGGACGGCGTGTGCCCACAGGGGGCTTCCGC CGATCCCCAACGCCAGGCCCGGGGGCCTGAGGGTTG TAATGACGCTCGAACAGGCATGCCCGCCAGAATACT GGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCA CTGAATTCTGCAATTCACATTACTTATCGCATTTCGCT GCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTG TTGAAAGTTTAATTTATTTGCTTGTTTACTCAGAAAA ACATTATAAAAACAGAGTTAGGGGTCCTCTGGCGGG GGCGGCCCGTTGTTACAGGGCCGTCTGTTCCCGCCGA AGCAACGTTTAGGTATGTTCACAGGGTTGATGAGTT GTATAACTCGGTAATGATCCCTCCGCTGGTTCACCAA CGGAGACCTTGTTACGACTTTACTTCCTCTA	100%	*Fusarium* sp.
22	Ml.4	AAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACC AGCGGAGGGATCATTACCGAGTTATACAACTCATCAA CCCTGTGAACATACCTAAAACGTTGCTTCGGCGGGAA CAGACGGCCCTGTAACAACGGGCCGCCCCCGCCAGA GGACCCCTAACTCTGTTTTTATAATGTTTTTCTGAGTA AACAAGCAAATAAATTAAAACTTTCAACAACGGATC TCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATG CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT CGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCT GGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTCA GGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCC CCCTGTGGGCACACGCCGTCCCTCAAATACAGTGGCG GTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACACC TCGCAACTGGAGAGCGGCGCGGCCATGCCGTAAAAC ACCCAACTTCTGAATGTTGACCTCGAATCAGGTAGGA ATACCCGCTGAACTTAAGCATA	100%	*Fusarium solani*
23	Ml. 6	GGAAGTAAAAGTGTAACAAGGTCTCCGTTGGTGAAC CAGCGGAGGGATCATTACCGAGTTATACAACTCATCA ACCCTGTGAACATACCTAAAACGTTGCTTCGGCGGGA ACAGACGGCCCTGTAACAACGGGCCGCCCCCGCCAG AGGACCCCTAACTCTGTTTTTATAATGTTTTTCTGAGT AAACAAGCAAATAAATTAAAACTTTCAACAACGGAT CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAAT GCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA TCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTC TGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTC AGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGC CCCCTGTGGGCACACGCCGTCCCTCAAATACAGTGGC GGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACAC CTCGCAACTGGAGAGCGGCGCGGCCATGCCGTAAAA CACCCAACTTCTGAATGTTGACCTCGAATCAGTAGGA	100%	*Fusarium solani*

		ATACCCGCTGAACTTAAGCATATC		
24	Zg.6	TCTTGGTCATTTAGAGGAAGTAAAAGTGAACAAGGTT CCGTTGGTGRACCAGCGGAGGGATCATTACCGAGTTA TACAACTCATCAACCCTGTGAACATACCTAAAACGTT GCTTCGGCGGGAACAGACGGCCCTGTAACAACGGGC CGCCCCCGCCAGAGGACCCCTAACTCTGTTTTTATAA TGTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTT TCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGA ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGT CATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGG ATCGGCGGAAGCCCCCTGTGGGCACACGCCGTCCCTC AAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGT AGTAGCTAACACCTCGCAACTGGAGAGCGGCGCGGC CATGCCGTAAAACACCCAACTTCTGAATGTTGACCTC GAATCAGGTAGGAATACCCGCTGAACTTAAGCATATC AATAAGCGGAGGAA	99%	*Fusarium* sp.
27	Ml.13	ATATGCTTAAGTTCAGCGGGTCTTCTACCTGATCCGA GGTCAACCTTGGGTTAAAAGGTGGTTTAACGGCCGGA ACCCGCAGCACGCCCAGAGCGAGATGTATGCTACTA CGCTCGGTGTGACAGCGAGCCCGCCACTGCTTTTCAG GGCCTGCGGCAGCCGCAGGTCCCCAACACAAGCCGG GGGCTTGATGGTTGAAATGACGCTCGAACAGGCATG CCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAA AGATTCGATGATTCACTGAATTCTGCAATTCACATTA CTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAA CCAAGAGATCCGTTGTTGAAAGTTTTGACTTATTCAG TACAGAAGACTCAGAGAGGCCATAAATTATCAAGAG TTTGGTGACCTCCGGCGGGCGCCCGCGGTGGGGCCCA GGGGCGCCCGGGGGGTAAACCCCGGGGCCGCCCGCC GAAGCAACGGTTTAGGTAACGTTCACAATGGTTTAGG GAGTTTTGCAACTCTGTAATGATCCCTCCGCTGGTTC ACCAACGGAGACCTTGTTACGACTTTTACTTCCTCA	99%	*Chaetomium* sp.
29	Zg.7	TAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGG TGAACCAGCGGAGGGATCATTACCGAGTTATACAACT CATCAACCCTGTGAACATACCTAAAACGTTGCTTCGG CGGGAACAGACGGCCCTGTAACAACGGGCCGCCCCC GCCAGAGGACCCCTAACTCTGTTTTTATAATGTTTTTC TGAGTAAACAAGCAAATAAATTAAAACTTTCAACAA CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGC GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT GAATCATCGAATCTTTGAACGCACATTGCGCCCGCCA GTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACA ACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCG GAAGCCCCCTGTGGGCACACGCCGTCCCTCAAATACA GTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCT AACACCTCGCAACTGGAGAGCGGCGCGGCCATGCCG TAAAACACCCAACTTCTGAATGTTGACCTCGAATCAG GTAGGAATACCCGCTGAACTTAAGCATATC	100%	*Fusarium* sp.
32	Zg.10	TTAAGTTCAGGGGTCTTCTACTGATCGAGGTCAACCT TGGGTTAAAAGGTGGTTTAACGGCCGGAACCCGCAG CACGCCCAGAGCGAGATGTATGCTACTACGCTCGGTG TGACAGCGAGCCCGCCACTGCTTTTCAGGGCCTGCGG	100%	*Chaetomium* sp.

		CAGCCGCAGGTCCCCAACACAAGCCGGGGGCTTGAT GGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGA ATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATG ATTCACTGAATTCTGCAATTCACATTACTTATCGCATT TCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATC CGTTGTTGAAAGTTTTGACTTATTCAGTACAGAAGAC TCAGAGAGGCCATAAATTATCAAGAGTTTGGTGACCT CCGGCGGGCGCCCGCGGTGGGGCCCAGGGGCGCCCG GGGGGTAAACCCCGGGGCCGCCCGCCGAAGCAACGG TTTAGGTAACGTTCACAATGGTTTAGGGAGTTTTGCA ACTCTGTAATGATCCCTCCGCTGGTTCACCAACGAGA CTTGTTACGACTTTTAC		
47	Ml.15	GCGGAGGTCTTACCGAAGTTCTCTTACCCTTGTGCCC TCTGCCGCGCGGCCGCGGGGAGGGCTGGGACCGCCA CTACATTTCGGGGACTGCGGGGGGGACGAGCCCCAC CCGTAGAGCCCCAACACCGGGCGACGGCAGGCCCCG TAGGGCTAGCCGCGCTCGAGGGAAGAAATGACGCTC GGACAGGCATGCCCGGCAGATTGCTGCCGGGCGCAA TGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGC AATTCACATTACTTATCGCATTTCGCTGCGTTCTTCAT CGATGCCAGAACCAAGAGATCCGTTGTTAAAAGTTTT GACTTGTTTTTTGTTTTGAATCAGAACGTGCAGTACG CTTTTTCAAATTTAGAGTTTGGCGCGGCCGGCGGGGA CGGGGACGGGGACGGCGGCCCGGTGAGGGGCGCGCC GACCCAGACCCCGCCTCCCCGCCGAGGCAACGCGCG GCAAGAGGTAAGGTTCACAATGGGTTTTGAAGAGTA ACTTCGGTAATGATCCCTCCGCTGGTTCACCAACGGA GACCTTGTTACGACTTTTACTTCCTCTAAGACCAAGA	99%	*Scopulariopsis brevicaulis*

Appendix 30*:* ANOVA summary of inhibition zones of the isolated endophytes against *X. axonopodis* pv. *Phaseoli.*

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3447.653	24	143.652	111.071	.000
Within Groups	64.667	50	1.293		
Total	3512.320	74			

Appendix 31: ANOVA summary of inhibition zones of isolated endophytes against *P. syringae* pv. *phaseolicola*

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3460.667	24	144.194	166.378	.0001

Within Groups	43.333	50	.867		
Total	3504.000	74			

Appendix 32: ANOVA summary of inhibition zones of the extracts against *X. axonopodis* pv. *phaseoli*

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1356.405	13	104.339	121.729	.000
Within Groups	24.000	28	.857		
Total	1380.405	41			

Appendix 33: ANOVA summary of zone of inhibition of the extracts against *P. syringae* pv. *phaseolicola*

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1625.238	13	125.018	403.905	.000
Within Groups	8.667	28	.310		
Total	1633.905	41			