ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC FUNGI ISOLATED FROM *Warburgia Ugandensis A*GAINST *Candida Albicans*

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Antimicrobial Activity of Endophytic Fungi Isolated from *Warburgia Ugandensis a*gainst *Candida albicans*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this thesis to my dear son Simeon and my beloved husband Timothy, the rest of my family members, spiritual parents who have been walking with me and friends as well. May the holy God of Israel bless you abundantly.

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LIST OF ABBREVIATIONS/ ACRONYMS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BSIs	Blood Stream Infections
CMR	Centre for Microbiology Research
KEMRI	Kenya Medical Research Institute
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
$FeCl_3$	Iron (III) Chloride
HIV	Human Immunodeficiency Virus
H_2SO_4	Sulfuric Acid
HSV-1	Herpes Simplex Virus 1
KOH	Potassium Hydroxide
LSD	Least Significant Difference
MEGA	Molecular Evolutionary Genetics Analysis
MIC	Minimum Inhibition Concentration
NCBI	National Centre for Biotechnology Information
PCR	Polymerase Chain Reaction

PDA	Potato Dextrose Agar
SAS	Statistical Analysis System
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USA	United States of America

ABSTRACT

Candida albicans is a dimorphic fungus which is part of normal microbial flora of an immunocompetent individual but becomes pathogenic in immunocompromised individuals causing infections such as candidiasis. Management of *Candida albicans* infections is by use of antifungals which are becoming increasingly resistant hence bioactive compounds from fungal endophytes can be of pharmacological value. Endophytes are microorganisms living in cells and tissues of plants without causing diseases. Both the endophyte and the host plant experience a symbiotic mode of nutrition where by the endophytes get shelter and nourishment from the host plant and in return they increase the ability of the host plant nutrients uptake and disease resistance. This study aimed at isolation of fungal endophytes from medicinal plant *Warburgia ugandensis* from Mt Kenya forest and determination of antimicrobial activity of their extracts against *Candida albicans*. Sterilized plant materials were incubated on tap water agar for six days at 27 ºC. Fungal positives were transferred to potato dextrose agar (PDA) plates followed by 4 days' incubation at the same temperature. Colony purification gave pure cultures which were subjected to macroscopic, microscopic and sequencing methods of identification. Extraction of bioactive compounds was done by use of organic solvent ethyl acetate. Extracts were subjected to preliminary toxicity testing using brine shrimp eggs. LC_{50} values were calculated using regression equations from graphs of percentage mortality of brine shrimps versus log10 of the fungal extracts generated using Microsoft Excel. Preliminary phytochemical screening of the extracts was done. Antimicrobial activity of the extracts was determined using disk diffusion method according to CLSI protocols. Seventeen endophytic fungi were isolated and identified up to molecular level as; *Nigrospora oryzae*, *Aspergillus flavus*, *Cladosporium spp*. (two), *Fusarium oxysporum*, *Phomopsis spp*.(two), *Colletotrichum acutatum*, *Altanaria spp.* (two), *Cochliobolus sativus*, *Bionectria ochroleuca*, *Phyllosticta gardeniicola*, *Guignardia mangiferae*, *Tricharina gilva*, *Diaporthe amygdali* and *Trichoderma harzianum*. All the seventeen fungal endophytes had DNA base pairs ranging from 1500-2000bp. All the fungal extracts had LC_{50} values >1000 µg/ml (greater than 1000 µg/ml) when tested for toxicity with brine shrimps hence nontoxic. *Colletotrichum acutatum* had the lowest LC_{50} value (2040 µg/ml) while *Phyllosticta gardeniicola* had the highest LC_{50} value (8500 µg/ml). Average LC_{50} value for all the extracts was 4451.8µg/ml. Phytochemical screening of the fungal extracts showed absence of phenols and anthraquinones in all the extracts; presence of saponins, tannins, alkaloids, flavonoids, sterols and glycosides in most of the extracts. Extracts of *Phomopsis mali* and *Alternaria alternata* showed activity against *Candida albicans* with 2.5 mm average diameter zone of inhibition; the rest of the extracts didn't show any activity against the test organisms. This study shows that fungal endophytes can be a potential source of metabolites which can be useful in pharmaceutical industry.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Endophytes are microorganisms which live within plant cells and tissues without causing adverse effects on the plant (Kalyanasundaram *et al*., 2015). Endophytes have been reported to be important source of bioactive compounds which are useful therapeutic materials for treatment of some of the infectious diseases and cancer (Premjanu *et al*., 2016). Both endophytes and the host plants experience symbiotic mode of nutrition where by the endophytes obtain nutrients from the plants while they improve nutrient uptake by the plant promoting vigorous growth and development (Suciatmih and Rahmansyah, 2013).

Candida albicans is a dimorphic fungus which is nonpathogenic and part of normal microbial flora inhabiting the upper respiratory tract, gastrointestinal tract, urinary tract and the skin. However, the organism invades the mucous membranes in the immunocompromised individuals causing opportunistic infections such as candidiasis, oral thrush, candiduria, candidemia and vulvovaginitis (Kalia *et al*., 2015). Individuals at risk of being infected include HIV and cancer patients, those in intensive care unit, and those undergoing major surgery or organ transplants (Dabas, 2013). Vaginal candidiasis was shown to increase in pregnant women according to previous study due to various factors such as immune suppression, stress, change of diet, hormonal changes which result to changes in vaginal content hence overgrowth of *Candida albicans* (Menza *et al*., 2013a).

Treatment of *Candida albicans* infections is done through administration of antifungals such as fluconazole, itraconazole, voriconazole, and posaconazole among others (Ngure *et al*., 2009). More antifungal agents in place for treatment include caspofungin. However, the drugs used today exhibit different sensitivity to various *Candida* isolates, some being resistant (Maroszyńska *et al*., 2013). Increased drug resistance over time is a threat to the public health because it leads to increased cost of health care, morbidity and mortality rates. Fungal endophytes could be an

alternative source of treatment hence susceptibility testing of endophytic bioactive compounds on *Candida albicans* can be of help in drug development

(Kalyanasundaram *et al*., 2015). The purpose of this study was to isolate endophytic bioactive compounds from *Warburgia ugandensis* and determine their effect on *Candida albicans*.

1.2 Statement of the Problem

Candidiasis is the main infection caused by *Candida albicans* and account to over 60% of infections in clinical practice. It is a significant cause of morbidity and mortality in immunocompromised individuals. The population of Kenya being over 40 million, 594,660 women are found to get over four episodes of *Candida albicans* infections yearly. Development of antifungal resistance has also led to increased fungal infections and significant morbidity especially in patients with Human Immunodeficiency Virus (HIV), cancer and diabetes. This calls for more research in alternative treatment of *Candida albicans* infections.

1.3 Justification

Candidiasis has emerged as an opportunistic infection over time with increased rates of cancer and HIV among other infections which compromise the immune system. There is also increase in drug resistance in treatment of *Candida albicans* infections hence need for alternative treatment. According to the available literature, the use of *Warburgia ugandensis* as a medicinal plant in various parts of Kenya and Africa for treatment of various infections is justified. Endophytes from *Warburgia ugandensis* when exploited can be potential source of secondary metabolites which can be used as compounds for manufacturing novel drugs like antifungals. The study therefore seeks to explore the potential of secondary metabolites of fungal endophytes isolated from *Warburgia ugandensis*. It also seeks to add to the existing knowledge and contribute towards development of novel antifungals which are more effective in management of candidiasis as well as curbing the issue of antifungal resistance.

1.4 Null Hypothesis

The bioactive compounds produced by *Warburgia ugandensis* fungal endophytes have no activity against *Candida albicans*.

1.5 Objectives

1.5.1 General Objective

To isolate endophytic fungi from *Warburgia ugandensis* and determine the activity of their extracts against *Candida albicans*.

1.5.2 Specific Objectives

1. To determine the occurrence of fungal endophytes in *Warburgia ugandensis*
2. To determine the phytochemicals present in the fungal endophytic extracts
3. To determine the antimicrobial activity of the extracts from the endophytic fungi on *Candida albicans*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Warburgia ugandensis

Warburgia ugandensis, also referred to as Kenyan green heart tree, is a spreading evergreen tree which is 4.5-30 m tall and 70 cm in diameter (Figure 2.1). The bark is smooth or scaly, pale green or brown (Figure 2.2), and it's clear of branches for about 3m height (http://www.worldagroforestry.org, 2009). It is commonly found in tropical Africa. The tree belongs to the *Canellaceae* family and has been widely used by the local communities in traditional medicine for treatment of various ailments such as stomach-ache, constipation, toothache, common cold, cough, fever, muscle pains, weak joints, measles, and malaria (Drage *et al*., 2014). The tree is said to be used in Kisii region which is in Southwest Kenya, for treatment of malaria, diabetes and pneumonia (Maobe & Nyarango, 2013).

Pharmacological studies have shown that *Warburgia ugandensis* extracts have antimicrobial activities. Abuto *et al.* (2016) reported that *Warburgia ugandensis* extracts collected from different populations across Kenyan Rift Valley showed activity against *Candida albicans* and *Staphylococcus aureus* but were resistant to *Escherichia coli*. The plant extract has been reported to have metabolites such as alkaloids, terpenes, flavonoids, cardiac glycosides, polyphenols and terpenoids among others which are of pharmacological importance (Were *et al*., 2015; Maobe & Nyarango, 2013). Research on a similar species of the plant, *Warburgia sulutaris*, has indicated that the tree has antimycobacterial and antiviral properties as a result of antioxidative properties of the extracts which may be due to drimane sesquiterpenoids namely; warburganal, isopolygodial, polygodial, mukaadial and ugandensidial (Kuglerova *et al*., 2011). Drimane sesquiterpenes and farnesol are reported to be biosynthesized by endophytes found in the *Warburgia spp*. through biotransformation process and is said to have insecticidal, molluscicidal, antimicrobial and cytotoxicity properties (Nermina, 2012).

Warburgia ugandensis was found to have antimicrobial properties against *Staphylococcus aureus, Shigella boydii, Escherichia coli, Klebsiella. pneumonia, Pseudomonas aeruginosa* and *Candida albicans* in a study done in Addis Ababa, Ethiopia (Merawie *et al*., 2013). A previous study done in Kenya has also showed that *Warburgia ugandensis* extract has antileishmania activity in addition to antifungal activity against *Fusarium oxysporum, Alternaria passiflorae*, and *Aspergillus niger* which are environmental fungi (Ngure *et al*., 2009).

Figure 2.1: Warburgia ugandensis Tree.

Photograph by world Agroforestry centre

Figure 2.2: Warburgia ugandensis bark,

Photograph by Scamperdale

2.2 Importance of Endophytes

Endophytes colonize various plant cells and tissues. The type of plant tissue determines the species of the endophyte and its frequency in that given tissue. Some endophytes will be more frequent in a root of a given plant than in the bark of the same plant (Kumar and Hyde, 2004). Some studies show that endophytes are not host specific hence a number of them can be isolated from different plants growing under different climatic conditions and with different ability to utilize various nutrients (Pavithra *et al*., 2012).

Studies have shown that endophytes are of great importance since they are able to produce bioactive compounds. Bioactive compounds from endophytes inhabiting various plant species can be used in agricultural, pharmaceutical and food industries since they have been reported to have antimicrobial, antimalarial activities and can also act as enzymes (Sunaryanto & Mahsunah, 2013). Fungal endophyte *Neotyphodium lolii* of perennial ryegrass has been found to produce bioactive alkaloids which help their host plant improve the rate of photosynthesis hence increased growth though this depends on the growth phase of the host plant (Spiering

et al., 2006). Previous research has it that a novel endophytic fungus *Muscodor vitigenus* was discovered from *Paullinia paullinioides*. This endophyte was found to produce naphthalene as a bioactive compound which is an insect repellant hence important for biological control methods in Agriculture (Bryn *et al*., 2002). Some endophytes like *Beauveria bassiana* and *Lecanicillium spp.* have been reported to have antagonistic activity against plant pathogens hence can be used in developing bio pesticides for carrying out Agricultural activities (Jaber & Ownley, 2018).

Endophytes have been found to produce metabolites, which act as anticancer agents. Such compounds include taxol which is produced by fungal endophyte *Taxomyces andreanae*, camptothecin extracted from *Fusarium solani* which was collected from *Camptotheca acuminate* and Ergoflavin extracted from endophytic fungi isolated from *Mimusops elengi* which is a medicinal plant in India. Other compounds extracted from endophytes from selected medicinal plants reported to have anticancer properties include phenylpropanoids, Podophyllotoxin, cytochalasins, Torreyanic acid, cytoskyrins, phomoxanthones A and B, photinides A-F and rubrofusarin B among others (Pimentel *et al*., 2011). In a study done on endophytic fungi isolated from Thai medicinal plants, quite a good number of the fungal extracts showed bioactivity against *Mycobacterium tuberculosis*, Herpes Simplex Virus 1 (HSV-1) and breast cancer cells in addition to the extracts having antimalarial activities (Wiyakrutta *et al*., 2004).

2.3 Resistance of *Candida albicans* to Antifungals

Candida albicans is a fungal pathogen which normally exists as a commensal in the gastrointestinal and urinary tract of healthy individuals without causing harm (Kabir *et al*., 2012). The organism has the ability to grow in different morphological forms which range from unicellular budding yeast to true hyphae depending on the temperature (Sudbery *et al*., 2004). The hyphae form is said to be the pathogenic form of *Candida albicans*.

Drug resistance is the persistence or progression of an infection despite appropriate drug therapy (Cowen *et al*., 2002). Antifungal resistance has occurred over time which is a challenge to the health care sector and is attributed to use of broad

spectrum antibiotics over a long period of time. Research reports that <2.5% and <9% of *Candida spp*. isolates analyzed worldwide were respectively resistant to fluconazole and itraconazole. A past 3-year study of *Candida spp.* infections of the bloodstream in both North America and Latin America found that resistance to azoles doesn't occur frequently (Perea & Patterson, 2002).

Azoles (fluconazole, voriconazole and Nystatin) have been found to show a lower effectiveness compared to caspofungin against clinical and food isolates of *Candida albicans* in Poland (Maroszyńska *et al*., 2013). Drug resistance among oral *Candida spp*. has been reported to be common in South Africa and Cameroon among HIV infected individuals (Abrantes *et al*., 2014). 2.2%, 7.7% and 4.7% of the identified isolates of *Candida albicans* in a study done in Ethiopia were found to be resistant to fluconazole, ketoconazole and itraconazole respectively; with frequent resistance to micafungin, amphotericin B and 5- Fluorocytosine (Mulu *et al*., 2013). According to Ooga *et al*., (2009) from Kenya, more than 70% of *Candida spp*. have been found to be susceptible to clotrimazole, itraconazole and posaconazole. Most of *Candida albican* isolates from pregnant women with signs of candidiasis have been reported to be susceptible to most of the azole drugs in Kenya (Menza *et al*., 2013b).

2.4 Mechanism of *Candida albicans* Resistance to Antifungals

Various factors have contributed to resistance of *Candida* to antifungals according to the available literature. For instance, resistance of *Candida* to azoles has been attributed to upregulation of cdr and mdr genes, which result to development of efflux pumps leading to decreased drug concentration. Mutation of erg gene has led to alteration of the target site interfering with binding of the drug. Upregulation of the target enzyme leads to its increased levels hence less activity of the drug (Kanafani & Perfect, 2008; Whaley et al., 2017). Various factors have led to resistance of *Candida spp*. to polyenes (amphotericin B). Such factors include; change in membrane sterols, cell membrane fatty acid composition, change in ergosterol structure as well as change in cell wall structure of the fungi (O'Shaughnessy *et al*., 2009). *Candida spp*. resistance to Echinocandins has occurred because of mutation of fks genes. *Candida* fks genes have naturally

occurring polymorphisms which make the fungi less sensitive to Echinocandins (Perlin, 2015). Resistance to Flucytosine is mainly due to mutation occurrence in cytosine permease which results to impaired cellular uptake hence defects in flucytosine metabolism (Kanafani & Perfect, 2008).

2.5 Prevalence of *Candida albicans* Infections in Clinical Practice

Candida albicans is mainly associated with different forms of candidiasis, which have different rates of prevalence. According to Yapar, (2014), the leading pathogen considered to be of significance is *Candida spp*. which is among the top five pathogens causing nosocomial blood stream infections (BSIs) in the USA (United States of America) with *Candida spp.* causing 8% to 10% of nosocomial BSIs. The Population-based surveillance studies indicate that the yearly incidence of *Candida spp.* infections is 8 per 100,000 population. A report by SENTRY Antimicrobial Surveillance Program stated that 1,354 infection episodes related to *Candida spp.* were detected between 2008 and 2009 out of which 36.5% were community-acquired. This community-acquired candidemia was found to be significantly higher in North America with 63.5% than in Europe which had 22.4% (Yapar, 2014).

Candida albicans infections have been reported to be the most prevalent infections compared to other *Candida spp*. in a study done in College Hospital in Ibadan, Nigeria (Jao *et al*., 2011). In Awka, Nigeria, occurrence of vaginal *Candida albicans* infections in the pregnant women is more prevalent compared to the rest of *Candida spp*. (Onuorah *et al*., 2015). *Candida albicans* carriage in HIV, cancer patients and normal individuals has been found to be higher compared to the rest of *Candida spp*. in the year 2015 in Johannesburg (Owotade *et al*., 2016). Vaginal candidiasis has been reported to be more prevalent in pregnant women in Thika, Kenya (Menza *et al*., 2013b).

2.6 Virulence Factors of *Candida albicans*

Candida albicans has several virulence factors which enhance its pathogenicity as well as the spread of the infections caused by the organism (Singh & Urhekar, 2013).

2.6.1 Polymorphism

Candida albicans has the ability to grow either as ovoid - shaped budding yeast, pseudohyphae or true hyphae. Various environmental factors affect the morphology of the organism, for instance, at pH less than 7, they grow as yeast while at pH higher than 7, they grow as hyphae. Quorum sensing also affect the morphology in that high cell densities promote yeast growth while low cell densities promote hyphae growth. Hyphae forms are more invasive compared to the yeast forms (Mayer *et al*., 2013).

2.6.2 Adhesion

Candida albicans have the ability to adhere to cells and surfaces such as catheters and denture materials which is important for the disease to establish. This is achieved by help of proteins such as mannan adhesins, fimbriae, plastic-binding protein and epithelial binding lectin-like protein among others. Adherence helps the *Candida albicans* to transform in to pseudohyphae which is a more virulent form (Nasution, 2013).

2.6.3 Biofilm Formation

Candida albicans has the ability to form biofilms which facilitate the influx of nutrients and removal of waste products. It is usually a process, which starts with adherence followed by colonization, proliferation, maturation and dispersion so the cycle can be repeated again. Cells in biofilm are resistant to antifungal agents hence contributes to the organism being more virulent (Uppuluri *et al*., 2009).

2.6.4 Proteinases

For *Candida albicans* to invade cells and tissues, it releases aspartyl proteinases which break down human proteins at the site of the lesion hence the organism is able to get nutrients and invade more cells and tissues (Yang, 2003).

2.6.5 Phenotypic Switching

Candida spp. are able to undergo phenotypic switching which involves morphological changes allowing them adapt to new environment during infection. Various morphological characteristics assumed by colonies of *Candida albicans* include smooth, rough, star, stippled, hat, wrinkle, and fuzzy. These changes are reversible and occur spontaneously during stress resulting in changes in cell surface behavior, colony appearance, metabolic, biochemical and molecular attributes. This affects virulence factors such as aspartyl proteinases regulation and even metabolic activities making the microorganism more virulent. Strains with high frequency of phenotypic switching are more virulent. Some strains change their morphological characteristics even under same environmental conditions (Khan *et al*., 2010).

2.6.6 Heat Shock Proteins

Candida albicans need to cope with stressful environments like high temperature, starvation and oxidative stress which may induce protein unfolding and nonspecific protein aggregation, eventually resulting to cell death. Heat shock proteins prevent such incidences by stabilizing the proteins (Mayer *et al*., 2013).

2.7 Forms of Candidiasis

2.7.1 Oropharyngeal Candidiasis

This is opportunistic candidiasis mostly seen in HIV positive patients. It is caused by *Candida albicans* though other *Candida spp.* May also be involved. Different forms of oropharyngeal candidiasis include acute pseudomembranous, erythematous, hyperplastic and denture-induced stomatitis. Symptoms include painful mouth with sore and a burning tongue with change in taste (Pankhurst, 2009).

2.7.2 Vulvovaginal Candidiasis

Vulvovaginal candidiasis is common problem which affects most of the women at least once in their lifetime and *Candida albicans* is the most common microbe responsible for causing it. The fungi have added advantage since it has the capacity

to survive and proliferate in physiological extremes of pH, osmolarity, nutrients availability and temperature. The symptoms of the infection are eczematoid dermatitis lesions that sometimes show vesicular and grey-white pseudo membrane, vulval pruritis, burning, erythema and curd like discharge (Dabas, 2013).

2.7.3 Cutaneous Candidiasis

This is secondary infection of the skin and nails which may be localized or generalized occurring mostly in warm, moist and creased areas like axillary folds, inguinal or intergluteal areas. It is commonly found in diabetic and obese individuals and brings about maceration and trauma in the skin (Dabas, 2013).

2.7.4 Invasive Candidiasis

Candidiasis has been ranked as the highest cause of death among the individuals who are immunocompromised and the ones in intensive care unit with *Candida albicans* being the most common isolate. Candidemia is the most frequent form of manifestation of invasive candidiasis with high mortality and morbidity rates among hospitalized patients. It spreads to other organs of the body through the blood stream such as kidneys, liver, spleen, eyes and the brain hence a complicated infection. The clinical signs of invasive candidiasis diagnosed mostly are endophthalmitis or chorioretinitis and the emergence of painless skin lesions (Zarrin & Mahmoudabadi, 2009).

2.8 Risk Factors for *Candida* Infections

Various factors which are either attributed to the host or to health care services have contributed to *Candida spp.* infections. For instance, significant blood stream infections among patients in ICU have been attributed to *Candida spp*. with some of the possible factors being receipt of antibiotic agents, chemotherapy, steroids, intravascular catheters, surgery, malignancy, neutropenia and prior fungal colonization among others (Blumberg *et al*., 2001). According to Dou *et al*. (2014) *Candida* infections were reported to increase with increase in age; pregnancy, induced abortions which lead to rapture of membranes, diabetes mellitus and use of

oral contraceptives with high estrogen dose were found to be risk factors; people with high level of education were less likely to have the infections. Other risk factors which have been reported as significant include presence of central venous catheters, parenteral nutrition, chronic renal insufficiency, immunosuppressive diseases and deteriorating clinical state due to underlying disease (Yapar, 2014).

2.9 Treatment of *Candida albicans* Infections

According to Ruhnke *et al*. (2011), polyenes, azoles, echinocandins and nucleoside analogues are licensed for invasive fungal infections treatment. Under the polyene class is amphotericin B. Some of the approved antifungals for treatment and prevention of *Candida* infections include two triazoles (voriconazole and posaconazole) and three echinocandins (anidulafungin, caspofungin and micafungin) of which only amphotericin B and echinocandins have demonstrated activity against *Candida albicans* consistently (Sardi *et al*., 2013). More drugs in use include itraconazole and fluconazole (Pappas *et al*., 2000). The kind of therapy one gets differs depending on the nature of infection. Increased rate of antifungal drug development has led to recent licensure of two more antifungal drugs, voriconazole and caspofungin along with the active development of 4 other antifungals; ravuconazole, posaconazole, micafungin, and anidulafungin. However, limited clinical data is available although these compounds are active towards *Candida albicans* and other *candida spp*.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

Warburgia ugandensis was collected from Mt. Kenya forest, which is situated at the Kenyan Central highlands, 180km north of Nairobi, and it is Kenya's highest mountain which covers Meru, Embu, Laikipia, Kirinyaga, Nyeri and Tharaka Nithi counties (Figure 3.1). It receives rainfall ranging from 900 mm in the north leeward side to 2300 mm on the windward side with January and February being the driest months. During March to June and October to November, maximum rainfalls are experienced. Temperature is dependent on the altitude with a decrease of temperature by 0.6 ºC for every 100 mm altitude increase (Kenya Forest Service, 2010). Other trees found in Mt. Kenya forest include bamboo, *Podocarpus milanjianus*, *Cassipourea malosana*, and *Juniperus procera* among others.

Figure 3.1: Mount Kenya location map (https://www.mountkenyaclimbing.com)

3.2 Study Design

The study design used was experimental study design. Samples for fungal isolation (leaves, stems, barks, roots) were divided in to three groups then one sample picked randomly from every group and processed for fungal isolation in three replicates. Three replicates were also done for each fungal extract during screening for antimicrobial activities.

3.3 Collection of Plant Material

Purposive sampling method was applied whereby two healthy *Warburgia ugandensis* trees were selected by help of local guides. Samples of mature, healthy leaves, stems, barks and roots, 500g each, of *Warburgia ugandensis* were collected giving a total weight of 4 kg. They were put in well-labeled sterile plastic paper bags and transported to Government of Kenya (GoK) laboratory, JKUAT, in an ice box. On arrival in the laboratory, the plant material was stored in a refrigerator at 4 ^{o}C temperature awaiting processing which was done after 48 hours of collection.

3.4 Isolation of Endophytic Fungi

Isolation of endophytic fungi was done according to (Tolulope *et al*. (2015) with slight modifications. The plant material was thoroughly rinsed with running tap water to remove dust, soil particles and debris. Surface sterilization was done by immersion of the plant material in 75% ethanol for 1 minute followed by immersion in 0.63% Sodium hypochlorite (12% of the original concentration which is 5.25% Sodium hypochlorite) for 1 minute then rinsed twice in sterile distilled water. The plant material was allowed to dry on a sterile filter paper after which it was cut in to pieces of 3cm with a sterile scalpel. Four pieces of each part were placed on tap water agar plate using a sterile forceps and incubated for 6 days at 27 ^{o}C. Fungal hyphae tips growing from the plant tissues were sub cultured on Potato Dextrose Agar (PDA) supplemented with 250 mg/L streptomycin to prevent growth of bacteria followed by incubation at 27 ^{o}C for 4 days (Gond *et al*., 2007). Colony purification

was done by further sub culturing the fungal colonies in PDA until pure isolates were acquired.

3.5 Identification of Endophytic Fungi

3.5.1 Phenotypic Identification

Pure cultures of isolated fungal endophytes were identified phenotypically up to genus level by observing the colony characteristics macroscopically and microscopically (Sarah *et al*., 2016; Barnett & Hunter, 1998; www.hayesmicrobial.com/library.php). For microscopic identification, adhesive tape slides were prepared by gently touching on the surface of the endophytic fungal colony with a piece of adhesive tape then placing it on a slide with a drop of lacto phenol cotton blue stain. The slides were observed under a light microscope at power ×100 and photos for clear fields with intact structures were taken. Sterile water was used in place of the lacto phenol cotton blue stain for fungi which did not take up the stain.

3.5.2 Molecular Identification

3.5.2.1 Fungal DNA Extraction

DNA was extracted from the fungal endophytes according to Ndahebwa, (2018), protocol with slight modifications. Pure *Aspergillus flavus* and sterile distilled water was used as positive and negative control respectively. Fungal endophytes were grown on PDA for 4 days at 27 ºC. Liquid nitrogen was used to freeze the fungal mycelia which was then ground in to powder using a mortar and pestle. The powder was transferred in to 2 ml Eppendorf tubes. 600µl of preheated fungal DNA extraction buffer (0.1M Tris-HCl pH 8, 10Mm EDTA pH 8, 2.5M NaCl, 3.5% CTAB, 150 µL 20 mg/ml proteinase K) was added to the powder followed by 30 minutes incubation in a water bath at temperature 65 ºC while mixing after every 10 minutes. 270 µl of 5M potassium acetate was added to the contents and centrifuged at 13000rpm for 10 minutes. The supernatant (700 µl) was transferred in to clean tubes then 5 µl RNase added to remove the RNA followed by incubation at 37 ºC for

30 minutes. An equal volume of a mixture of chloroform and iso-amyl alcohol which was prepared in the ratio of 24:1 was added to the contents. 600 µl of the supernatant was pipetted in to clean tubes. Precipitation of the DNA was done by addition of a tenth of the volume of 3M potassium acetate and two thirds of the volume of isopropanol. It was incubated at -20 ºC for 30 minutes and centrifuged at 13000rpm for 10 minutes. 70% ethanol was used to wash the DNA pellet which was then centrifuged for 10 minutes. Elution of the DNA was done in to 50 µl of Rnase-free water followed by storage at -20 ºC.

3.5.2.2 Amplification and Sequencing of Endophytic fungal DNA

Amplification of fungal DNA was done according to Ndahebwa, (2018) with some modifications. The primers used were; NS1 5' (GTA GTC ATA TGC TTG TCT C) 3' and NS24 3' (AAA CCT TGT TAC GAC TTT TA) 5' which were the forward and reverse primers respectively. This was achieved on a GeneAmp PCR system 9600 (Applied Biosystems) using 1µl Taq Polymerase (Applied Biosystems), 1µl each of 10 Pm concentrations of forward and reverse primers, 27 µl sterile deionized water, 8 µl PCR buffer containing dNTPs and $MgCl_2$, and 2 µl DNA template, for a total reaction volume of 40 µl. The following cycling program was used: 1 cycle of 95 °C for 5 minutes, 35 cycles of 95 °C for 30 seconds, 60 °C for 45 seconds, and 72 °C for 40 seconds; and a final extension of 72 °C for 5 minutes. Visualization of the PCR products was achieved through gel electrophoresis on a 1% agarose gel whereby ethidium bromide was added directly. Sequencing of the products was done by Macrogen, Sequencing Company (Netherlands) by subjecting them to Sanger dideoxy sequencing. Both the forward and reverse sequences were assembled using SeqMan Pro. The resulting sequences were edited. BLAST (Basic Local Arithmetic Search Tool) analysis was done on edited sequences to get the identity of the isolates from NCBI (National Center for Biotechnology Information) website depending on the maximum score, total score, query cover and the percentage identity. Assembled sequences were submitted to NCBI for accession numbers.

3.6 Extraction of Metabolites

Mass cultivation of endophytes and fungal metabolites extraction was done according to (Selvi & Balagengatharathilagam, 2014) with slight modifications. Agar blocks of actively growing pure fungi (3 mm diameter) were inoculated in a 200 ml universal bottle containing 100 ml of sterile nutrient broth followed by incubation at 27 ^{o}C in a shaker for 14 days. The cultures were filtered after the incubation period by use of Whatman filter papers to remove the mycelia. Part of the media sample was sterilized through microfiltration to remove the spores. This formed the crude extract which was preserved at -20 ^{o}C for further analysis.

Metabolites from endophytic fungi were extracted using ethyl acetate where, equal volumes of ethyl acetate were added to the filtrates then shaken well for 10 minutes to mix the contents. The solutions were transferred to separating funnel, allowed to settle for 5 minutes such that two layers (media layer and ethyl acetate layer) were formed which were collected separately. The ethyl acetate was evaporated to dryness using a vacuum rotary evaporator at 36 ^{o}C. The extracts were reconstituted using 0.1% DMSO (Dimethyl Sulfoxide) then sterilized through microfiltration and preserved for further analysis. This formed the ethyl acetate extract.

3.7 Toxicity Testing

Toxicity testing was done using brine shrimp larva which were obtained after hatching brine shrimp eggs in artificial sea water. The eggs were hatched according to (Olowa & Nuñeza, 2013) protocol. Brine shrimp eggs were bought from an aqua pet shop in Nairobi town. 38 g of sea salt were dissolved in 1 litre of tap water for preparation of artificial sea water in order to hatch the shrimp eggs. The sea water was filtered then poured in to a hatchery with three partitions, the inner most partition, middle and the outer partition. Brine shrimp eggs were poured in to the inner most partition and the hatchery was covered with a lid which has an opening to the inner most partition such that it allows light while the other parts are dark. A bulb was lit above the other side of the hatchery. This bulb helps raise the temperature around the hatchery slightly above the room temperature and also provides light which attracts the hatched shrimp to the dark parts of the hatchery. The eggs hatched

in to larva (nauplii) after two days. Solutions for performing the toxicity assay were made by diluting the extracts in to various concentrations of 5ml volumes using 0.1% DMSO through serial dilution. 10 nauplii were picked using a dropper and added to each solution. A solution of 0.1% DMSO and sea water was used as a negative control and fluconazole, (an antifungal) for positive control. The alive nauplii were counted and recorded after 24 hours against a lighted background with a magnifying glass lens. A graph was generated by plotting the mean percentage mortality against the logarithm of the concentrations. The percentage of deaths at each dose was determined using the formula:

$$Percentage\ Death = \frac{No.of\ Dead\ Shrimps}{No.\ of\ surviving\ Shrimps\ in\ control} \times 100$$

Regression equations were generated from the data of mean results of percentage mortality of the brine shrimps versus the log10 of concentrations using Microsoft Excel which were used to give the LC_{50} values (Zakari & Kubmarawa, 2016).

3.8 Screening of Endophytic Fungal Extracts for Phytochemicals

Primary screening of the crude extract for the following phytochemicals was done according to the standard protocols described by Auwal *et al*. (2014) with slight modifications; tannins, saponins, sterols, glycosides, alkaloids, phenols, flavonoids and anthraquinones.

3.8.1 Test for Tannins

2 ml of the extract was added to 10% Ferric chloride solution. Occurrence of either black-blue colour or green-black colour indicated presence of tannins.

3.8.2 Test for Saponins

3ml of the extract was mixed with 2 ml of distilled water and shaken vigorously till a stable persistent froth was formed. 2 drops of olive oil were mixed with the froth and shaken vigorously then observed for formation of stable emulsion.

3.8.3 Test for Sterols

2 ml of the extract was mixed with 2 ml of chloroform. 1ml of acetic anhydride was added to the mixture followed by 2 drops of concentrated H_2SO_4. The test tube was observed for green colour which is indicates presence of sterols.

3.8.4 Test for Glycosides

2 ml of glacial acetic acid were added to 2 ml of the extract followed by addition of 3 drops of $FeCl_3$ solution. 1 ml of concentrated sulphuric acid was added carefully along the side of the test tube. Appearance of brown ring at the interface indicated presence of glycosides. Beneath the brown ring, a purple ring may appear while in the acetic acid layer a green ring may form gradually throughout the layer (Singh & Bag, 2013).

3.8.5 Test for Alkaloids

3 drops of Dragendorff's reagent were added in to 2 ml extract. Formation of orange brown precipitate indicated the presence of alkaloids.

3.8.6 Test for Phenols

3 drops of 10% lead acetate solution were added to 2 ml of the extract. Formation of white precipitate was an indication of presence of phenols (Singh & Bag, 2013).

3.8.7 Test for Flavonoids

2 ml of the extract was mixed with 0.05 g of metallic zinc and 3 ml of concentrated sulphuric acid. It was observed for red colour as indicative of flavonoids.

3.8.8 Test for Anthraquinones

2 ml of chloroform was added to 1 ml of the extract. Equal volume of 10% ammonia solution was added then shaken. The upper layer was observed for bright pink colour which indicates presence of anthraquinones.

3.9 Screening of the Endophytic Fungi for Antimicrobial Activities

Screening for antimicrobial activities was done both for the crude extract and the ethyl acetate extract using disk diffusion technique. Pure test organisms of *Candida albicans* ATCC 90018 were obtained from Center for Microbiology Research - Kenya Medical Research Institute (CMR – KEMRI) laboratory. The test organism was sub cultured in nutrient agar and after 24 hours incubation at 37 ^{o}C, 0.5 McFarland solution of the test organism was prepared (Andrews, 2001) to make the test inoculum. The test organism was inoculated on well labeled nutrient agar plates by spreading the inoculum uniformly on the agar using sterile cotton sticks. Six millimeters (6mm) diameter sterile discs were impregnated with 20 µl of the fungal extracts then transferred to the plates inoculated with the test organism (Radu and Kqueen, 2002). 20 µl of fluconazole (Diflucan I.V-Pfizer Company, 2 mg/ml concentration, total volume 100ml) was used as a standard control for *Candida albicans*. The negative control used was 0.1% DMSO. Three replicates were made for each fungal extract. The plates were incubated for 24 hours at 37 ^{o}C after which the diameters of the zones of inhibition were measured to the nearest millimeter (mm) using a ruler, the diameter of the disc excluded. Antimicrobial activity graphs were generated using Ms Excel spread sheet to help compare the inhibition zones of the fungal extracts against the test organism. Data analysis was carried out using SAS Software, version 9.4, for analysis of variance (ANOVA) and mean separation using least significant difference (LSD) test.

CHAPTER FOUR

RESULTS

4.1 Isolation and Identification of Endophytic Fungi

A total of sixty fungal isolates were isolated from *Warburgia ugandensis* collected from Mount Kenya forest. Twenty isolates (33.3%) were from the leaves, Seventeen (28.3%) from the stem, fourteen (23.3%) from the bark and nine (15%) from the root (Table 4.1, Appendix II). The leaf had majority of the fungal isolates (20/60) followed by the stem (17/60); the root gave the minimum number of isolates (9/60) (Table 4.1).

Table 4.1: Endophytic Fungi Isolated from *Warburgia ugandensis*

	Number	**Percentage (%)**
Leaf	20	33.3
Stem	17	28.3
Bark	14	23.3
Root	9	15
Total	60	100

Majority of the fungal endophytes isolated were *Phomopsis spp*., 14/60, (23.3%) followed by *Fusarium oxysporum*, 8/60, (13.3%) and *Alternaria spp.*, 7/60, (11.7%). *Tricharina gilva, Diaporthe amygdali*, *Phyllosticta gardeniicola*, *Guignardia mangiferae* and *Trichoderma harzianum* were the least isolated fungal endophytes, 1/60, (1.7%) per isolate, (Table 4.2).

Phomopsis spp. was the only isolate isolated from all the four plant parts. *Cladosporium spp*. and *Fusarium oxysporum* were isolated from the leaf, stem and the root. *Nigrospora oryzae* and *Bionectria ochroleuca* were isolated from leaf, stem and the bark. *Alternaria spp*. was isolated from the bark and the leaf. *Cochliobolus sativus* and *Tricharina gilva* were only isolated from the bark. *Phyllosticta*

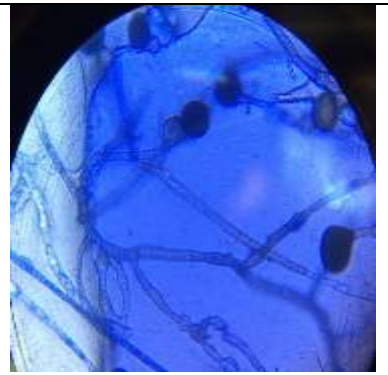
gardeniicola and *Diaporthe amygdali* were only isolated from the stem. *Guignardia mangiferae* was only isolated from the leaf and *Trichoderma harzianum* from the root (Table 4.2).

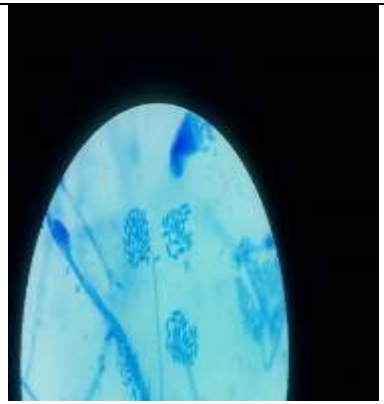
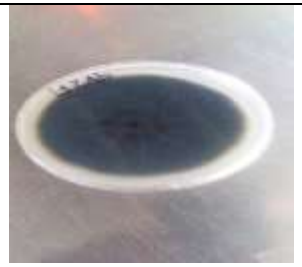
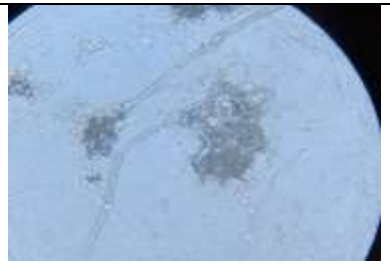
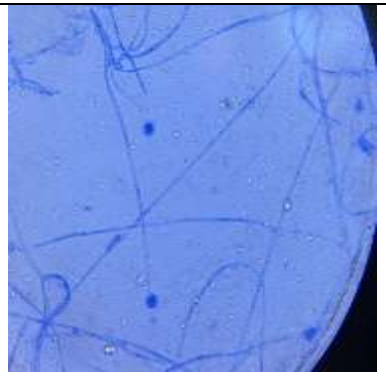
Table 4.2: Distribution of Fungal Endophytes isolated from *Warburgia ugandensis*

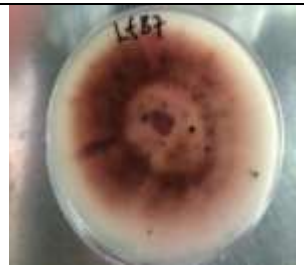
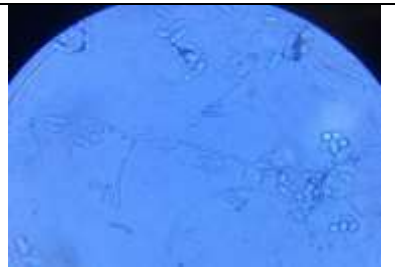
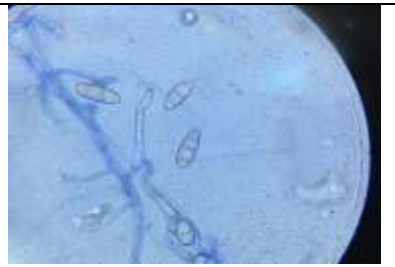
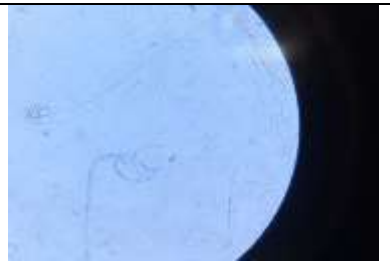
Fungal Isolate	Leaf	Stem	Bark	Root	Total
Nigrospora oryzae	3	1	1		**5**
Aspergillus flavus	3	2			**5**
Cladosporium spp.	2	3		1	**6**
Fusarium oxysporum	3	3		2	**8**
Phomopsis spp.	3	6	2	3	**14**
Colletotrichum acutatum	3			1	**4**
Alternaria spp.	1		6		**7**
Cochliobolus sativus			3		**3**
Bionectria ochroleuca	1		1	1	**3**
Phyllosticta gardeniicola		1			**1**
Guignardia mangiferae	1				**1**
Tricharina gilva			1		**1**
Diaporthe amygdali		1			**1**
Trichoderma harzianum				1	**1**
Total	20	17	14	9	**60**

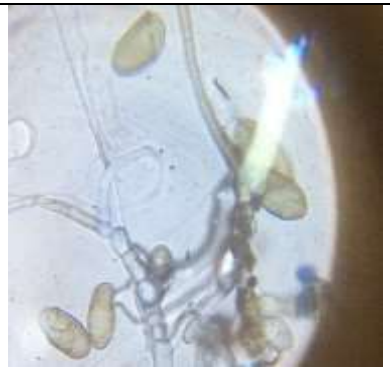
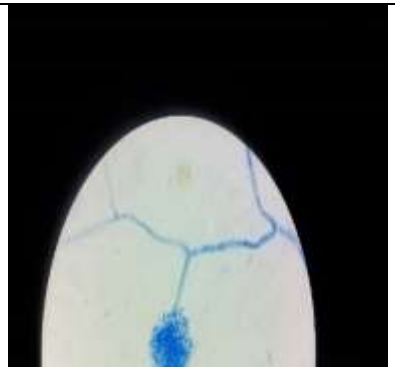
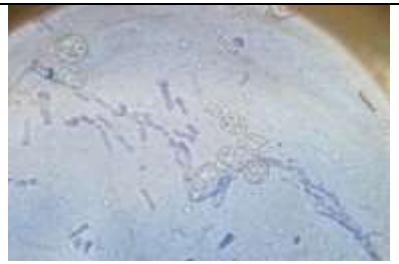
All the isolates were characterized using phenotypic and microscopic methods. They were preliminary placed in fourteen different genera namely *Nigrospora, Aspergillus, Cladosporium, Fusarium, Phomopsis, Colletotrichum, Alternaria, Cochliobolus, Bionectria, Phyllosticta, Guignardia, Tricharina, Diaporthe* and *Trichoderma* (Table 4.3).

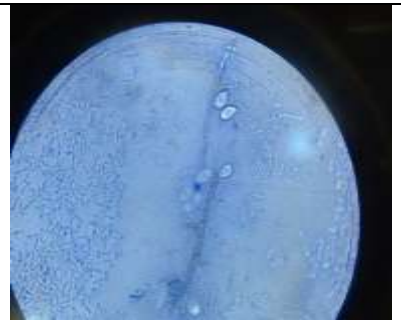
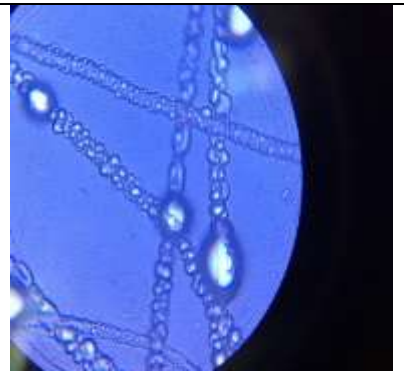
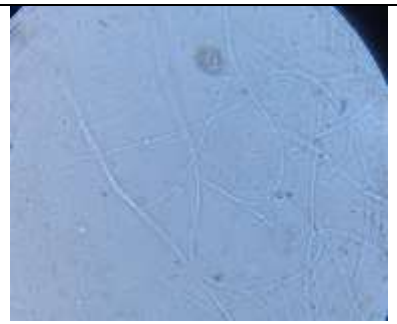
Table 4.3: Microscopic and morphological identities of the fungal endophytes isolated from *Warburgia ugandensis*

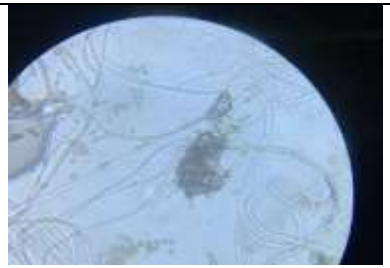
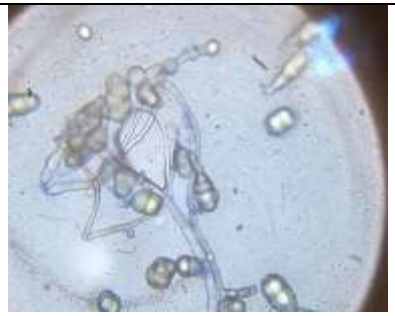
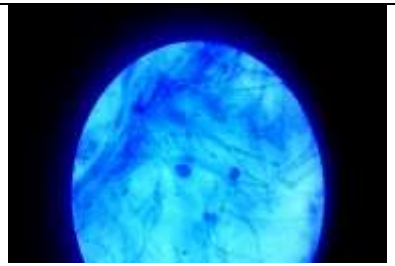
Isolate Code	Phenotypical Characteristics	Front photo of the fungi on PDA plate	Reverse photo of the fungi on PDA plate	Microscopic Photo of the fungi (X100) Objective Lense)	Genus
Lf3b	Cotton white colonies, Floccose, the reverse is white. Short conidiophores, the conidia are one-celled, shiny black, globose, situated on flattened hyaline vesicle.				*Nigrospora*
Lf3a2	Cream white colonies, powdery texture with granules, reverse is cream-yellow. Spores grow in chains from phialides that emerge from a central vesicle.				*Aspergillus*

LfZA2	White colonies with aerial growth, becoming purple. Reverse is cream. Short conidiophores, macroconidia slightly curved, pointed at the tip, microconidia straight of often curved.				*Fusarium*
LfZAb	Grey colonies with regularly folded surface from the middle, powdery. Blastocatenate conidia that readily disarticulate.				*Cladosporium*
Bk2b	Cotton white colonies, almost colourless at the edges. Reverse is cream white, almost colourless at the edges. Simple conidiophores, one celled conidia, nearly globose.				*Phomopsis*

LfB7	Pink pigmented colonies with white aerial growth. Reverse is pink with a brown shade. Banana shaped conidia.				*Colletotrichum*
Bkba2	Fast-growing grey-black colonies, the reverse is black. Ovoid septate conidia pale brown in colour				*Alternaria*
StZA8	Cotton white colonies, almost colourless towards the edges. Simple conidiophore, one celled ovoid conidia.				*Phomopsis*

Bk5b	Dark olive brown colonies, raised with aerial growth. Reverse is black with pale brown edges. Simple brown conidiophore, brown conidia, oval shaped with round ends.				*Cochliobolus*
BkZA3	Cotton white colonies, densely floccose, reverse is cream. Hyaline conidiophore, one celled cylindrical conidia which aggregate at the apex of the phyalides.				*Bionectria*
St2b	Raised grey black colonies, reverse is black with a grey part. Short conodiophores, one celled conidia, ovoid, almost hyaline.				*Phyllosticta*

Lf7b1	Slow growing gray black dome shaped colonies. Reverse is green black with cream edges. Simple conidiophores, one celled hyaline conidia.				*Guignardia*
Bk3a	Brown colonies with white aerial growth. Reverse is deep brown at the middle, the other part is pale brown. Conidiophore has a ring like structure, oval shaped conidia				*Tricharina*
St1a	Cotton white colonies with aerial growth. The reverse is cream white. Simple conidiophore, one celled conidia.				*Diaporthe*

Lf12b	Grey colonies with regularly folded surface from the middle, powdery. Blastocatenate conidia that readily disarticulate.				*Cladosporium*
Lf6a2	Grey colonies, reverse is black. Club shaped spores; highly septate with divisions on both vertical and horizontal planes.				*Alternaria*
RtZB3	White downy colonies, reverse is cream. Branched conidiophores, conidia clustered at the tip of the vesicle.				*Trichoderma*

A further characterization using sequencing methods of identification targeting 18s rRNA gene region classified the isolates in to species level.

All the fungal isolates had DNA of base pairs ranging from 1500-2000bp after running the gel electrophoresis (Figure 4.1).

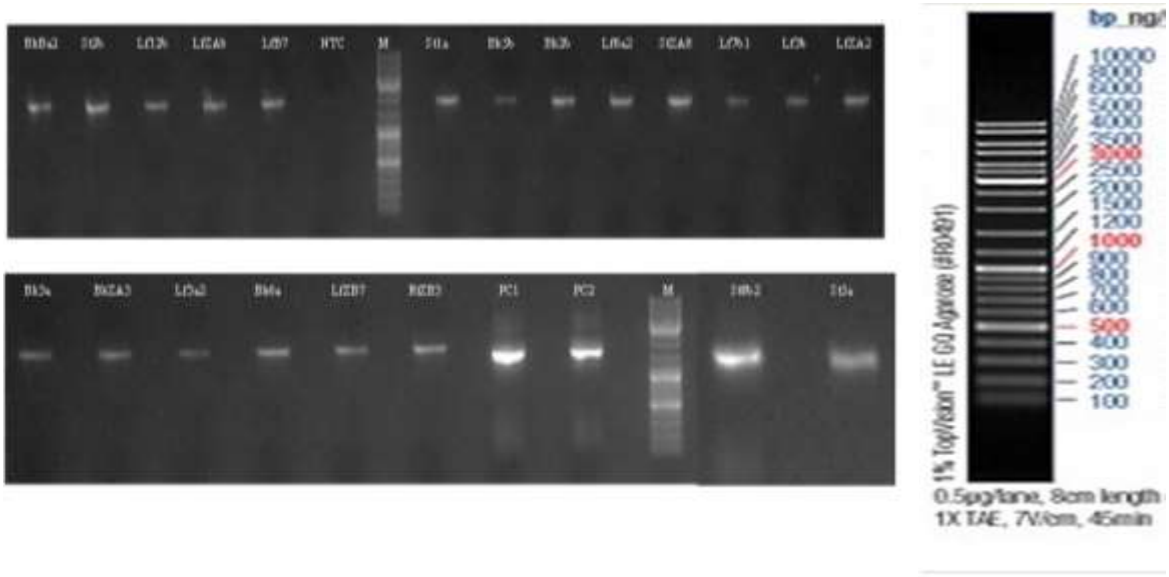


Figure 4.1: Gel Electrophoresis of Endophytic fungal isolates from *Warburgia ugandensis*

M – Molecular ladder of 1 kb; NTC – No template control; P1 and P2 – positive controls, BkBa2: *Alternaria spp*., St2b: *Phyllosticta gardeniicola*, Lf12b: *Cladosporium spp*., LfZAb: *Cladosporium bruhnei*, LfB7: *Colletotrichum acutatum* St1a: *Diaporthe amygdali*, Bk5b: *Cochliobolus sativus*, Bk2b: *Phomopsis spp*., Lf6a2: *Alternaria alternata*, StZA8: *Phomopsis mali*, Lf7b1: *Guignardia mangiferae*, Lf3b: *Nigrospora oryzae*, LfZA2: *Fusarium oxysporum*, Bk3a: *Tricharina gilva*, BkZA3: *Bionectria ochroleuca*, Lf3a2: *Aspergillus flavus*, Bk6a: *Phomopsis mali*, LfZB7: *Bionectria ochroleuca*, RtZB3: *Trichoderma harzianum*, St8b2: *Phomopsis mali*, St5a: *Phomopsis mali.*

The sequences obtained were given Accession numbers after having been deposited in NCBI (Table 4.4).

Table 4.4: Fungal Endophytes isolated from *Warburgia ugandensis* Identified to species level and their Accession Numbers

Isolate Code	Identity	Max Score	Total Score	Query cover	Ident	Accession No.
Lf3b	*Nigrospora oryzae strain: IFO 32860*	2212	2212	98%	98%	MH014997
Lf3a2	*Aspergillus flavus strain Ya1*	2179	2179	99%	98%	MH014996
LfZA2	*Fusarium oxysporum strain M1-EGY*	2174	2174	99%	98%	MH015007
LfZAb	*Cladosporium bruhnei strain USN 11*	2094	2094	98%	98%	MH015009
Bk2b	*Phomopsis spp. M-32 strain*	2013	2013	99%	96%	MH013432
LfB7	*Colletotrichum acutatum strain BBA68396*	2174	2174	99%	99%	MH015004
Bkba2	*Alternaria spp. isolate KSA-SGY-12*	2058	2058	99%	99%	MH025761
StZA8	*Phomopsis mali strain IFO 31031*	2048	2048	99%	99%	MH016188
Bk5b	*Cochliobolus sativus strain NBRC 100205*	2069	2069	99%	99%	MH014993
BkZA3	*Bionectria ochroleuca strain WY-1*	2192	2192	99%	99%	MH014995
St2b	*Phyllosticta gardeniicola isolate: MUCC0117*	2003	2003	99%	99%	MH020175
Lf7b1	*Guignardia mangiferae isolate: MUCC0215*	2109	2109	96%	98%	MH015001
Bk3a	*Tricharina gilva voucher HMAS61180*	2093	2093	99%	99%	MH013964
St1a	*Diaporthe amygdali isolate MUCC0101*	2008	2008	99%	99%	MH015011
Lf12b	*Cladosporium spp. strain ALEF-C1*	2102	2102	99%	99%	MH015002
Lf6a2	*Alternaria alternata strain S-f6*	2105	2105	98%	98%	MH014998
RtZB3	*Trichoderma harzianum isolate BCS8A*	2217	2217	99%	99%	MH015010

4.2 Toxicity Testing Results

Eight serial dilutions were done and after 24 hours of incubation with the *Artemia salina*, the dead brine shrimps per concentration were counted and recorded. The values were used to generate graphs with regression equations of percentage (%) mortality of brine shrimps versus Log 10 concentration of the fungal extracts using MS Excel spread sheet (figures 4.3, 4.4, Appendices III, IV, V).

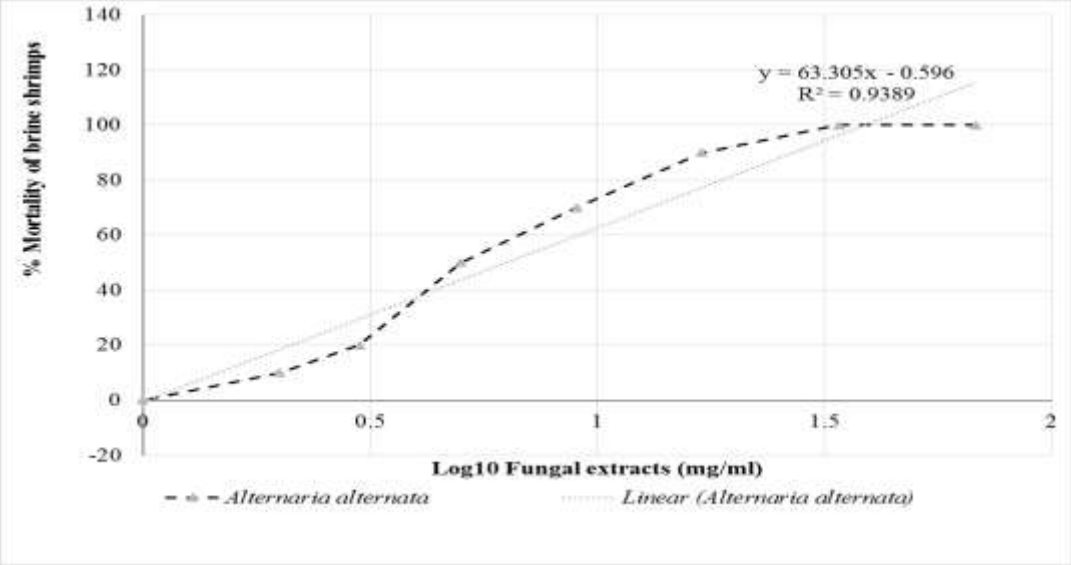


Figure 4.2: Percentage Mortality of brine shrimps versus Log10 concentration of *Alternaria alternata* extract isolated from *Warburgia ugandensis*

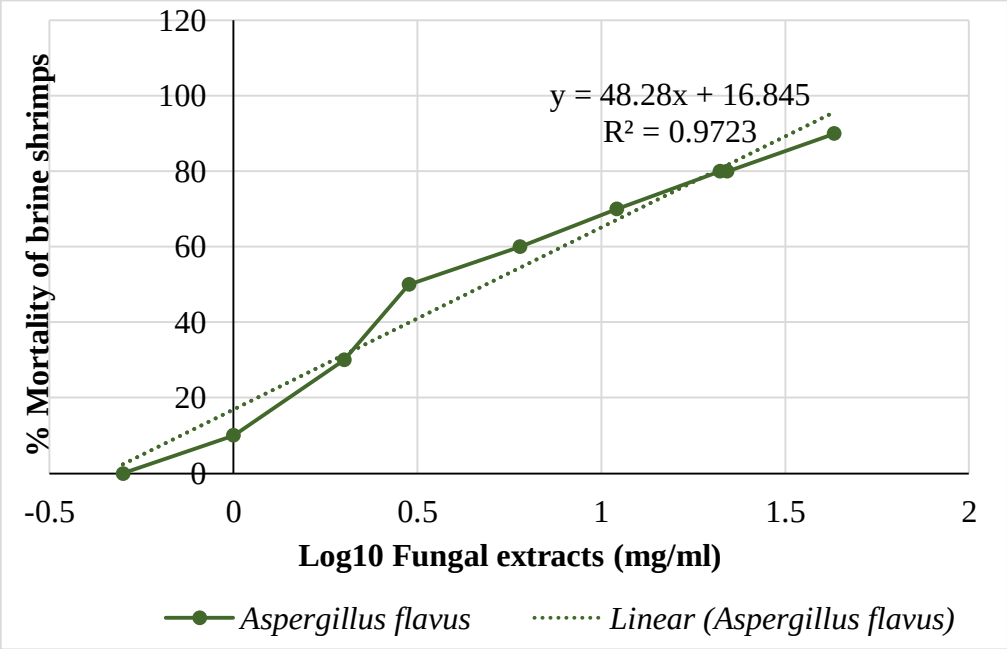


Figure 4.3: Percentage Mortality of brine shrimps versus Log 10 concentration of *Aspergillus flavus* extract Isolated from *Warburgia ugandensis*

The regression equations generated together with the graphs were used to arrive at the LC_{50} values shown below (Table 4.5). All the extracts had LC_{50} values >1000 µg/ml hence non-toxic according to (Zakari & Kubmarawa, 2016).

Table 4.5: LC50 (in µg/ml) of Extracts of Fungal Endophytes isolated from *Warburgia ugandensis*

Fungi	LC_{50} (µg/ml)
Fusarium oxysporum	5130
Colletotrichum acutatum	2040
Cladosporium spp.	3280
Tricharina gilva	4160
Nigrospora oryzae	5400
Cladosporium bruhnei	2210
Phomopsis spp.	4380
Guignardia mangiferae	3090
Alternaria spp.	2940
Cochliobolus sativus	4960
Phyllosticta gardeniicola	8500
Aspergillus flavus	6080
Diaporthe amygdali	4110
Trichoderma harzianum	4240
Alternaria alternata	6300
Bionectria ochroleuca	4050
Phomopsis mali	4810

4.3 Screening for Phytochemicals

Preliminary phytochemical analysis on the crude extracts gave positive results for saponins, tannins, alkaloids, flavonoids, sterols and glycosides. However, the extracts showed negative results for phenols and anthraquinones (Table 4.6, Appendix VI).

Table 4.6: Phytochemical Analysis of Crude Extracts of Endophytic Fungal Isolated from *Warburgia ugandensis*

Code	Fungal Extract	Sap	Tan	Alka	Flav	Ster	Glyco	Anthra	Phen
BkZA3	*Bionectria ochroleuca*	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
StZA8	*Phomopsis mali*	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
LfZA2	*Fusarium oxysporum*	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
LfB7	*Colletotrichum acutatum*	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
Lf12b	*Cladosporium spp.*	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
Bk3a	*Tricharina gilva*	+ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve
Lf3b	*Nigrospora oryzae*	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
LfZAB	*Cladosporium bruhnei*	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
Bk2b	*Phomopsis spp.*	+ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve
Lf7b1	*Guignardia mangiferae*	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
Bkba2	*Alternaria spp.*	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
Bk5b	*Cochliobolus sativus*	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve
St2b	*Phyllosticta gardeniicola*	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
Lf3a2	*Aspergillus flavus*	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
St1a	*Diaporthe amygdali*	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
RtZB3	*Trichoderma harzianum*	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve
Lf6a2	*Alternaria alternata*	+ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve

-ve: absence, +ve: presence, Sap: Saponins, Tan: Tannins, Alka: Alkaloids, Flav: Flavonoids, Ster: Steroids, Glyco: Glycosides, Anthra: Anthraquinones, Phen: Phenols

4.4 Screening of Endophytic Fungi for Antimicrobial Activities

Out of the seventeen (17) extracts from the 17 isolates, (both crude and Ethyl acetate extracts) only two isolates showed antimicrobial activity in their crude extracts after 24 hours' incubation under 37 ^{o}C temperature (Table 4.7).

The crude extracts of both *Phomopsis mali* (coded as 8 in the culture plate) and *Alternaria alternata* (coded as 9 in the culture plate) had antimicrobial activity against *Candida albicans* with 3 mm and 2 mm diameters of zones of inhibition respectively (Figure 4.4). Their Ethyl acetate extracts however didn't show any activity. The positive control had 17 mm diameter zone of inhibition.

Figure 4.4: Antimicrobial Activivity of Extacts of Fungal Endophytes isolated from *Warburgia ugandensis* against *Candida albicans*

After ANOVA and mean separation using LSD test, it was found that there was a significant difference between fungal extracts and the positive and negative controls in regard to inhibition of *Candida albicans.* Highest significant inhibition was recorded for Fluconazole (Positive control) while the lowest was recorded for DMSO (Negative control). *Phomopsis mali* and *Alternaria alternata* fungal extracts had intermediate inhibition which was significantly different from one another with *Phomopsis mali* showing higher inhibition capacity than *Alternaria alternata* (Table 4.7).

Table 4.7: Inhibition significance and Susceptibility Category of the Extracts of Fungal Endophytes *Phomopsis mali* and *Alternaria alternata*

Test Organism	Fungal Extract	Diameter of Inhibition (mm)	Susceptibility
Candida albicans	*Phomopsis mali* (8m)	3.0 ± 0.1^{b}	Resistant
	Alternaria alternata (9m)	2.1 ± 0.2^{c}	Resistant
	Fluconazole	17.1 ± 0.1^{a}	Sensitive
	DMSO	0.0^{d}	No activity

(Susceptibility categories: ≤14 mm – Resistant, 15 – 18 mm – Intermediate, and ≥19 mm – Susceptible (Barry and Brown, 1996); a-d means with different superscripts are significantly different at P = 0.05 level of significance).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Isolation and Identification of Endophytic Fungi

The current study found that the most isolated endophyte was *Phomopsis spp.* (13 isolates) followed by *Fusarium oxysporum* (8 isolates) and *Alternaria spp.* (7 isolates). However, this results contradicts Katoch *et al*. (2017) findings on endophytes from *Monarda citriodora* plant which showed that *Fusarium spp*. was the most dominant isolate followed by *Cladosporium spp., Alternaria spp.* was less dominant and *Phomopsis spp.* was not isolated although some isolates were similar to the ones in the current study (*Colletotrichum spp., Fusarium spp., Alternaria alternata, Aspergillus flavus* and *Cladosporium spp*.). Bogner *et al*. (2016) isolated similar isolates to the ones in the present study from tomato roots in a study done in Kenya though different species namely; *Trichoderma asperellum, Fusarium nygamai, Fusarium spp., Aspergillus sclerotiorum, Altenaria solani* and *Cochliobolus spp.* which were reported to have nematode bio control potential. The present study also corresponds with Nasimiyu *et al*. (2018) who also isolated *Fusarium, Colletotrichum, Phomopsis, Cladosporium* and *Aspergillus* from selected medicinal plants in Kenya. Various *Fusarium spp*. (*Fusarium verticillioides, Fusarium boothii* and *Fusarium poae*) have been previously identified as mycotoxigenic fungi contaminating maize samples in a previous study carried out in Kenya. *Lasiodiplodia theobromae, Mucor nidicola,* and *Nigrospora oryzae* were also found among the mycotoxigenic fungi though in small counts (Kibe, 2015). Some of the endophytes isolated in this study have been isolated elsewhere as environmental fungi. For instance, *Aspergillus flavus* and *Trichoderma harzianum* have been among the soil fungi isolated from rice growing regions in Kenya (Mwashasha *et al*., 2014). *Aspergillus flavus* and *Cladosporium spp*. have been recently isolated from medicinal plant *Kigelia africana* is a similar study done in Sudan (Idris *et al*., 2013). Similar endophytic fungal

isolates realized in the present study were among the endophytes isolated from the plant *Diospyros crassiflora* in a study done in Cameroon where by such isolates include; *Colletotrichum spp., Diaporthe spp., Phomopsis spp., Guignardia mangiferae and Phyllosticta spp.* (Meli & Ewald, 2012). In this study, endophytes colonized all the plant parts collected for isolation (leaves, stem, bark and roots), similar observation was made by Meli and Ewald, (2012) meaning endophytes can inhabit any part of a healthy plant.

Endophytes are of great importance to the host plant and the environment as well. For instance, a new strain of *Cladosporium sphaerospermum* was found to increase the growth rate of rice and soybean due to bioactive compound GA3, GA4 and GA7 in a review article (Nair & Padmavathy, 2014). Endophytes also increase growth of the host plant through release of hormones, activation of expression of certain enzymes and genes (Min *et al*., 2016). Endophytes such as *Aspergillus, Cladosporium, Fusarium, Nigrospora* and *Phomopsis* among others have been reported to have potential to release enzymes such as pectinases, cellulases, xylanases and proteases useful in biotechnological processes as well as decomposition of plant material which is important for recycling of nutrients. Other compounds released by endophytes enhance defense of the host plant from pathogens (Dhanya & Padmavathy, 2014). *Guignardia mangiferae* isolated from *Taxus media* has been reported to produce bioactive compound taxol which is a compound used for cancer treatment. Taxol has also been found to be released by *Phyllosticta spp*. Endophyte released by the plant *Ocimum basilicum* (Souza & Santos, 2017; Kaul *et al*., 2014). Both *Alternaria alternata* and *Phomopsis spp.* Endophytes isolated from *Miquelia dentate* have been found to produce bioactive compound camptothecine which is an anticancer agent. *Cochliobolus spp.* From host plant *Piptadenia adiantoides* was reported to release bioactive compound cochlioquinone-A which is antiparasitic (Kaul *et al*., 2014).

5.1.2 Toxicity Testing

The percentage mortality of *Artemia salina* increased with increase in concentration of the extracts and vice versa. The observed mortality of *Artemia salina* in the present study shows that the endophytic fungal extracts have cytotoxic components (Olowa & Nuñeza, 2013). Since all the extracts had LC_{50} values greater than 1000 µg/ml, the extracts were not toxic according to a study done by Zakari and Kubmarawa, (2016) in Nigeria. However, there is no recommended LC_{50} dose for fungal endophyte extracts since there is a diversity in the phytochemicals in extracts of endophytes depending on the strain of the endophyte. This study contradicts Wu *et al*. (2013) who carried out LC_{50} tests on extracts of endophytic fungi from *Scapania verrucosa* plant in China, and reported that the highest LC_{50} value from the fungal endophytes extracts was 622.69 µg/mL and the extract with the lowest value being less than 20 µg/mL meaning that the endophytes have the potential to produce potent cytotoxic compounds and more toxic than the endophytic fungal extracts in this study. On the contrary, (Shoeb *et al*., 2014) who did a similar experiment in University of Dhaka, using extracts of fungal endophyte *Penicillium thiomii* isolated from *Terminalia chebula Retz* medicinal plant had 24.29 µg/mL as the highest LC_{50} value with the lowest value being 2.39 µg/mL hence all the extracts were lethal to the brine shrimps. LC_{50} values of extracts of endophytic fungi from *Dysosma pleiantha* plant, one of the endangered Chinese species, ranged from 4.86 µg/mL to more than 1000 µg/mL (Lu *et al*., 2009). Lu *et al*. (2012) also worked on fungal endophytes extracts of *Actinidia macrosperma* in China and the range of the LC_{50} values was similar to the one for the previous study he had done (4.86 µg/ml to more than 1000 µg/ml) but with higher cytotoxicity potential compared to the extracts in the present study.

5.1.3 Screening for Phytochemicals

This study showed that the extracts were negative for phenols and anthraquinones but positive for the rest of the phytochemicals screened for (saponins, tannins, alkaloids, flavonoids, sterols and glycosides). A similar study done in Cameroon by Yemeda *et al*.

(2015) reported the presence of flavonoids, anthraquinones, tannins, phenols, steroids, coumarins and terpenoids, absence of alkaloids and saponins in ethyl acetate extracts of the endophytes *Aspergillus*, *Penicillium*, *Fusarium* and *Trichoderma*. According to Bhardwaj *et al*. (2015), extracts of fungal endophyte *Alternaria alternata* from *Pinus roxburghii* had saponins, steroids, cardiac glycosides and tannins. According to the findings of the present study, *Trichoderma harzianum* extract, a root endophyte, is negative for flavonoids which contradicts a recent study done in Tripura University by Bhattacharya *et al*. (2018) who reported that a similar root endophyte extract (*Trichoderma asperellum*) from *Ananus comosus* plant was positive for flavonoids which were attributed to its antioxidant potential. Extract of the same fungal endophyte, (*Trichoderma harzianum*) from *Tabebuia argentea* tree tested positive for flavonoids, phenols and anthraquinones after phytochemical screening unlike in the present study (Govindappa *et al*., 2013). *Fusarium oxysporum* extracts showed presence of tannins, alkaloids, flavonoids, steroids and glycosides and absence of phenols. However Ramesha and Scrinivas, (2014), report on phytochemicals present in an extract of a similar fungal extract from *Plumeria acuminata L.* and *Plumeria obtusifolia L.*, in Bangalore, India, indicate that the extract was positive for phenols and phenolic compounds hence contradicting the present study. Desaraju *et al*., (2013) also reported that extracts of *Fusarium oxysporum* from *Rhynchosia beddomei* and *Terminalia pallida* endemic medicinal plants of Eastern Ghats, India, had phenols, alkaloids and flavonoids phytochemicals, both *Alternaria alternata* and *Cladosporium spp*. from the same plant had flavonoids and alkaloids while *Aspergillus flavus* had phenols but no tannins compared to the present study.

Metabolites such as alkaloids, terpenes, flavonoids, cardiac glycosides, polyphenols and terpenoids in *Warburgia ugandensis* extract have been reported to contribute to its antiplasmodial activities in Kenya (Were *et al*., 2015; Maobe & Nyarango, 2013). Flavonoids and phenolic compounds which are antioxidants have been found to enhance the host plant survive abiotic stresses. Alkaloid compound has been associated with protection of the host plant against insect attack according to a review done by Min *et al*.

(2016). Some ergot alkaloids have been reported to be toxic to both cattle and humans hence when produced by endophytes, they play a role in protecting the host plant against herbivores (Giménez *et al*., 2007). According to Satari *et al*. (2018), phytochemicals such as phenols and saponins have been found to possess both antimicrobial and antioxidant activities; Flavonoids have antibacterial, anti-inflammatory, anti-allergic and antiviral activities in a study done in Western Himalayas. Extracts of medicinal plant *Diospyros lotus* have been found to have terpenoids, tannins and anthraquinones which contribute to it having pesticide and anti-inflammatory activities in a study done in Peshawar University (Uddin *et al*., 2011).

5.1.4 Screening of Endophytic Fungi for Antimicrobial Activities

Phomopsis mali and *Alternaria alternata* crude extracts showed activity against *Candida albicans* meaning its metabolites have antifungal activity. However, the activity was insignificant while the rest of the endophytes didn't show any activity. This could be because of diversity in phytochemicals present and also not all strains of fungal endophytes have antimicrobial activities. During sterilization, some of the compounds may have stuck on the micro filter layer or even some compounds could have been unstable hence dissociated before the antimicrobial tests. Growth conditions for the endophytes in the experimental set up may also have affected the antimicrobial effect potential. If maybe many different endophytic fungal isolates like a hundred and above were tested for various activities such as antiviral, antibacterial, anticancer activities among others using different test organisms, more activities would have been recorded. According to the work of Tong *et al*. (2014) done in Malaysia, extracts of *Phomopsis* isolated from *Orthosiphon stamineus Benth* have antimicrobial activities against both Gram-positive and Gram-negative bacteria hence can be a good source of broad spectrum antibiotic. In a review done by Kaul *et al*. (2014), *Phomopsis spp.* Endophyte isolated from *Plumeria acutiforia poiret* was found to release terpenoids which were antibacterial. The same endophyte isolated from *Erythrina crista-galli* was reported to release isoflavonoids which were antimicrobial. *Alternaria africana* crude extract also

had activity against *Candida albicans*. Bhardwaj *et al*. (2015) who did a similar study in Garhwal region of Uttrakhand, on extracts of fungal endophytes isolated from spikes of *Pinus roxburghii*, reported sensitivity against *Staphylococcus aureus, E. coli* and *Candida albicans*. Kamal *et al*. (2015) reported that extracts of *Fusarium oxysporum* endophyte isolated from leaf of *Nothapodytes foetida* exhibited activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* while in the present study it didn't show any activity against *Candida albicans*. Both *Aspergillus flavus* and *Cladosporium spp.* extracts didn't show any activity against the test organism in the current study while Katoch *et al*. (2017) reported that extracts from similar isolates (*Aspergillus fumigatus* and *Cladosporium tenuissimum*) isolated from *Monarda citriodora* plant in India had significant activity against *E. coli*, *Staphylococcus aureus* and *Candida albicans*. *Aspergillus flavus* and *Cladosporium spp*. Extracts from *Kigelia africana* plant were sensitive against *Staph aureus* and *E. coli* in a study done in Sudan (Idris *et al*., 2013). Yet another study done in Tamilnadu in India by Prabavathy and Nachiyar, (2012) on endophytes of *Justicia adathoda* showed that extract of *Aspergillus spp*. had antifungal activity against *Candida albicans*.

This study corresponds with Mousa *et al*. (2015) who reported that *Aspergillus spp.* Extracts isolated from finger millet didn't show any antifungal activity. According to Martin and Dombrowski, (2015), *Alternaria spp*. and *Fusarium spp*. Extracts isolated from grass, in a study done in Oregon Coast, didn't show any antimicrobial activity which contradicts with the current study. However, both studies correspond together since *Cladosporium spp*., *Diaporthe spp*. and *Trichoderma spp*. didn't show any antimicrobial activity in both studies.

5.2 Conclusions

1 Fungal endophytes were isolated from roots, stem, bark and leaves of *Warburgia ugandensis* which were the parts of target.
2 *Phomopsis spp*. Were the most common fungal endophytic species followed by *Fusarium spp*. and *Alternaria spp*.

3 Endophytic fungal extracts contained phytochemicals namely: saponins, tannins, flavonoids, sterols and glycosides.

4 *Phomopsis mali*, *Alternaria alternata* and *Fusarium oxysporum* extracts had metabolites with antimicrobial activities hence can be used in the pharmaceutical industry.

5.3 Recommendations

1 There is need to use various test organisms (fungi, bacteria, protozoa, viruses) for antimicrobial screening of the extracts and also increasing the number of endophytes tested for can increase the chances of getting more endophytes with antimicrobial activity since some strains lack antimicrobial activities.

2 Clinical isolates of *Candida albicans* can also be used in future in order to compare the activity with the pure test organisms.

3 A study should be carried out to check the synergistic action of *Warburgia ugandensis* with the endophytes.

REFERENCES

Abrantes, S. M. P., McArthur, P. C. & Wilma, J. A. C. (2014). Multi-drug resistannt (MDR) oral *Candida* species isolated from m HIV-positive patients in South Africa and Cameroon. *Diagnostic Microbiology and Infectious Disease*, *79*(2), 222–227.

Abuto, J. O., Muchugi, A., Mburu, D., Machocho, A. K. & Karau, G. M. (2016). Variation in antimicrobial activity of *Warburgia ugandensis* extracts from different populations across the Kenyan Rift Valley. *Journal of Microbiology Research*, *6*(3),55–64.

Andrews, M. J. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, *48*, 5–16.

Auwal, S. M., Saka, S., Mairiga, I. A., Sanda, K. A., Shuaibu, A. & Ibrahim, A. (2014). Preliminary phytochemical and elemental analysis of aqueous and fractionated pod extracts of *Acacia nilotica* (*Thorn mimosa*). *Veterinary Research Forum*, *5*(2), 95–100.

Barnett, H. L. & Hunter, B. H. (1998). *Title: Illustrated Genera of Imperfect Fungi* (4th ed.). Amer Phytopathological Society.

Barry, A. L. & Brown, S. D. (1996). Fluconazole disk diffusion procedure for determining susceptibility of *Candida* species, *34*(9), 2154–2157.

Bhardwaj, B., Deeksha, S., Jadon, N. & Agrawal, K. P. (2015). Antimicrobial and phytochemical screening of endophytic fungi isolated from spikes of *Pinus roxburghii*. *Archives of Clinical Microbiological*, *6*(3:1), 1–9.

Bhattacharya, Debnath, S. & Saha, K. A. (2018). Evaluation of antioxidant activity of two endophytic fungi isolated from *Ananus Comosus L*. *European Journal of*

Biomedical and Pharmaceutical Sciences, *5*(1), 896–899.

Blumberg, H. M., Jarvis, W. R., Soucie, J. M., Edwards, J. E., Patterson, J. E., Pfaller, M. A., Rangel-Frausto, M. S., … & the NEMIS Study Group. (2001). Risk factors for candidal blood stream infections in surgical intensive care unit Patients : The NEMIS prospective multicenter study. *NEMIS SICU Candidal Study*, *6*(33), 177–186.

Bogner, C. W., Kariuki, M. G., Elashry, A., Sichtermann, G., Buch, A. K., Mishra, B., Thines, M., … & Schouten, A. (2016). Fungal root endophytes of tomato from Kenya and their nematode biocontrol potential. *Mycological Progress*, *15*(30), 39–50..

Bryn, D., Gary, S., Ezra, D., Castillo, U., Gary, B. & Hess, W. M. (2002). *Muscodor vitigenus Anam. Spp.* Nov., An Endophyte from *Paullinia paullinioides*. *Mycotaxon*, *84*, 39–50.

Cowen, E. L., Anderson, J. B. & Kohn, M. L. (2002). Evolution of drug resistance in *Candida albicans*. *Annual Review of Microbiology*, *56*, 139–165.

Dabas, P. S. (2013). An approach to etiology , diagnosis and management of different types of candidiasis. *Journal of Yeast and Fungal Research*, *4*(August), 63–74.

Desaraju, B. S., Tartte, V., Nagam, V., Kalla, C., Sarma, K. & Nagaraju, C. (2013). Endophytic Fungal Diversity from Endemic Plants of Tirumala Hills in Eastern Ghats and their Anti-Candidal Activity. *World Journal of Pharmaceutical Research*, *3*(1), 834–847.

Dhanya, N. N. & Padmavathy, S. (2014). Impact of endophytic microorganisms on plants, environment and humans. *The Scientific World Journal*, *2014*, 11.

Dou, N., Weiping, L., Enfeng, Z., Chan, W., Zhaozhao, X. & Honghui, Z. (2014). Risk factors for *candida* infection of the genital tract in the tropics. *African Health Sciences*, *14*(4), 835–837.

Drage, S., Mitter, B., Tröls, C., Muchugi, A., Jamnadass, H. R., Sessitsch, A. & Hadacek, F. (2014). Antimicrobial drimane sesquiterpenes and their effect on endophyte communities in the medical tree *Warburgia ugandensis*. *Frontiers in Microbiology*, *13*(10), 1–17.

Giménez, C., Cabrera, R., Reina, M. & Coloma, G. A. (2007). Fungal endophytes and their role in plant protection. *Current Organic Chemistry*, *11*, 707–720.

Gond, S. K., Verma, V. C., Kumar, A., Kumar, V. & 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*(10), 1371–1375.

Govindappa, M., Channabasava, R., Kumar, S. R. K. & Pushpalatha, K. C. (2013). Antioxidant Activity and Phytochemical Screening of Crude Endophytes Extracts of *Tabebuia argentea* Bur . & K. Sch. *American Journal of Plants Sciences*, *4*, 1641–1652.

http://www.worldagroforestry.org. (2009). *Warburgia ugandensis* Sprague. *Database*, *1*, 1–5.

Idris, A., Al-tahir, I. & Idris, E. (2013). Antibacterial activity of endophytic fungi extracts from the medicinal plant *Kigelia africana*. *Egyptian Academic Journal of Biological Sciences*, *5*(1), 1–9.

Jaber, R. L. & Ownley, B. H. (2018). Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? *Biological Control*, *116*, 36–45.

Jao, A., Io, O., Odu, N. N., Kolade, A. F. & Nwanze, J. C. (2011). Detection and prevalence of *Candida* isolates among patients in Ibadan, Southwestern Nigeria. *Journal of Microbiology and Biotechnology Research*, *1*(3), 176–184.

Kabir, A. M., Hussain, A. H. & Ahmad, Z. (2012). *Candida albicans*: a model organism for studying fungal pathogens. *International Scholarly Research Network Microbiology*, *2012*, 1–15.

Kalia, N., Singh, J., Sharma, S., Kamboj, S. S., Arora, H. & Kaur, M. (2015). Prevalence of vulvovaginal infections and species specific distribution of vulvovaginal candidiasis in married women of north India. *International Journal of Current Microbiology and Applied Sciences*, *4*(8), 253–266.

Kalyanasundaram, I., Nagamuthu, J. & Muthukumaraswamy, M. (2015). Antimicrobial activity of endophytic fungi isolated and identified from salt marsh plant in Vellar Estuary. *Journal of Microbiology and Antimicrobials*, *7*, 13–20.

Kamal, A. S., Hamza, F. L. & Hameed, H. I. (2015). Antibacterial activity of secondary metabolites isolated from *Alternaria alternata*. *African Journal of Biotechnology*, *14*(43), 2972–2994.

Kanafani, A. Z. & Perfect, R. J. (2008). Resistance to antifungal agents: mechanisms and clinical impact. *Clinical Infectious Diseases*, *46*(1), 120–128.

Katoch, M., Phull, S., Vaid, S. & Singh, S. (2017). Diversity, Phylogeny, anticancer and antimicrobial potential of fungal endophytes associated with *Monarda citriodora L*. *BMC Microbiology*, *17*(44), 1–13.

Kaul, S., Ahmed, M., Sharma, T. & Dhar, M. K. (2014). Unlocking the myriad benefits of endophytes: an overview. *Microbial Diversity and Biotechnology in Food Security*, 41–57.

Kenya Forest Service. (2010). *Mt. Kenya Forest Reserve Management Plan*. Nairobi: Kenya Forest Service

Khan, A. S. M., Ahmad, I., Aqil, F., Owais, M., Shahid, M. & Musarrat, J. (2010). Virulence and pathogenicity of fungal pathogens with special reference to *Candida albicans*. *Combating Fungal infections*, Berlin, Heidelberg: Springer.

Kibe, N. E. (2015). *Occurrence of Mycotoxigenic Fungi in Maize from Food Commodity Markets in Kenya*. Gent: University of Gent.

Kidd, S., Halliday, C., Alexiou, H. & Ellis, D. (2016). *Descriptions of Medical Fungi* (3rd ed.). Adelaide: Dr. Sarah E. Kidd.

Kuglerova, M., Tesarova, H., Grade, T. J., Halamova, K., Maganyi, W. O., Damme V. P. & Kokoska L. (2011). Antimicrobial and antioxidative effects of Ugandan medicinal barks. *African Journal of Biotechnology*, *10*(18), 3628–3632.

Kumar, S. S.D. & Hyde, D. K. (2004). Biodiversity and tissue-recurrence of endophytic fungi in *Tripterygium wilfordii*. *Fungal Diversity*, *17*(1994), 69–90.

Lu, Y., Chen, C., Chen, H. Zhang, J. & Chen, W. (2012). Isolation and identification of endophytic fungi from *Actinidia macrosperma* and investigation of their bioactivities. *Evidence-Based Complementary and Alternative Medicine*, *2012*, 8.

Lu, Y., Chen, S. & Wang, B. (2009). Cytotoxic activities of endophytic fungi isolated from the endangered, Chinese endemic species *Dysosma pleiantha*. *Journal of Biosciences*, *64*(7–8), 518–520.

Maobe, A. G. M. & Nyarango, M. R. (2013). Fourier transformer infra-red spectrophotometer analysis of *Warburgia ugandensis* medicinal herb used

for the treatment of diabetes, malaria and pneumonia in Kisii region, Southwest Kenya. *Global Journal of Pharmacology*, *7*(1), 61–68.

Maroszyńska, M., Kunicka-Styczyńska, A., Rajkowska, K. & Maroszyńska, I. (2013). Antibiotics sensitivity of *Candida* clinical and food-borne isolates. *Acta Biochimica Polonica*, *60*(4), 719–724.

Martin, R. C. & Dombrowski, J. E. (2015). Isolation and identification of fungal endophytes from grasses along the Oregon Coast. *American Journal of Plant Sciences*, *6*, 3216–3230.

Mayer, F. L., Wilson, D. & Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence*, *4*(2), 119–128.

Meli, D. C. & Ewald, L. (2012). Diversity and molecular phylogeny of fungal endophytes associated with *Diospyros crassiflora*. *Mycology*, *3*(3), 175–187.

Menza, N., Wanyoike, W. & Muturi, M. (2013). Prevalence of vaginal candidiasis and determination of the occurrence of *Candida* species in pregnant women attending the antenatal clinic of Thika District Hospital, Kenya. *Open Journal of Medical Microbiology*, *3*(4), 264–272.

Merawie, Y., Sahile S., Moges, F. & Husen, A. (2013). Antimicrobial activity of crude and semi-purified fractions of *Warburgia ugandensis* against some pathogens. *Journal of Coastal Life Medicine*, *1*(3), 233–240.

Min, J., Ling, C., Hai-Liang, X., Cheng-Jian, Z., Khalid, R., Ting, H. & Lu-Ping, Q. (2016). A friendly relationship between endophytic fungi and medicinal plants: a systematic review. *Frontiers in Microbiology*, *7*(June), 1–14.

Mousa, K. W., Schwan, A., Davidson, J., Strange, P., Liu, H., Zhou, T., Auzanneau, I. F. & Raizada, M. N. (2015). An endophytic fungus isolated from finger millet

(*Eleusine coracana*) produces anti-fungal natural products. *Frrontiers in Microbiology*, *6*(1157), 1–16.

Mulu, A., Kassu, A., Anagaw, B., Moges, B., Gelaw, A., Alemayehu, M., Belyhun, Y., … & Isogai, E. (2013). Frequent detection of "azole" resistant *Candida* species among late presenting AIDS patients in northwest Ethiopia. *BMC Infectious Diseases*, *13*, 82.

Mwashasha, R. M., Murage, H., Tani, A., Kahangi, M. E. & Makonde, M. H. (2014). Molecular characterization of bacteria and fungi from rice growing regions in Kenya. *International Journal of Biosciences*, *5*(3), 7–14.

Nair, N. D. & Padmavathy, S. (2014). Impact of endophytic microorganisms on plants, environment and humans. *The Scientific World Journal*, *2014*, *11*.

Nasimiyu, W. V., Wagara, N. I., Obonyo, M. A. & Matasyoh, J. C. (2018). Isolation , identification and bioactivity of fungal endophytes from selected Kenyan medicinal plants. *African Journal of Microbiology Research*, *12*(17), 405–412.

Nasution, I. A. (2013). Virulence Factor and Pathogenicity of *Candida albicans* in Oral Candidiasis. *World Journal of Dentistry*, *4*(4), 267–271.

Ndahebwa, C. M. (2018). *Isolation and molecular characterization of low density poly-ethene degrading bacteria and fungi from Dandora dumpsite-Nairobi*. Nairobi: Pan African Universisty.

Nermina, S. (2012). *Biotransformation and utilization of drimane sesquiterpenes by endophytic microorganisms*. Wien: Wien University.

Ngure, K. P., Tonui, K. W., Ingonga, J., Mutai, C., Kigondu, E., Ng'ang'a, Z., Rukunga, G. & Kimutai, A. (2009). In vitro antileishmanial activity of extracts of

Warburgia ugandensis (*Canellaceae*), a Kenyan medicinal plant. *Journal of Medicinal Plants Research*, *3*(2), 61–66.

O'Shaughnessy, M. E., Lyman, A. C. & Walsh, J. T. (2009). Amphotericin B: Polyene resistance mechanisms. *Antimicrobial Drug Resistance*, 183–193.

Olowa, L. F. & Nuñeza, M. O. (2013). Brine shrimp lethality assay of the ethanolic extracts of three selected species of medicinal plants from Iligan City, Philippines. *International Research Journal of Biological Sciences*, *2*(11), 74–77.

Onuorah, S., Ifeanyi, O. & Ugochukwu, O. (2015). Prevalence of *candida* species among vaginitis symptomatic pregnant women attending ante-natal clinic of Anambra State University Teaching Hospital, Awka, Nigeria. *Bioengineering and Bioscience*, *3*(2), 23–27.

Ooga, V. B., Gikunju, J. K. & Bii, C. C. (2009). Characterization and antifungal drug susceptibility of clinical isolates of *Candida* species . *African Journal of Health Sciences*, *19*, 80–87.

Owotade, F. J., Gulube, Z., Ramla, S. & Patel, M. (2016). Antifungal susceptibility of *Candida albicans* isolated from the oral cavities of patients with HIV infection and cancer. *The South African Dental Journal*, *71*(1), 8–11.

Pankhurst, L. C. (2009). Candidiasis (Oropharyngeal). *Clinical Evidence*, 1–36.

Pappas, G. P., Rex, H. J., Sobel, J. D., Filler, G. S., Dismukes, W. E., Walsh, T. J. & Edwards, J. E. (2000). Practice guidelines for the treatment of candidiasis. *Clinical Infectious Diseases*, *30*(4), 662–678.

Pavithra, N., Sathish, L. & Ananda, K. (2012). Antimicrobial and enzyme activity of endophytic fungi isolated from Tulsi. *Journal of Pharmaceutical and*

Biomedical Sciences, 16(12), 12–17.

Perea, S. & Patterson, F. T. (2002). Antifungal resistance in pathogenic fungi. *Clinical Infectious Diseases, 35*(9), 1073–1080.

Perlin, S. D. (2015). Echinocandin resistance in *Candida*. *Clinical Infectious Diseases, 61*(6), 612–617.

Pimentel, R. M., Molina, G., Dion´ısio, P. A., Mar ´ostica, R. M. J. & Pastore, M. G. (2011). The use of endophytes to obtain bioactive compounds and their application in biotransformation process. *Biotechnology Research International, 2011*, 1–11.

Prabavathy, D. & Nachiyar, V. C. (2012). Study on the antimicrobial activity of *Aspergillus spp.* isolated from *Justicia adathoda*. *Indian Journal of Science and Technology, 5*(9), 3317–3320.

Premjanu, N., Jaynthy, C. & Diviya, S. (2016). Antifungal activity of endophytic fungi isolated from *lannea coromandelica* – an insilico approach. *International Journal of Pharmacy and Pharmaceutical Sciences, 8*(5), 207–210.

Radu, S. & Kqueen, C. Y. (2002). Preliminary screening of endophytic fungi from medicinal plants in Malaysia for antimicrobial and antitumor activity. *The Malaysian Journal of Medical Sciences, 9*(2), 23–33.

Ramesha, A. & Scrinivas, C. (2014). Antimicrobial activity and phytochemical analysis of crude extracts of endophytic fungi isolated from *Plumeria acuminata L.* and *Plumeria obtusifolia L. European Journal of Experimental Biology, 4*(2), 35–43.

Ruhnke, M., Rickerts, V., Cornely, A. O., Buchheidt, D., Glo¨ckner, A., Heinz, W., Ho¨ hl, R.,… & Groll, H. A. (2011). Diagnosis and therapy of *Candida*

infections: Joint recommendations of the German Speaking Mycological Society and the Paul-Ehrlich-Society for Chemotherapy. *Mycoses*, *54*(4), 279–310.

Sardi, J. C. O., Scorzoni, L. Bernardi, T., Fusco-Almeida, A. M. & Giannini, M. M. J. S. (2013). *Candida* species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *Journal of Medical Microbiology*, *62*, 10–24.

Satari, H. A., Zargar, I. M., Shah, W. A., Bansal, R. & Bhat, F. M. (2018). Isolation , molecular identification , phytochemical screening and in vitro anti-oxidant activity of endophytic fungi from *Achilea millefolium Linn*. *Journal of Pharmacognosy and Phytochemistry*, *7*(4), 87–92.

Selvi, B. K. & Balagengatharathilagam, P. (2014). Isolation and screening of endophytic fungi from medicinal plants of Virudhunagar District for antimicrobial activity. *International Journal of Science and Nature*, *5*(1), 147–155.

Shoeb, M., Haque, M. & Nahar, N. (2014). Bioactive compounds from endophytic fungus *Penicillium thiomii* isolated from *Terminalia chebula Retz*. *Journal of Natural Product and Plant Resources*, *4*(3), 65–70.

Singh, L. K. & Bag, G. C. (2013). Phytochemical analysis and determination of total phenolics content in water extracts of three species of Hedychium. *International Journal of PharmTech Research*, *5*(4), 1516–1521.

Singh, G. & Raksha, A.D.U. (2013). Candidal Infection: epidemiology, pathogenesis and recent advances for diagnosis. *Bulletin of Pharmaceutical and Medical Sciences (BOPAMS)*, *1*(1), 1–8.

Souza, S. B. & Santos, T. T. (2017). Endophytic fungi in economically important plants: ecological aspects, diversity and potential biotechnological applications.

Biotechnology and Biodiversity, *4*, 113–126.

Spiering, J. M., Greer, H. D. & Schmid, J. (2006). Effects of the fungal endophyte, *Neotyphodium lolii*, on net photosynthesis and growth rates of perennial ryegrass (*Lolium perenne*) are independent of in planta endophyte concentration. *Annals of Botany*, *98*, 379–387.

Suciatmih & Rahmansyah, M. (2013). Endophytic fungi isolated from Mangrove plant and have antagonism role against *Fusarium* wilt. *Journal of Agricultural and Biological Science*, *8*(3), 251–257.

Sudbery, P., Gow, N. & Berman, J. (2004). The distinct morphogenic states of *Candida albicans*. *Trends in microbiology*, *12*(7), 317-324.

Sunaryanto, R. & Mahsunah, H. A. (2013). Isolation, purification, and characterization of antimicrobial substances from endophytic actinomycetes. *Makara Journal of Science*, *17*(3), 78–81.

Tolulope, A. R., Adeyemi, I. A., Erute, A. M. & Abiodun, S. T. (2015). Isolation and screening of endophytic fungi from three plants used in traditional medicine in Nigeria for antimicrobial activity. *International Journal of Green Pharmacy*, *9*(1), 58.

Tong, W. Y., Zaadah. N. J., Nurhaida, Tan, W. N., Melati, K., Latiffah, Z. & Darah, I. (2014). Antimicrobial activity of *Phomopsis spp*. ed2 residing in medicinal plant *Orthosiphon stamineus Benth*. *Annual Research & Review in Biology*, *4*(9), 1490–1501.

Uddin, G., Rauf, A., Siddiqui, S. B. & Shah, Q. S. (2011). Preliminary comparative phytochemical screening of *Diospyros lotus Stewart*. *Middle-East Journal of Scientific Research*, *1*(10), 78–81.

Uppuluri, P., Pierce, G. C. & López-Ribot, L. J. (2009). *Candida albicans* biofilm formation of its clinical consequences. *Future Microbiology*, *4*, 1235–1237.

Were, P. S., Waudo, W., Ozwara, H. S. & Kutima, H. L. (2015). Phytochemical analysis of *Warburgia ugandensis* sprague using Fourier Transform Infra-red (FT-IR) Spectroscopy. *International Journal of Pharmacognosy and Phytochemical Research*, *7*(2), 201–205.

Whaley, S. G., Berkow, E. L., Rybak, J. M., Nishimoto, T. A., Barker, S. K. & Rogers, P. D. (2017). Azole antifungal resistance in *Candida albicans* and emerging non-albicans *candida* species. *Frontiers in Microbiology*, *7*(2173), 1–12.

Wiyakrutta, S., Sriubolmas, N., Panphut, W., Thongon, N., Kanjana, D. K., Ruangrungsi, N. & Meevootisom, V. (2004). Endophytic fungi with anti-microbial, anti-cancer and anti-malarial activities isolated from Thai medicinal plants. *World Journal of Microbiology and Biotechnology*, *20*(3), 265–272.

Wu, J. G., Peng, W., Zeng, P. Y., Wu, Y. B., Yi, J. & Wu, J. Z. (2013). Antimicrobial activity and cytotoxicity of endophytes from *Scapania verrucosa Heeg*. *Genetics and Molecular Research*, *12*(2), 916–924.

www.hayesmicrobial.com/library.php. (n.d.). Hayes Microbial Consulting - Library.

Yang, Y. (2003). Virulence factors of *Candida* species. *Journal of Microbiology, Immunology and Infection*, *36*, 223–228.

Yapar, N. (2014). Epidemiology and risk factors for invasive candidiasis. *Therapeutics and Clinical Risk Management*, *10*(1), 95–105.

Yemeda, C. F. L., Nyegue, M. A., Ngene, J. P., Benelesse, G. E., Lenta, B., Wansi, J. D., Mpondo, M. E., & Dibong, S. D. (2015). Identification and phytochemical

screening of endophytic fungi from stems of *Phragmanthera capitata* (*Sprengel*) *S. Balle* (*Loranthaceae*). *Journal of Applied Biosciences*, *90*, 8355–8360.

Zakari, A. & Kubmarawa, D. (2016). Acute toxicity (LD50) studies using swiss albino mice and brine shrimp lethality (LC50 and LC90) determination of the ethanol extract of stem bark of *Echnaceae angustifolia DC*. *Natural Products Chemistry & Research*, *4*(6), 4–7.

Zarrin, M. & Mahmoudabadi, Z. A. (2009). Invasive candidiasis ; a review article. *Jundishapur Journal of Microbiology*, *2*(1), 1–6.

APPENDICES

Appendix I: Media Preparation

Agar agar

Typical Formula: Acid insoluble Ash – max 1.0%, Foreign Organic matter- max 1.0%, Foreign insoluble matter – Max 1.0%, Sulphated ash – Max 5.0%, Loss on drying – Max 18.0%.

1. 15g of Agar agar is dissolved in 1Litre of tap water followed by autoclaving at 121 ºC for 15minutes
2. Cool the media to 45 – 50 ºC then dispense.

Nutrient Broth

Typical Formula: 'Lab – lemco' powder 1.0, Yeast Extract 2.0, Peptone 5.0, Sodium chloride 5.0

1. Dissolve 13g of the broth in to one litre of distilled water
2. Mix well then distribute in to the final containers
3. Sterilize by autoclaving at 121 ºC for 15 minutes

Potato Dextrose Agar (PDA)

Ingredients in Gms/Litre

Potato Infusion from – 200.00, Dextrose – 20.00, Agar – 15.00, Final pH (at 2.5 ºC) 5.6 ± 0.2

1. Suspend 39.0g of PDA in 1Litre of distilled water.
2. Heat to boiling to dissolve the media completely
3. Sterilize by autoclaving at 15 Ibs pressure (121 ºC) for 15 minutes

4. Cool to 45 – 50 ºC
5. Mix well before dispensing

Nutrient Agar

1. Suspend 28g of the media in 1 litre of distilled water.

2. Heat to boiling to dissolve the media completely

3. Sterilize by autoclaving at 15 Ibs pressure (121 ºC) for 15 minutes.

4. Cool the media to 45-50 ºC then dispense.

Appendix II: Codes given to the Sixty (60) Fungal Isolates from the four parts (Leaf, Stem, Bark and Root) of *Warburgia ugandensis*

Leaf	Stem	Bark	Root
LfZAb	St1a	Bk3a	RtZA1
Lf6a2	St5a	BkZA3	RtZA2
LfZA2	StZA8	Bk6a	RtZA3
Lf3a2	StZA7a	Bk4a	RtZA4
Lf1a	St1a2	Bk2a	RtZA5
Lf2a	St2a	Bk1a	RtZB3
Lf3a	St3a	Bk5a	RtZB2
Lf4a	St4a	Bkba2	RtZB1
Lf5a	St2b	Bk5b	RtZB4
Lf12b	St8b2	Bk2b	
Lfb7	St1b	Bkba1	
Lf7b1	St3b	Bk1b	
Lf3b	St4b	Bk3b	
Lf7b7	St5b	Bk4b	
Lf8b	St6b		
Lf10b	St7b		
Lf4b	St8b1		
Lf11b			
Lf1b			
LfZB7			

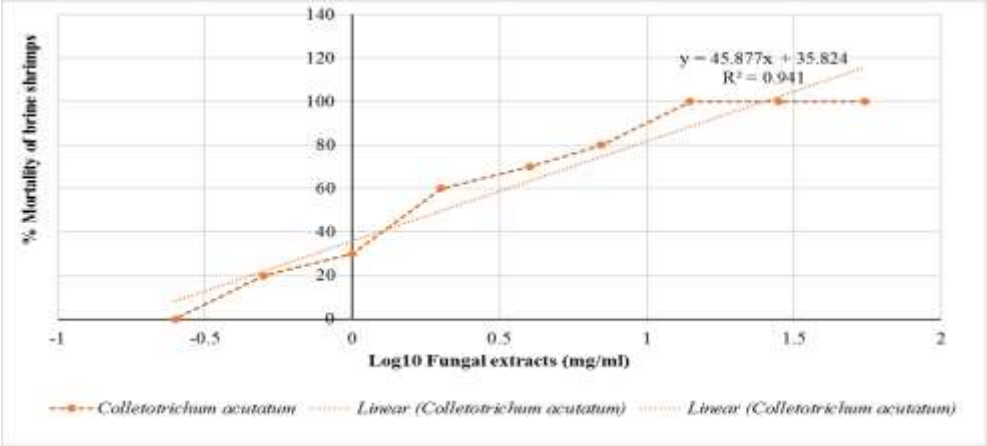
Appendix III: Number of Dead Brine Shrimps per Dilution after 24 Hours' Incubation

Extract	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
LfZA2	10	10	9	7	4	2	0	0	0
LfB7	10	10	10	8	7	6	3	2	0
Lf12b	10	10	8	8	7	6	4	2	0
Bk3a	10	10	5	4	3	2	0	0	0
Lf3b	10	10	7	5	3	2	1	0	0
LfZAb	10	8	8	5	4	4	2	0	0
Bk2b	10	10	7	6	5	3	0	0	0
LfZb1	10	10	10	8	5	3	1	0	0
BkBA2	10	10	9	7	6	4	1	0	0
Bk5b	10	10	8	7	6	1	0	0	0
St2b	10	8	6	3	2	1	0	0	0
Lf3a2	9	8	8	7	6	5	3	1	0
St1a	10	9	9	8	2	2	1	0	0
RtZB3	10	9	8	6	5	3	1	0	0
Lf6a2	10	10	9	7	5	2	1	0	0
BkZA3	10	10	8	6	5	3	1	0	0
StZA8	10	10	9	5	3	2	0	0	0
-ve ctrl	0	0	0	0	0	0	0	0	0

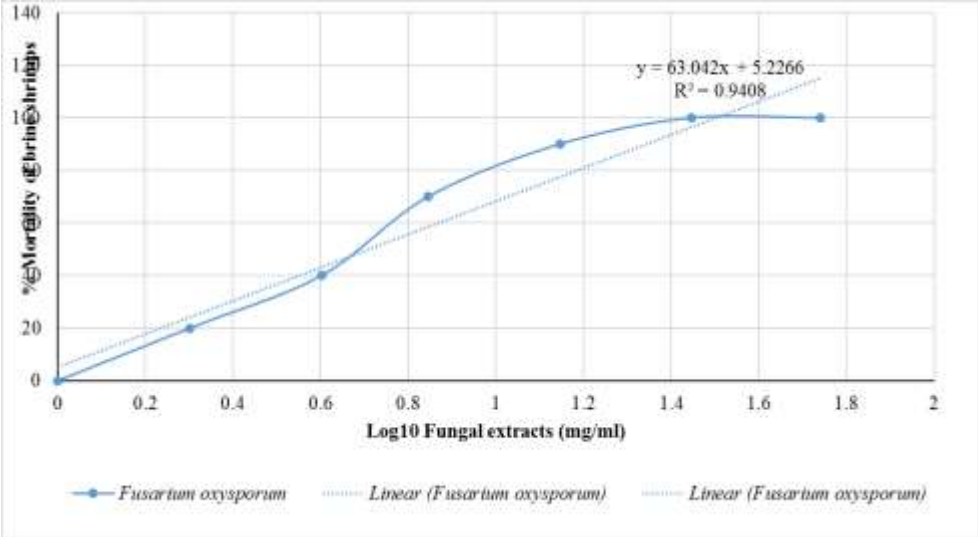
Appendix IV: Concentration of the Fungal Extracts (mg/ml) versus the Percentage Mortality of Brine Shrimps

	Concentration in mg/ml per dilution versus the %mortality								
Fungal Extracts	**10^{0}**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**
Fusarium oxysporum(LfZA2)	55/100	28/100	14/90	7/70	4/40	2/20	1/0		
Colletotrichum acutatum(LfB7)	100/55	100/28	100/14	80/7	70/4	60/2	30/1	20/0.5	0/0.25
Cladosporium sp. (Lf12b)	43/100	22/100	21/80	11/80	6/70	3/60	2/40	1/20	0.5/0
Tricharina gilva (Bk3a)	30/100	15/100	8/50	4/40	2/30	1/20	0.5/0		
Nigrospora oryzae (Lf3b)	53/100	27/100	14/70	7/50	4/30	2/20	1/10	0.25/0	
Cladosporium bruhnei (LfZAb)	20/100	10/80	5/80	3/50	2/40	1/40	0.5/20	0.3/0	
Phomopsis sp. (Bk2b)	40/100	20/100	10/70	5/60	3/50	2/30	1/0		
Guignardia mangiferae (LfZb1)	38/100	19/100	10/100	5/80	3/50	2/30	1/10	0.5/0	
Alternaria sp.(BkBA2)	33/100	17/100	9/90	5/70	3/60	2/40	1/10	0.5/0	
Cochliobolus sativus(Bk5b)	50/100	25/100	13/80	7/70	4/60	2/10	1/0		
Phyllosticta gardeniicola (St2b)	48/100	24/80	12/60	6/30	3/20	2/10	1/0		
Aspergillus flavus (Lf3a2)	43/90	22/80	21/80	11/70	6/60	3/50	2/30	1/10	0.5/0
Diaporthe amygdali(St1a)	40/100	20/90	10/90	5/80	3/20	2/20	1/10	0.5/0	
Trichoderma harzianum (RtZB3)	48/100	24/90	12/80	6/60	3/50	2/30	1/10	0.5/0	
Alternaria alternata (Lf6a2)	68/100	34/100	17/90	9/70	5/50	3/20	2/10	1/0	
Bionectria ochroleuca (BkZA3)	48/100	24/100	12/80	6/60	3/50	2/30	1/10	0.5/0	
Phomopsis mali (StZA8)	38/100	19/100	10/90	5/50	3/30	2/20	1/0		
-ve control	0	0	0	0	0	0	0	0	0

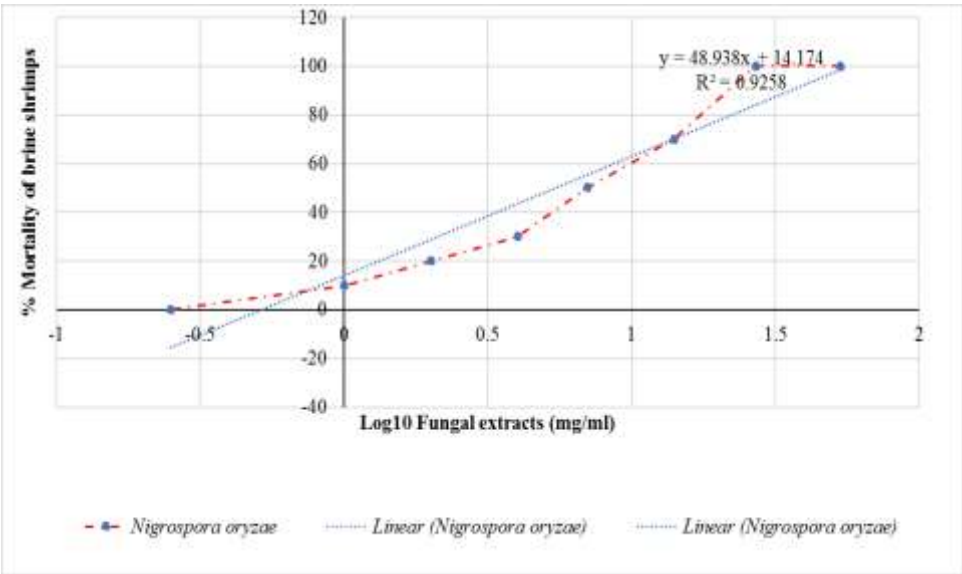
Appendix V: Graphs showing the Percentage mortality of Brine Shrimps versus Log 10 concentration of Fungal Endophytes isolated from *Waburgia ugandensis*



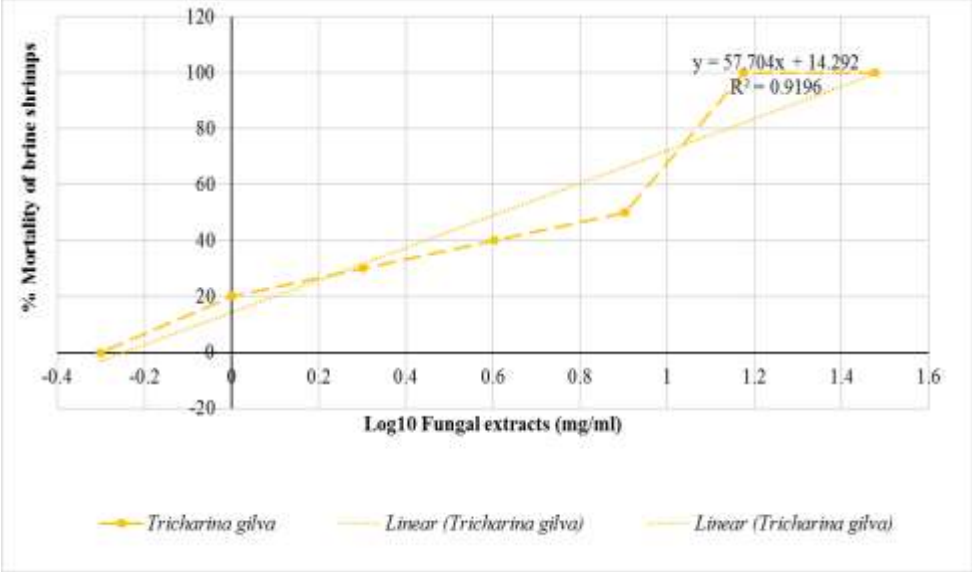
Percentage Mortality of brine shrimps versus Log 10 concentration of *Colletotrichum acutatum* extract isolated from *Warburgia ugandensis*



Percentage Mortality of brine shrimps versus Log 10 concentration of *Fusarium oxysporum* extract isolated from *Warburgia ugandensis*

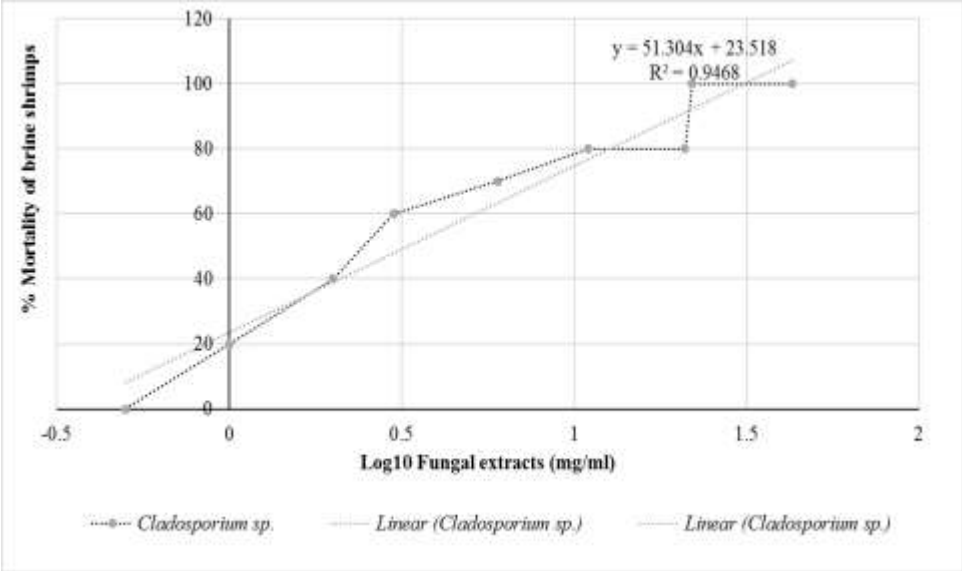


Percentage Mortality of brine shrimps versus Log 10 concentration of *Nigrospora oryzae* extract isolated from *Warburgia ugandensis*

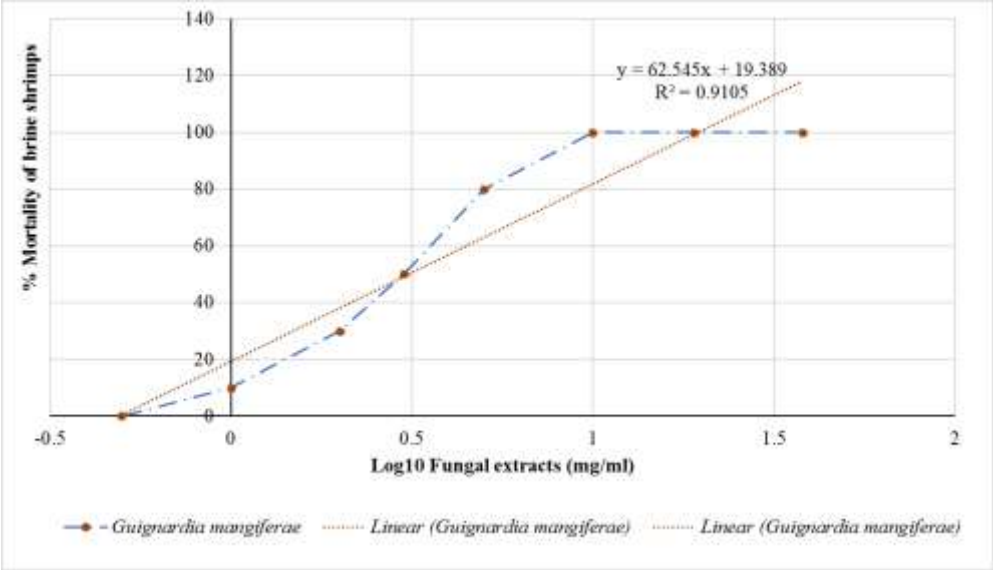


Percentage Mortality of brine shrimps versus Log 10 concentration of *Tricharina gilva* extract isolated from *Warburgia ugandensis*

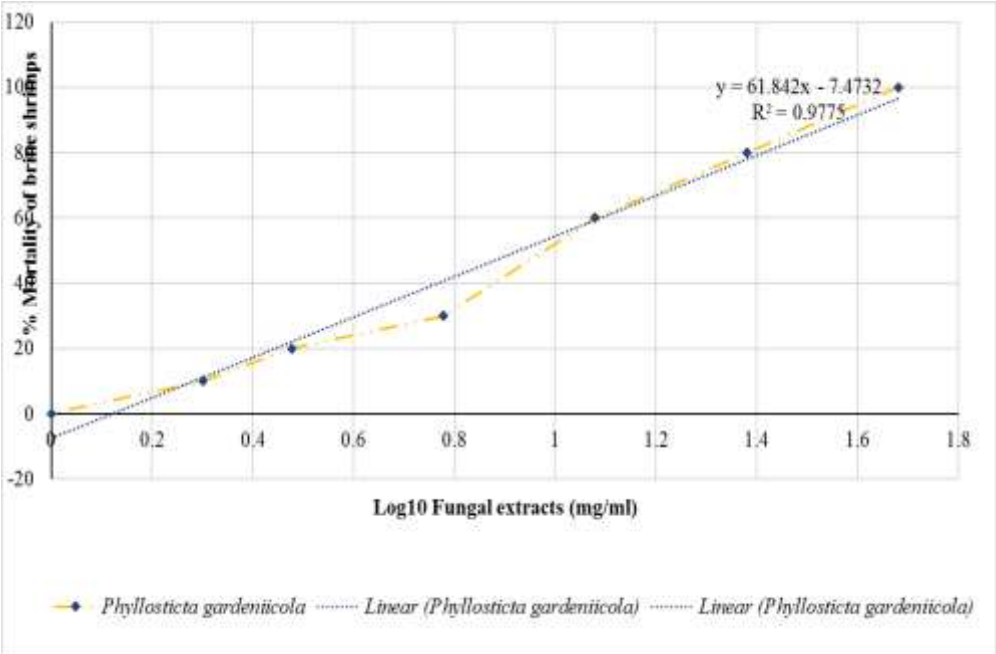
Percentage Mortality of brine shrimps versus Log 10 concentration of *Cladosporium bruhnei* extract isolated from *Warburgia ugandensis*



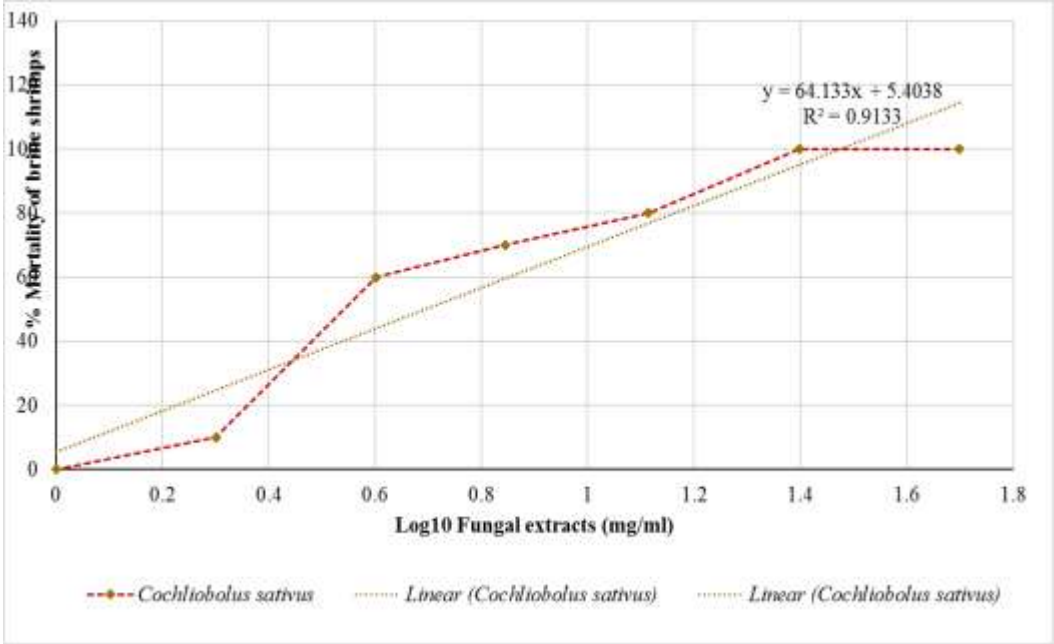
Percentage Mortality of brine shrimps versus Log 10 concentration of *Cladosporium spp.* extract isolated from *Warburgia ugandensis*



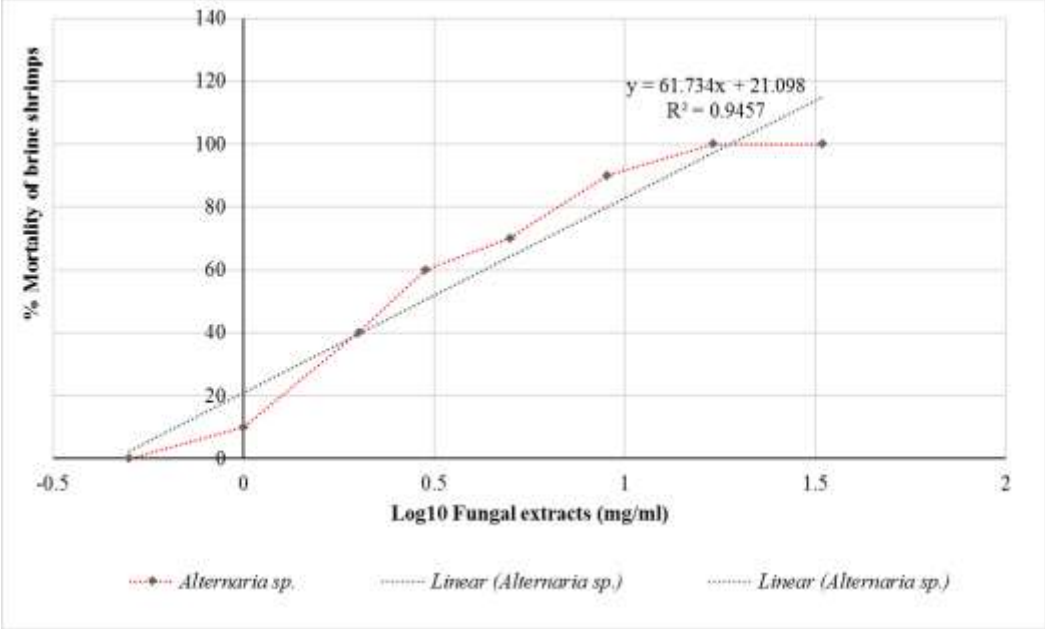
Percentage Mortality of brine shrimps versus Log 10 concentration of *Guignardia mangiferae* extract isolated from *Warburgia ugandensis*



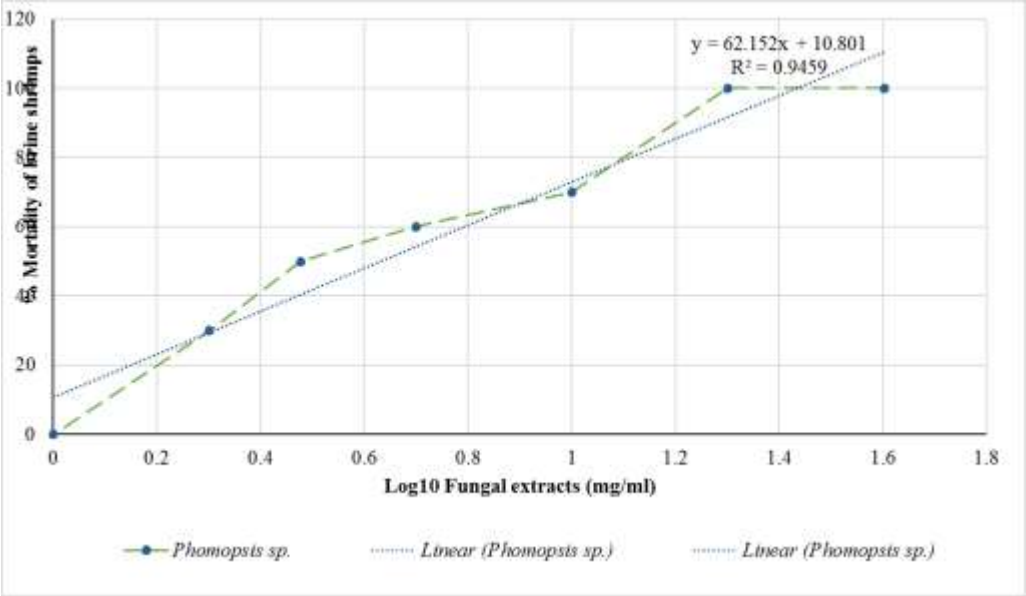
Percentage Mortality of brine shrimps versus Log 10 concentration of *Phyllosticta gardeniicola* extract isolated from *Warburgia ugandensis*



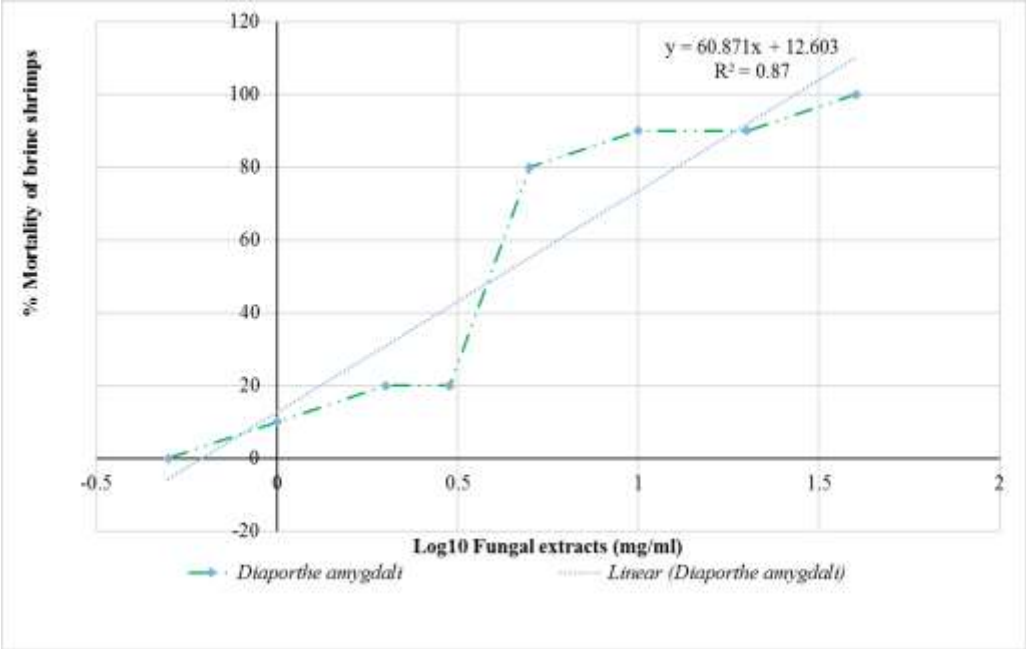
Percentage Mortality of brine shrimps versus Log 10 concentration of *Cochliobolus sativus* extract isolated from *Warburgia ugandensis*



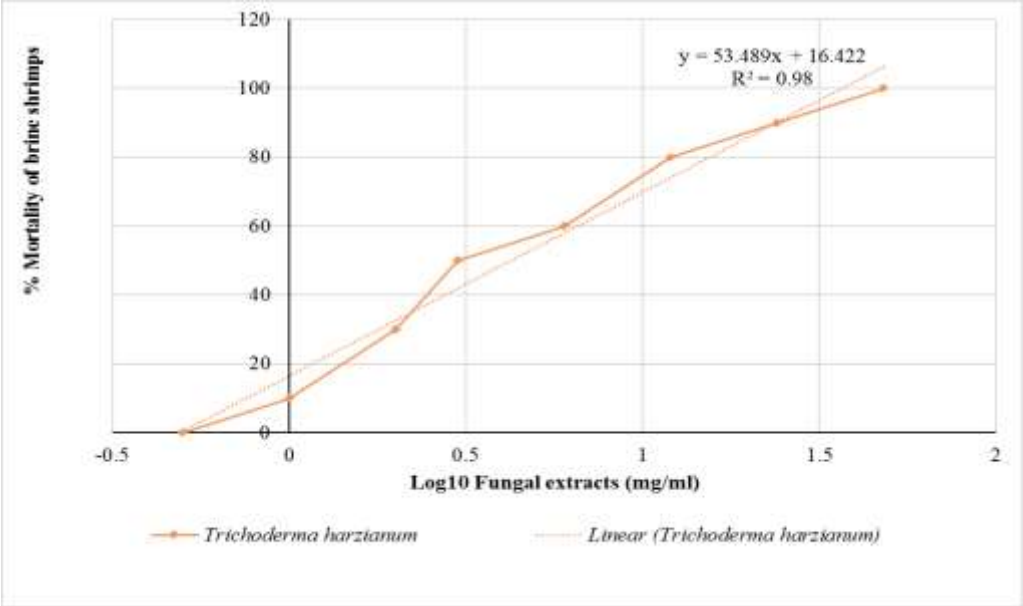
Percentage Mortality of brine shrimps versus Log 10 concentration of *Alternaria spp.* extract isolated from *Warburgia ugandensis*



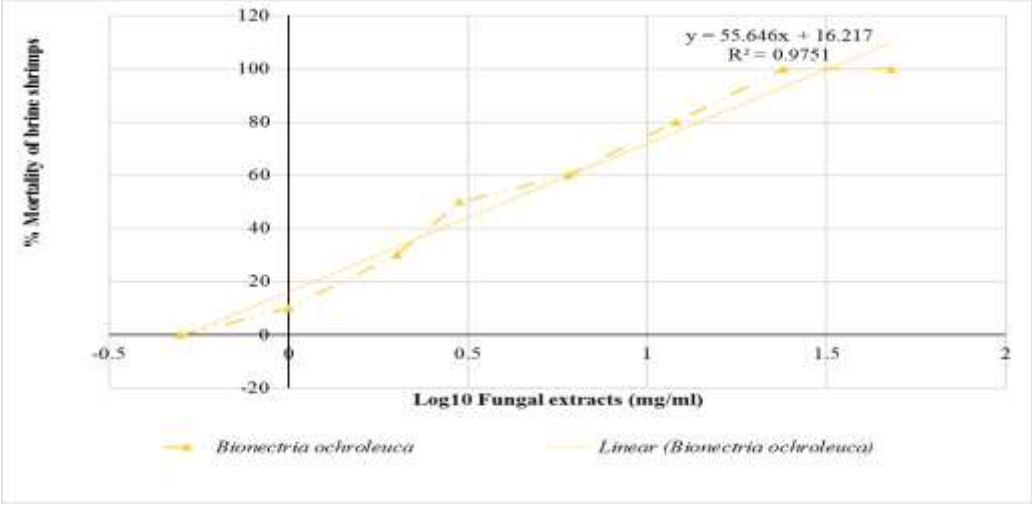
Percentage Mortality of brine shrimps versus Log 10 concentration of *Phomopsis spp.* extract isolated from *Warburgia ugandensis*



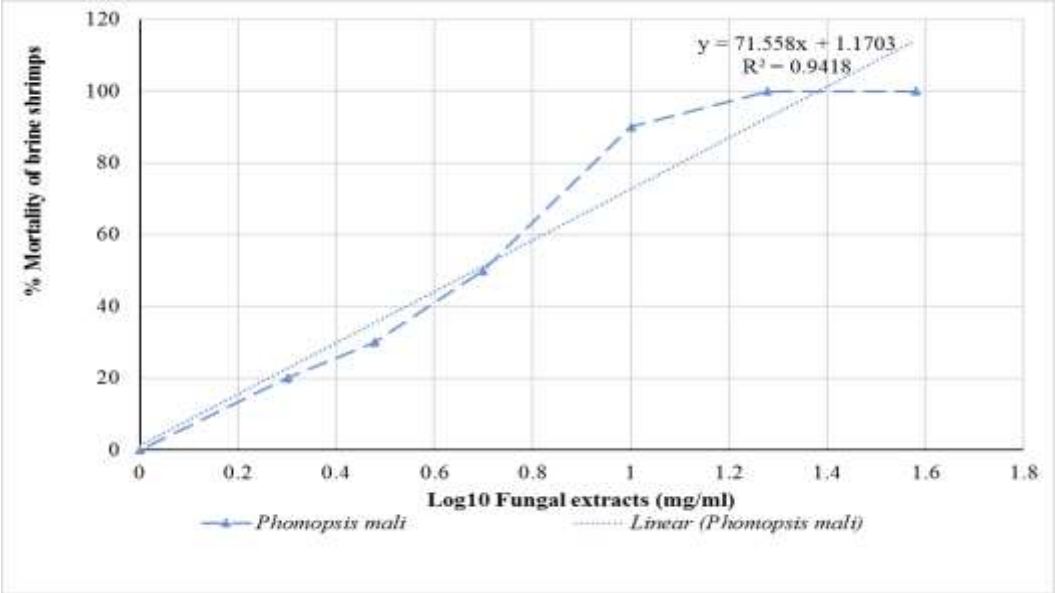
Percentage Mortality of brine shrimps versus Log 10 concentration of *Diaporthe amygdali* extract isolated from *Warburgia ugandensis*



Percentage Mortality of brine shrimps versus Log 10 concentration of *Trichoderma harzianum* extract isolated from *Warburgia ugandensis*



Percentage Mortality of brine shrimps versus Log 10 concentration of *Bionectria ochroleuca* extract isolated from *Warburgia ugandensis*



Percentage Mortality of brine shrimps versus Log 10 concentration of *Phomopsis mali* extract isolated from *Warburgia ugandensis*

Appendix VI: Photo for Phytochemical Screening of the Fungal Extracts

Appendix VIII: ***Warburgia ugandensis*** **leaf and stem samples in tap water agar respectively**