FUNGAL ENDOPHYTES: ISOLATION, IDENTIFICATION AND ASSESSMENT OF BIOACTIVE POTENTIAL OF THEIR NATURAL PRODUCTS

By

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PREFACE

The work contained in this thesis has not been previously submitted for a degree or diploma at any other higher education institutions to the best of my knowledge and belief. This thesis contains no material previously published or submitted for publication by another person except where due reference has been made.

Signed: ……. Date: 20 October 2017

Edson Panganayi Sibanda

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DECLARATION 2: PUBLICATIONS

The publications (in print, in press and submitted) that constitute this thesis and the contribution I made to each of the manuscripts are presented here.

Publication 1

Sibanda EP, Mabandla M, Mduluza T. 2017. A Review of Endophytic Fungi Bioprospecting in Africa – 1994 to 2014. *Current Biotechnology*. 2018; 7 (2): 80-88.
DOI: 10.2174/2211550106666170221152036.

Author contributions

The supervisors (Prof T Mduluza and Prof M Mabandla) and I conceptualised the paper and I did the literature search and drafted the paper. The supervisors critically reviewed the paper.

Publication 2

Sibanda EP, Mabandla M, Chisango T, Nhidza AF, Mduluza T. 2017. Endophytic fungi associated with *Annona senegalensis*: identification, antimicrobial and antioxidant potential. *Current Biotechnology*. E-pub Ahead of Print DOI: 10.2174/2211550107666180129154838.

Author contributions

I designed the study, collected and analysed the data, compiled and wrote the manuscript. The supervisors guided the design, provided logistical support during data collection and thought leadership, reviewed the manuscript and provided critical comments. The co-authors T Chisango and AF Nhidza made conceptual contributions reviewed the manuscript and provided critical comments.

Publication 3

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Author contributions

I designed the study, collected and analysed the data, compiled and wrote the manuscript. The supervisors guided the design, provided logistical support during data collection and thought leadership, reviewed the manuscript and provided critical comments. The co-authors T Chisango and AF Nhidza made conceptual contributions reviewed the manuscript and provided critical comments.

Publication 4

Sibanda EP, Mabandla M, Chisango T, Nhidza AF, Mduluza T. 2017. Endophytic fungi isolated from the medicinal plants *Kigelia africana* and *Warburgia salutaris. Current Biotechnology.* E-pub Ahead of Print. DOI: 10.2174/2211550107666180308154448.

Author contributions

I designed the study, collected and analysed the data, compiled and wrote the manuscript. The supervisors guided the design, provided logistical support during data collection and thought leadership, reviewed the manuscript and provided critical comments. The co-authors T Chisango and AF Nhidza made conceptual contributions reviewed the manuscript and provided critical comments.

DEDICATION

To Kundai, Batsirai, Tutsirai and Ashirai for your love patience and support

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TABLE OF CONTENTS

LIST OF APPENDICES

ACRONYMS

HTS	High-throughput screening
WHO	World Health Organisation
PDA	Potato dextrose agar
ITS	Nuclear ribosomal internal transcribed spacer region
EtOAc	Ethyl acetate
MeOH	Methanol
SET	Single electron transfer
CRE	Copper reducing equivalents
ATCC	American Type Culture Collection
FT-IR	Fourier-transform infrared spectroscopy
GC-MS	Gas chromatography–mass spectrometry
IUCN	International Union for Conservation of Nature and Natural Resources
CDC	Centers for Disease Control and Prevention

ABSTRACT

Fungal endophytes produce a broad variety of bioactive compounds with potential to address some of the unmet human needs. Medicinal plants have an important role to play in the search for new strains of endophytes fungi, as it is possible that their beneficial characteristics are as a result of the metabolites produced by their endophytic community. However, inspite of this potential as repositories of bioactive compounds, the fungal endophytes of African medicinal plants remain largely underexplored. This thesis reports on studies that were conducted to bioprospect for endophytic fungi with antioxidant and antimicrobial activity hosted by the plants *Warburgia salutaris*, *Annona senegalensis*, *Kigelia africana* and *Vitex payos* used in Zimbabwean traditional medicine. The surface sterilization technique was used to isolate the endophytic fungi that were identified by ribosomal DNA sequencing of the nuclear ribosomal internal transcribed spacer region. Crude extracts obtained from the fermentation of the isolated endophytic fungi were screened for antimicrobial activity using the agar diffusion method and evaluated for total antioxidant activity using a commercial kit that used the single electron transfer mechanism. Fourier-transform infrared spectroscopy (FT-IR) and Gas Chromatography – Mass Spectrometry (GC-MS) were used to provide a snapshot of the metabolites present in the endophyte fungi extracts. A total of 33 endophytic fungi were isolated from the medicinal plants and the fungal endophyte colonisation rates varied by plant species and plant tissue. The isolated fungi across the different plant species and tissue types were found to be dominated by members of the phylum Ascomycota. The endophytic fungi *Penicillium chloroleucon* was isolated from all the plant species except for *Cladosporium uredinicola* and *Myrothecium gramineum* (both isolated from *Kigelia africana*) which had an inhibitory effect against *Escherichia coli* (ATCC1056). Whilst *Epicoccum sorghinum* isolated from *Annona senegalensis* exhibited the most potent antioxidant activity, a significant number of the screened endophytic fungi from the different plant species were also found to have some antioxidant activity. The total phenolic content was found to have a positive correlational relationship with total antioxidant activity of the screened endophytic fungi crude extracts. The endophytic fungi were shown to produce a diverse range of metabolites including phenolic and polyphenolic compounds through FT-IR and GC-MS analysis. The isolate *Cladosporium uredinicola* has potential as a source of antimicrobial compounds whilst the isolate *Epicoccum sorghinum* has potential as a source of natural antioxidant. Antioxidant activity is a common phenomenon in the studied endophytic fungi and the fungal endophytes of the medicinal plants of Zimbabwe have potential as sources of bioactive compounds.

Keywords: Bioprospecting, antioxidant, antimicrobial, endophytic fungi, medicinal plants, Zimbabwe

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Background and the context of the study

Natural products and their derivatives have been recognized for many years as a valuable source of therapeutic agents and structural diversity (Kinghorn et al., 2011; Newman and Cragg, 2012; Lahlou, 2013). However, the intrinsic difficulties associated with natural product-based drug discovery resulted in the pharmaceutical industry shifting its focus towards synthetic compound libraries and HTS for discovery of new drug leads (Beutler, 2009; David et al., 2015). Recently, there has been renewed interest in the bioprospecting for natural products with potential therapeutic applications due to the failure of alternative drug discovery methods to deliver lead compounds in therapeutic areas such as immunosuppression, anti-infectives and metabolic diseases (Lahlou, 2013).

Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease (Stone et al., 2000). Bioprospection of endophytes is considered a new frontier for the discovery of useful natural products as endophytes have been shown to produce a broad variety of bioactive secondary metabolites with potential agricultural, pharmaceutical and industrial applications (Pimentel et al., 2011; Zhang et al., 2006). The number of secondary metabolites produced by fungal endophytes is larger than that of any other group of endophytic microorganisms (Zhang et al., 2006) and to date the vast majority of microbes isolated as endophytes have been fungi (Bascom-Slack et al., 2009). Fungal metabolites have served as lead compounds for the development of anticancer, antifungal and antibacterial agents (Rosa et al., 2011). It is estimated that globally there might be 1-1.5 million species of endophytic fungi, the majority of which are yet to be identified and studied (Subbulakshmi et al., 2012). The estimate is based on the assumption that individual higher plant (there are approximately 250 000 different plant species on the earth) hosts an average of four endophytes (Zhang et al., 2006).

Medicinal plants are rich sources of bioactive natural compounds and studies have shown that some medicinal properties of these plants may be related to the endophytic fungi that they host (Alvin et al., 2014). The endophytic fungi may participate in some of the plant metabolic pathways or may gain some genetic information to produce specific biologically active compound like those produced by the host plant (Golinska et al., 2015). Therefore, medicinal plants have an important role to play in the search for new strains of endophytic fungi, as it is possible that their beneficial characteristics are a result of the metabolites produced by their fungal endophytic community (Kusari et al., 2013; Kaul et al., 2012). Despite the potential benefits that can be derived from fungal endophytes, only a tiny fraction of medicinal and other plant species have

been investigated for their potential as repositories of endophytic fungi that produce bioactive compounds (Pimentel et al., 2011).

Considering the above, this study determined the diversity of endophytic fungi isolated from the medicinal plants *Annona senegalensis*, *Kigelia africana*, *Vitex payos* and *Warburgia salutaris* by rDNA sequencing of the ITS region. The antioxidant and antimicrobial potential of the isolated endophytic fungi was assessed using conventional methods. This study also identified the metabolites and functional groups of the metabolites produced by some of the endophytic fungi using GC-MS and FT-IR. The information resulting from this study will contribute to bridging the existing knowledge gap concerning fungal endophyte diversity in African medicinal plants and to also identify fungal endophytes that produce metabolites with potential as lead compounds for natural antioxidant and antimicrobial agents.

1.2 Literature review

1.2.1 Endophytic fungi

The term endophyte was initially coined by de Bary who defined endophytes as microorganisms that colonise the internal plant tissue (de Bary, 1866). A widely accepted definition defines endophytes as being microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease (Stone et al., 2000). Endophytic fungi have been shown to have a global distribution as they are found in all ecological zones of the world and across all plant families (Zhang et al., 2006). In addition, endophytic fungi have been reported to be more metabolically active than their free-living counterparts and this explains their ability to produce a large number of secondary metabolites (Zhang et al., 2006). It is also important to note that endophytic fungi have been shown to produce some of the important plant derived drugs such as the anti-cancer drug paclitaxel (Taxol®) from *Taxomyces* (Cragg and Newman, 2013). Despite the potential of endophytic fungi as sources of novel natural products and their global distribution, endophytic fungi are a poorly investigated group of microorganisms (Chandra, 2012).

Table 1: Examples of Bioactive compounds obtained from fungal endophytes

Fungus	Compound (s)	Activity	Reference
Aspergillus terreus	Lovastatin	Cholesterol lowering	(Alberts et al., 1980)
Coleophoma empetri	Micafungin	Antifungal	(Frattarelli et al., 2004)
Tolypocladium inflatum	Cyclosporine	Immunosuppressant	(Borel and Kis, 1991)
Hypocrea lixii	Cajanol	Anticancer	(Zhao et al., 2013)
Fusarium oxysporum	Fusarium oxysporum	Biopesticide	(Akello et al., 2008)
Phomopsis archeri	Phomoarcherins A–C	Anti-malarial	(Hemtasin et al., 2011)
Penicillium janthinellium	Citrinin	Anti leishmanial	(Marinho et al., 2005)
Xylaria sp.	Mellisol	Anti-viral	(Joseph & Priya, 2011)
Cephalosporium sp	4,6-dihydroxy-5-methoxy-7-methylphthalide	Antioxidant	(Kaul et al., 2012)
Phomopsis sp.	Phomoxanthone A and B	Anti TB	(Isaka et al., 2001)

There has been resurgence in the past 15 years in investigating the potential of endophytic fungi as sources of novel, biologically active products (Maheswari and Rajagopal, 2013). This is shown by heightened research interest as borne out by an analysis of endophytic fungi related publication output in the Pubmed – NCBI (National Center for Biotechnology Information, 2017) and Scopus databases (Elsevier, 2017) and patenting activity in the Patentscope – World Intellectual Property Organisation – WIPO website. The publications activity analysis up to December 31, 2016 reveals that approximately 90% of the publications related to endophytic fungi have been published in the period 2000 – 2016.

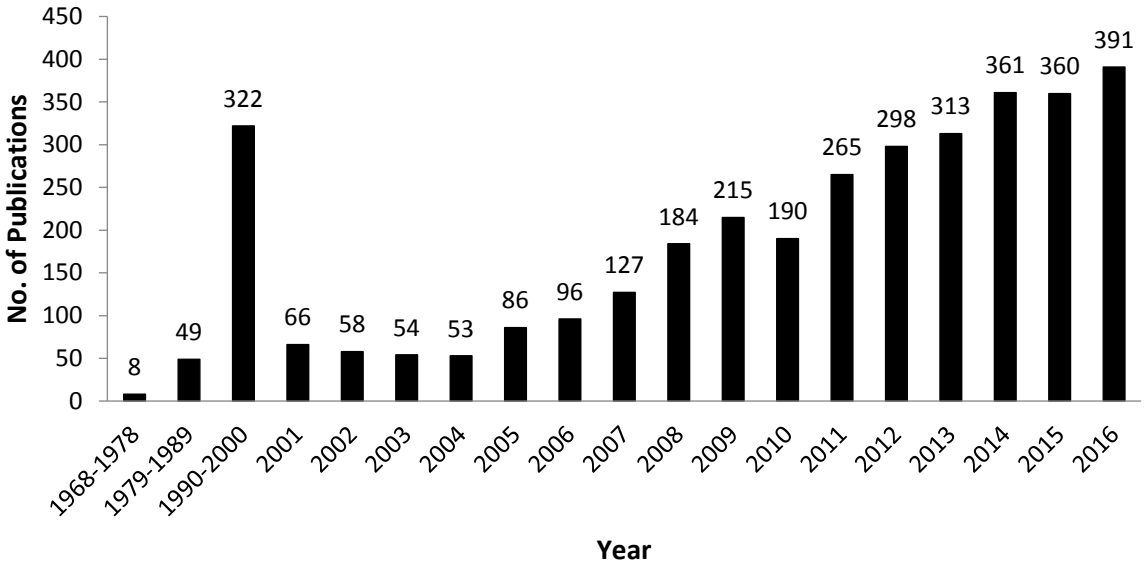

Figure 1: Endophytic fungi publications trends 1968-2016

Source Scopus, October 2017

The patenting activity analysis on the WIPO website shows a similar trend to the publication activity and reveals that more than 95% of the patents related to endophytic fungi have been granted in the period 2001 – 2016 (World Intellectual Property Organization, 2017). This resurgence has been largely driven by the advent of drug-resistant pathogens (Bascom-Slack et al., 2009) and other unmet needs in the treatment of fungal and viral infections. This has led to the search for new therapeutic agents to meet these unmet needs (Pimentel et al., 2011). Apart from elaborating novel bioactive compounds (such as the the palmarumycins and benzopyranone), endophytes have also been shown to have the ability to bring about stereoselective biotransformations of chemicals thus aiding in compound modifications and development of new lead molecules for pharmaceuticals (Suryanarayanan et al., 2009).

1.2.2 Medicinal plants

Medicinal plants can be defined as any plant which has curative properties or provides temporary relief from symptomatic problems; usually the identification, preparation, method of use, safety and efficacy of the medicinal plants and medicinal plant-based products are based on ethno botanical information. *Warburgia salutaris*, *Annona senegalensis*, *Kigelia africana* and *Vitex payos* are plants found in Zimbabwe that are used in ethnomedicine to treat a variety of ailments (Figure 2).

Figure 2: Photographs of some of the plant specimens from which endophytic fungi were isolated: a. *Warburgia salutaris*; b. *Kigelia africana*; c. *Vitex payos*; d. *Annona senegalensis*

1.2.2.1 Warburgia salutaris

Warburgia salutaris (*W. salutaris*) is endemic to a few sites in the north-eastern parts of South Africa, Swaziland, south-eastern Zimbabwe, southern Mozambique, Malawi and Zambia (Palgrave, 2002). In Zimbabwe, the plant is commonly called the pepper bark tree (English) and muranga (Shona). The plant species is classified as follows: Kingdom: **Plantae**; Phylum: **Tracheophyta**; Class: **Magnoliopsida**; Order: **Magnoliales**; Family: **Canellaceae**. The plant species is on the IUCN Red List for endangered species and is thought to be now extinct in the wild in Zimbabwe (IUCN, 2017). The species is generally slow growing in the wild; and its limited distribution and low abundance makes it vulnerable to human-induced habitat degradation and over-exploitation as a medicinal plant (Maroyi, 2013a). In Zimbabwe, the plant species is used in traditional medicine to treat a variety of ailments including pneumonia, abdominal pain, fever, headaches and different types of cancer and is also used as an aphrodisiac and aid to divination (Maroyi, 2013a).

1.2.2.2 Annona senegalensis

Annona senegalensis (*A. senegalensis*) is native to the African continent and is widespread in West, East and Southern Africa including Madagascar, the Comoros and Cape Verde Islands (Hyde et al., 2017). The plant is commonly known as the wild custard apple (English) and muroro (Shona) in Zimbabwe. The plant species is classified as follows: Kingdom: **Plantae**; Phylum: **Tracheophyta**; Class: **Magnoliopsida**; Order: **Magnoliales**; Family: **Annonaceae**. The plant species is used in traditional medicine to treat a variety of ailments such as guinea worms and other worms, diarrhea, dermatological diseases, pneumonia, venereal diseases, gastroenteritis, snakebite, toothache and respiratory infections (Orwa et al.,2009).

1.2.2.3 Vitex payos

Vitex payos (*V. payos*) is endemic to East Africa and parts of Southern Africa (Hyde et al., 2017). The plant is commonly known as the chocolate berry (English) and mutsvubvu (Shona) in Zimbabwe. The plant species is classified as follows: Kingdom: **Plantae**; Phylum: **Tracheophyta**; Class: **Magnoliopsida**; Order: **Lamiales**; Family: **Lamiaceae**. The plant species is used in Zimbabwean traditional medicine a remedy for coughs (Maroyi, 2013b).

1.2.2.4 Kigelia africana

Kigelia africana (*K. africana*) is wide spread in tropical Africa and parts of north eastern South Africa (Hyde et al., 2017). The plant is commonly known as the sausage tree (English) and mubveve (Shona) in Zimbabwe. The plant species is classified as follows: Kingdom: **Plantae**; Phylum: **Tracheophyta**; Class: **Magnoliopsida**; Order: **Lamiales**; Family: **Bignoniaceae**. *K. africana* is used in traditional medicine to treat a variety of skin ailments (fungal infections, boils, leprospy, syphilis and skin cancer), as wound dressing, as a cosmetic and aphrodisiac (Phytotrade, 2017).

1.2.3 Antimicrobial resistance

There is no denying the indispensable role that antimicrobial drugs have played in decreasing illnesses and deaths associated with infectious diseases in animals and humans (Tadesse et al., 2012). However, the emergence and spread of drug-resistance traits among pathogenic and commensal bacteria largely driven by selective pressure exerted by antimicrobial drug use has resulted in the antimicrobials becoming ineffective (WHO, 2017; Aarestrup et al., 2008). Antibiotic resistance by pathogenic bacteria is present in every country and the world is facing key challenges due to this increasing bacterial resistance against current antibiotics and lack of new molecules to combat the bacterial resistance (Fiers et al., 2017). Currently it is estimated that around 700 000 people die of resistant infections each year and that by 2050, the figure will have risen to 10 million lives at a cumulative economic output loss of USD 100 trillion (Review on Antimicrobial Resistance; 2016). Modern medicine is underpinned by antibiotics and if they lose their effectiveness, routine medical procedures such as caesarean sections could become too dangerous to perform (Review on Antimicrobial Resistance; 2016). It is important to note that most of the direct and indirect impacts of antimicrobial resistance will fall on low and middle-income countries such as the majority of African countries (Review on Antimicrobial Resistance; 2016).

Klebsiella pneumoniae (*K. pneumoniae*) are Gram-negative common intestinal bacterium that is a major cause of hospital-acquired infections such as pneumonia, bloodstream infections, and infections in new-borns and intensive-care unit patients. The bacterium can cause life-threatening infections and antimicrobial resistant *K. pneumoniae* (which is resistant even to last the resort

treatment – carbapenem antibiotics) has spread to all regions of the world. In some countries due to resistance, carbapenem antibiotics are ineffective in more than 50% of the people treated for *K. pneumoniae* infections (WHO, 2017).

Escherichia coli (*E. coli*) are Gram-negative commensal bacteria that are normally found in the intestines of people and animals. However, some variants of *E. coli* are pathogenic and can cause diarrhea, urinary tract infections, respiratory illness and pneumonia, and other illnesses (CDC, 2017). The types of *E. coli* that can cause illnesses can be transmitted through contaminated water or food, or through contact with infected animals or persons (CDC, 2017). Resistance in *E. coli* to fluoroquinolone antibiotics (widely used to treat urinary tract infections) is now widespread and there are many parts of the world where this treatment is now ineffective in more than 50% of patients (WHO, 2017).

Staphlylococcus aureus (*S. aureus*) are Gram-positive bacteria that about 30% of people carry in their noses (CDC, 2017). *S. aureus* is usually not harmful but, in some cases, it can cause severe and fatal infections especially in healthcare facilities (WHO, 2017). The infections that can be caused by *S. aureus* include bacteremia, pneumonia, endocarditis and osteomyelitis (CDC, 2017). *S. aureus* has also developed resistance to certain antibiotics and these drug-resistant *S. aureus* infections include: Methicillin-resistant *S. aureus* (MRSA), Vancomycin-intermediate *S. aureus* (VISA), and Vancomycin-resistant *S. aureus* (VRSA). People with MRSA are estimated to be 64% more likely to die than people with a non-resistant form of the infection (CDC, 2017; WHO, 2017).

1.2.4 Antioxidants

Antioxidants are compounds that can donate electrons to free radicals and terminate free radical mediated reactions before oxidative damage can occur to biomolecules (Ascêncio et al., 2014). Free radicals are by products of normal metabolism and play important roles in metabolic reactions within the body (Ascêncio et al., 2014; Salini et al, 2015). However, an imbalance in cell redox reactions may result in the unregulated generation of free radicals or decreased antioxidant defenses and this can lead to oxidative damage of biomolecules (Halliwell, 1994; Gülçin, 2007). The oxidative damage to the biomolecules has been implicated in many degenerative human diseases such as diabetes mellitus, cancer, Alzheimer's disease and other neurodegenerative disorders, ageing and inflammatory diseases (Gülçin, 2007). In the food industry, artificial antioxidants such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) are commonly used as neutralizing agents of free radicals. However, their use has been linked with toxicity or mutations problems and therefore there is a need to find alternative safe and effective antioxidants (Augustyniak et al., 2010).

1.3 Research problem and significance

The failure of alternative drug discovery methods to deliver lead compounds has resulted in renewed interest in the bioprospecting for natural products with potential therapeutic applications. Antimicrobial resistance is an emerging global health issue, if not addressed has the potential to usher the world into a post-antibiotic era in which currently treatable common infections and injuries will be untreatable and fatal (WHO, 2014). Therefore, there is a constant need to discover and develop new anti-infective therapeutic agents (Ascêncio et al., 2014). Free radicals are byproducts of normal metabolism and play important roles in metabolic reactions within the body (Ascêncio et al., 2014). Pathophysiological or environmental interference have been shown to cause unregulated generation of free radicals that result in oxidative damage to biomolecules (Halliwell, 1994; Gülçin, 2007). The oxidative damage to the biomolecules has been implicated in many degenerative human diseases such as diabetes mellitus, cancer, Alzheimer's disease and other neurodegenerative disorders, ageing and inflammatory diseases (Gülçin, 2007). Currently there are a few antioxidant compounds that can be used as therapeutics and synthetic antioxidants have been reported to have toxic side effects, therefore, there is a need for new, safe and efficient molecules that can be used as antioxidants (Ascêncio et al., 2014; Salini et al, 2015). Bioactive molecules from natural sources such as fungal endophytes are an attractive option to consider as they may lead to the discovery of safe and effective lead compounds or compounds with structures that can be modified to produce safe and effective anti-infectives and antioxidants.

As indicated in Section 1.1, medicinal plants have an important role to play in the search for new strains of endophytic fungi, as it is possible that their beneficial characteristics are a result of the metabolites produced by their fungal endophytic communites (Kusari et al., 2013; Kaul et al., 2012). However, despite their potential as repositories of endophytic fungi that produce bioactive compounds, medicinal plants of Zimbabwe are yet to be studied with regards to their endophytic fungi diversity and bioactivity potential. This suggests that an opportunity exists to unravel the endophytic fungal diversity of medicinal plants found in Zimbabwe and discover novel biologically active natural products produced by these endophytes. The unravelling of the endophytic fungal diversity of medicinal plants of Zimbabwe will contribute to the body of knowledge on fungal diversity. Furthermore, the determination of the antimicrobial and antioxidant potential of the isolated fungal endophytes might lead to the identification of new, safe and effective lead molecules or compounds with structures that can be modified to produce safe and effective anti-infectives and antioxidants.

1.4 Research questions

1.4.1 General research question

Do the medicinal plants of Zimbabwe host endophytic fungi with antioxidant and antimicrobial activity?

1.4.2 Specific research Questions

i. What are the endophytic fungi hosted by *Annona senegalensis*, *Kigelia africana*, *Vitex payos* and *Warburgia salutaris*?
ii. What is the antioxidant activity of these endophytic fungi?
iii. What is the antibacterial activity of these endophytic fungi?

1.4.3 General hypothesis

If endophytic fungi are isolated from selected Zimbabwean plant species, they will be shown to have the ability to produce compounds with antioxidant and antimicrobial activity.

1.4.4 General objective

To identify endophytic fungi hosted by *Annona senegalensis*, *Kigelia africana*, *Vitex payos* and *Warburgia salutaris* and determine their antioxidant and antimicrobial activity potential.

1.4.5 Specific objectives

1. To isolate endophytic fungi hosted by *Annona senegalensis*, *Kigelia africana*, *Vitex payos* and *Warburgia salutaris*
2. To identify the isolated endophytic fungi
3. To evaluate the antimicrobial activities of the isolated fungi
4. To evaluate the antioxidant potential of the isolated fungi

1.5 General methodology

1.5.1 Schematic outline of the study design

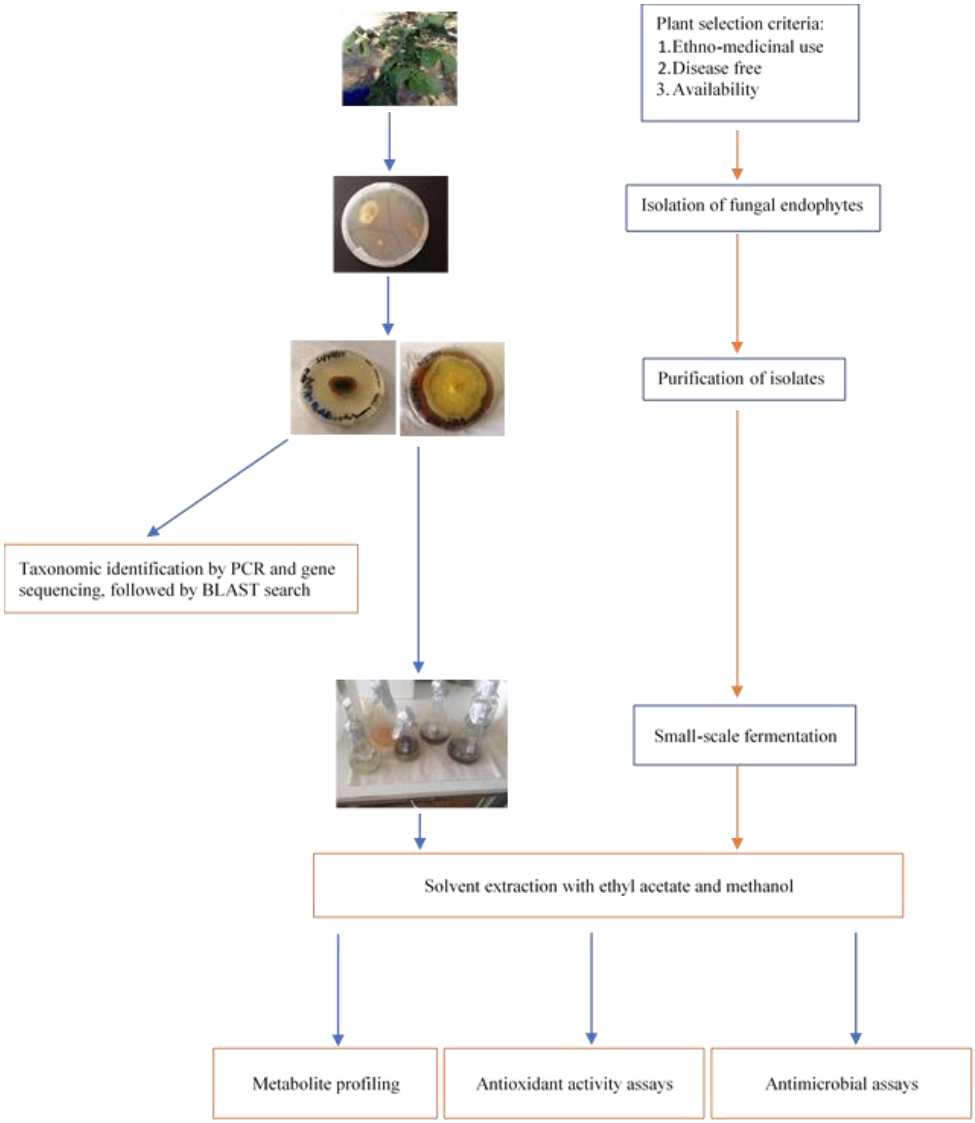

Figure 3: Schematic outline of the study design

1.5.2 Plant selection and sampling

The plant species investigated in this study were selected based on their history of use in Zimbabwean traditional medicine. Fresh leaf and stem tissue of *A. senegalensis, K. africana, V. payos* and *W. salutaris* were obtained from the National Herbarium and Botanical Gardens (Harare, Zimbabwe) plant collection in June 2015. The tissue samples were pretreated by cleaning them under running water to remove dirt and soil particles and were then air-dried to remove any surface moisture before they were packaged into labeled sterile sample collecting bags. The samples were then transported to the laboratory and stored at 4 °C until endophyte isolation procedures could be instituted (Strobel, 2003).

1.5.3 Tissue preparation

Endophytic fungi were isolated from the different tissues collected from the medicinal plants using a modified surface sterilization procedure as described by Kjer *et al*., 2010. Briefly, the plant tissue samples were removed from storage and thawed by briefly putting them under running tap water and then washed using 0.1% (v/v) Tween 80 for 15 minutes followed by another wash for 1 hour 30 minutes using running water. The cleaned plant tissues were then transferred to a laminar airflow cabinet. The plant tissue samples were dipped in 70% ethanol: 30 seconds for leaf samples and 2 minutes for stem samples. The ethanol was drained out after the required amount of time and the plant tissue was then washed using a 2% sodium hypochlorite solution for 15 minutes which was then followed by four washes using double distilled water after which the samples were dried using sterile paper towels. The effectiveness of the sterilization procedure was tested by plating 0.1 ml of the final sterile water rinse onto Petri dishes containing potato dextrose agar (PDA) and by rolling the sterilized sample onto Petri dishes containing PDA.

1.5.4 Isolation of endophytic fungi

The surface sterilized tissues were cut into smaller segments (1-2 cm) using sterile razor blades and placed onto Petri dishes containing PDA supplemented with 200U ml^{-1}Penicillin-Streptomycin (Lonza, Cat # 17-602E) and incubated at 28±2 °C until fungal growth was initiated. The colonization rate of endophytic fungi was determined as the total number of segments yielding ≥ 1 isolate in a host sample divided by total number of segments incubated in that sample multiplied by 100. The growing hyphal tips which grew out of the samples were isolated and sub cultured onto Petri dishes containing PDA. The cycle of sub culturing was repeated until pure cultures of the isolates were obtained (Kjer et al., 2010).

1.5.5 Identification of the endophytic fungi

Genomic DNA was extracted from the cultures using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using EconoTaq® PLUS GREEN 2X Master Mix (Lucigen) with the primers presented in Table **2**. The PCR products were run on a gel and gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit, Catalogue No. D4001). The extracted fragments were purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050) and sequenced in the forward and reverse directions (Applied Biosystems, ThermoFisher Scientific, Big Dye terminator kit v3.1). The purified fragments were run on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). CLC Bio Main Workbench v7.6 was used to analyze the .ab1 files generated by the ABI 3500XL Genetic Analyzer and endophytic fungi isolates were identified on the basis of similarity of amplified sequence with those found in the US National Centre for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (nBLAST).

Table 2: ITS primer sequences

Name of Primer	Target	Sequence (5' to 3')	Reference
ITS1	Small Sub-Unit	TCCGTAGGTGAACCTGCGG	White et al., 1990
ITS4	Large Sub-Unit	TCCTCCGCTTATTGATATGC	White et al., 1990

1.5.6 Secondary metabolite extraction

The small-scale fermentations and solvent extractions were carried as described by Kjer *et al*., 2010 with modifications. Briefly, the fungi were cultivated in 500 mL of potato dextrose broth and were incubated at 28±2 ^{O}C for 30 days in a shaker at 180 rev min^{-1}. The fungal mycelia were then separated from the culture broth by filtration and extracted with analytical grade methanol (MeOH) solvent 1:10. The filtrate was then extracted three times at the liquid-liquid partition with analytical grade ethyl acetate (EtOAc) solvent 1:1 (v/v). The resulting crude extracts were collected and concentrated to dryness in a vacuum rotary evaporator at 40–45^{O}C, then dissolved into 1 mL of MeOH solvent, followed by drying under vacuum to obtain the EtOAc and MeOH extracts. The crude extracts were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 1mg ml^{-1} and kept at 4°C (Sharma et al., 2016).

1.5.7 Determination of total phenolic content of the crude extracts

The total phenolic content of the extract was determined by the Folin–Ciocalteu method. Briefly, 200 µL of the different crude extract (1 mg ml^{-1}) were made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 minutes, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was left to stand for a further 60 minutes in the dark and absorbance was measured at 650 nm (Lasany I-290, Panchkula, India). The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight. Each test was repeated three times and the mean values (±SD) were calculated (Baba and Malik, 2015).

1.5.8 Determination of the antioxidant activity of the crude extracts

The total antioxidant capacity of the endophytic fungal extracts at 1mg ml^{-1} concentration was determined using the OxiSelect™ Total Antioxidant Capacity Assay Kit (Cell Biolabs, Inc, San Diego, USA), per the manufacturer's instructions. The method is based on the single electron transfer (SET) mechanism and involves the reduction of Cu^{2+} to Cu^{+} by the endogenous antioxidants and by other reducing equivalents in the sample. The Cu^{+} interacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. The absorbance value is proportional to the total antioxidant (respectively reducing) capacity of extracts. The samples were analyzed spectrophotometrically at 490 nm using the microplate

reader SPECTROstar^{Nano} (BMG LABTECH, Ortenberg, Germany). The total antioxidant capacity was determined using a calibration curve based on uric acid standards. The results were expressed as µM copper reducing equivalents (CRE). Each test was repeated three times and the mean values (±SD) were calculated.

1.5.9 Determination of the antimicrobial activity of the crude extracts

The microorganisms were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The extracts were tested against the Gram-positive bacteria *Staphylococcus aureus* (ATCC 11632); Gram-negative bacteria, *Escherichia coli* (ATCC1056) and *Klebsiella pneumoniae* (ATCC 13883) using the agar disk diffusion method per the Clinical and Laboratory Standards Institute (Wikler, 2006). Briefly, the test microorganisms were grown in nutrient broth medium until 1 X 10^8 colony-forming units were attained and then used to inoculate sterile 90 mm Petri dishes containing nutrient agar medium using the spread-plate technique. Dried and sterile filter paper discs (6.0 mm diameter) were impregnated with 40 µl of the extracts (containing 500 µg fungal extracts), air dried under the laminar airflow hood and placed on test microorganism inoculated plates. The plates were incubated at 37^{O}C for 24 hours and three sets of controls were used. One control was the organism control and consisted of a seeded Petri dish with no sample. In the second control, samples were introduced to the unseeded Petri dishes to check for sterility. Disks impregnated with 40 µl DMSO were run simultaneously as a third control. Standard antibiotics were also run simultaneously as reference agents to understand the comparative antimicrobial efficacy. The antimicrobial potency of the extracts was measured by their ability to prevent the growth of the microorganisms surrounding the discs.

1.5.10 FT-IR analysis

Infrared spectra were collected on a Nicolet 6700 FT-IR spectrometer (ThermoScientific, Madison, WI USA) equipped with a diamond crystal attenuated total reflectance sampling accessory (ThermoScientific, Madison, WI USA). 100 to 200 milligrams of the samples were placed on the ATR and scanned from 500 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. Each recorded spectrum was the result of 36 co-added scans. The FT-IR was performed to predict the presence of various functional groups in the isolates.

1.5.11 GC-MS analysis

The crude ethyl acetate extracts of the fungi were subjected to GC-MS analysis for further chemical characterization of metabolites. GC-MS analysis was performed using an Agilent 7890A GC system coupled to an Agilent 5975C VL MSD mass spectrophotometer chromatograph equipped with an Agilent ion trap mass-spectrometer. Samples were separated using the Agilent 19091S - 433 column (30m x 250µm x 0.25µm). The gas chromatographic oven temperature was programmed from 50°C to 310°C at a rate of 5°C min-1and the run time was 54

minutes. Helium was used as the carrier gas. The inlet pressure was 25 kPa and the flow rate was 0.68099 mL min-1. The injector temperature was 275°C and the injection mode was splitless. The mass spectrometric scan conditions were as follows: source temperature, 230°C; interface temperature, 250°C and mass scan range, 10-550 amu. Interpretation on mass-spectrum GC-MS-MS was conducted using the database of National Institute of Standards and Technology (NIST) and the spectrum of the extracts was compared with the spectrum of known compounds stored in the NIST library. The name, molecular weight and molecular formula of the extracts samples were ascertained based on the comparison with the known compounds.

1.5.12 Statistical analysis

The data was statistically analyzed using Microsoft Excel 2013 (Microsoft, USA).

1.6 Thesis outline

This thesis comprises of preliminary sections followed by Chapter 1 comprising of the introduction and literature review. Chapter 2 is a review of the status of endophytic fungi bioprospecting in Africa (1994-2014) and data is presented in Chapters 3 to 4 in the form of manuscripts as submitted to the different journals. Chapter 5 is the final chapter of the thesis and it presents a synthesis of the data chapters.

1.6.1 Chapter 1: Introduction and literature review

This chapter introduces the work that was conducted and provides an overall review of literature on the subject matter.

1.6.2 Chapter 2: A review of endophytic fungi bioprospecting in Africa – 1994 to 2014

Chapter 2 provides a review article focussed on the on the status of bioprospecting of endophytic fungi in Africa. The manuscript from this review paper has been accepted for publication in the journal *Current Biotechnology* with the title "A Review of endophytic fungi bioprospecting in Africa – 1994 to 2014". Current Biotechnology. 2018; 7 (2): 80-88.

DOI: 10.2174/2211550106666170221152036.

1.6.3 Chapter 3: Endophytic fungi associated with **Annona senegalensis***: identification, antimicrobial and antioxidant potential*

The fungal endophytes associated with *A. senegalensis* have not yet been described and the study presented in this chapter describes the fungal endophytes isolated from *A. senegalensis* and their antioxidant and antimicrobial potential. The manuscript from this study has been accepted for publication in the journal *Current Biotechnology* with the title "Endophytic fungi associated with *Annona senegalensis*: identification, antimicrobial and antioxidant potential". Current Biotechnology. E-pub Ahead of Print DOI: 10.2174/2211550107666180129154838.

1.6.4 Chapter 4: Endophytic fungi from Vitex payos*: identification and bioactivity assessmen*

The fungal endophytes isolated from *V. payos* have not yet been described and the study presented in this chapter describes the fungal endophytes isolated from *V. payos* and their antioxidant and antimicrobial potential. The manuscript from this study titled "Endophytic fungi from *Vitex payos*: identification and bioactivity assessment" has been submitted to the journal *Acta Mycologica.*

1.6.5 Chapter 5: Endophytic fungi isolated from the medicinal plants Kigelia africana *and* Warburgia salutaris

The endophytic fungi associated with *W. salutaris* have not yet been described; whilst endophytes isolated from *K. africana* are yet to be investigated for their antioxidant activity potential. The study presented in this chapter describes the fungal endophytes isolated from *W. salutaris* and a profile of their natural products and the antioxidant and antimicrobial potential of fungal endophytes isolated from *K. africana*. The manuscript from this study has been accepted for publication in the journal *Current Biotechnology* with the title "Endophytic fungi isolated from the medicinal plants *Kigelia africana* and *Warburgia salutaris*". Current Biotechnology. E-pub Ahead of Print. DOI: 10.2174/2211550107666180308154448.

1.6.6 Chapter 6: Synthesis of research findings

This is the last chapter of the thesis and it provides an overview of the study including general discussion, summary of findings and the general conclusions. This chapter also contains implications and possible application of the study as well suggested areas of future research.

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The introduction and background highlighted the need for new bioactive molecules that can be used as medicines or lead molecules for the development of new medicines. The literature review indicated the potential that medicinal plants have as repositories of bioactive endophytic fungi and the potential that endophytic fungi have as sources of bioactive molecules. Chapter 1 therefore presents the need for bioprospecting and the logic for targeting endophytic fungi found in medicinal plants. Thus, Chapter 2 presents a detailed study conducted to determine the status of endophytic fungi bioprospecting in Africa to identify gaps in focus areas of research and medicinal plants to target.

CHAPTER 2: A REVIEW OF ENDOPHYTIC FUNGI BIOPROSPECTING IN AFRICA – 1994 TO 2014.

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A review of endophytic fungi bioprospecting in Africa – 1994 to 2014

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Abstract

Background: Despite the recognition that endophytic fungi are an important source of diverse and unique natural bioactive products, there are no reviews outlining how African researchers have been tapping into this bioresource. **Objective:** This review aims to consolidate and provide insights into the research on fungal endophytes in Africa during the period 1994-2014. **Methods:** A predefined search protocol was used to undertake a structured search of the bibliographic databases for peer-reviewed research literature. Literature that met the inclusion criteria was then analysed using semi-quantitative and qualitative techniques. **Results:** One hundred and thirty – nine papers from only 20 African countries met the inclusion criteria, with Egypt (35 papers) and South Africa (31 papers) being the leading countries. The main areas of research were biology and diversity determination (38.1%), bioprospecting for compounds with pharmaceutical potential (35.3%) and assessment of the ability of endophytic fungi to act as biopesticides (21.6%). A diversity of new and known compounds were shown to be secondary metabolites produced by the endophytic fungi and most of these compounds were investigated for their potential as antimicrobial and anticancer agents. Research was observed to have evolved from a focus on endophyte diversity in the 1990s and early 2000s to exploring the possibility of using endophyte fungi as sources of compounds with pharmaceutical and other commercial applications post 2005. **Conclusion**: In Africa, whilst fungal endophytes remain largely an untapped reservoir of potentially useful natural products, research on the fungal endophytes has also evolved in line with trends elsewhere.

Keywords: Endophytic Fungi, Bioprospecting, Natural Products, Africa, Endophyte, Fungi

1. INTRODUCTION

Endophytes are widely defined as microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease [1]. Bioprospection of fungal endophytes (and other endophytes) is considered a new frontier for natural products lead discovery as fungal endophytes have been shown to produce a broad variety of bioactive secondary metabolites with potential medicinal, industrial and agricultural applications [2]. Despite the potential benefits that can be derived from fungal endophytes, only a tiny fraction of plant species have been investigated for their fungal endophyte biodiversity [3]. It is estimated that globally there might be 1-1.5 million species of endophytic fungi, most which are yet to be identified and studied [4].

Africa is endowed with approximately 40,000 – 60,000 plant species [5] and it is speculated that only a few have been studied relative to their endophytic biology in part due to lack of up to date information on the status of endophytic fungi research in African countries. There is therefore a need to review the work that has been done on fungal endophytes in Africa to gain an insight on the status quo and identify research gaps and suggest perspectives for future research. The intent of this review article is to provide insights into the research on fungal endophytes that has been conducted in Africa in the period 1994 – 2014.

2. MATERIALS AND METHODS

2.1. Literature Selection Procedure

The research output on endophytic fungi in different African countries (1994 – 2014) was collated. Available references and/or reports on endophytic fungi research from peer reviewed published scientific journals, books, reports, theses and conference papers were consulted. Literature was accessed from the bibliographic databases Scopus, Google Scholar and PubMed – NCBI using specific search terms such as “endophytic fungus and specific African country name”, “endophyte (s) and specific African country name”. Sources that were considered for the study had to have been published in the period 1994 – 2014 and were those that had the research work conducted in an African country and/or used plant materials sourced from an African country and/or at least one of the authors had to have been affiliated to an organisation based in an African country. Data collected from the literature search included the year of publication; the name of the plant species studied, identity of the isolated endophytic fungi, research focus and key findings.

2.2. Reviewed Literature

Based on the inclusion and exclusion criteria, 139 publications were selected for this review article. Fig. **1** provides a flow chart summary of the selection process for the reviewed literature.

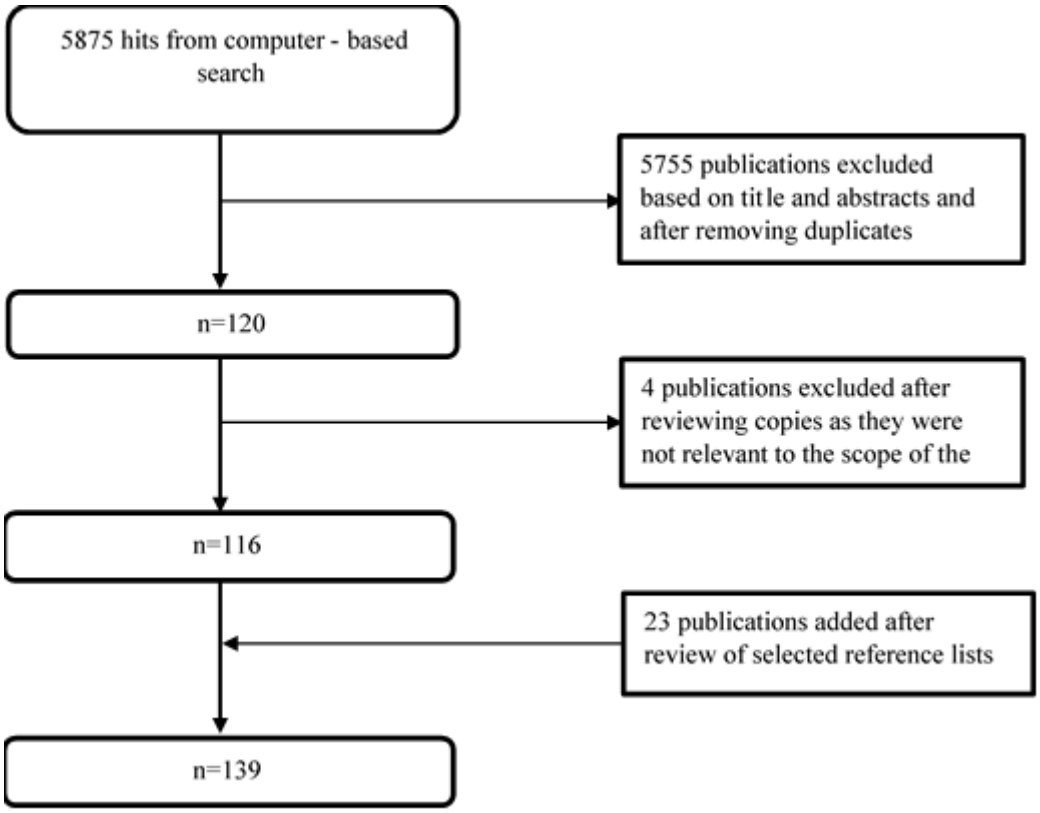

Fig. (1). Flow chart summary of the publications selection process

3. RESULTS AND DISCUSSION

3.1. African countries involved in endophytic fungi research

In the period under review, some research on endophytic fungi was conducted in only 20 African countries (Fig. **2**) and based on the number of hits on online databases Africa only contributed approximately 4% to the global research output on endophytic fungi. Egypt, South Africa and Cameroon were the leading African countries in fungal endophytes research as they contributed 25.2%, 22.3% and 12.9% respectively to the research output from Africa.

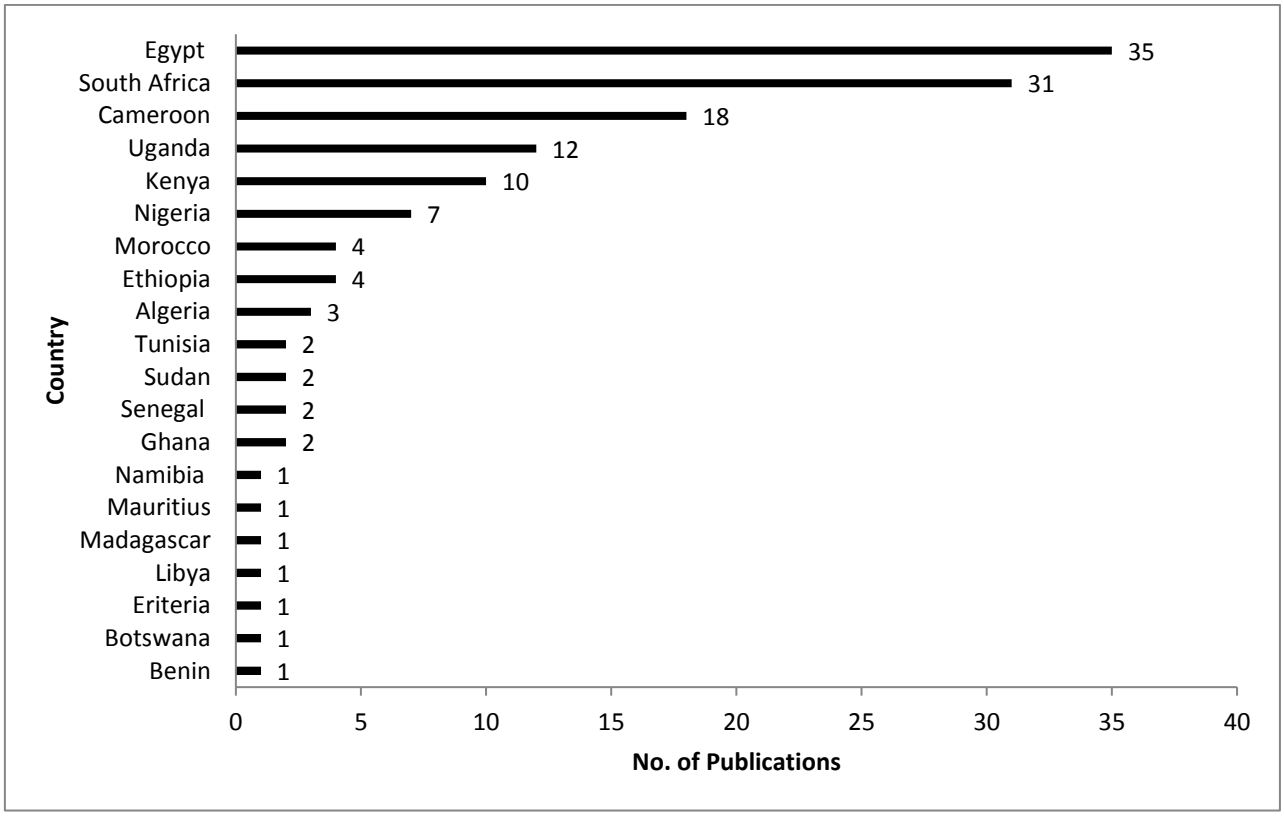

Fig. (2). Distribution of the articles published on endophytic fungi associated with Africa over the period 1994-2014, per the origin of the research teams

A study of the patenting activity (among other things) over the same period would be probably interesting to understand how the research on endophytic fungi within the African countries is being financed and determine the influence of private grants in the financing of the research work.

3.2. Trends in Publications

An analysis of the publication activity in the PubMed and Scopus websites (as measured by the number of hits) up to December 31, 2014 revealed that globally 86% of the publications related to endophytic fungi were published in the period 2004-2014. The situation in Africa also mirrors the global trends as approximately 91.4% of the publications related to endophytic fungi were also published in the period 2004-2014 (Fig. **3**). These observations support the findings by Maheswari and Rajagopal who in 2013 stated that there has been a global resurgence in investigating the potential of endophytic fungi as sources of novel biologically active products in the past 10 years [6].

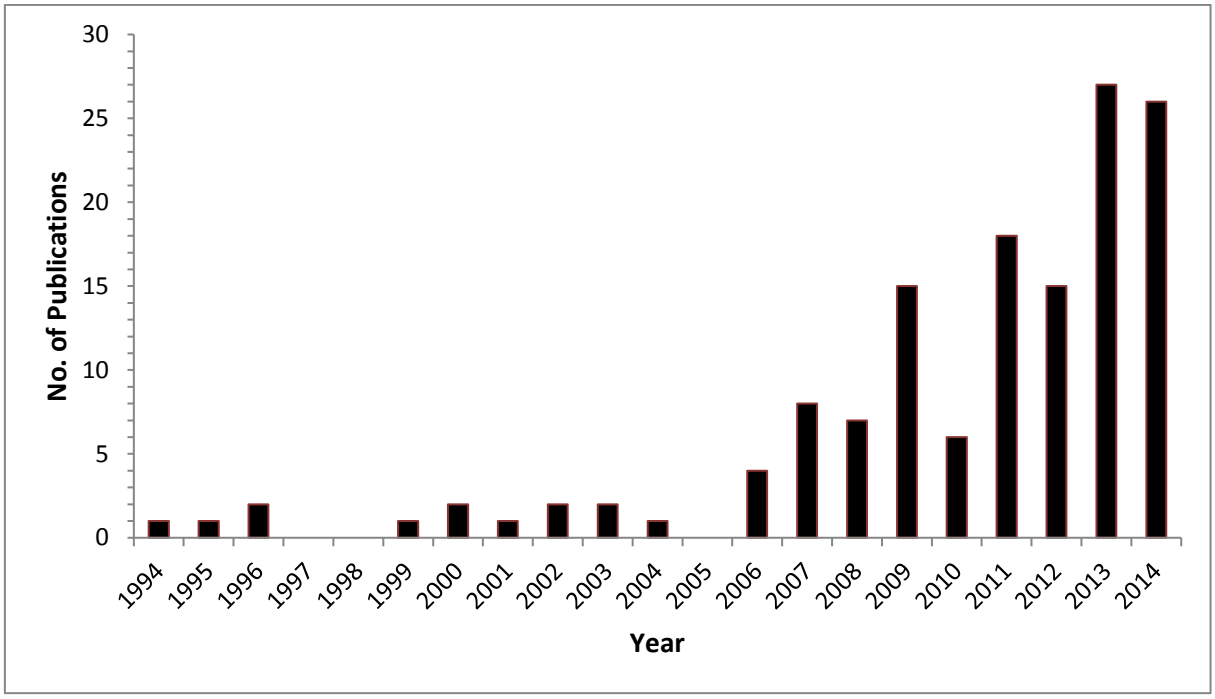

Fig. (3). Evolution of the number of articles from Africa published on subjects involving endophytic fungi, over the period 1994-2014.

The evolution of research on endophytic fungi in Africa from the period 1994 to 2014 allowed us to identify three major periods which correspond to different ways of thinking and interest in endophytic fungi. The first period covering 1994-2000 correspond to what we have called the "exploratory period". From 1994 to 2000, the number of publications per year was approximately 1 and this period was dominated by one major research theme, the study of the diversity and biology of endophytic fungi. Research work dedicated to understanding the diversity and biology of endophytic fungi in general accounted for 86% of the publications whilst the remainder (14%) was focused on investigating the potential of endophytic fungi as biocontrol agents against root-node nematodes. The paper with the most significant impact during this period as measured by the number of citations in the Scopus and Google Scholar websites originated from South Africa (Universities of Free State and Stellenbosch working in collaboration). The research theme of the paper was centred on investigating fungal endophyte diversity and host plant-fungal endophyte interactions [7]. Concerning the origin of the research works, it was observed that few African countries (4) were involved in endophytic fungi research and this period may be termed the "South African period" as 57% of the articles produced originated from South Africa research teams (University of Free State and of University Stellenbosch). The remaining articles were produced equally by research teams in Egypt, Nigeria and Kenya.

The second period started from 2001 to around 2007 and we have termed it the "expansion period" as more African countries became involved in endophytic fungi research and saw an increase in the number of publications per year from 1 in the period 1994 to 2000 to approximately 2.6 articles per year. If we consider specifically the research themes which were studied during this period, by crossing titles and keywords, we mainly observe that research was dedicated to understanding the diversity and biology of endophytic fungi. Articles that sought to understand endophyte diversity and biology in general accounted for 78% of the publications whilst articles that sought to investigate the potential of endophytic fungi as biocontrol agents against plant pests and diseases in general accounted for 22% of the publications. The publication with the greatest impact (as measured by number of citations in Scopus and Google Scholar) originated from the University of Pretoria in South Africa. The publication was a review article and the theme of the paper was focused on exploring recent developments in understanding the role of Botryosphaeriaceae as endophytes and latent pathogen [8]. Concerning the origins of the work, it was observed that more African countries (9) became involved in endophytic fungi research and the period can be termed the "Ugandan period" as 33% of the articles produced originated from Ugandan research teams (with the International Institute of Tropical Agriculture in Uganda and Makerere University being prominent). The remaining articles originated from South Africa (22%), Cameroon (17%), Benin (5.6%), Botswana (5.6%), Egypt (5.6%), Ethiopia (5.6%), and Mauritius (5.6%).

The third period started around 2008 and is currently in progress and we have termed it the "enlargement period" as there was an extension in the investigation of the potential applications of endophytic fungi in other fields: pharmaceutical applications, biofuels production, bioremediation, method development. The number of publications per year in the period 2008 to 2014 increased to 16.3 articles per year boosted by increased output from research teams that were previously involved in endophytic fungi research and contributions made by new research teams from other African countries that were not previously involved in endophytic fungi research. The most prominent research theme during this period (which is currently in progress) was the investigation of the potential application of endophytic fungi as sources of lead molecules with pharmaceutical applications (antimicrobial, antiparasitic, anticancer, antioxidant, antiviral, anti-inflammatory). Articles that sought to investigate the potential applications of endophytic fungi as sources of lead molecules with pharmaceutical applications in general accounted for 43.1% of the publications. The potential for endophytic fungi to produce lead molecules with potential antimicrobial and anticancer activities were particularly studied. The other research themes were: potential application of endophytic fungi as biocontrol agents against plant pest and diseases (21.9%), endophyte diversity and biology (28.9%), potential applications of endophytic

fungi as agents of bioremediation (2.6%), method development and validation (2.6%), and potential application of endophytic fungi in the production of biofuels (0.9%).The publication with the greatest impact during this period (as measured by number of citations in Scopus and Google Scholar) originated from the Université Mohammed V Agdal in Morocco working in collaboration with research institutes in Germany (Heinrich-Heine-Universität and Johannes-Gutenberg-Universität) and Scotland (University of Aberdeen). The publication was focused on identifying secondary metabolites produced by the endophytic fungi *Stemphylium globuliferum* isolated from the plant *Mentha pulegium* and assessing their potential application as lead molecules for anticancer pharmaceuticals [9]. Concerning the origins of the work, it was observed that more African countries (17) became involved in endophytic fungi research and the period can be termed the "Egyptian period" as 28.9% of the articles produced originated from Egyptian research teams (with the National Research Centre, Dokki being prominent as it was associated with 42.4% of the publications from Egypt during this period). The remaining articles originated from South Africa (20.2%), Cameroon (13.2%), Kenya (7.9%), Uganda (5.3%), Nigeria (5.3%), Morocco (3.5%), Ethiopia (2.6%), Algeria (2.6%), Ghana (1.8%), Senegal (1.8%), Sudan (1.8%), Tunisia (1.8%), Madagascar (0.9%), Namibia (0.9%), Eretria (0.9%), and Libya (0.9%).

3.3. Investigated research areas

In this part of the review, we present an overview of the fields that were research upon in the articles associated with Africa over the period under study. However, it is important to note that for our analysis we decided that the major theme indicated in the title would determine how we classified an article to avoid the difficulties associated with classifying a pool of articles (as frequently a paper can fall into two or more research themes). Though country specific differences in the focus areas of research were noted, in general the main areas of endophytic fungi research in Africa (1994 – 2014) were to determine fungal endophyte diversity and biology (38.1%), to assess the potential of endophytic fungi as sources of lead molecules with pharmaceutical applications (35.3%), and to determine the potential of endophytic fungi as biocontrol agents against plant pests and diseases (21.6%). There were a few publications that could not be classified under the above research themes (bioremediation, biofuels production and method development and validation) and these were grouped under miscellaneous and these contributed approximately 5% to the total of the publications associated with Africa.

In the period under review, a notable success story in endophyte research was recorded in Africa where because of the work (on investigating the potential of endophytic fungi as biocontrol agents against plant pests and diseases) conducted by a consortium led by the International Institute of Tropical Agriculture (IITA) in Uganda, endophyte (*Beauveria bassiana* and *Fusarium*

oxysporum) enhanced banana tissue culture was successfully developed [10]. This technology is being promoted in East and Central Africa to boost banana production [11].

3.4. Diversity of studied fungal endophytes and plant species

In the period 1994 – 2014, the majority (99.2%) of the isolated and investigated fungal endophytes in Africa belonged to the phylum Ascomycotina and the top five most studied endophytic fungi genera were *Fusarium* (12.2%), *Aspergillus* (11.4%), *Penicillium* (8.1%), *Beauveria* (5.7%) and *Alternaria* (5.7%). *Musa* was the most studied plant species in the period under review with approximately 22% of the research output from Africa on endophytic fungi being associated with the plant. Approximately 57 plant species and two algal species were investigated for their endophytic fungi associations in African countries (1994 – 2014) and the top three most studied plant families were Fabaceae, Poaceae and Rutaceae. This suggests that less than 0.2% of the plant species found in Africa have been investigated for their fungal endophyte diversity.

It is important to note that intra and inter species differences in fungal endophyte diversity were observed in the plant species that were studied for their endophyte diversity. The environmental conditions (in which the plant species was grown in) and the age and type of plant tissue (used in the isolation of the fungal endophytes) were shown to influence the fungal endophyte diversity and succession patterns.

While investigating the diversity of fungal endophytes some studies were also conducted in Africa to validate and develop new methods of endophyte isolation and diversity determination. The physical abrasion surface sterilization technique was found to be superior to the chemical technique in removing non-endophytic DNA [12]. Using *Eucalyptus grandis* as a case study, semiconductor sequencing (implemented using the Ion Torrent Personal Genome Machine) was shown to be an ideal strategy for environmental sequencing (and hence determination of the diversity) of endophytic fungal communities [13].

3.5. Reported examples of bioactive compounds

In this part of the review we present an overview on new and known natural products that were shown to be produced by endophytic fungi and their reported bioactivities and potential applications. In the period under review, the research on endophytic fungi associated with Africa resulted in the elucidation of the structures and bioactivity potential of several new and known natural compounds produced by endophytic fungi. Most these new and known compounds showed potential as antimicrobial and anticancer agents.

3.5.1. Recently Discovered Compounds

This section describes new compounds that were isolated from endophytic fungi isolated from research associated with African countries in the period 1994 to 2014 (Table **1**). It is important to note that a diversity of new compounds such as polyketides, chromenes, xanthones and epoxyl quinols were shown to be secondary metabolites produced by the isolated endophytic fungi. The structures of the compounds that were discovered in the period under review are highlighted in the supplementary materials section.

Table 1. New compounds and their reported bioactivities

Host Plant	Fungal Endophyte	Compound Name	Reported Activity	References
-	*Fusarium equiseti* SF-3-17	Fusaequisin A	Moderate antimicrobial activity against *Staphylococcus aureus* NBRC 13276 and *Pseudomonas aeruginosa* ATCC 15442.	[14]
Piper guineense	*Fusarium species CAMKT24b1*	(S) – Banchromene	Inhibition of the motility of phytopathogenic *Plasmopara viticola* zoospores at 20 µg/mL and 50 µg/mL.	[15]
-	*Microsphaeropsis* species	Microsphaeropsones A to C	Microsphaeropsones A and C (at concentrations of 50 µg per test filter disk) were shown to have some antibacterial, fungicidal, and algicidal properties.	[16]
Turraeanthus longipes	*Pestalotiopsis theae*	Cytosporins F to K	The compounds did not exhibit any significant cytotoxic activity (IC50>10µg/mL) against the mouse lymphoma cell line L5178Y and were also not active against Staphylococcus aureus (ATCC 25922), Streptococcus pneumoniae (ATCC 49619), and Escherichia coli (ATCC 25922) even at a concentration of 64µg/mL.	[17]
Garcinia nobilis	*Penicillium* species	Penialidins A to C	Significant efficacy against *Staphylococcus aureus* and *Bacillus subtilis*(MIC values of 5 µg/mL against both microorganisms)	[18]

Limonium tubiflorum	*Penicillium* species	Penilactone; 10, 11-epoxycurvularin; Neobulgarone G; and Sulfimarin	Compounds did not show promising anti-trypanosomal activity and inhibitory effects on TNFα-induced NF-κB activity.	[19]
Zanthoxylum leprieurii	*Chaetomium* species	Chaetosidone A	Antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* at concentrations of 40 µg per test disk and moderate toxicity towards brine shrimp larvae.	[20]
Mentha pulegium	*Stemphylium globuliferum*	Alterporriols G and H; Altersolanol K and L; and Stemphypyrone	The mixture of alterporriols G and H was shown to be a potent inhibitor of a panel of 24 kinases and displayed significant cytotoxicity against L5178Y cells with an EC_{50} value of 2.7µg/mL.	[9]
Juncus actus	*Stemphylium globuliferum*	Stemphylanthranols A and B	-	[21]
Bidens pilosa	*Botryosphaeria obtuse*	Botryorhodine E	Antiproliferative effects against HUVECs (GI50 value of 62.04 µM) and K-562 cell line (GI50 value of 40.15 µM); Cytotoxicity activity against the HeLa cancer cell line (CC50 value of 43.80 µM); Antifungal activity against *Penicillium notatum* (MIC value of 16 µg/mL) and *Aspergillus terreus* (MIC value of 12 µg/mL)	[22]
Ceratonia siliqua	*Penicillium citrinum*	Citriquinochroman; Tanzawaic acids G and H 6-methylcurvulinic acid; and	Citriquinochroman was shown to have cytotoxicity activity against mouse L5178Y cells with an IC_{50} value of 6.1 µM	[23]

		8-methoxy-3,5-dimethylisoquinolin-6-ol		
Halimeda opuntia	*Aspergillus versicolor*	Isorhodoptilometrin-1-methyl ether	Isorhodoptilometrin-1-methyl ether was shown to have moderate activity against *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* at a concentration of 50µg	[23]
Citrus aurantum	*Bartalinia pondoensis*	[N- (ethyloxy, hydroxymethyl) phenylethylamine]	The methanol extract and fractions obtained by solid phase extraction were shown to have an inhibitory effect against *Mycobacterium tuberculosis* shikimate kinase at a concentration of 50µg/mL.	[24]
Vinca rosea	*Nigrospora sphaerica*	Nigrosphaerin A	Though the antileukemic, antileishmanial, antifungal and antibacterial activities of nigrosphaerin A and other known isolated compounds were studied, the bioassays data obtained for nigrosphaerin A was not presented in the publication	[25]
Ipomoea batatas	*Aspergillus glaucus*	2, 14-dihydrox-7-drimen-12, 11-olide	This compound was shown to have moderate cytotoxic activity against Hep-G2 cells (IC_{50} value of 61 µg/mL) and strong cytotoxic effects against MCF-7 cells (IC_{50} value of 41.7 µg/mL)and high anti-oxidant activity	[26]
Vinca rosea	*Alternaria phragmospora*	5-butyl-4-methoxy-6-methyl-2H-pyran-2-one; 5-butyl-6-(hydroxymethyl)-4-methoxy-2H-pyran-2-one;	5-butyl-6-(hydroxymethyl)-4-methoxy-2H-pyran-2-one and 4-methoxy-6-methyl-5-(3-oxobutyl)-2H-pyran-2-one were shown to have moderate antileukemic activities against	[27]

		5-(1-hydroxybutyl)-4-methoxy-6-methyl-2H-pyran-2-one; and 4-methoxy-6-methyl-5-(3-oxobutyl)-2H-pyran-2-one	HL60 cells (IC_{50} values of 2.2 and 0.9 µM respectively)and K562 cells with IC_{50} values of 4.5 and 1.5 µM respectively	
Mentha suaveolens	*Epicoccum nigrum*	Epicocconigrones A and B; 3-methoxyepicoccone B; 3-methoxyepicoccone; and 2,3,4-trihydroxy-6-(methoxymethyl)-5-methylbenzaldehyde	Epicocconigrones A was shown to be an inhibitor of histone deacetylase (IC_{50} = 9.8µM) and displayed potent inhibitory activity against a panel of different protein kinases.	[28]
Gongronema latifolium	*Corynespora cassiicola*	Corynesidone D; Corynether lactone A; and Corynether B	Corynesidone D was shown to have potential to serve as a lead molecule for the development of novel anti-inflammatory and/or anti-cancer agent through its effects on the tumour necrosis factor-α, inducible nitric oxide, and reactive oxygen species and reactive nitrogen species production by stimulated RAW264.7 macrophagesHowever, at concentrations of 20µM, corynesidone D was shown not to influence the chaperoning activity of the Hsp90 chaperoning machine	[29-30]
Jatropha gossypiifolia	*Aspergillus japonicus*	Microsphaerone C	Moderate cytotoxic activity towards the mouse cell line L5178Y	[31]

3.5.2. Known Compounds

In the period under review, the bioactivity potentials of some known compounds produced by isolated fungal endophytes were also determined. This section will detail the known compounds and their bioactivities (Table **2**).

Table 2. Known compounds and their reported bioactivities

Host Plant	Fungal Endophyte	Compound Name	Reported Activity	Reference
Zanthoxylum leprieurii	*Cryptosporiopsis* species	Cryptosporiopsin A	Biopesticide against grapevine downy mildew pathogen	[32]
		Natural cyclic pentapeptide		
		Hydroxypropan-20,30-diol orsellinate		
Bryophyllum pinnatum	*Diaporthe phaseolorum*	Cytochalasin D	Anticancer	[31]
Vinca rosea	*Nigrospora sphaerica*	Ergosta-4,6,8(14), 22-tetraene-3one	Antileukemic, Antileishmanial	[25]
		Ergosta-7,9(14),22-triene-3β-ol	Antileukemic, Antifungal	
		4-(hydroxymethyl)-3,5-dimethyl dihydrofuran-2(3H)-one	Antileukemic	
		3-(1-hydroxyethyl)-4-methyl dihydrofuran-2(3H)-one		
		Ergosta-7,22-diene-3β-ol	Antileishmanial	
		Ergosta-5(6),7,22-triene-3β-ol		
Limonium tubiflorum	*Penicillium* species	11β-methoxycurvularin and 11α-methoxycurvularin	Antitrypanosomal, Anticancer, Regulation of the NF-κB pathway	[19]
		5-chloro-6,8,10-trihydroxy-1-methoxy-3-methyl-9(*10H*)-anthracenone	Antitrypanosomal	

		Trichodimerol	Antitrypanosomal	
		Trans-dehydrocurvularin	Anticancer	
		1-chloro-2,4-dihydroxy-5-methoxy-7-methylanthraquinone	Anticancer, Regulation of the NF-κB pathway	
Bidens pilosa	*Botryosphaeria obtuse*	Preussomerin C	Anticancer	[22]
Halimeda opuntia	*Aspergillus versicolor*	Siderin	Antibacterial	[33]
Laggera alata	*Podospora* species	Sterigmatocystin and 13-hydroxyversicolorin B	Larvicidal against *Anopheles gambiae*	[34]
Mentha suaveolens	*Epicoccum nigrum*	Epicoccolide B	Protein kinase inhibitor, Histone deacetylase inhibitor	[28]
Salvia officinalis	*Chaetomium species*	Cochliodinol	Anticancer	[35]
Mentha pulegium	*Stemphylium globuliferum*	Altersolanol A	Protein kinase inhibitor, Anticancer	[36]
		6-O-methylalaternin	Protein kinase inhibitor	
		Macrosporin		
Piper guineense	*Fusarium species*CAMKT24b1	Beauvericin and Fusaproliferin	Biopesticide against grapevine downy mildew pathogen, Cytotoxic	[15]
		Radicinin	Biopesticide against grapevine downy mildew pathogen	

It is important to note that quite a number of publications during this period also reported on the bioactivities of uncharacterised extracts obtained from the fermentation broth of isolated endophytic fungi.

3.6. African research institutes involved in fungal endophyte research

In this part of the review we present an overview of the research African institutes that were found to be involved in endophytic fungi research in the period 1994-2014 (Table **3**). In general, the increase in publication output from 1994 to 2014 was also mirrored by an increase in the number of African research teams (and institutes) involved in endophytic fungi research i.e. from about five research institutes in the period 1994 to 2000 to greater than 40 research institutes in the period 2008 to 2014.However, it appears that there is a need to encourage and strengthen research collaborations between research institutes (both local and foreign) and leverage on the different expertise that may exist in the different laboratories to ensure meaningful endophytic fungi research output from Africa.

A study of the patenting activity over the same period would be probably interesting to determine if there is a link between the numbers of publications generated per institute (or research team) and technology developed by these institutes based on fungal endophytes.

Table 3. Top five African research institutes involved in fungal endophyte research based on publications output (1994-2014)

Research Institute	Country	*Number of Publications
International Institute of Tropical Agriculture	Uganda	19
University of Pretoria	South Africa	18
National Research Centre – Dokki	Egypt	15
University of Stellenbosch	South Africa	12
Makerere University	Uganda	9

**Number of publications in which the institutes were cited in author affiliation by one or more of the authors*

CONCLUSIONS

Even though this study is limited in that it excludes publications that are not found on line and patenting activity, the review of the publication activity in the period 1994-2014 suggests that in

Africa, fungal endophyte research is still in its infancy as in a majority (63%) of the African countries there was no record of research work on fungal endophytes. This infers that in Africa fungal endophytes remain largely an untapped reservoir of natural products; therefore, there is a need for more African research teams (and countries) to get on board and be involved in endophytic fungi research. Fungal endophyte research output has the potential to address some of the challenges faced in Africa as shown by the application of endophyte enhanced banana tissue culture in East Africa to boost agricultural production. Regional groups such as the Southern African Development Community and networks such as the Southern Africa Network for Biosciences (NEPAD SANBio) should be lobbied to ensure that endophyte bioprospecting is included as a key thematic area in their scientific research programs.

The type of plant species, environment in which the plant species were grown and plant part (i.e. leaves, stems and roots) used for endophyte isolation influenced the fungal endophyte diversity and bioactive potential. Despite these differences, novel and bioactive compounds were shown to be produced by fungal endophytes isolated from plant species found in Africa. However, there is need to ensure that this basic research knowledge is translated into beneficial practical applications. This could be achieved through participation by African researchers in platforms like the Indian Open Source Drug Discovery (OSDD) or development of such platforms by individual African countries or regional groupings/networks.

It was observed that though variable methods were employed in the isolation and study of fungal endophytes; limited work was done in Africa to investigate the production of bioactive volatile organic compounds produced by endophytic fungi. This suggests that investigation of bioactive volatile organic compounds produced by endophytic fungi is an area that needs further attention within the African research community. Moreover, there is a need for African countries to catalogue and make collections of the isolated endophytic fungi (and other microbes) for referencing and future use.

As a development to this article, it would be interesting to study the patenting activity related to endophytic fungi associated with identified African countries (and research institutes) over the same period. This would help to explain how this work is being funded and the rate at which research on endophytic fungi in Africa is being translated into technology with practical beneficial applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Chemical structures of new compounds that were discovered because of research on endophytic fungi associated with Africa in the period 1994 to 2014.

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The status quo review study in Chapter 2 highlighted that whilst Africa in general was lagging behind in fungal endophyte research, in the countries of southern Africa (excluding South Africa) there was hardly any research on fungal endophytes. This presented an opportunity as it meant that most of the medicinal plants found in southern Africa were yet to be investigated for their endophytic fungi diversity. An analysis of the research focus areas revealed that despite the need, few researchers where investigating the antioxidant potential of endophytic fungi. Thus, Chapter 3 presents a detailed study conducted to determine the endophytic fungi associated with the medicinal plant *Annona senegalensis* their identification and bioactivity potential (antimicrobial and antioxidant). This chapter addresses objectives 1 to 4.

CHAPTER 3: ENDOPHYTIC FUNGI ASSOCIATED WITH *ANNONA SENEGALENSIS*: IDENTIFICATION, ANTIMICROBIAL AND ANTIOXIDANT POTENTIAL

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Endophytic fungi associated with *Annona senegalensis*: identification, antimicrobial and antioxidant potential

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Abstract

Background: The endophytes of African medicinal plants are largely underexplored despite their potential as repositories of bioactive compounds. **Objective:** To identify the endophytic fungi associated with *Annona senegalensis* and evaluate the antimicrobial and antioxidant potential of their crude ethyl acetate extracts. **Method:** The surface sterilization technique (using ethanol and sodium hypochlorite) was used to isolate the endophytic fungi that were identified by rDNA sequencing of the ITS region. The extracts were screened for antimicrobial activity using the agar diffusion method and evaluated for antioxidant activity using a commercial kit. The total phenolic content of the extracts was determined using the Folin-Ciocalteu method and functional groups present in the extracts were predicted using Fourier-transform infrared spectroscopy. **Results:** Eight fungal isolates identified as *Phoma* species, *Epicoccum nigrum*, *Epicoccum sorghinum*, *Alternaria alternata*, *Alternaria tenuissima*, *Phaeosphaeria* species and *Penicillium chloroleucon* were isolated from the tissues of *Annona senegalensis*. The extracts did not exhibit any potential antimicrobial activity and the extract obtained from *Epicoccum sorghinum* demonstrated both the highest total phenolic content (28.85±1.14 mg GAE/g dry weight) and total antioxidant capacity (593.46 ± 1.86 μM CRE). A strong positive linear correlation (r = 0.9556) was found between antioxidant capacity and phenolic content. The Fourier-transform infrared spectral analysis of the crude extracts from *Epicoccum sorghinum* confirmed the presence of molecules carrying bonded hydroxyl functional groups characteristic of phenolic compounds. **Conclusion:** The preliminary results indicate that the isolated fungal endophytes from *Annona senegalensis* belong to the phylum Ascomycota and have potential as sources of natural antioxidants.

Keywords: Antioxidant, Endophytic Fungi, Phenolic Content, Bioprospecting, Antimicrobial, FT-IR, ITS, *Annona senegalensis*

1. INTRODUCTION

Bioprospecting interest for natural compounds with the potential for use as therapeutics has been heightened due to several factors that include the emergence of new life-threatening diseases and the rapid development of anti-microbial resistant pathogenic microbes [1, 2] Antimicrobial resistance is an emerging global health issue, if not addressed it has the potential to usher the world into a post-antibiotic era in which currently treatable common infections and injuries will be untreatable and fatal [3]. Free radicals are byproducts of normal metabolism and play important roles in metabolic reactions within the body [4]. Pathophysiological or environmental interference have been shown to cause unregulated generation of free radicals that result in oxidative damage to biomolecules [5, 6]. The oxidative damage to the biomolecules has been implicated in many degenerative human diseases such as diabetes mellitus, cancer, Alzheimer's disease and other neurodegenerative disorders, ageing and inflammatory diseases [6]. Antioxidants are compounds that can donate electrons to free radicals and terminate free radical mediated reactions before oxidative damage can occur to biomolecules [7].

Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease [8]. Bioprospection of endophytes is considered a new frontier for screening of natural products as endophytic fungi have been shown to produce a broad variety of bioactive secondary metabolites including compounds with antioxidant and antimicrobial activity [9, 10]. Studies have also shown that some medicinal properties of plants may be related to endophytic fungi hosted by these plants [2]. Therefore, medicinal plants have an important role to play in the search for new bioactive strains of endophytic fungi, as it is possible that their beneficial characteristics are a result of the metabolites produced by their endophytic community [11, 12]. Despite the potential, only a tiny fraction of plant species have been studied regarding their endophytic fungi diversity [9]. *Annona senegalensis* an ethnomedicinal plant used to treat a variety of ailments in traditional African medicine such as diarrhea, dermatological diseases, pneumonia, venereal diseases, gastroenteritis, snakebite, toothache and respiratory infections [13].

As part of our contribution to ongoing efforts to describe the endophytic fungi diversity and search for novel antimicrobial and antioxidant agents from fungal endophytes we isolated and identified the endophytic fungi found in the leaf and stem tissues of *A. senegalensis*. We further investigated the

antibacterial activity, total phenolic content (TPC) and total antioxidant potential (TAC) of some endophytic fungi strains isolated from *A. senegalensis*. In addition, the presence of various functional groups responsible for the biological activities of the endophytic fungi crude extracts was predicted using Fourier transform infra-red (FT-IR) spectroscopy.

2. MATERIALS AND METHODS

2.1. Collection and identification of plant materials

Fresh leaf and stem tissues of *A. senegalensis* were obtained from the National Herbarium and Botanical Gardens (Harare, Zimbabwe) plant collection. The tissue samples were pretreated by cleaning them under running water to remove dirt and soil particles and were then air-dried to remove any surface moisture before they were packaged into labeled sterile sample collecting bags. The samples were then transported to the laboratory and stored at 4 °C until endophyte isolation procedures could be instituted [14].

2.2. Isolation and establishment of *in vitro* culture of endophytes

Endophytic fungi were isolated from the different tissues collected from the medicinal plants using a modified surface sterilization procedure as described by Kjer *et al*. 2010 [15]. Briefly, the plant tissue samples were removed from storage and thawed by briefly putting them under running tap water and then washed using 0.1% (v/v) Tween 80 for 15 minutes followed by another wash for 1 hour 30 minutes using running water. The cleaned plant tissues were then transferred to a laminar airflow cabinet. The plant tissue samples were dipped in 70% ethanol: 30 seconds for leaf samples and 2 minutes for stem samples. The ethanol was drained out after the required amount of time and the plant tissue was then washed using a 2% sodium hypochlorite solution for 15 minutes which was then followed by four washes using double distilled water after which the samples were dried using sterile paper towels. The effectiveness of the sterilization procedure was tested by plating 0.1 ml of the final sterile water rinse onto Petri dishes containing potato dextrose agar (PDA) and by rolling the sterilized sample onto Petri dishes containing PDA. The surface sterilized tissues were cut into smaller segments (1-2 cm) using sterile razor blades and placed onto Petri dishes containing PDA supplemented with Penicillin-Streptomycin antibiotic at a concentration of 200U ml^{-1} (Lonza, Cat # 17-602E) and incubated at 28±2 °C until fungal growth was initiated. The colonization rate of endophytic fungi was determined as the total number of segments yielding ≥ 1 isolate in a host sample divided by total number of segments incubated in that sample multiplied by 100. The growing hyphal

tips which grew out of the samples were isolated and sub cultured onto Petri dishes containing PDA. The cycle of sub culturing was repeated until pure cultures of the isolates were obtained.

2.3. Identification of the endophytic fungi

Genomic DNA was extracted from the cultures using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using EconoTaq® PLUS GREEN 2X Master Mix (Lucigen) with the primers presented in Table **1**. The PCR products were run on a gel and gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse directions (Applied Biosystems, ThermoFisher Scientific, Big Dye terminator kit v3.1) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were run on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). CLC Bio Main Workbench v7.6 was used to analyze the .ab1 files generated by the ABI 3500XL Genetic Analyzer and endophytic fungi isolates were identified on the basis of similarity of amplified sequence with those found in the US National Centre for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (nBLAST).

Table 3: ITS primer sequences

Name of Primer	Target	Sequence (5' to 3')	Reference
ITS1	Small Sub-Unit	TCCGTAGGTGAACCTGCGG	[16]
ITS4	Large Sub-Unit	TCCTCCGCTTATTGATATGC	[16]

2.4. Secondary metabolite extraction

The small-scale fermentations and solvent extractions were carried out as described by Kjer *et al*. 2010 with modifications [14]. Briefly, the fungi were cultivated in 500 mL of potato dextrose broth and were incubated at 28±2 °C for 30 days in a shaker at 180 rev min^{-1}. An equal volume of analytical grade ethyl acetate (EtOAc) was added to the fermentation broth and the mixture was homogenized. Fungal mycelia were separated from the homogenised culture broth by filtration and the filtrate was then extracted three times with analytical grade EtOAc 1:1 (v/v). The resulting crude extracts were collected and concentrated to dryness in a vacuum rotary evaporator at 40–45^{O}C, then dissolved into 1 mL of methanol solvent, followed by drying under vacuum to obtain the EtOAc crude extracts. The dried crude extracts were weighed before being dissolved in dimethyl sulphoxide (DMSO) at a concentration of 1mg ml^{-1} and kept at 4°C [17].

2.5. Determination of total phenolic content of the crude extracts

The total phenolic content of the extract was determined by the Folin–Ciocalteu method. Briefly, 200 µL of the different crude extracts (1 mg ml^{-1}) were made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 minutes, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was left to stand for a further 60 minutes in the dark and absorbance was measured at 650 nm (Lasany I-290, Panchkula, India). The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight. Each test was repeated three times and the mean values (±SD) were calculated.

2.6. Determination of the antioxidant activity of the crude extracts

The total antioxidant capacity (TAC) of the endophytic fungal extracts at 1mg ml^{-1} concentration was determined using the OxiSelect™ Total Antioxidant Capacity Assay Kit (Cell Biolabs, Inc, San Diego, USA), as per the manufacturer's instructions. The method is based on the single electron transfer (SET) mechanism and involves the reduction of Cu^{2+} to Cu^{+} by the endogenous antioxidants and by other reducing equivalents in the sample. The Cu^{+} interacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. The absorbance value is proportional to the total antioxidant (respectively reducing) capacity of extracts. The samples including the negative control (DMSO sample) were analyzed spectrophotometrically at 490 nm using the microplate reader SPECTROstar^{Nano} (BMG LABTECH, Ortenberg, Germany). The TAC was determined using a calibration curve based on uric acid standards. The results were expressed as µM copper reducing equivalents (CRE). Each test was repeated three times and the mean values (±SD) were calculated.

2.7. Determination of the antimicrobial activity of the crude extracts

The microorganisms were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The extracts were tested against the Gram-positive bacteria *Staphylococcus aureus* (ATCC 11632); Gram-negative bacteria, *Escherichia coli* (ATCC1056) and *Klebsiella pneumoniae* (ATCC 13883) using the agar disk diffusion method as per the Clinical and Laboratory Standards Institute [18]. Briefly, the test microorganisms were grown in nutrient broth medium until 1 X 10^8 colony-forming units were attained and then used to inoculate sterile 90 mm Petri dishes filled with nutrient agar medium using the spread-plate technique. Dried and sterile filter paper discs (6.0 mm diameter) were impregnated with 40 µl of the extracts (containing 500 µg fungal extracts), air dried

under the laminar airflow hood and placed on test microorganism inoculated plates. The plates were incubated at 37 ^{O}C for 24 hours and three sets of controls were used. One control was the organism control and consisted of a seeded Petri dish with no sample. In the second control, samples were introduced to the unseeded Petri dishes to check for sterility. Disks impregnated with 40 µl DMSO were run simultaneously as a third control. Standard antibiotics were also run simultaneously as reference agents to understand the comparative antimicrobial efficacy. The antimicrobial potency of the extracts was measured by their ability to prevent the growth of the microorganisms surrounding the discs.

2.8. FT-IR analysis

Infrared spectra were collected on a Nicolet 6700 FT-IR spectrometer (ThermoScientific, Madison, WI USA) equipped with a diamond crystal attenuated total reflectance sampling accessory (ThermoScientific, Madison, WI USA). A few grams of the samples were placed on the ATR and scanned from 500 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. Each recorded spectrum was the result of 36 co-added scans. The FT-IR was performed to predict the presence of various functional groups in the isolates.

2.9. Statistical analysis

The data was statistically analyzed using Microsoft Excel 2013 (Microsoft, USA). Pearson correlation was used to analyze the relationship between total phenolic content and total antioxidant activity. Results are expressed as means ±standard deviation.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of endophytic fungal strains

A total of eight endophytic fungi strains were isolated from *A. senegalensis* and the endophytic fungi colonization rate was 100%. The isolated strains were identified (Table **2**) and all belong to the phylum Ascomycota.

Table 4: Endophytic fungi isolated from the stems and leaves of *A. senegalensis*

Isolate Code*	Sequence similarity with	% sequence similarity	Accession number
ML1	*Phoma* species	99	HQ630999
ML2	*Epicoccum nigrum*	100	KY318481
ML3A	*Epicoccum sorghinum*	99	KY454467
ML3B	*Phoma* species	99	KM259932
ML4	*Alternaria tenuissima,*	100	MF055666
MS1	*Alternaria alternata*	100	KU997022
MS2	*Phaeosphaeria* species	96	HQ631018
MS3	*Penicillium chloroleucon,*	99	KP016813

**MS = endophyte isolated from stem tissues, ML=endophyte isolated from leaf tissues*

The observation that the isolated endophytic fungi belong to the phylum Ascomycota agrees with results from other studies which have shown that endophytic fungi are dominated by different lineages of Ascomycota [19, 20, 21]. To the best of our knowledge, this is the first study to report on the endophyte colonization rate and diversity in *A. senegalensis* and hence there was no data from similar work that we could use for comparison.

3.2. Determination of total phenolic compounds and total antioxidant capacity

Epicoccum nigrum (ML2), *Epicoccum sorghinum* (ML3A) and *Phoma* species (ML3B) were the endophytic fungi that were investigated for their total phenolic content and total antioxidant capacity. Our total phenolic content determination results (Table **3)** showed that there was variation in the total phenolic content of the endophytic fungi extracts which ranged from 5.39 to 28.85 mg gallic acid equivalent (GAE) g^{-1} of dry weight. All the studied extracts at a concentration of 1 mg ml^{-1} had some antioxidant activity that ranged from 223.90 to 593.46 µM copper reducing equivalent (CRE).

Extracts obtained from the endophytic fungi *Epicoccum sorghinum* (ML3A) exhibited both the highest TPC and TAC and there was a strong positive correlation (r = 0.9556) between total phenolic content and total antioxidant capacity for the studied endophytic fungi.

Table 5. Total phenolic content and total antioxidant capacity of endophytic fungi ethyl acetate extracts. *

Isolate	Isolate code	Total Phenolic Content (TPC) mg $GAEg^{-1}$ Extract	Total Antioxidant Capacity (TAC) 1mg ml^{-1} CRE^{b} (µM)
Epicoccum sorghinum	ML3A	28.85 ± 1.14	593.46 ± 1.86
Epicoccum nigrum	ML2	5.39 ± 0.29	223.90 ± 1.77
***Phoma* species**	ML3B	8.01 ± 0.39	369.26 ± 2.40

**Results are represented as means ± standard deviation (n = 3). [a]Gallic acid equivalent; [b]Copper reducing equivalent*

The strong positive linear correlation shown between total phenolic content and total antioxidant activity supports observations made in other studies which also showed strong correlation between total phenolic content and total antioxidant activity in fungal endophyte and plant extracts [7, 4]. The findings suggest that the phenolic content had a significant influence on the antioxidant activity of these crude extracts as phenolic compounds have redox properties and are antioxidants due to their hydroxyl groups which confer to them their free radical scavenging ability [22, 23]. Plant extracts obtained from *A. senegalensis* have been reported to have antioxidant activity [24] and some endophytes have been shown to produce one or more bioactive compounds originally from their host plants [25, 26]. Therefore, it would be interesting to identify the compounds responsible for the antioxidant activity by the endophytic fungi extracts and compare them with those responsible for the antioxidant activity by the host plant extracts. Further work is planned to determine the antioxidant activity of the extracts from the other fungi that were not investigated for antioxidant activity during this preliminary study.

3.3. Determination of antimicrobial activity

All the extracts did not exhibit antibacterial activity against the test microorganisms: *E. coli* (ATCC1056), *K. pneumoniae* (ATCC 13883) and *S. aureus* (ATCC11632) as well as strains of the same bacteria obtained from clinical samples. The preliminary results obtained for the antimicrobial

activity assays suggests that the studied endophytic fungi do not have potential as sources of new antimicrobial agents against the bacterial species assessed. The results obtained from this study, whilst not confirming activity support the findings from other studies that not all endophytic fungi have antibacterial activity [27]. However, before these endophytic fungi are totally discounted as potential sources of new antimicrobial agents, there is a need to explore their antibacterial activity against other test microorganisms and screen them for antifungal activity.

3.4. Fourier transform infra-red (FT-IR) analysis

FT-IR spectral analysis of the extracts from *Epicoccum nigrum* (ML2), *Epicoccum sorghinum* (ML3A) and *Phoma* species (ML3B) revealed the occurrence of multiple functional groups. Spectral data of the extracts confirmed the presence of functional groups such as -OH, -CHO, -COOH and –COOR [28]. Important infra-red absorption frequencies obtained from the extracts are tabulated in Table **4**.

The functional groups present in an extract influence the biological activity of the extract and hence functional group analysis plays an important role in understanding the physicochemical properties of an extract and helps to evaluate the structure-activity relationship of an extract [29]. In the present study, the FT-IR spectral analysis of the crude ethyl acetate extracts of the studied endophytes revealed the presence of molecules carrying a bonded hydroxyl (–OH) functional group. It is well established that the hydroxyl group is an integral part of most of the phenolic compounds such as flavonoids and tannins [30]. The FT-IR results therefore also served to provide supporting evidence to the observations made in the total phenolic content analysis. Furthermore, the FT-IR spectra analysis suggests that diverse groups of functional groups (and hence diverse classes of compounds) are potentially present in the extracts. This is in line with previous findings by other researchers who have shown that endophytic fungi produce diverse classes of metabolites [31, 32, 33]. It is important to note that whilst the observed different functional groups reflect the complex nature of the metabolites in the crude ethyl acetate extracts, there is a need to carry out further investigations to determine and identify the individual metabolites present in the extracts.

Table 4. Major bands observed in the FT-IR spectra of the of the five ethyl acetate extracts

Wave number (cm^{-1})	**Vibration band/group**	**Probable Compound Classes**
3570-3200	Hydrogen-bonded O-H stretch	Phenols, alcohols
	N-H stretch	Amine, amides
3000-2800	H-C-H asymmetric and symmetric stretch	Saturated aliphatic
	O–H stretch	Carboxylic acid
1820-1680	C=O stretch	Carbonyls, lactones
	Dialkyl/aryl sulphones	Sulphur-oxy compounds
1600-1550	C-C=C symmetric stretch	Aromatics
	-C=N-	Thiols and thio-substituted compounds
	C=O	Carbonyls
	N-H bend	Amines
	Nitrogen-oxy	Aromatic nitro compounds
1500-1450	C-C=C asymmetric stretch	Aromatics
1420-1300	C=O	Carbonyls
	Aliphatic nitro compounds	Hetero-oxy compounds
	Dialkyl/aryl sulphones	Sulphur-oxy compounds
1250-1000	C-O stretch	Alcohols, Phenols
	C-N	Aliphatic amines
	N-O	Aromatic amine oxide
	C=S	Thiocarbonyl
	Φ-O-H	Aromatic ethers

CONCLUSION

The results of this preliminary study suggest that the assessed fungal endophytes of *A. senegalensis* have potential as sources of natural antioxidants. However, further research is required to isolate and identify the bioactive molecules from the crude extracts and evaluate *in vivo* the biological activities of the isolated compounds. Furthermore, there is also a need to screen the other fungal endophyte isolates for their antioxidant activity. To the best of our knowledge, this is the first reported work on the antioxidant activities of fungal endophytes isolated from *A. senegalensis.*

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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In Chapter 3, the endophytic fungi isolated from *A. senegalensis* were isolated, identified and screened for their antioxidant and antimicrobial potential and to the best of our knowledge is the first study to present data on the antioxidant potential of endophytic fungi isolated from *A. senegalensis*. The metabolite extraction method used has an impact on the on molecules that are extracted and the type of plant tissue from which endophyte is extracted from also influences the bioactivity of endophytic fungi. Chapter 4 therefore presents a study using fungal endophytes isolated from *V. payos* where the metabolite solvent extraction method is varied to determine if the antioxidant and antimicrobial activity in *V. payos* is due to extracellular or intracellular metabolites. The chapter covers all the objectives of the study.

CHAPTER 4: ENDOPHYTIC FUNGI FROM *VITEX PAYOS*: IDENTIFICATION AND BIOACTIVITY ASSESSMENT

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Endophytic fungi from *Vitex payos*: identification and bioactivity assessment

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Abstract

Endophytic fungi isolated from medicinal plants have an important role to play in the search for new bioactive natural compounds; however, despite their potential as repositories of bioactive compounds the endophytes of African medicinal plants are largely underexplored. The aim of this study was to isolate and identify the endophytic fungi associated with *Vitex payos* and evaluate their antimicrobial and antioxidant potential. The surface sterilization technique was used to isolate the endophytic fungi that were identified by rDNA sequencing of the ITS region. Crude methanol and ethyl acetate extracts were screened for antimicrobial activity using the agar diffusion method and evaluated for antioxidant activity using a commercial total antioxidant capacity assay kit. The total phenolic content of the extracts was determined using the Folin-Ciocalteu method and functional groups present in the extracts were predicted using Fourier-transform infrared spectroscopy. Seven endophytic fungi isolates identified as *Glomerella acutata*, *Epicoccum nigrum*, *Diaporthe* species, *Penicillium chloroleucon*, *Diaporthe endophytica*, *Mucor circinelloides* and *Epicoccum nigrum* were isolated from the tissues of *Vitex payos*. All the extracts did not exhibit antimicrobial activity and the crude ethyl acetate extract obtained from *Epicoccum nigrum* demonstrated both the highest total phenolic content (2.97±0.13 mg GAE g^{-1} dry weight) and total antioxidant capacity (231.23 ± 2.03 μM CRE). The Fourier-transform infrared spectral analysis of the crude extracts from *Epicoccum nigrum* confirmed the presence of molecules carrying bonded hydroxyl functional group characteristic of phenolic compounds. The preliminary results indicate that most of the isolated fungal endophytes from

Vitex payos belong to the phylum Ascomycota and that the isolated strain of *Epicoccum nigrum* has potential as a source of natural antioxidants.

Keywords

Vitex payos; endophytic fungi; antimicrobial; antioxidant, bioprospecting

Introduction

There is heighted interest in the bioprospecting for natural compounds with potential use as therapeutics due to several factors that include the rapid development of anti-microbial resistant pathogenic microbes and emergence of new life-threatening diseases [1, 2]. Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease [3]. Endophytic fungi have been shown to produce a broad variety of bioactive secondary metabolites and bioprospection of endophytes is considered a new frontier in the search for natural products with potential agricultural, pharmaceutical and industrial applications [4, 5]. Medicinal plants are rich sources of bioactive natural compounds and studies have shown that some medicinal properties of these plants may be related to the endophytic fungi that they host [2]. The endophytic fungi may participate in some of the plant metabolic pathways or may gain some genetic information to produce specific biologically active compound like those produced by the host plant [6]. Therefore, endophytic fungi isolated from medicinal plants have an important role to play in the search for new bioactive natural compounds. Despite this potential of medicinal plants and the rich plant biodiversity in Africa, only a tiny fraction of African medicinal plant species have been studied with regards to their endophytic fungi diversity. *Vitex payos* (*V. payos*) is an African ethnomedicinal plant used to treat several ailments in Zimbabwean traditional medicine [7]. As part of our contribution to the ongoing efforts to understand the diversity of endophytic fungi we isolated and identified the endophytic fungi found in the leaf and stem tissues of *V. payos*. Furthermore, we determined the antibacterial activity and antioxidant potential for some of the identified endophytic fungi strains. In addition, Fourier transform infra-red (FT-IR) spectroscopy was used predict the presence of various functional groups in the endophytic fungi crude extracts.

Materials and methods

Collection and identification of plant materials

Fresh leaf and stem tissue of Endophytic fungi were isolated from leaf tissue samples (5 samples) and stem tissue samples (2 samples) obtained from a single *V. payos* plant grown under natural (wild) conditions at the National Herbarium and Botanical Gardens (Harare, Zimbabwe). The tissue samples were pretreated by cleaning them under running water to remove dirt and soil particles and were then air-dried to remove any surface moisture before they were packaged into labeled sterile sample collecting bags. The samples were then transported to the laboratory and stored at 4 °C until endophyte isolation procedures could be instituted [8].

Isolation and establishment of in vitro culture of endophytes

Endophytic fungi were isolated from the leaf and stem tissues collected from the medicinal plant using a modified surface sterilization procedure as described by Kjer *et al*. 2010 [9]. The plant tissue samples were removed from storage and thawed by washing them using running tap water. The thawed plant tissue samples were then washed using 0.1% (v/v) Tween 80 for 15 minutes followed by another wash for 1 hour 30 minutes using running water. The cleaned plant tissues were then transferred to a laminar airflow cabinet where the surface sterilization and endophyte isolation was conducted under aseptic conditions. The plant tissue samples were cut into 5 cm segments and were surface sterilized with 70% ethanol: 30 seconds for leaf samples and 2 minutes for stem samples, soaked in 2% NaOCl solution for 15 minutes, rinsed four times with sterile double distilled water and finally blot dried using sterile paper towels. The effectiveness of the sterilization procedure was tested by plating 0.1 ml of the final sterile water rinse onto Petri dishes containing potato dextrose agar (PDA) and by rolling the sterilized sample onto Petri dishes containing PDA. The surface sterilized tissues were cut into smaller segments (1-2 cm) using sterile razor blades and placed onto Petri dishes containing PDA supplemented with 200U ml^{-1} Penicillin-Streptomycin (Lonza, Cat # 17-602E) and incubated at 28±2 °C until fungal growth was initiated. The fungal mycelia growing out of the sample segments were subcultured and maintained on Petri dishes containing PDA.

Identification of the endophytic fungi

Genomic DNA was extracted from the cultures using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using EconoTaq® PLUS GREEN 2X Master Mix (Lucigen) with the primers using the universal primers ITS1 and ITS4.

The PCR reactions were carried out under the following conditions: Initial denaturation at 95^{o}C for 15 minutes, 35 cycles at 95^{o}C (denaturation) for 1 minute, 56^{o}C (annealing) for 30 seconds, 72^{o}C (extension) for 1 minute and then a final extension for 10 minutes at 72^{o}C. The PCR products were run on a gel and gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse directions (Applied Biosystems, ThermoFisher Scientific, Big Dye terminator kit v3.1) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were run on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). CLC Bio Main Workbench v7.6 was used to analyze the .ab1 files generated by the ABI 3500XL Genetic Analyzer and endophytic fungi isolates were identified on the basis of similarity of amplified sequence with those found in the US National Centre for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (nBLAST).

Secondary metabolite extraction

The small-scale fermentations and solvent extractions were carried as described by Kjer *et al*. 2010 with modifications [9]. Briefly, the fungi were cultivated in 500 mL of potato dextrose broth and were incubated at 28±2°C for 30 days in a shaker at 180 rev min^{-1}. The fungal mycelia were then separated from the culture broth by filtration and extracted with analytical grade methanol (MeOH) solvent 1:10. The filtrate was then extracted three times at the liquid-liquid partition with analytical grade ethyl acetate (EtOAc) solvent 1:1 (v/v). The resulting crude extracts were collected and concentrated to dryness in a vacuum rotary evaporator at 40–45°C, then dissolved into 1 mL of MeOH solvent, followed by drying under vacuum to obtain the EtOAc and MeOH extracts. The crude extracts were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 1mg ml^{-1} and kept at 4°C [11].

Determination of the antimicrobial activity of the crude extracts

The microorganisms were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The extracts were tested against the Gram-positive bacteria *Staphylococcus aureus* (ATCC 11632); Gram-negative bacteria, *Escherichia coli* (ATCC1056) and *Klebsiella pneumoniae* (ATCC 13883) using the agar disk diffusion method per the Clinical and Laboratory Standards Institute [12]. Briefly, the test microorganisms were grown in nutrient

broth medium until 1 X 10^8 colony-forming units were attained and then used to inoculate sterile 90 mm Petri dishes filled with nutrient agar medium using the spread-plate technique. Dried and sterile filter paper discs (6.0 mm diameter) were impregnated with 40 µl of the extracts (containing 500 µg fungal extracts), air dried under aseptic conditions in a laminar airflow cabinet and placed on test microorganism inoculated plates. The plates were incubated at 37°C for 24 hours and three sets of controls were used. One control was the organism control and consisted of a seeded Petri dish with no sample. In the second control, samples were introduced to the unseeded Petri dishes to check for sterility. Disks impregnated with 40 µl DMSO were run simultaneously as a third control. Standard antibiotics were also run simultaneously as reference agents to understand the comparative antimicrobial efficacy. Three replicates per extract were used for the antimicrobial activity assays. The antimicrobial potency of the extracts was measured by their ability to prevent the growth of the microorganisms surrounding the discs.

Determination of total phenolic content of the crude extracts

The total phenolic content of the extracts was determined by the Folin–Ciocalteu method. Briefly, 0.2 mL of the different crude extract (dissolved in DMSO at a concentration of 1 mg ml^{-1}) were added to 2.8 mL of distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 minutes, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was left to stand for a further 60 minutes in the dark and absorbance was measured at 650 nm (Lasany I-290, Panchkula, India). The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight. Three replicates per extract were used for the determination of total phenolic content and the mean values (±SD) were calculated.

Determination of the antioxidant activity of the crude extracts

The total antioxidant capacity (TAC) of the endophytic fungal extracts at 1mg ml^{-1} concentration was determined using the OxiSelect™ Total Antioxidant Capacity Assay Kit (Cell Biolabs, Inc, San Diego, USA), per the manufacturer's instructions. The method is based on the single electron transfer (SET) mechanism and involves the reduction of Cu^{2+} to Cu^{+} by the endogenous antioxidants and by other reducing equivalents in the sample. The Cu^{+} interacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. The absorbance value is proportional to the total antioxidant (respectively reducing) capacity of

extracts. The samples were analyzed spectrophotometrically at 490 nm using the microplate reader SPECTROstar^{Nano} (BMG LABTECH, Ortenberg, Germany). The TAC was determined using a calibration curve based on uric acid standards. The results were expressed as μM copper reducing equivalents (CRE). Three replicates per extract were used for the determination of the antioxidant activity of the crude extracts and the mean values (±SD) were calculated.

FT-IR analysis

Infrared spectra were collected on a Nicolet 6700 FT-IR spectrometer (ThermoScientific, Madison, WI USA) equipped with a diamond crystal attenuated total reflectance sampling accessory (ThermoScientific, Madison, WI USA). A few grams of the samples were placed on the ATR and scanned from 500 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. Each recorded spectrum was the result of 36 co-added scans. The FT-IR was performed to predict the presence of various functional groups in the isolates.

Data analysis

The colonization rate of endophytes was determined as the total number of segments yielding ≥ 1 isolates divided by the total number of segments from which endophytes were isolated (expressed as a percentage). The results for the antimicrobial and antioxidant activity and total phenolic content determination assays are expressed as the mean ± SD of triplicates (n = 3). Difference analysis (one-way ANOVA) of the antioxidant activity results was conducted using Microsoft Excel 2013 (Microsoft, USA). The 'P value' found to be < 0.05, was considered as significant.

Results

Isolation of endophytic fungi

A total of seven endophytic strains were isolated from *V. payos* and the endophytic colonization rate was 100%. The isolated strains were identified (Table 1) and were all found to belong to the phylum Ascomycota except for *Mucor circinelloides* which is a member of the phylum Zygomycota.

Table 1: Endophytic fungi isolated from the stems and leaves of *V. payos*

Isolate Code*	Sequence similarity with	% sequence similarity	Accession number
TL1	*Glomerella acutata*	99	AM991137
TL2	*Epicoccum nigrum*	99	KX869952
TL3	*Diaporthe sp.*	98	KU671340
TL4	*Penicillium chloroleucon*	99	KP016813.
TL5	*Diaporthe endophytica*	96	AB899789
TS1	*Mucor circinelloides*	100	KC461495
TS2	*Epicoccum nigrum*	99	KX869952

**TL= endophyte isolated from leaf tissues, TS=endophyte isolated from stem tissues*

Antimicrobial activity

All the extracts did not exhibit antibacterial activity against the test microorganisms: *E. coli* (ATCC1056), *K. pneumoniae* (ATCC 13883) and *S. aureus* (ATCC11632) as well as strains of the same bacteria obtained from clinical samples.

Antioxidant capacity and total phenolic content

In the present study, the antioxidant activity of ethyl acetate and methanol extracts of two representative isolates obtained from stem (*Epicoccum nigrum*, isolate TS2) and leaf (*Diaporthe* species, isolate TL3) was determined. The ethyl acetate and methanol extracts of the endophytic fungi *Epicoccum nigrum* both exhibited antioxidant activity whilst for the endophytic fungi *Diaporthe* species only the methanol extract showed some antioxidant activity (Table 2). Data analysis revealed that whilst significant differences [$F(2, 6) = 8429.77$, $p < 0.00001$ at the 0.05 alpha level] were observed in the antioxidant activities of the studied extracts, there was no significant differences [$F(1, 4) = 5.495$, $p = 0.07902$ at the 0.05 alpha level] in the total phenolic content between the ethyl acetate extract from *Epicoccum nigrum* and the methanol extract from *Diaporthe* species.

Table 2: Total phenolic content and antioxidant capacity of extracts obtained from *Epicoccum nigrum* and *Diaporthe* species*

Endophyte	Isolate code	Total Phenolic Content (TPC)	Total Antioxidant Capacity (TAC) 1mg/ml
		mg GAE/g Extract[a]	CRE[b] (μM)
Ethyl acetate extracts			
Epicoccum nigrum	TS2	2.97±0.13	231.23±2.03
Methanol extracts			
Epicoccum nigrum	TS2	0.50±0.01	32.28±2.96
Diaporthe species	TL3	2.76±0.07	60.97±2.53

**Results are represented as means ± standard deviation (n = 3). [a]Gallic acid equivalent; [b]Copper reducing equivalent*

Fourier transform infra-red (FT-IR) analysis

The ethyl acetate extracts from *Epicoccum nigrum* which exhibited both the highest antioxidant and total phenolic content were further analyzed using FT-IR spectroscopy. FT-IR spectroscopy is a well-established tool for the characterization and identification of functional groups present extracts. The FT-IR spectral analysis of the ethyl acetate extract of *Epicoccum nigrum* revealed the presence of occurrence of multiple functional groups such as -OH, -CHO, -COOH and –COOR [13]. Important IR absorption frequencies obtained from the extracts are tabulated in Table 4.

Table 4. Major bands observed in the FT-IR spectra of the *Epicoccum nigrum* acetate extracts

Wave number (cm^{-1})	Vibration band/group	Probable Compound Classes
3600-3300	Hydrogen-bonded O-H stretch	Phenols, alcohols
3000-2500	H-C-H asymmetric and symmetric stretch	Saturated aliphatic
	O–H stretch	Carboxylic acid
	S-H stretch	Thiols
1820-1680	C=O stretch	Carbonyls, lactones
1600-1550	C-C=C symmetric stretch	Aromatics
	-C=N-	Thiols and thio-substituted compounds
	C=O	Carbonyls
	N-H bend	Amines
	Nitrogen-oxy	Aromatic nitro compounds
1500-1450	C-C=C asymmetric stretch	Aromatics
1420-1300	C=O	Carbonyls

	Aliphatic nitro compounds	Hetero-oxy compounds
	Dialkyl/aryl sulphones	Sulphur-oxy compounds
1250-1000	C-O stretch	Alcohols, Phenols
	C-N	Aliphatic amines
	N-O	Aromatic amine oxide
	C=S	Thiocarbonyl
	Φ-O-H	Aromatic ethers

Discussion

The observation that most of the isolated endophytic fungi from this study belong to genera that are found under the phylum Ascomycota tallies with observations from other studies which show that to date most of the isolated endophytic fungi are dominated by different lineages of Ascomycota [14, 15, 16]. It is important to note that all the genera of endophytic fungi isolated from *V. payos* in this study have previously been isolated from a wide range of other different plant hosts in diverse environments and this suggests that these genera are not host and environment specific.

The obtained preliminary antimicrobial activity results suggest that the studied endophytic fungi do not have potential as sources of new antimicrobial agents against the assessed bacterial species. Whilst this is disappointing, the results support the findings from other studies that not all endophytic fungi strains have antibacterial activity [17]. However, there is a need to assess the antibacterial activity of these strains against other test microorganisms; screen them for antifungal activity and vary the fermentation conditions before we totally discount these strains as potential sources of new antimicrobial agents.

The ethyl acetate extract of the endophytic fungi *Epicoccum nigrum* exhibited both the highest antioxidant activity and total phenolic content. Endophytic and marine derived strains of *Epicoccum nigrum* have been shown to have antioxidant activity [18, 19]. This suggests that this species might be generally predisposed to produce natural antioxidants. Phenolic compounds have redox properties and are antioxidants due to their hydroxyl groups which confer to them their free radical scavenging ability [20, 21]. The total phenolic content and antioxidant activities results obtained for the extracts obtained from the stem isolate of *Epicoccum nigrum* (TS2) suggests that phenolic content influenced antioxidant activity, however there is a need for further studies to confirm this observation. Endophytic species of the genus *Diaporthe* have been reported to have antioxidant activity and our results for the methanol extract of our *Diaporthe* leaf isolate tally with those observations.

Biological activity of extracts is influenced by the functional groups present in the extracts and hence functional group analysis plays an important role in understanding the biological activity of extracts [22]. In the present study, the FT-IR spectral analysis of the *Epicoccum nigrum* crude ethyl acetate extracts revealed the presence of molecules carrying the bonded hydroxyl (–OH) functional group. The hydroxyl group is an integral part of most of the phenolic compounds such as flavonoids and tannins and therefore, the FT-IR results serve to provide further supporting evidence for the presence of phenolic compounds in the crude ethyl acetate extracts. The FT-IR spectra analysis also suggests that diverse groups of functional groups (and hence diverse classes of compounds) are potentially present in the extracts. This is in line with previous findings by other researchers who have shown that endophytic fungi produce diverse classes of metabolites [23, 24, 25]. However, it is important to note that further investigations using different techniques are required to identify the compounds present in the *Epicoccum nigrum* crude ethyl acetate extracts. To the best of our knowledge, this is the first reported work on the identification, antibacterial and antioxidant activities of fungal endophytes isolated from *V. payos.* The results of this study suggest that whilst fungal endophyte isolates obtained from *V. payos* have potential as sources of natural antioxidants, further research is required to isolate and identify the bioactive molecules from the crude extracts and evaluate *in vivo* their biological activities.

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Authors' contribution

T.M and M.M were the project leaders; E.S was responsible for the experimental design and data collection; T.C and A.T.N made conceptual contributions; T.M and E.S wrote the manuscript.

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The manuscript in Chapter 4 suggests that metabolites with antioxidant potential are produced either as intracellular or extracelleular metabolites by different endophytic fungi and that endophytic fungi from *V. payos* are potential sources of natural antioxidants. However, none of the endophytes displayed antimicrobial activity and the metabolites produced by the endophytes were not identified. This motivated further research on endophytes isolated from *Kigelia africana* and *Warburgia salutaris* in the search for endophytic fungi with antimicrobial activity and also identification of metabolites produced by the endophytes. The chapter covers all the objectives of the study.

CHAPTER 5: ENDOPHYTIC FUNGI ISOLATED FROM THE MEDICINAL PLANTS KIGELIA AFRICANA AND WARBURGIA SALUTARIS.

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Endophytic fungi isolated from the medicinal plants *Kigelia africana* and *Warburgia salutaris*

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Abstract

Background: Medicinal plants play an important role in the search for bioactive strains of endophytic fungi. **Objective:** To isolate and identify the endophytic fungi associated with *Kigelia africana* and *Warburgia salutaris* and evaluate their bioactivity. **Methods:** Endophytic fungi were isolated from the stem and leaf tissues of *Kigelia africana* and *Warburgia salutaris* and identified by rDNA sequencing of the ITS region. Crude extracts were screened for antimicrobial activity using the agar diffusion method and evaluated for total antioxidant activity using a commercial kit. The metabolites present in the extracts were predicted using Gas Chromatography – Mass Spectrometry (GC-MS). **Results:** Eleven endophytic fungi identified as *Penicillium chloroleucon*, *Alternaria solani*, *Cryptococcus cf. taibaiensis*, *Coprinellus micaceus*, *Vishniacozyma victoriae*, *Alternaria alternata* and *Cystobasidium larynges* were isolated from *Warburgia salutaris.* Seven more endophytic fungi identified as *Penicillium chloroleucon*, *Myrothecium gramineum*, *Phomopsis* sp., *Alternaria brassicae*, *Cercospora chrysanthemi*, *Cladosporium uredinicola*, and *Aureobasidium leucospermi* were isolated from *Kigelia africana.* The isolate *Cladosporium uredinicola* from *Kigelia africana* was shown to have both antioxidant (175.42±2.53 µM copper reducimg equivalent) and antimicrobial activity (against *E.coli* ATCC1056). The main constituents in the crude ethyl acetate extract of the isolate *Vishniacozyma victoriae* from *Warburgia salutaris* were identified as N- hydroxymethylacetamide (33.14%), 26-hydroxycholesterol (8.21%), formic acid, butyl ester (1.14%), cyclotetracosane (0.70%), 1-heptacosanol (0.66%), benzo [h] quinolone, 2, 4-dimethyl (0.57%), and pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(9-phenylmethyl) – (0.50%) through GC-MS spectra analysis. **Conclusion:** The isolate *Cladosporium uredinicola* has potential as a source of antimicrobial and

antioxidant bioactive compounds and the isolate *Vishniacozyma victoriae* produces a diverse range of metabolites.

Keywords: Antioxidant, Endophytic fungi, Bioprospecting, Antimicrobial, Bioactivity, GC-MS

1. INTRODUCTION

Natural products and their derivatives have been recognized for many years as a valuable source of therapeutic agents and structural diversity [1, 2, 3] However, the intrinsic difficulties associated with natural product-based drug discovery resulted in the pharmaceutical industry shifting its focus towards synthetic compound libraries and high-throughput screening (HTS) for discovery of new drug leads [4, 5].

Recently, there has been renewed interest in the bioprospecting for natural products with potential therapeutic applications due to the failure of alternative drug discovery methods to deliver lead compounds in therapeutic areas such as immunosuppression, anti-infectives and metabolic diseases [3].

Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease [6]. These microorganisms have been shown to produce a broad variety of bioactive secondary metabolites and are considered a new frontier for the discovery of useful natural products [7, 8]. The number of secondary metabolites produced by fungal endophytes is larger than that of any other group of endophytic microorganisms [8] and to date most microbes isolated as endophytes have been fungi [9]. Fungal metabolites have served as lead compounds for the development of anticancer, antifungal and antibacterial agents [10]. It is estimated that globally there might be up to 1.5 million species of endophytic fungi, the majority of which are yet to be identified and studied [11]. The estimation assumes that each individual higher plant (approximately 250 000 different plant species on earth) hosts an average of four endophytes [8].

Medicinal plants are rich sources of bioactive natural compounds and studies have shown that some medicinal properties of these plants may be related to the endophytic fungi that they host [12]. The endophytic fungi may participate in some of the plant metabolic pathways or may gain some genetic information to produce specific biologically active compound like those produced by the host plant [13]. Therefore, medicinal plants have an important role to play in the search for new strains of endophytic fungi, as it is possible that their beneficial characteristics are a result of the metabolites produced by their fungal endophytic community [14, 15]. Despite the potential

benefits that can be derived from fungal endophytes, only a tiny fraction of medicinal and other plant species have been investigated for their potential as repositories of endophytic fungi that produce bioactive compounds [7].

As part of our contribution to the ongoing efforts unravel the endophytic fungi diversity and search for novel antimicrobial and antioxidant agents from fungal endophytes we isolated and identified the endophytic fungi found in the leaf and stem tissues of *Kigelia africana* and *Warburgia salutaris*. We further investigated the antibacterial activity and total antioxidant potential of some endophytic fungi strains isolated from *Kigelia africana*. In addition, we screened the chemical composition of metabolites produced by the endophytes isolated from *Warburgia salutaris* using Gas chromatography-Mass spectrometry (GC-MS).

2. MATERIALS AND METHODS

2.1. Collection, identification of plant materials

Fresh leaf and stem tissues from mature and healthy *W. salutaris* and *K. africana* plants grown under natural (wild) conditions were obtained from the plant collection at the National Herbarium and Botanical Gardens (Harare, Zimbabwe). The tissue samples were pretreated by cleaning them under running water to remove dirt and soil particles and were then air-dried to remove any surface moisture before they were packaged into labeled sterile sample collecting bags. The samples were then transported to the laboratory and stored at 4 °C until endophyte isolation procedures could be conducted [16].

2.2. Isolation and establishment of in vitro culture of endophytes

A total of 17 plant tissue segments from *W. salutaris* and 10 plant tissue segments from *K. africana* were used for endophytic fungi isolation using a modified surface sterilization procedure as described by Kjer *et al*. (2010) [17]. Briefly, the plant tissue samples were removed from storage and thawed by briefly putting them under running tap water and then washed using 0.1% (v/v) Tween 80 for 15 minutes followed by another wash for 1 hour 30 minutes using running water. The cleaned plant tissues were then transferred to a laminar airflow cabinet. The plant tissue samples were dipped in 70% ethanol: 30 seconds for leaf samples and 2 minutes for stem samples. The ethanol was drained out after the required amount of time and the plant tissue was then washed using a 2% sodium hypochlorite solution for 15 minutes which was then followed by four washes using double distilled water after which the samples were dried using sterile paper towels. The effectiveness of the sterilization procedure was tested by plating 0.1 ml of the final

sterile water rinse onto Petri dishes containing potato dextrose agar (PDA) and by rolling the sterilized sample onto Petri dishes containing PDA. The surface sterilized tissues were cut into smaller segments (1-2 cm) using sterile razor blades and placed onto Petri dishes containing PDA supplemented with 200U ml^{-1} Penicillin-Streptomycin (Lonza, Cat # 17-602E) and incubated at 28±2 °C until fungal growth was initiated. The colonization rate of endophytic fungi was determined as the total number of segments yielding ≥ 1 isolate in a host sample divided by total number of segments incubated in that sample multiplied by 100. The fungal mycelia growing out of the sample segments were continuously subcultured and maintained on fresh PDA plates. The cycle of sub culturing was repeated until pure cultures of the isolates were obtained.

2.3. Identification of the endophytic fungi

Genomic DNA was extracted from the isolated endophytic fungi cultures using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using EconoTaq® PLUS GREEN 2X Master Mix (Lucigen) using the universal primers ITS1 and ITS4. The PCR reactions were carried out under the following conditions: Initial denaturation at 95ºC for 15 minutes, 35 cycles at 95ºC (denaturation) for 1 minute, 56ºC (annealing) for 30 seconds, 72ºC (extension) for 1 minute and then a final extension for 10 minutes at 72ºC. The PCR products were run on a gel and gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse directions (Applied Biosystems, ThermoFisher Scientific, Big Dye terminator kit v3.1) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were run on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). CLC Bio Main Workbench v7.6 was used to analyze the .ab1 files generated by the ABI 3500XL Genetic Analyzer and endophytic fungi isolates were identified on the basis of similarity of amplified sequence with those found in the US National Centre for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (nBLAST).

2.4. Secondary metabolite extraction

The small-scale fermentations and solvent extractions were carried as described by Kjer *et al*. 2010 with modifications [17]. Briefly, the fungi were cultivated in 500 mL of potato dextrose broth and were incubated at 28±2 °C for 30 days in a shaker at 180 rev min^{-1}. An equal volume of analytical grade ethyl acetate (EtOAc) was added to the fermentation broth and the mixture

was homogenized. Fungal mycelia were separated from the homogenised culture broth by filtration and the filtrate was then extracted three times with analytical grade EtOAc 1:1 (v/v). The resulting crude extracts were collected and concentrated to dryness in a vacuum rotary evaporator at 40–45^{O}C, then dissolved into 1 mL of methanol solvent, followed by drying under vacuum to obtain the EtOAc crude extracts. The dried crude extracts were weighed before being dissolved in dimethyl sulphoxide (DMSO) at a concentration of 1mg ml^{-1} and kept at 4°C [19].

2.5. Determination of total phenolic content of the crude extracts

The total phenolic content of the extract was determined by the Folin–Ciocalteu method. Briefly, 0.2 mL of the different crude extracts (dissolved in DMSO at a concentration of 1 mg ml^{-1}) were added to 2.8 mL of distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 minutes, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was left to stand for a further 60 minutes in the dark and absorbance was measured at 650 nm (Lasany I-290, Panchkula, India). The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight. Each test was repeated three times and the mean values (±SD) were calculated [19].

2.6. Determination of the antioxidant activity of the crude extracts

The total antioxidant capacity (TAC) of the endophytic fungal extracts dissolved in DMSO at a concentration of 1mg ml^{-1} was determined using the OxiSelect™ Total Antioxidant Capacity Assay Kit (Cell Biolabs, Inc, San Diego, USA), per the manufacturer's instructions. The method is based on the single electron transfer (SET) mechanism and involves the reduction of Cu^{2+} to Cu^{+} by the endogenous antioxidants and by other reducing equivalents in the sample. The Cu^{+} interacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. The absorbance value is proportional to the total antioxidant (respectively reducing) capacity of extracts. The samples were analyzed spectrophotometrically at 490 nm using the microplate reader SPECTROstar^{Nano} (BMG LABTECH, Ortenberg, Germany). The TAC was determined using a calibration curve based on uric acid standards. The results were expressed as μM copper reducing equivalents (CRE). Each test was repeated three times and the mean values (±SD) were calculated.

2.7. Determination of the antimicrobial activity of the crude extracts

The microorganisms were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The extracts were tested against the Gram-positive bacteria *Staphylococcus aureus* (ATCC 11632); Gram-negative bacteria, *Escherichia coli* (ATCC1056) and *Klebsiella pneumoniae* (ATCC 13883) using the agar disk diffusion method per the Clinical and Laboratory Standards Institute [21]. These microbes were selected for the study because antimicrobial resistant strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphlylococcus aureus* have become widespread and there is a need for new molecules to combat this resistance. Briefly, the test microorganisms were grown in nutrient broth medium until 1 X 10^8 colony-forming units were attained and then used to inoculate sterile 90 mm Petri dishes filled with nutrient agar medium using the spread-plate technique. Dried and sterile filter paper discs (6.0 mm diameter) were impregnated with 40 µl of the extracts (containing 500 µg of the crude fungal extracts), air dried under the laminar airflow hood and placed on test microorganism inoculated plates. The plates were incubated at 37 ^{O}C for 24 hours and three sets of controls were used. One control was the organism control and consisted of a seeded Petri dish with no sample. In the second control, samples were introduced to the unseeded Petri dishes to check for sterility. Disks impregnated with 40 µl DMSO were run simultaneously as a third control. Standard antibiotics were also run simultaneously as reference agents to understand the comparative antimicrobial efficacy. Three replicates per extract were used for the antimicrobial activity assays. The antimicrobial potency of the extracts was measured by their ability to prevent the growth of the microorganisms surrounding the discs.

2.8. GC-MS analysis

The crude ethyl acetate extracts of the fungi were subjected to GC-MS analysis for further chemical characterization of metabolites. GC-MS analysis was performed using an Agilent 7890A GC system coupled to an Agilent 5975C VL MSD mass spectrophotometer chromatograph equipped with an Agilent ion trap mass-spectrometer. Samples were separated using the Agilent 19091S - 433 column (30m x 250µm x 0.25µm). The gas chromatographic oven temperature was programmed from 50°C to 310°C at a rate of 5°C min-1and the run time was 54 minutes. Helium was used as the carrier gas. The inlet pressure was 25 kPa and the flow rate was 0.68099 mL min-1. The injector temperature was 275°C and the injection mode was splitless. The mass spectrometric scan conditions were as follows: source temperature, 230°C; interface temperature, 250°C and mass scan range, 10-550 amu. Interpretation on mass-spectrum GC-MS-

MS was conducted using the database of National Institute Standard and Technology (NIST) and the spectrum of the extyracts was compared with the spectrum of known compounds stored in the NIST library. The name, molecular weight and molecular formula of the extracts samples were ascertained based on the comparison with the known compounds.

2.9. Data analysis

Species richness of the isolated endophytic fungi was determined by calculating the Menhinick's index (D*mn*) using the equation: number of different endophytic species (s) divided by the square root of the total number of isolated endophytic fungi (N) from tissues obtained from one plant species.

$$\mathrm{D}mn = \frac{s}{\sqrt{N}}$$

The endophytic fungi diversity was quantified using the Shannon's diversity index (H′).

$$\mathrm{H}' = -\sum_{i} Pi\ln(Pi)$$

The data was analyzed using Microsoft Excel 2013 (Microsoft, USA) to calculate the index values and perform the Hutcheson t-test. Where applicable, results are expressed as means ±standard deviation.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of endophytic fungal strains

A total of ten endophytic fungi strains were isolated from *W. salutaris* whilst seven strains were isolated from *K. africana*. The endophytic fungi colonization rate was 65% in *W. salutaris* and 70% in *K. africana*. The isolated strains were identified (Table **2**) and it is important to note that in *K. africana* all the isolates belonged to the phylum Ascomycota whilst in *W. salutaris* in addition to genera found in the phylum Ascomycota, genera found in the phylum Basidiomycota (*Cryptococcus*, *Coprinellu*s and *Vishniacozyma*) were also isolated.

Table 1: Endophytic fungi isolated from the stems and leaves of *W. salutaris and K. africana*

Isolate Code	**Closest blast match (GeneBank accession number)**	**% sequence similarity**
W. salutaris*		
WS1	*Penicillium chloroleucon (KP016813)*	100
WS2	*Alternaria solani (LN879928)*	96
WS3	*Cryptococcus cf. taibaiensis (KM246154)*	97
WS4	*Coprinellus micaceus (KY548384)*	100
WS5	*Vishniacozyma victoriae (LC203739)*	89
WS6	*Penicillium chloroleucon (KP016813)*	100
WS7	*Alternaria alternata (KX078456)*	83
WS9	*Alternaria alternata (KY814634)*	100
WL1	*Cystobasidium larynges (AF190014)*	99
WL3	*Coprinellus micaceus (KY548384)*	100
WL6	*Epicoccum nigrum (KX869965)*	98
K. africana*		
SS1	*Penicillium chloroleucon (KP016813)*	99
SS4	*Myrothecium gramineum (KJ780796)*	99
SS5	*Phomopsis sp. (AB302247)*	96
SL2	*Alternaria brassicae (KP641144)*	99
SL3	*Cercospora chrysanthemi (KJ696542)*	99
SL4	*Cladosporium uredinicola (KC876518)*	98
SL5	*Aureobasidium leucospermi (JN712487)*	100

WS = endophyte isolated from stem tissues, WL=endophyte isolated from leaf tissues

****SS= endophyte isolated from stem tissues, SL=endophyte isolated from leaf tissues***

The observation that the majority isolated endophytic fungi belong to the phylum Ascomycota tallies with results from other studies which have shown that endophytic fungi are dominated by different lineages of Ascomycota [22, 23, 24].

Table 2: Diversity indices of endophytic fungi isolated from *K. africana* and *W. salutaris*

	K. africana	***W. salutaris***
Shannon diversity (H')	1.946	2.02
Menhinick's species richness (D*mn*)	2.65	2.41

The Shannon diversity index (H') values obtained for the plant species were compared using the Hutcheson t-test and the obtained *p* value (p=0.8192) was greater than 0.05 implying that the difference in diversity between the endophytes obtained from the two plants was not statistically significant. However, there is a need to undertake further research using a larger sample size and plant species grown in different environments to validate the obtained preliminary diversity results.

The species richness determined by calculating the Menhinick's index (D*mn*) suggests that plant tissue samples obtained from *K. africana* were richer in endophytic fungal species (D*mn* 2.65) compared to those obtained from *W. salutaris.*

To the best of our knowledge, this is the first study to report on the endophyte colonization rate and diversity in *W. salutaris*. Several studies have been conducted to determine the endophytic fungi communities present in *K. africana* and similarly as in our study all the isolated endophytic fungi were members of the phylum Ascomycota [25, 26; 27]. A comparison of the reported endophytic fungi genera isolated from *K. africana* further reveals that *Cladosporium* species have also been previously reported as being endophytic fungi of the plant species [25].

3.2. Bioactivity potential

Preliminary screening of the endophytes from *K. africana* revealed that the ethyl acetate extracts of *Cladosporium uredinicola* had the highest total antioxidant activity at 1mg ml^{-1} (175.42±2.53 µM copper reducimg equivalent). The ethyl acetate extracts from *Cladosporium uredinicola* were further analysed for total phenolic content and were found to have total phenolic content of 3.41±0.08 gallic acid equivalent per gram of extract (GAE g^{-1} extract**).** To the best of our knowledge, this study represents the first attempt to investigate the antioxidant potential of

endophytes isolated from *K. africana* and thus it provides a basis for further bioprospecting for antioxidant activity from endophytic fungi isolated from this plant.

Furthermore, the crude ethyl acetate extracts of the isolates *Cladosporium uredinicola* and *Myrothecium gramineum* were found to have an inhibitory effect on the test microorganism *E. coli*. Their zones of inhibition at a concentration 0.1 mg ml^{-1}were 20±0.25 mm and 22±0.5 mm respectively suggesting that the endophytic fungi have potential as sources of natural antimicrobials. Fungal endophytes isolated from *K. africana* have previously been reported to have varying levels of antimicrobial activity [25, 26]. Therefore, our results and the previous findings taken together suggest that fungal endophytes isolated from *K. africana* have potential as sources of antimicrobials and there is a need to continue bioprospecting for antimicrobials within endophytic fungi communities isolated from the plant species. Further work is planned to identify the bioactive molecules responsible for the antioxidant and antimicrobial activities from the endoophytic fungi.

3.3. GC-MS analysis

Metabolite profiling of the ethyl acetate extracts of the endophytic fungi *Vishniacozyma victoriae* isolated from *W. salutaris* was conducted using GC-MS. This particular endophyte was selected on the basis that its genera is not commonly isolated as the isolation of a novel taxonomy of an endophyte or the acquisition of one that is only rarely seen enhances the prospects for also discovering novel bioactive natural products [28]. Analysis of the GC-MS spectra of the crude ethyl acetate extracts predicted the presence of many metabolites and the main predicted compounds were N-hydroxymethylacetamide (33.14%), 26-hydroxycholesterol (8.21%), formic acid, butyl ester (1.14%), cyclotetracosane (0.70%), 1-heptacosanol (0.66%), benzo [h] quinolone, 2, 4-dimethyl (0.57%), and pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(9-phenylmethyl) - (0.50%). The other compounds were found below 0.50%. To the best of our knowledge, this is the first reported identification by GC-MS analysis of metabolites produced by endophytes isolated from *W. salutaris.* It is important to note that whilst the predicted molecules reflect the complex nature of the metabolites in the crude ethyl acetate extracts, there is a need to carry out further investigations using different techniques to confirm the identity of the molecules present in the ethyl acetate extracts of *Vishniacozyma victoriae* and also profile the extracts from the other endophytic fungi that were not profiled during this present study. Furthermore, there is a need to access the bioactivity potential of the identified metabolites.

CONCLUSION

The endophytic fungi *Cladosporium uredinicola* isolated from *K. africana* has potential as a source of antimicrobial and antioxidant bioactive compounds. However, further research is required to isolate and identify the bioactive molecules and also, there is a need to screen the other fungal endophytes isolates that were not investigated upon during the course of this study for their bioactivity potential. To the best of our knowledge, this is the first reported work on the endophyte diversity in *W. salutaris* and antioxidant activities of fungal endophytes isolated from *K. africana.*

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

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CHAPTER 6: SYNTHESIS OF RESEARCH FINDINGS

6. Synthesis of research findings

This thesis reports on the various sub-projects that were conducted to answer the overall objective of identifying and determining the antioxidant and antimicrobial potential of endophytic fungi hosted by the Zimbabwean medicinal plants *Annona senegalensis*, *Kigelia africana*, *Vitex payos* and *Warburgia salutaris*. The work reported in this thesis shows that though bioprospecting for bioactive endophytic fungi is still in its infancy in Africa; the studied medicinal plants harbor endophytic fungi that produce diverse groups of metabolites with different antioxidants and antimicrobial bioactivity potentials. The following sections discuss the key findings reported in this thesis in the context of the objectives of the study, which are stated in Chapter 1, section 1.4.

6.1. Isolation of endophytic fungi

A total of 33 fungal endophyte isolates were obtained from the studied medicinal plant species, the fungal endophyte colonization rate was used as an indirect indicator to measure the research objective of endophyte isolation. Information on the endophyte colonization rate was presented in all the data chapters. The data from the studies showed that the fungal endophyte colonization rates varied by plant species and plant tissue used in the isolation of the fungal endophytes. This agrees with results from previous fungal endophyte isolation studies which have shown that the colonization rates of endophytic fungi in addition to being a function of the environment (for example tropical climate) and host plant species are also influenced by the type of plant tissue used in the endophyte isolation [1]. It is important to note that the colonization rates presented for the medicinal plants *Annona senegalensis*, *Vitex payos* and *Warburgia salutaris* provide the first insights into the host-endophyte associations in these plants. Though fungal endophytes have been reportedly isolated from *Kigelia africana*, the data presentedin this thesis is the first to provide insights into the fungal endophyte colonization rates for the tissues obtained from this plant species [2, 3, 4]. The colonization rates data presented in this thesis contributes to the body of knowledge concerning fungal endophyte-host biology and aid in the deciphering of the nature of the relationships between endophytes and their hosts.

6.2. Identification of isolated endophytic fungi

The isolated fungi across the different plant species and tissue types were found to be dominated by members of the phylum Ascomycota. This observation tallies with results from other studies which have shown that the isolated endophytic fungi to date are dominated by different lineages of Ascomycota [1, 5, 6]. The endophytic fungi *Penicillium chloroleucon* was isolated from all the

plant species and this suggests that it is a culturable fungal endophyte with a wide host range. An interesting observation from the obtained data is that the endophytic fungi that belonged to the other phyla (Zygomycota and Basidiomycota) were isolated from stem tissues in the different plant species. The observation tallies with other studies that have shown that stem tissues generally harbour more diverse endophytic taxa than the leaf tissues across different plant species [7]. To the best of our knowledge, whilst the fungal endophyte diversity in *Kigelia africana* has previously been reported on, the studies in this thesis are the first to report on the isolation and identification of fungal endophytes from *Warburgia salutaris*, *Annona senegalensis* and *Vitex payos* and contribute to the general body of knowledge concerning fungal endophyte diversity and provides information that will assist in gaining a better understanding of the host range of fungal endophyte. Fungal endophytes of the genera *Penicillium* and *Cladosporium* were identified as endophytic fungi isolated from *Kigelia africana* in our studies and have also been previously been reported as fungal endophytes isolated from the same plant species [2, 3] suggesting that members of these genera might be common fungal endophytes of the plant species. The fungal endophyte identification results presented in this thesis are significant in that they show that the endophytes associated with the studied medicinal plants represent different genera and are diverse.

6.3. Screening for antimicrobial activity

The majority of the isolated fungal endophytes did not have antimicrobial activity against the test microorganisms suggesting that the endophytic fungi do not have potential as sources of new antimicrobial agents against the assessed bacterial species. The results obtained from this study, whilst not confirming activity support the findings from other studies that not all endophytic fungi have antibacterial activity [8]. However, before these endophytic fungi are totally discounted as potential sources of new antimicrobial agents, there is a need to explore their antibacterial activity against other test microorganisms and screen them for antifungal activity. The crude ethyl acetate extracts of the *Kigelia. africana* isolates *Cladosporium uredinicola* and *Myrothecium gramineum* were found to have an inhibitory effect on the test microorganism *E. coli* (ATCC1056) and this suggests that these endophytic fungi have potential as sources of natural antimicrobials and should be further assessed for the development of new antimicrobials against *E. coli*. The antimicrobial activity screening results for fungal endophytes isolated from *Kigelia africana* tally with findings from other studies which have reported different fungal endophytes isolated from the same plant species having varying levels of antimicrobial activity [2, 3]. The obtained results are significant

in that they provide intial information on the antimicrobial potential of endophytic fungi isolated from *Annona senegalensis* and *Vitex payos* and suggests (in conjuction with data from studies by others) that antimicrobial activity is a relatively common phenomenon within fungal endophytes isolated from *Kigelia africana*.

6.4. Antioxidant potential of the isolated fungi

Epicoccum sorghinum isolated from *Annona senegalensis* exhibited the most potent antioxidant activity when compared to the other screened endophytic fungi. However, in general, a significant number of the screened endophytic fungi from the different plant species were also found to have some antioxidant activity. This suggests that ablity to produce antioxidants by the screened endophytic fungi is a relatively more common trait (compared to the studied antimicrobial activity). However, an analysis of the publications trends (publications output) on the Scopus website shows that antimicrobial activity potential is a more commonly investigated trait of fungal endophytes than antioxidant activity potential. The interest in antimicrobial bioprospecting in fungal endophytes compared to bioprospecting for antioxidants might be related to availability of more research funding targeting antimicrobial resistance or narrow research focus by the researchers involved in endophyte bioprospecting. The total phenolic content was found to have a positive correlational relationship with total antioxidant activity of the screened endophytic fungi crude extracts. In general, in the screened endophytic fungi, endophytic fungi crude ethyl acetate extracts were found to have higher antioxidant activity and total phenolic content compared to methanol extracts. This suggests that the active compounds primarily responsible for the antioxidant activity are low and high molecular weight polyphenolic compounds as ethyl acetate is a selective solvent for polyphenols [9]. The Fourier-transform infrared spectroscopy confirmed the presence of presence of –OH group characteristic of phenolic compounds across the extracts from the plant species. It is important to note that the Gas chromatography-Mass spectrometry analysis of the studied extracts did not provide a complete picture of the metabolites present in the extracts as the method only identified the volatile compounds within the extracts and it must be combined with other methods that can be used to identify the none volatile compounds within the extracts. To the best of our knowledge, the results from this thesis are the first to report on the antioxidant activity of endophytic fungi isolated from the studied plant species. The antioxidant activity results presented in this thesis are significant in that they show

that the fungal endophytes isolated from the different medicinal plants have wide-ranging levels of antioxidant activity and that antioxidant activity that is a far common trait in the endophytic fungi than antimicrobial activity.

6.5. Conclusions

The fungal endophytes of African medicinal plants are an under investigated reservoir of natural products and my study recorded many firsts in terms of endophyte diversity description and bioactive potential assessment. The type of plant species and plant tissue used for endophyte isolation were shown to influence the endophyte colonisation rates and diversity. The culturable endophytic fungi are dominated by members of the phylum Ascomycota and the stem tissue harbours more diverse endophytic fungi taxa across different plant species. *Penicillium chloroleucon* is a culturable fungal endophyte with a wide host range as shown by its isolation from all studied medicinal plants. Ability by endophytic fungi to produce metabolites with antioxidant activity appears to be more widespread than the research output on the bioprospecting for antioxidant activity in endophytic fungi. There is a positive correlational relationship between total phenolic content and total antioxidant activity of the endophytic fungi extracts. The isolate *Cladosporium uredinicola* has potential as a source of antimicrobial compounds whilst the isolate *Epicoccum sorghinum* from *Annona senegalensis* has potential as a source of natural antioxidant. Therefore, there is a need to further assess the two fungal endophytes for the development of new antimicrobial and antioxidants. In conclusion, whilst endophytic fungi isolated from the studied medicinal are potential repositories of bioactive molecules, further research is required to confirm and expand on the intial data provided in this thesis.

6.6. Future research

It was not possible to investigate the ability of the endophytic fungi to produce bioactive mnolecules for other conditions and illnesses and hence this brings in the issue on the need for contries like Zimbabwe to catalogue and build easily accessible collections of isolated endophytic fungi (and other microbes) not only for referencing but also for future investigations by different researchers. This in my view will hasten the identification of useful biomolecules from the endophytic fungi.

For those endophytic fungi that were shown to have some bioactivity potential, further research is required to isolate and identify the bioactive molecules (using a combination of different techniques) from the crude extracts and evaluate *in vivo* the biological activities of the isolated compounds. Furthermore, studies will need to be undertaken to ascertain fully the toxicity profile of the bioactive metabolites to provide insights on the safety of the bioactive metabolites.

6.7. References

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APPENDICES

Appendix A1: Representative FT-IR spectrums

Thu Aug 10 15:16:33 2017 (GMT-07:00)
FIND PEAKS:
Spectrum: EPS ML3B
Region: 4000.00 513.00
Absolute threshold: 114.737
Sensitivity: 52
Peak list:

Position:	518.68	Intensity:	64.154
Position:	536.13	Intensity:	68.619
Position:	556.28	Intensity:	69.773
Position:	571.49	Intensity:	70.021
Position:	579.15	Intensity:	74.119
Position:	592.44	Intensity:	68.768
Position:	625.09	Intensity:	74.279
Position:	644.43	Intensity:	74.610
Position:	684.27	Intensity:	76.172
Position:	833.39	Intensity:	75.526
Position:	894.75	Intensity:	87.932
Position:	969.26	Intensity:	73.493
Position:	1041.02	Intensity:	79.028
Position:	1079.48	Intensity:	70.419
Position:	1123.41	Intensity:	74.484
Position:	1188.16	Intensity:	78.637
Position:	1346.02	Intensity:	89.029
Position:	1465.03	Intensity:	87.818
Position:	1493.62	Intensity:	90.048
Position:	1595.95	Intensity:	82.475
Position:	2854.01	Intensity:	100.739
Position:	2923.15	Intensity:	93.954
Position:	3569.88	Intensity:	112.877

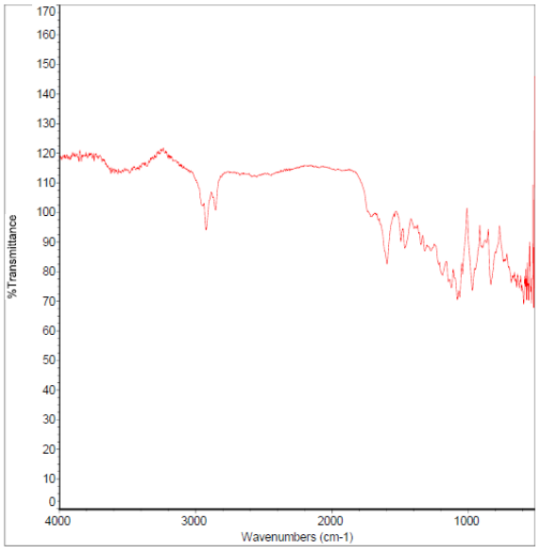

Figure 1A: Spectra of extract obtained from *Phoma* species (ML3B).

Thu Aug 10 15:10:45 2017 (GMT-07:00)
FIND PEAKS:
Spectrum: EPS ML2 13 07 2017
Region: 4000.00 513.00
Absolute threshold: 114.216
Sensitivity: 52
Peak list:

Position:	523.45	Intensity:	39.070
Position:	529.49	Intensity:	59.052
Position:	535.84	Intensity:	60.354
Position:	548.81	Intensity:	61.521
Position:	566.66	Intensity:	55.712
Position:	574.89	Intensity:	61.536
Position:	586.40	Intensity:	59.293
Position:	596.31	Intensity:	62.230
Position:	616.87	Intensity:	64.376
Position:	629.79	Intensity:	66.574
Position:	652.06	Intensity:	68.946
Position:	1018.36	Intensity:	70.222
Position:	1079.89	Intensity:	66.491
Position:	1373.30	Intensity:	90.416
Position:	1456.80	Intensity:	93.006
Position:	1735.79	Intensity:	95.497
Position:	2854.57	Intensity:	97.730
Position:	2929.35	Intensity:	92.592
Position:	3118.83	Intensity:	106.882
Position:	3219.03	Intensity:	106.510
Position:	3319.39	Intensity:	107.631
Position:	3330.31	Intensity:	107.727
Position:	3398.40	Intensity:	108.141
Position:	3444.38	Intensity:	110.032
Position:	3525.46	Intensity:	110.931
Position:	3568.34	Intensity:	112.659

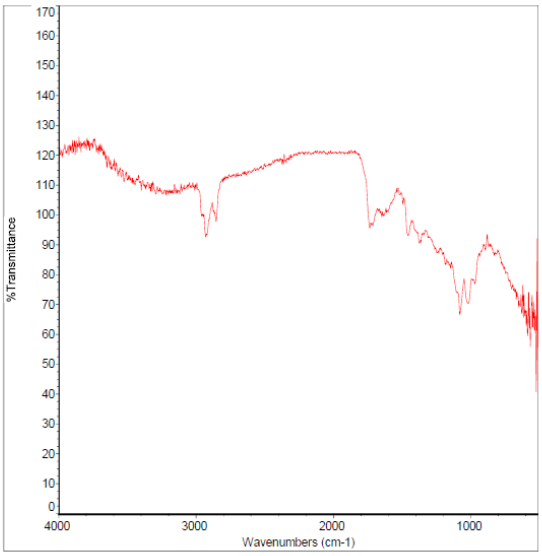

Figure 2A: Spectra of extract obtained from *Epicoccum nigrum* (ML2).

Thu Aug 10 15:12:54 2017 (GMT-07:00)
FIND PEAKS:
Spectrum: EPS_ML3A
Region: 4000.00 513.00
Absolute threshold: 111.439
Sensitivity: 52
Peak list:

Position:	515.36	Intensity:	74.742
Position:	524.31	Intensity:	70.005
Position:	532.18	Intensity:	52.396
Position:	536.19	Intensity:	51.258
Position:	555.59	Intensity:	48.102
Position:	568.79	Intensity:	55.370
Position:	578.20	Intensity:	56.583
Position:	597.29	Intensity:	52.681
Position:	631.09	Intensity:	58.827
Position:	682.27	Intensity:	57.100
Position:	742.12	Intensity:	65.903
Position:	794.63	Intensity:	66.803
Position:	832.71	Intensity:	59.620
Position:	968.41	Intensity:	61.142
Position:	1027.26	Intensity:	57.112
Position:	1120.92	Intensity:	65.343
Position:	1149.18	Intensity:	61.467
Position:	1203.72	Intensity:	71.769
Position:	1318.00	Intensity:	79.927
Position:	1450.01	Intensity:	82.568
Position:	1492.12	Intensity:	83.974
Position:	1584.81	Intensity:	77.916
Position:	2854.99	Intensity:	101.601
Position:	2923.35	Intensity:	96.970
Position:	3259.76	Intensity:	99.659

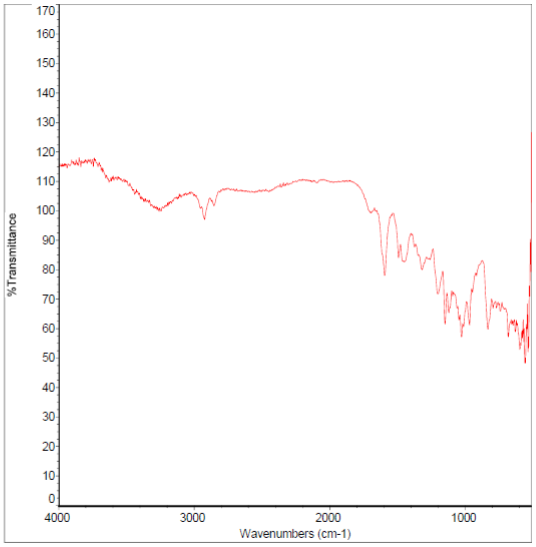

Figure 3A: Spectra of extract obtained from *Epicoccum sorghinum* (ML3A).

Thu Aug 10 15:23:11 2017 (GMT-07:00)
FIND PEAKS:
Spectrum: EPS_TS2
Region: 4000.00 513.00
Absolute threshold: 119.829
Sensitivity: 52
Peak list:

Position:	517.33	Intensity:	83.179
Position:	526.07	Intensity:	86.694
Position:	535.90	Intensity:	73.845
Position:	562.34	Intensity:	80.498
Position:	569.47	Intensity:	81.260
Position:	579.67	Intensity:	79.865
Position:	593.35	Intensity:	77.398
Position:	618.18	Intensity:	81.082
Position:	629.64	Intensity:	81.190
Position:	645.60	Intensity:	83.617
Position:	725.75	Intensity:	91.387
Position:	831.96	Intensity:	82.929
Position:	872.30	Intensity:	91.325
Position:	968.39	Intensity:	81.550
Position:	1040.64	Intensity:	89.567
Position:	1061.90	Intensity:	81.535
Position:	1077.65	Intensity:	82.354
Position:	1123.46	Intensity:	84.476
Position:	1195.41	Intensity:	81.298
Position:	1346.10	Intensity:	91.462
Position:	1399.92	Intensity:	92.430
Position:	1465.98	Intensity:	93.242
Position:	1493.30	Intensity:	95.001
Position:	1556.02	Intensity:	85.274
Position:	1596.42	Intensity:	87.637
Position:	1716.94	Intensity:	96.344
Position:	2551.56	Intensity:	114.718
Position:	2855.94	Intensity:	108.047
Position:	2923.81	Intensity:	102.222
Position:	3544.72	Intensity:	118.510

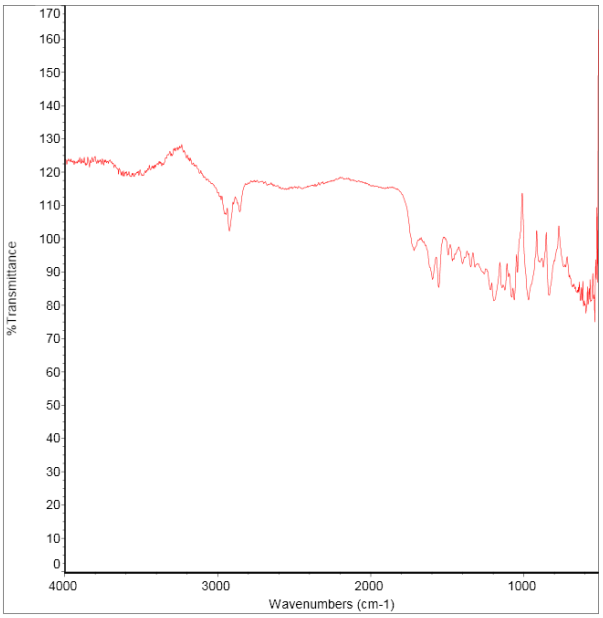

Figure 4A: Spectra of extract obtained from *Epicoccum nigrum* (TS2).

Appendix A2: Representative GC-MS spectra

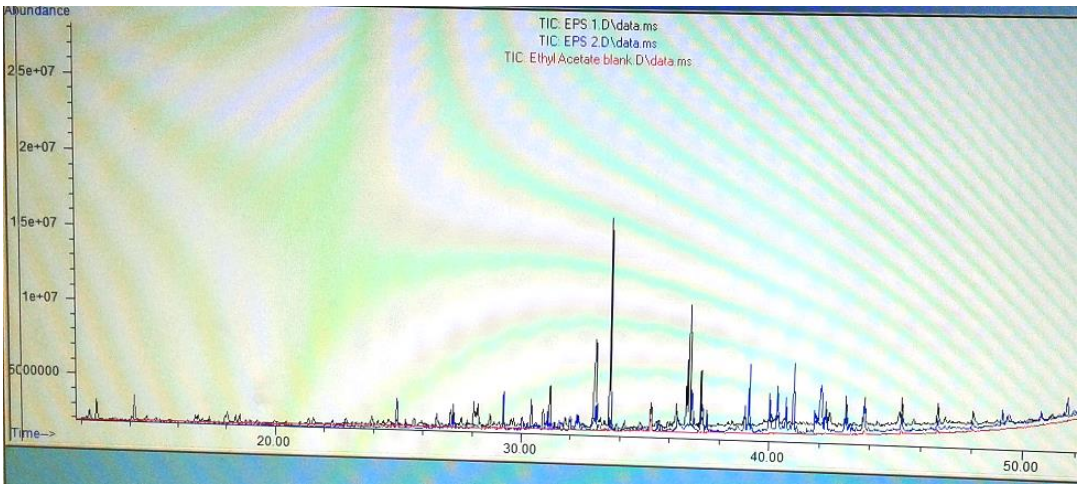

Figure 5A: GC-MS spectra obtained for the crude ethyl acetate obtained from *Vishniacozyma victoriae* (WS5).

Appendix A3. Representative output from the BLAST search

```
BLASTN 2.6.1+
Reference: Stephen F. Altschul, Thomas L. Madden, Alejandro A. Sch&auml;ffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped
BLAST and PSI-BLAST: a new generation of protein database search programs",
Nucleic Acids Res. 25:3389-3402.

RID: T329FN5T014


Database: nr
Query= ML1 Consensus
Length=542


ALIGNMENTS
>gb|HQ630999.1| Phoma sp. 1 TMS-2011 voucher SC13d50p14-6 18S ribosomal RNA gene, partial
sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer
2, complete sequence; and 28S ribosomal RNA gene, partial sequence
 gi|355321692|emb|HE608794.1| Phoma sp. MS-2011-F25 genomic DNA containing ITS1, 5.8S rRNA gene
and ITS2, strain F25
 gi|1002635746|gb|KT768295.1| Uncultured Phoma clone FYr19 18S ribosomal RNA gene, partial
sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer
2, complete sequence; and 28S ribosomal RNA gene, partial sequence
          Length=580
```

```
 Score = 951.7 bits (1054),  Expect = 0E00
 Identities = 537/542 (99%), Gaps = 1/542 (0%)
 Strand = Plus/Minus

Query  1    TTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  580  TTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAG  521

Query  61   AGTGTAAAAATGTACTTTTGGACGTCGTCGTTATGAGTGCAAAGCGCGAGATGTACTGCG  120
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  520  AGTGTAAAAATGTACTTTTGGACGTCGTCGTTCTGAGTGCAAAGCGCGAGATGTACTGCG  461

Query  121  CTCCGAAATCAATACGCCGGCTGCCAATTGTTTTAAGGCGAGTCTACACGCAGAGGCGAG  180
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  460  CTCCGAAATCAATACGCCGGCTGCCAATTGTTTTGAGGCGAGTCTACACGCAGAGGCGAG  401

Query  181  ACAAACACCCAACACCAAGCAGAGCTTGAAGGTACAAATGACGCTCGAACAGGCATGCCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  400  ACAAACACCCAACACCAAGCAGAGCTTGAAGGTACAAATGACGCTCGAACAGGCATGCCC  341

Query  241  CATGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  340  CATGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCA  281

Query  301  ATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  280  ATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCG  221

Query  361  TTGTTGAAAGTTGTAACTATTATGTTTTTTCAGACGCTGATTGCAACTGCAAAGGGTTTA  420
            |||||||||||||||||||||| |||||||||||||||||||||||||||| ||||||
Sbjct  220  TTGTTGAAAGTTGTAACTATTAAGTTTTTTCAGACGCTGATTGCAACTGCAAATGGTTTA  161

Query  421  AATTTGTCCAATCGGCGGGCGGACCCGCCGAGGAAACGAAGGTACTCAAAAGACATGGGT  480
            || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  160  AA-TTGTCCAATCGGCGGGCGGACCCGCCGAGGAAACGAAGGTACTCAAAAGACATGGGT  102

Query  481  AAGAGATAGCAGGCAAAGCCTACAACTCTAGGTAATGATCCTTCCGCAGGTTCACCTACG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  101  AAGAGATAGCAGGCAAAGCCTACAACTCTAGGTAATGATCCTTCCGCAGGTTCACCTACG  42

Query  541  GA  542
            ||
Sbjct  41   GA  40
```