Biodiversity and antimicrobial activity of endophytic fungi isolated from native *Sutherlandia frutescns* (cancer bush)

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Dissertation accepted in fulfilment of the requirements for the degree Masters of Science in Biology at the North West University

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DECLARATION

I, Anathi Sishuba hereby declare and confirm that the work done for this dissertation is my own work, unless where acknowledged. It has not been submitted to any institution for the purpose of obtaining a qualification. All materials used and quoted herein have been duly acknowledged.

Signed……NWU MAFIKENG……this the……11th……. Day of……July……..…2022

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DEDICATION

I dedicate this dissertation to my wonderful and amazing mother, **Nokhwezi Sishuba** who has loved me without measure, prayed for me and made sure I have all that I need. This is also dedicated to my siblings who have always supported and loved me.

ACKNOWLEDGEMENTS

I would like to thank **God** for making it all possible, for everything was because of his approval and favour. (Grateful! Thankful! Blessed! -John 1:16).

I also acknowledge my supervisors Prof CN Ateba and Dr MC Manganyi for their endless efforts, patience, and for always being there throughout this project. Their encouragement and motivation kept me going.

The assistance and cooperation received from my colleagues at "**ATEBALAB"** is highly appreciated.

I also acknowledge the North West University and the Department of Microbiology for giving me the opportunity to pursue a Master's Degree and also providing the space and resources for bench work.

The National Research Foundation of South Africa is acknowledged for providing financial support in the form of a postgraduate bursary. Lastly, I thank my family for their support throughout this journey.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

ARC **:** Agricultural Research Council

Blast **:** Basic Alignment Search Tool

EDTA **:** Ethylenediamine-tetraacetic acid

NCBI **:** National Centre for Biotechnology Information

NCF **:** National Collection of Fungi

PCA **:** Potato Carrot Agar

PDA **:** Potato Dextrose Agar

rRNA **:** Ribosomal Ribonucleuotide Acid

WA **:** Water Agar

WHO **:** World Health Organization

DEFINITION OF CONCEPTS

Antibiotic resistance **:** The capability of a microbes to survive the exposure to a defined concentration of an antimicrobial agent.

Phylogeny **:** A process in which the lineage of organisms evolved by separation from common ancestors.

Polymerase Chain Reaction **:** A molecular method that is used to amplify specific regions of DNA many times over using primers.

Species **:** A collection of microbial cells which share an overall similar pattern of traits in contrast to other microbial whose pattern differ significantly

Secondary metabolites **:** Are compounds such as terpenes, alkaloids, polyketides and pigments, that may not be essential for the growth and health of the organism but often provide them with competitive advantages over other species competing for nutrients by eliciting their biological activities.

Endophytes **:** Are micro-organisms that live within the plant tissues

SUMMARY

The current study focused on finding alternative bioactive compounds with the potentials to mitigate or control antimicrobial resistance. Natural products such as medicinal plants; are being studied for their therapeutic potentials in the race against antimicrobial resistance. The plant kingdom offers surfeit of biologically active compounds and most studied plants have been found to at least host one endophyte. In addition, studies on establishing the relationship between endophytes and plants and the products of their interaction have since gained traction. Endophytes are endosymbionts (bacterial or fungal) that produce bio-compounds that aid in plant protection and growth. The bioactive compounds produced by endophytes in symbiosis are used in the pharmaceutical industry for manufacturing secondary products. South Africa is well-known for its valuable and untapped information on medicinal plants. Since ancient times, people have been using plants for their essential wellbeing.

This study centered around the medicinal plant, *Sutherlandia frutescens*, commonly known as cancer bush indigenous to Southern Africa, and produces bioactive compounds of medicinal value. The study aimed at evaluating the biodiversity and antimicrobial activity of possible endophytic fungi isolated from the plant. A total of fifty-one (51) fungal endophytes were isolated, identified and classified into various genera. The predominant genus found was *Penicillium* (25%), followed by the *Mucor* (12%), *Alternaria* (10%) and *Coniochateta* (10%) genera. The endophytes were further characterized to species-level based on known identities in the GenBank database, and their relatedness and evolutionary lines were determined by phylogenetic analysis with a total of four (4) clusters and six (6) sub-clusters constructed. The endophytes were evaluated for their

antibacterial activity against environmental Gram-negative and Gram-positive bacteria. The antibacterial properties of the secondary metabolites were investigated using the disc diffusion and agar plug assays.

A disk diffusion assay showed *Salmonella enterica* (environmental strain) was the most sensitive and with its growth being inhibited by the activity of the secondary metabolites from the sixteen (16) out of the fifty-one (51) endophytes isolated. In overall, 51% of the endophytic fungi produced bioactive compounds that exhibited antimicrobial activity against at least one tested bacterial. However, the agar plug assay showed less activity compared to the latter with only eight (8) endophytic fungal plugs that managed to inhibit the growth of four (4) bacteria. The largest zone of inhibition with a diameter of 21.0 mm was exhibited by *Coniochaeta hoffmannii* (CB016) observed against *E. coli* 017.

Some fungal isolates were further tested for their antifungal activity against plant pathogenic fungi. The dual culture test gave 84% antifungal effectiveness against fungal pathogens, with *Mucor*, *Penicillium* genera and *Aspergillus brasiliensis* exhibing broad-spectrum antifungal activities for most plant pathogenic fungi tested in this assay. During the culture filtrate test, 78% showed inhibition activity against one or more pathogens. The culture filtrate of the endophytic fungus CB011 (*Purpureocillium lilacinum*) exhibited a broad range of antifungal activity against all the pathogens. Under salt conditions, most isolates were able to grow at a 3% concentration, giving a total of 90% fungal growth rate with a maximum within the range of 75-80.5 mm growth in diameter. At pH of 5, an overall 44% of the total fungal growth was observed, with most growth

noted at a temperature of 25 °C. The antimicrobial activity showed 19% of growth activity. The results suggest endophytic fungi isolated from *Sutherlandia frutescens* may be good sources of bioactive molecules, including those capable of inhibiting or controlling both human and plant pathogens.

Keywords *Sutherlandia frutescens*, antimicrobial activity, endophytic fungi, secondary metabolites, bioactive compounds

CHAPTER 1
INTRODUCTION AND PROBLEM STATEMENT

1.1 GENERAL INTRODUCTION

The effectiveness of antibiotics against pathogenic infections over the past centuries is an important discovery for mankind as the agents were directed at inactivating disease causing organisms (Rios and Recio, 2005). The main functions of antibiotics are to improve on public health and veterinary medicine by contributing to sustainable development goal (SDG) 3, which aspires to ensure health and well-being for all. However, the undifferentiated and continuous misuse of antibiotics in both human and veterinary medicine has led to the emergence and dissemination of multidrug resistant pathogens. Antibiotic resistance is thus posing increasing therapeutic challenges in both human and veterinary medicine (Tekwu *et al*., 2012).

Against this background, there is the need to search for alternative methods to address this ever-increase bacteria resistance to antimicrobial agents. This drive also contributes to the World Health Organization (WHO) approved global action plan on antimicrobial resistance with strategic objectives designed to i) enhance awareness and understanding of antimicrobial resistance; ii) to strengthen surveillance and research; iii) reduce the incidence of infection; iv) optimize the use of antimicrobial medicines; and v) ensure sustainable investment in countering antimicrobial resistance (WHO, 2015). Studies have shown that plants are able to produce a variety of compounds that serve as defense mechanisms against a variety of pathogens and insect pests (Sen and Batra, 2012; Tekwu *et al.*, 2012). It has therefore been proposed that antibiotic resistance inhibitors from plants should be investigated constantly with the aim of searching for alternative antimicrobial agents to reduce the burden of diseases in humans (Tekwu *et al.*, 2012). Medicinal

plants are a great source of antimicrobial agents worldwide. Approximately 60 to 90% of humans populations in developing countries rely on antimicrobial agents from plants for healing purposes (Khan *et al.*, 2013). Moreover, in Africa where resources and access to modern medicine are limited, many rely solely on traditional medicine in the treatment of infectious diseases (Harnett *et al.*, 2005; Khan *et al.*, 2013).

There is evidence to suggest that biologically active compounds extracted from the leaves, roots, and in some cases, from the stems (Chadwick *et al*., 2006) have been highly effective against multidrug resistant pathogens and associated infectious diseases. Furthermore, these biologically active agents from plants also display attributes of natural preventatives against foodborne pathogens and thus have the potential to increase their shelf-life of food (Mostafa *et al*., 2018).

Sutherlandia frutescens also called "cancer bush" has been used in traditional medcine for a very long time especially by individuals in the Khoi and Nama clan in Western cape and Kacroo regions of the Southeran Africa (Fernandes *et al*., 2004; Tai *et al.*, 2004). Native to South Africa, the plant contains triterpenoid glycosides as the most common active ingredient and this metabolite has been reported to exhibit anticancer and antiviral activities (Van Wyk and Wink, 2017).

Amongst the Zulu, Xhosa, Cape Dutch, Khoi-San tribes, cancer bush is commonly used as a remedy against various- infirmities ranging from trivial illnesses such as fever, common colds, cancer and HIV (Mncwangi and Viljoen, 2012) to chickenpox, stomachaches, backaches, liver problems and rheumatism (Chadwick *et al.*, 2006). Additionally, Lakshman and Indika (2008) reported that the plant has adaptogenic properties. Furthermore, the active ingredients (tannins, alkaloids, triterpenoids and flavonoids) from cancer bush have been documented to exhibit

antimicrobial properties against a number of pathogens (Khan *et al.*, 2013). Despite the fact that numerous studies have successfully isolated fungal endophytes with unique antimicrobial properties from various medicinal plants (Ahmed *et al*., 2012; Khalil *et al*., 2021; Sishuba *et al*., 2021), King (2021), only isolated a bacteria endophyte from *S. frutescens*. . With the aim of strengthening surveillance and research on the search for alternative antimicrobial agents, this study seeks to isolate and characterize fungal endophytes from *S. frutescens*. This willl contribute to bioprospecting of cancer bush and specificlly its extracts for use as alternative therapeutic agents (Gibson, 2011).

This is motivated by the fact that the initial isolation of paclitaxel (taxol) compounds from the endophytic fungus *Taxomyces andreanae* in 1993 increased interest on the search for bioactive compounds from other endophytes (Zhao *et al*., 2010). Endophytes are microorganisms that colonize internal plant tissues and therefore spend all or part of their life cycle within the host but without causing disease-like symptoms (Khan *et al.*, 2010). They possess bioactive secondary metabolites and enzymes that may be valuable for the production of pharmaceutical products (Zou *et al.*, 2000; Strobel *et al.*, 2004; Krishnamurthy *et al.*, 2008).

Over the last century, a broad spectrum of antimicrobial compounds such as penicillin (Fleming, 1929), lovastatin (Alberts *et al.*, 1980), and paclitaxel (taxol) (3) have been isolated from endophytic fungi (Brakhage, 2013). Additionally, *Trichoderma* species are currently utilized as growth promoters or bio-fertilizers in the agricultural industry (Vinale *et al.*, 2014). Given that the relationship between the plant and endophytes is symbiotic, the fungi receive shelter and nutrients from the plant while plant is protected from attack by pathogens and herbivores as well as increased

resistance to abiotic stress and toxicity to high concentrations of heavy metals (Ramesh *et al*., 2017).

1.2 PROBLEM STATEMENT

The recent emergence of multidrug resistance (MDR) has raised concerns in public health care systems worldwide, due to this phenomenom rendering the current antimicrobial drugs ineffective (Wiyakrutta *et al,* 2004). Moreover, the constant and misuse of synthetic antibiotics results to the accumilation of antibiotic recidues within the environment thus increasing the selective pressure required to build up resistant determinants. This amplifies the need to search for new antimicrobial agents from natural sources such as plants in order to combat this universal public health challenge remains a top priority (Bhaskarwar *et al.,* 2008; Martinez-Klimova *et al.,* 2017). The current trends indicates the need to devise more sustainable and natural approaches to improve public health. This therefore serves to motivate the focus of this study which is aimed at exploring natural secondary metabolites from *S. frutescens* as alternative antimicrobial agents for microorganisms. Moreover, endophytic fungi have been identified as a treasure of undiscovered useful bioactive secondary metabolites and enzymes that may be valuable for the drug discovery in the pharmaceutical industry (Manganyi *et al.*, 2018). Bioactive compounds are well-known for their biological properties, including antibacterial, antifungal, anti-cancer, anti-inflammatory, antioxidant activity etc. This study therefore aims to search for new, affordable, efficacious antimicrobial compounds that may be very useful in pharmaceutical and agricultural applications.

1.3 RESEARCH AIM AND OBJECTIVES

1.3.1 Aim

This study aimed to evaluate the biodiversity and antimicrobial activities of endophytic fungi isolated from *S. frutescens* plants.

1.3.2 Objectives

The specific objectives of the study were;

i. To isolate endophytic fungi from *S. frutescens* plants

ii. To identify and confirm the identies of endophytic fungi by converntional means and molecular techniques, respectively;

iii. To determine the antimicrobial activities of the isolated endophytes against some selected bacterial and fungal pathogenic organisms; and

iv. To assess the stress tolerance of endophytic fungi against unfavourable salt, temperature and pH conditions

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

Pathogenic multidrug-resistant microbial strains are rapidly spreading in hospitals where patients are immunocompromised. Novel antimicrobials are required to combat these bacterial pathogens; however, progress made in developing them is slow. Before the realization of the existence of microbes by mankind, plants were being used with the knowledge that they have healing potentials. Man has been using plants for the treatment of common ailments because they possess what would be characterized as antimicrobial properties (Rios and Recio, 2005). The demand for new and safe bioactive compounds to assist in all aspects of life is forever escalating. Scientists and pharmaceutical companies are exploring natural components in search of new medically relevant antimicrobials to deal with antimicrobial resistance and the emergence of superbugs. Lately, scholars have directed their attention on the relationship and interaction between microbes and vectors (Hopkins *et al*., 2014). Fungal metabolites production is induced by selective pressure exerted on the fungus by other organisms. These metabolites often perform other bioactivities but equally act as chemical protectors against predation to survive in hostile conditions.

2.2 ANTIBIOTIC RESISTANCE: A PERSISTENT CHALLENGE IN THE HEALTH AND AGRICULTURAL INDUSTRIES

The introduction of antibiotics in the preceding century had an immense impact on reducing mortality and morbidity resulting from infectious diseases. However, their misuse has led to antimicrobial resistance (Haque *et al*., 2016). Alterations caused by enzymatic degradation and active efflux of drugs have been reported to increase resistance to antibiotics (Terzi *et al*., 2014).

Primary resistance can naturally occur in certain microorganisms independent of the actual exposure to the antimicrobial agent, for example, the resistance of *Cryptococcus neoformans* to echinocandins and that of *Candida krusei* to fluconazole (Cuenca-Estrella *et al*., 2001). Secondary resistance, unlike primary resistance, occurs as a result of strains previously susceptible to the antimicrobials, however, due to exposure, have now developed resistance, such as evolutionary resistance of fluconazole in *Cryptococcus neoformans* and *Candida albicans* (Kanafani and Perfect, 2008; Tanwar *et al*., 2014).

2.2.1 History of antimicrobial resistant

Since the advancement of primary antimicrobials during the 1940s, antimicrobial resistance has been a growing issue. The discovery of modern antimicrobials was by testing of soil organisms, then later by chemical alteration of existing drugs. The rate of discovery of new antibacterial classes declined within the late 1960s. However, the rate at which bacteria become resistant to antibiotics, compared to the slow rate of new drug development, has driven a few scientists to warn of a "post-antibiotic era" (Song, 2012; Bisacchi and Manchester, 2015), Figure 2.1 displays the development trend of antimicrobial agents and emergence of drug-resistant bacteria.

Advancements have been made for antimicrobial agents in different viewpoints in addition to the range and activity of antimicrobials. The effectiveness of drugs relies on pharmacodynamics such as the absorption capacity of oral drugs, concentration within the blood, and distribution to tissue inflammatory responses (Asín-Prieto *et al*., 2015). In search of an ideal drug, earlier antimicrobial drugs with adverse side effects are being replaced with newer drugs with minimal side effects. Although a large number of companies in various countries have competed within the advancement of more current antimicrobial agents, the number of new drugs has been astoundingly diminishing in recent times, with few antimicrobial agents of modern classes being discovered and

made accessible. The rising and re-emerging infectious disease continue to strike humans with drug resistant organisms (Sag and Yamaguchi, 2009).

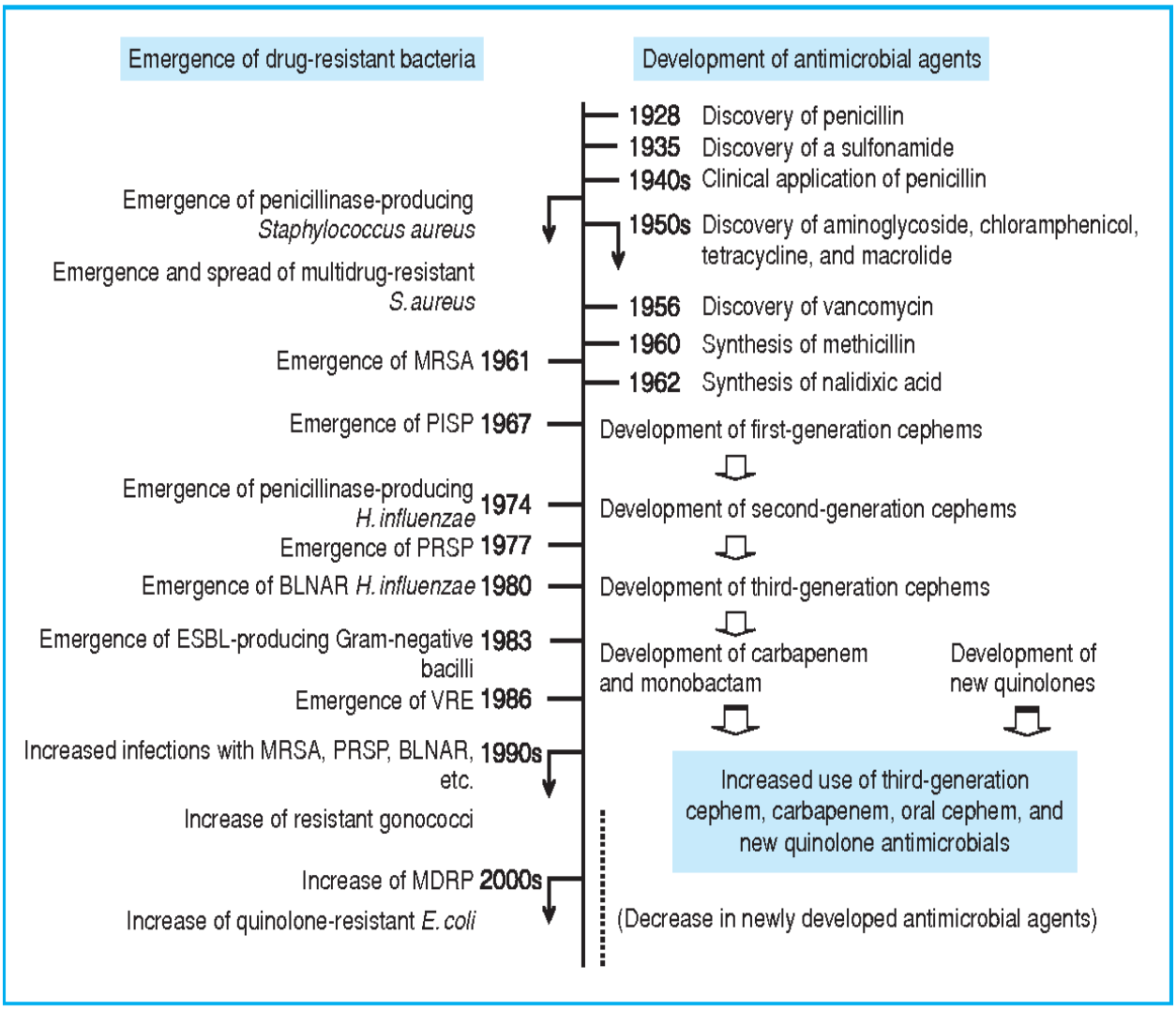


Figure 2.1: Trend of development of antimicrobial agents and emergence of drug-resistant bacteria (Saga and Yamaguchi, 2009)

2.2.2 Impact of antimicrobial resistance

Antimicrobial resistance (AMR) is a significant problem for healthcare systems worldwide. This might lead to negative impacts on outcomes such as lengthened periods spent in hospitals, utilitarian decline, extended healthcare use and all-cause mortality. It has been predicted that by 2050, AMR will cause 10 million deaths around the world each year (Nguyen *et al*., 2019).

Mortality increases more among patients infected by multidrug-resistant pathogens compared to those infected by sensitive pathogens (Niederman, 2019). The majority of patients with invasive mycoses experience treatment failure because of clinical resistance, which is a concept critical to the outcome of a fungal infection. Clinical resistance can occur under several circumstances (Kanafani and Perfect, 2008) as listed in Table 2.1.

Table 2.1: Principal factors determining antifungal clinical resistance.

Factor	**Implication**
Wrong diagnosis	Weak diagnostics
Net state of immunosuppression	Improvement in immunity of host is essential
High burden of fungus at initiation of treatment	Earlier treatment intervention improves outcome
Strain acquisition of increased virulence	Probably less of a problem than host factors but can be measured
Pharmacokinetics and/or pharmacodynamics	Drug toxicity, drug-drug interaction, drug levels
Site of infection	Drug penetration, tissue necrosis, foreign body
Length of treatment and /or compliance	Precision is not certain; patient and clinician may lose focus on long-term drug administration
Underlying disease	Final arbitrator in most invasive mycoses

2.3 MEDICINAL PLANTS

Plants that possess therapeutic properties or beneficial pharmacological effects; are referred to as medicinal plants (Namdeo, 2018). The effectiveness and influence of these plants in both animal and human health have been studied. Plant-derived drugs are effective in treating anxiety, insomnia, and fatigue. Several studies have been conducted in various fields such as ethnomedicine, ethnoveterinary medicine and phytomedicine to assess the usefulness of a wide

variety of indigenous plants. In doing so, some discoveries were made with respect to the biological activities, such as anti-inflammatory, anticancer, antioxidants, antimalarial, anti-HIV and antiviral activities of these plants against a broad spectrum of bacteria, fungi, parasites and viruses (Arceusz *et al*., 2010; Abdalla and McGaw, 2018). Medicinal plants produce a wide array of bioactive compounds for pharmaceuticals. They can generate endless bioactive compounds with some examples listed in Table 2.2. These plants can protect themselves against macro and microorganisms by their chemical products (secondary metabolite) with significant pharmaceutical characteristics. Since ancient times, plants have been fighting the incessant attacks of parasites, fungi, bacteria and viruses by secreting secondary metabolites. Among medicinal plants, is *Sutherlandia frutescens* indigenous to Southern Africa and is regarded as a popular medicinal plant with numerous known functions.

Table2.2: Some promising plants having antimicrobial activity against multidrug-resistant strains.

Plant	**Name/Type of extract**	**Susceptible microorganisms**	**Reference**
Garcinia mangostana	Alpha-magostin	Vancomyvine resistant enterococci	Sakagami *et al*., 2005
Caesalpinia coriaria	Methanolic extracts	*Klebsiella pneumoniae*	Mohana *et al*., 2008
Psidium guajava	Methanolic extracts	MDR *staphylococcus aureus*	Anas *et al*., 2008
Commiphora molmol and Boswellia papyrifera	Methanolic extracts	Methicillin-resistant *staphylococcus aureus* (MRSA)	Abdallah *et al*., 2009
Pelargonium sidoides	Ethanolic extracts	*Aspergillus niger*	Mativandlela *et al*., 2006
Acacia nilotica, cinnamomum zeylanicum and Syzygium aromaticum	Ethanolic extracts	*Escherichia coli, Klebsiella pneumoniae* and *Candida albicans*	Khan *et al*., 2009

2.4 CANCER BUSH (*Sutherlandia frutescens*): THE IDEAL MEDICINAL PLANT

2.4.1 Botanical description

Sutherlandia frutescens plant is a well-known native medicinal plant also referred to as cancer bush or "Kankerbos", Umnwele (Xhosa), Insizwa (Zulu) and Phetola (Sotho). It was named after James Sutherland, the first director of Edinburgh botanic garden. *Sutherlandia frutescens* is a soft small shrub with a height of 0.5 to 1 m high with prostrate to erect stems of flowering plants belonging to the legume family (Fabaceae) (Van Wyk *et al*., 2008). The plant has pinnate leaves of about 4-10 mm long. The petals of the flower are orange-red and appear to be 35 mm long as shown in Figure 2.2 image C&D, sprouting annually from September to January (spring to summer) in the Southern hemisphere. The plant is characterized by having a bitter taste (Albrecht *et al*., 2012).

Figure 2.2: *Sutherlandia frutescens* (A) commercial plantation; (B) fruiting plant of commercial type ("subsp. *microphylla*"); (C) flowers of "subsp. *frutescens*"; (D) flowers and pods of "subsp. *frutescens*"; (E) dried product (sutherlandia herb)

2.4.2 Geographical distribution

Sutherlandia frutescens is indigenous to Southern Africa, Figure 2.3 highlights the geographic distribution of the plant in South Africa where it grows in the savannah and hillsides near streams. The plant can tolerate different environmental conditions, including drought, it can also grow on rocky, sandy soils along coastal areas and favors the Western Cape and Northern Cape provinces (South African National Biodiversity Institute, 2018; Korthy, 2021), but also found in the Eastern cape and certain parts of KwaZulu-Natal, differing in its chemical and genetic makeup across different geographic areas (Aboyade *et al*., 2014)

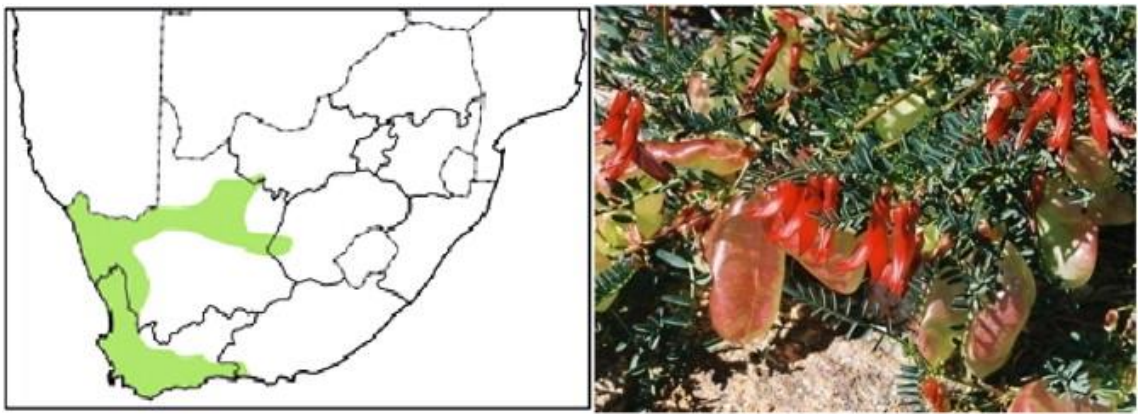


Figure 2.3: Geographical distribution of *Sutherlandia* species in South Africa (sahealthinfo.org) (*left*) and *S. frutescens* growing in the wild at Goegap Nature Reserve, Springbok, Northern Cape (photo taken by N. Harding) (*right*) (Faleschini *et al*., 2013)

2.4.3 Traditional origin and uses

The Khoisan and the Nama people have been using this plant since ancient times as an essential part of the indigenous culture and materia medica, hence the common name "cancer bush" as the Dutch and Khoisan people traditionally used the plant to treat internal cancer, and it got distributed

along the west coast of the Western Cape (Chinkwo, 2005). The plant is traditionally known for its medicinal uses in the treatment of stomach cancer, stress, diabetes, wounds, colds and decoctions consumed as a blood tonic, uterine diseases and eye infection (Lei *et al*., 2015). Traditional healers prepare decoctions by infusing the leaves, flowers, stem or roots in boiling water depending on the patient's ailment (Aboyade *et al*., 2014).

2.4.4 Medicinal value of cancer bush

S. frutescens has normally been used to treat a broad spectrum of disorders such as inflammation, anxiety and stress as well as diabetes and cancer. The *Sutherlandia* is available in different forms, such a capsules and tablets containing raw material in powder form, gels for topical application, creams, liquid extracts and ointments and are found in herbal shops and pharmacies. Through extracts from leaves, seed pods and capsules of *S. frutescens*, several secondary metabolites such as flavonoids and terpenoid compounds have been isolated. Research shows that terpenoid displays anti-cancer, anti-inflammatory and anti-hypertensive properties (Yadav *et al*., 2010; Chen *et al*., 2015; Bai *et al*., 2016). In addition, it is used in Asian medicine to treat chronic ailments such as cardiovascular atherosclerosis, diabetes, depression and arthritis (Sergeant *et al*., 2017).

Leaves of *S. frutescens* plants were air-dried and supplied to a health shop in Port Elizabeth, Eastern Cape, with cultivation and commercialization that started in 1990. Years later, other companies initiated large-scale cultivation and from the powdered herb, they manufactured tablets. *Sutherlandia* tablets became a well-known adaptogenic tonic and appeared to stimulate appetite and reported to work against the muscle-wasting effects of HIV-AIDS. It was shown to have hypoglycemic action andused to assist with the control of sugar homeostasis in diabetics (Albrecht *et al*., 2012; Aboyade *et al*., 2014).

The safety study on *S. frutescens*, although no serious side effects have been reported in ancient times, some people experience side effects when taking *Sutherlandia* on an empty stomach. These include loose stools, dizziness, and dry mouth. Although this herb is commonly used during pregnancy, scientific data regarding its use is not available (Mills *et al*., 2005). The dosage that traditional practitioners give is determined depending on the patient's age, illness and the nature of the complaint (Van Wyk and Albrecht, 2008). There have been various reports of varying dosage of this herbal. A scientific study conducted on the daily dosage of *Sutherlandia* leaf powder indicated that it should be taken at a daily dosage of around 9 mg. This is equivalent to two tablets of the plant per day. A toxicology study conducted on vervet monkeys revealed that the daily dosage of 18 mg of *Sutherlandia* leaf powder did not cause significant changes in the animal's physiological and biochemical properties. A similar study was also conducted on healthy individuals. The results of the study, which was conducted through a double-blind study, showed that 400 mg of leaf powder was tolerated for three months. South Africa's health ministry has also encouraged the use of this herbal for the treatment of AIDS (Johnson *et al*., 2007).

2.4.5 Bioactive compounds isolated from cancer bush

Sutherlandia leaves have non-protein amino acids, and amongst, them the most common compound there is L-Canavanine, pinitol, gamma-aminobutyric acid (GABA), arginine, asparagine and Figure 2.4 shows some of the chemical structure of compounds from the plant. The discovery of high levels of canavanine provided rational reason to the traditional use of *Sutherlandia* against cancer (King, 2021).

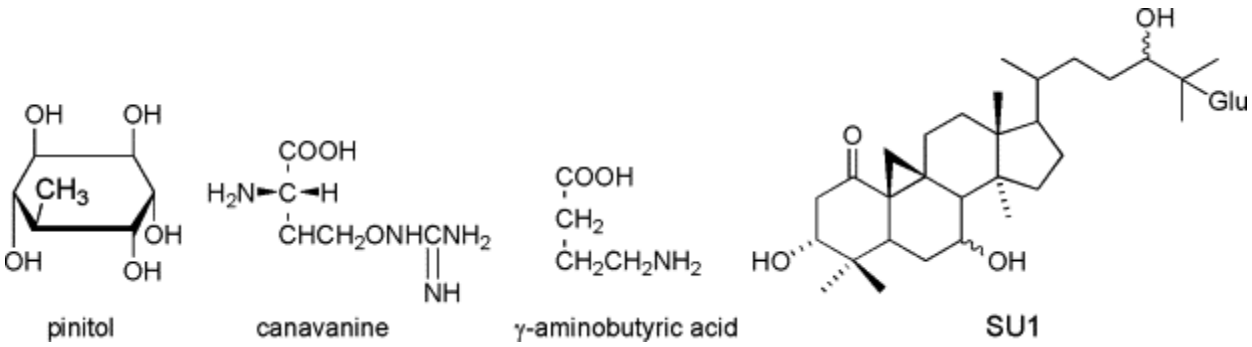


Figure 2.4: Chemical structures of compounds from *Sutherlandia frutescens*.

The documented bioactivities of pinitol justify the traditional uses against diabetes and inflammation as a potentially significant compound in *Sutherlandia*. Pinitol is classified as an anti-diabetic agent, which might have an application in the treatment of cancer and AIDS. It produces an insulin-like effect, leading in low blood sugar levels and elevating glucose available for cell metabolism. Therefore, pinitol appears to play a role in regulating cellular energy, leading to elevated energy levels and a reduction in fatigue. Canavanine has been recognized to possess antiviral and anticancer activity, together with inhibition of retroviruses and influenza virus. The compound -aminobutyric acid (GABA) is an inhibitory neurotransmitter that partly justifies the use of *Sutherlandia* in treating stress and anxiety, and also inhibit tumour cell migration (Van Wyk and Albrecht, 2008).

Flavonoids were detected in the Lessertia genus by Moshe in 1998. Flavonoids can occur in a free state or as glycosides, two constituents known as the biggest group of naturally occurring polyphenolic compounds (Evans, 1989). The use of flavonoids for different illness can be accredited to the wide range of activities namely; anti-cancer, antimicrobial, antioxidant, anti-allergic, antiviral, ect (Evans, 1989; Mills and Bone, 2000).

Other research studies on the leaves of *S. frutescens* led to the discovery of four cycloartanol glycosides, namely; sutherlandiosides A, sutherlandiosides B, sutherlandiosides C, sutherlandiosides D (Fu *et al*., 2008). Another isolation study led to the discovery of four flavonoid glycosides namely; sutherlandin A, sutherlandin B, sutherlandin C, sutherlandin D (Fu *et al*., 2010)

These compounds are associated with the medicinal properties of this plant. The medicinal properties of this plant make it a good subject plant to investigate for new pharmaceuticals in drug development (King, 2021). Medicinal plants harbour a diversity of endophytic microbes, which produce several bioactive compounds. Due to this, there is a necessity to discover the novel and useful bioactive substances to improve human and animal health. New solutions can be investigated to reduce the problems faced by mankind such as antibiotic resistance. Endophytic microbes have vast potential to synthesise different novel compounds, which can be exploited in the pharmaceutical and or agricultural industries (Kaul *et al*., 2012; Golinska *et al*., 2015; Egamberdieva *et al*., 2017).

2.5 ENDOPHYTES

2.5.1 What are endophytes?

Plants may be reservoirs for immeasurable microorganisms, commonly referred to as endophytes (Uzma *et al*., 2019). “Endophytes” originate from the Greek words “endo”, meaning inside or within, and “phyton”, which means plant (Manganyi, 2018). They are a highly diverse group of microorganisms, including fungi, bacteria and actinomycetes, and exist in symbiosis with plants (Sufya, 2014). These endosymbionts are a source of diverse bioactive metabolites with various properties that are useful as antibiotics, immunosuppressants, anticancer compounds, and antimycotics (Musavi and Balakrishnan, 2013). They live within various plant parts such as the

stems, petioles, roots and leaves. They offer a variety of unknown advantages to the host and produce higher yields of stimulating growth hormones for the plant, such as gibberellic acid and indole acetic acid (Gao *et al*., 2018).

2.5.2 Classification of endophytes

The biodiversity of fungal endophytes has been divided into two major groups, Balansiaceous and non- Balansiaceous and four classes, withthe criteria used shown in Table 2.3. Class I endophytes provide the plant with drought tolerance, protect the plant from animals by producing chemicals toxic to animals, and regularly increase the biomass of the plant and these endophytes include Clavicitaceous endophytes. Class II consists of a diversity of mycorrhizal fungi, restricted to an unusual number of plants. They can cause the host plant to be tolerant to habitant specific stress. The occurrence and horizontal transmission is the basis used to differentiate it from Class III endophytes. Class III endophytes inhibit above-ground plant tissues found in vascular and nonvascular. Class IV endophytes are limited to the roots of the plants, and most common are Ascomycetous fungi, in which the roots create melanized structures and microsclerotia. This group of endophytes is found in host plants such as non-mycorrhizal from subalpine, arctic, alpine, temperate zones, Antarctic and tropical ecosystems (Mishra *et al.,* 2014).

Table 2.3: Criteria used to characterize fungal endophytic classes

	Clavicipitaceous	Nonclavicipitaceous		
Criteria	Class I	Class II	Class III	Class IV
Host range	Narrow	Broad	Broad	Broad
Tissue(s) colonized	Shoot and rhizome	Shoot, root and rhizome	Shoot	Root
In planta colonization	Extensive	Extensive	Limited	Extensive
In planta biodiversity	Low	Low	High	Unknown
Transmission	Vertical and horizontal	Vertical and horizontal	Horizontal	Horizontal
Fitness benefits	NHA	NHA and HA	NHA	NHA

*Nonhabitat-adapted (NHA) benefits such as drought tolerance and growth enhancement are common among endophytes regardless of the habitat of origin. Habitat-adapted (HA) benefits result from habitat- specific selective pressures such as pH, temperature and salinity.

2.5.3 PLANT-FUNGAL INTERACTION, CO-EVOLUTION AND RELATIONSHIP

Fungal endophytes are capable of forming relationships with single or multiple plant hosts. Their host-range specificity is due to complex biochemical interactions with their host plants (Huang *et al*., 2008; Selim *et al*., 2011). One recorded phenomenon in endophytes is host recurrence, in which a fungal endophyte occurring in a single host is present in other hosts in the same habitat. Additionally, they showcase host selection over related plant species, and under conducive conditions will favour interaction with a paticular host over the other (Huang *et al*., 2008). However, previous studies suggest that fungal endophytes are not host-specific (Khan, 2007) but rather indicate a selective preference. Their distribution is closely related to the distribution of plants, and the difference in their metabolic profiles and activity, even amongst related species, may be a result of the chemical differences amongst host plants (Selim *et al*., 2011). Fungal endosymbionts benefit from their host by receiving nutrients and protection (Figure 2.5) and in turn, provide their host with tolerance to environmental stresses, diseases and growth factors (Pavithra *et al*., 2020).

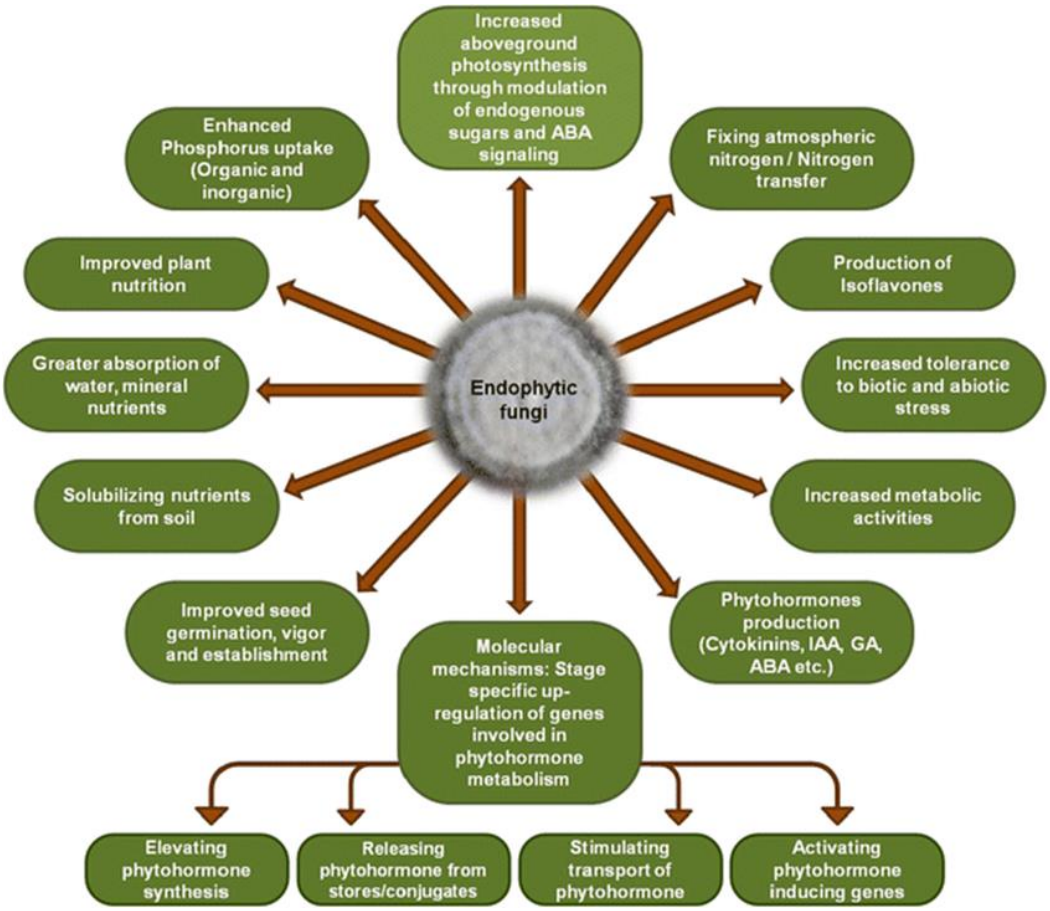


Figure 2.5 Different mechanisms of plant growth promotion by endophytic fungi

Globally, there are approximately 300 000 land plant species and it is estimated that these have larger populations of endophytes inhabiting them. Fungal endophytes are isolated from various plants such as trees (pine and yew), fruits, cereal grains, vegetables, fodders and other crops (Lu *et al*., 2012). Knowledge concerning plant-endophyte interaction is still not fully understood despite fungal endophytes being significant elements in plant micro-ecosystems. A better understanding of their interactions would possibly lead to the discovery of new drugs and improve drug quality (Jia *et al*., 2016).

An endophytes appear as a defensive bio resource, which has inspired researchers to look more

into the mechanism by which it protects the associated host. The ability of endophytes to act as biocontrol agents is an important contribution to the agricultural sector, furthermore, the ability of endophytes to produce bioactive compounds play a significant role as biocontrol agents against plant pathogens. Fungal endophytes have acted against a wide range of microbial pathogens, nematodes, insects and pests (Russo *et al*., 2016). Most significantly, fungal endophytes facilitate induced systemic resistance in plants, which is a vital mechanism for disease management and plant protection (Patshangba, 2017).

2.5.4 ROLE OF ENDOPHYTES IN THE ECOSYSTEM

The interaction between microbial and plant communities drives the ecosystem functioning, maintenance of biodiversity, and community stability. This occurs when the fungal endophyte confers resistance to abiotic and biotic insults to its host, promotes growth and is available as a bio-inoculant and biocontrol agent. Fungal endophytes are role players in carbon and nutrient recycling, attributable to their ability to decompose organic materials (Arora and Ramawat, 2017; Yao *et al*., 2019). Endophytic infection increases microbial root colonisation in plants by triggering the production of root exudates that attract rhizospheric microbes, making it convenient for complex minerals to be extracted and facilitating smooth mineral transport from soil to plant (Chhipa and Deshmukh, 2019).

Apart from supplementing extensive fertilizer use, the relationship of fungal endophytes with crops can play an important role in improving crop growth and yield (Naik *et al*., 2008). Fungal endophytes offer tolerance to drought, metals, disease, heat, and herbivory and/or encourage growth and acquisition of nutrients. This suggests that fungal symbiont integration is a beneficial technique in both alleviating climate change impacts on major crops and extending agricultural

production on marginal lands (Redman *et al*., 2011). Colonization by fungal endophytes involves increased nutrient availability, pathogen and predator protection, stress tolerance, and phytoremediation and rhizoremediation. Different endophytes stabilize, solubilize and mobilize the plant micro-and macro-elements by conferring resistance to stress on host plants (Khan *et al*., 2012; Anitha *et al*., 2013; Arora and Ramawat, 2017; Jain and Pundir 2017; Lata *et al*., 2018; Khare *et al*., 2018; Kumar *et al*., 2019).

Endophytic fungi play a crucial ecological role in the succession of plants through the beneficial interaction between them which has evolved over a long period (Ali *et al*., 2018). Due to the synthesis of a wide variety of bioactive compounds, fungal endophytes have important roles on human health as well as in the ecosystem (Yadav, 2019).

2.5.5 ROLE OF ENDOPHYTES IN AGRICULTURE

Endophytes protect the host from pathogens, insects, pests, nematodes, etc. The natural surroundings of plants expose them to various environmental challenges that influence the development and growth of plants (Bamisile *et al*., 2018). Hormones and many endogenous signals in combination with genetic information regulate plant growth and development. Under severs environmental conditions, fungal endophytic phytohormones are capable ofaffecting the production of phytohormones such as gibberellic acid, auxin abscisic acid, etc, together with secondary metabolites such as flavonoids to protect the plant against stress (Bilal *et al*., 2017).

Agricultural success suffers extensively due to plant pathogens, insects and several abiotic stresses. Agribusiness being one of the world's biggest financial divisions, requires time to discover and set up appropriate methodologies for maintainable agriculture and enhancement of crop development and production. Endophytic fungi live in advantageous association with plants and play a critical

part in plant development advancement, higher seed yield and plant resistance to different biotic and abiotic stresses. Numerous endophytic fungi can produce antimicrobial compounds, plant development hormones and different agrochemical bioactive metabolites. These mycoendophytes hold immense potential for the advancement of eco-friendly and financially sound agrarian items (Rai *et al*., 2014).

The development of resistance to fungicides is among the critical causes of poor disease control in agriculture (Chang *et al.*, 2008) and most fungicides act directly on essential fungal functions such as respiration, sterol biosynthesis or cell division like other pesticides. They also influence populations or activity of other non-target organisms and have been reported to have greater effects on soil organisms than herbicides or insecticides, with the potential to affect beneficial soil fungi (Bi Fai and Grant, 2009).

The attack of different types of fungal pathogens causes a drastic reduction in crop production. For example, one of the world's most important oil crops, Sunflower, is attacked by many diseases. The major diseases of sunflower are caused by fungi, which result in rust, Sclerotinia stalk, head rot, phoma black stem etc., in which their severity reduce the crop yield and quality significantly (Mukhtar., 2009). An estimate of 20% of crop reduction was caused by fungal infection worldwide and has led to the use of fungicides being crucial in the last few decades. This has decreased the effective control they have on plant disease. Because of their low cost and efficiency, fungicides became the primary way of controlling fungi (Maria, 2012). Another method for protecting plants is to activate their internal defense mechanisms through the use of specific biotic or abiotic elicitors (Wagas *et al*., 2015).

Fungal endophytes have insecticidal activities because of their ability to protect the host plant against pests and pathogens. Some of the alkaloids produced by endophytes can also reduce herbivory as they are toxic to insects and vertebrates (Shymanovich *et al*., 2015). From the coffee tree, various genera of entomopathogenic fungibelonging to the *Clonostachys*, *Beauveria*, *Acremonium, Cladosporium* and *Paecilomyces* genera were recovered (Posada and Vega, 2006; Posada *at al*., 2007; Vega *et al*., 2008) and found to be pathogenic to coffee berry borer and control the borer insects in coffee seedling. Endophytic fungi are also known to act as plant growth stimulants, i.e., *Fusarium tricinctum* and *A. alternata* production of indole acetic acid enhanced the plant growth. *Rhizoctonia cerealis, Gaeumannomyce graminis, Phytophthora capsici* and *Pyricularia oryzae* are phytopathogens were found to be active against fungal endophytes such as *Cryptosporiopisis cf. qauercina* and *Colletotrichum sp.* (Aime *et al*., 2008; Sudha *et al*., 2016).

2.6 ENDOPHYTIC FUNGI AS PROMISING POTENTIAL SOURCES OF BIOACTIVE COMPOUNDS FROM

Studies have shown the potential of plant-endophyte interactions to produce pharmacologically significant natural products. These studies have brought about the notion that endophytes might be facilitators in plant's ability to produce medicinal chemicals. Various chemicals and biologically active compounds have been discovered (Uzma *et al*., 2019). Endophytes are valuable for the pharmaceutical production of substance as a result of their ability to yield secondary metabolites and enzymes. Furthermore, they consist of rich and reliable sources of genetic diversity and biological novelty (Lu *et al*., 2012).

2.7 PHARMACOLOGICAL APPLICATIONS OF VARIOUS ENDOPHYTIC FUNGI

Fungal endophytes are a significant source of novel metabolites with therapeutic potentials (Rodrigues *et al*., 2000). More than 20,000 bioactive metabolites are of microbial origin. Additionally, fungi have led to the discovery of novel metabolic pathways, drugs and synthetic modifications.

The success of recovering several medicinal drugs from microbial origin such as the antibiotic penicillin from *Penicillium* sp., the immunosuppressant cyclosporine from *Tolypocladium inflatum* and *Cylindrocarpon lucidum*, the antifungal agent griseofulvin from *Penicillium griseofulvum*, the cholesterol biosynthesis inhibitor lovastatin from *Aspergillus terreus* fungus, and β-lactam antibiotics from various fungi, has shifted focus on drug discovery from plants to microorganisms (Selim *et al*., 2011).

Endophytic fungi can produce the same bioactive compounds produced by their host plants. Bioactive compounds such as podophyllotoxin, paclitaxel, camptothecine, hypericin, vinblastine and diosgenin are available on both the endophyte and the plant. The secondary metabolites found in medicinal plants could serve as a potential source of resistance modification and antimicrobial characteristics. Plant extracts can fix protein domains resulting on inhibition or modification protein-protein interactions (Jia *et al*., 2016).

2.7.1 ANTICANCER ACTIVITY

Paclitaxel (taxol) is an anticancer compound isolated from endophytic fungi. This active anticancer drug was discovered in the bark of a yew tree species and has a distinctive manner in which it prevents depolymerization of tubulin during cell division. There are various reports on the

production of other potential anticancer compounds besides paclitaxel, such as torreyanic acid isolated from an endangered tree *Torreya taxifolia* (Sudha *et al*., 2016); Figure 2.6 presents some of the compounds produced by endophytes.

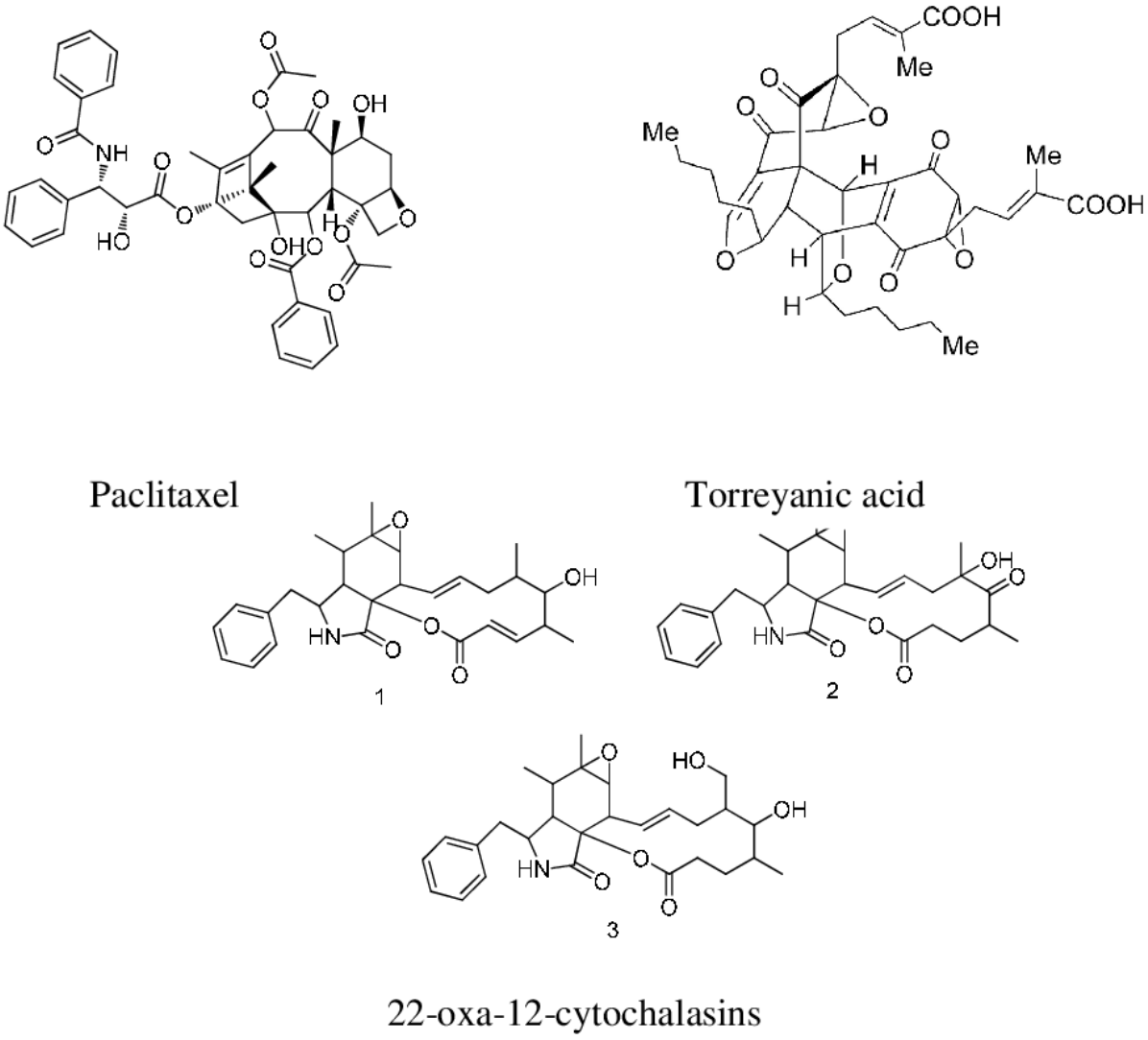


Figure 2.6: Anticancer compounds produced by endophytes (Siriwach, 2013)

Taxomyces andreanae is an endophyte discovered to produce taxol. The isolation through microbial fermentation was more cost-effective and produced a higher concentration in

comparison to plant taxol. Several anticancer compounds such as paclitaxel have been isolated from fungal endophytes as well as medicinal plants. Torreyanic acid, a specific cytotoxic quinone dimer, was identified from *Pestalotiopsis microspora* isolated from the endangered tree *Torreya taxifolia* and caused cell death via apoptosis (Pimentel *et al*., 2011; Siriwach, 2013). Sclerotiorin isolated from endophytic fungus *Cephaotheca faveolata* demonstrated activity against cancer cells and promoted apoptosis in cancer cells (Giridharan *et al*., 2012).

2.7.2 ANTIOXIDANT ACTIVITY

Medicinal plants, fruits, and vegetables are the common source of natural antioxidants compounds. However, endophytes produce new natural metabolites that exhibit antioxidant activity (Chandra *et al*., 2020). Figure 2.7 shows the chemical structure of pestacin and isopestacin which have antioxidant properties and they were isolated from *Pestalotiopsis* microspore, endophytes isolated from the Papua New Guinea plant, *Terminalia merobenisis* (Jalgaonwala *et al*., 2011)

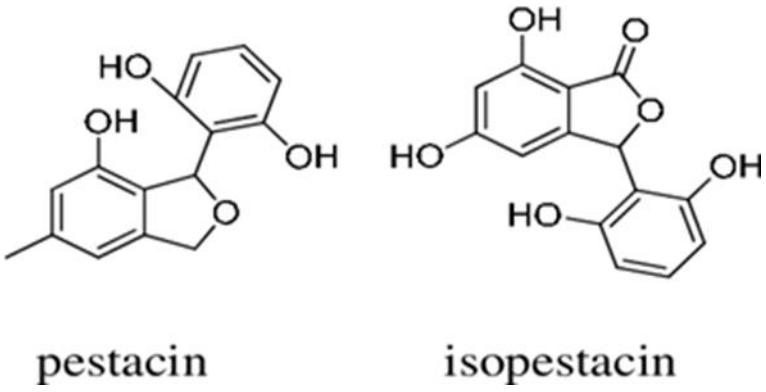


Figure 2.7: antioxidant compounds produced by endophytes (Siriwach, 2013)

2.7.3 ANTIMICROBIAL ACTIVITY

2.7.3.1 Metabolites as antibacterial compound

A large number of metabolites showing antimicrobial activity have been isolated from endophytes, which belong to several structural classes like alkaloids, terpenoids, quinines, phenols, flavonoids and steroids. The endophytes are known to produce a resistance mechanism for plants to withstand pathogenic attacks by producing secondary metabolites. New antimicrobial metabolites isolated from endophytes are a good way to enhancing the treatment of human and plant pathogens and provision of effective antibiotics against different bacterial species. In the food industries, antimicrobial compounds have been utilized in the preservation of food, to reduce food-borne diseases.

2.7.3.2 Metabolites as a source of antifungal compound

Various metabolites are attained from fungal endophytes with the ability of antifungal agents (figure 2.8). Microbial natural products served as an alternative natural pool for the isolation of distinctive molecule for different therapeutic applications (Bhardwaj and Agrawal, 2014).

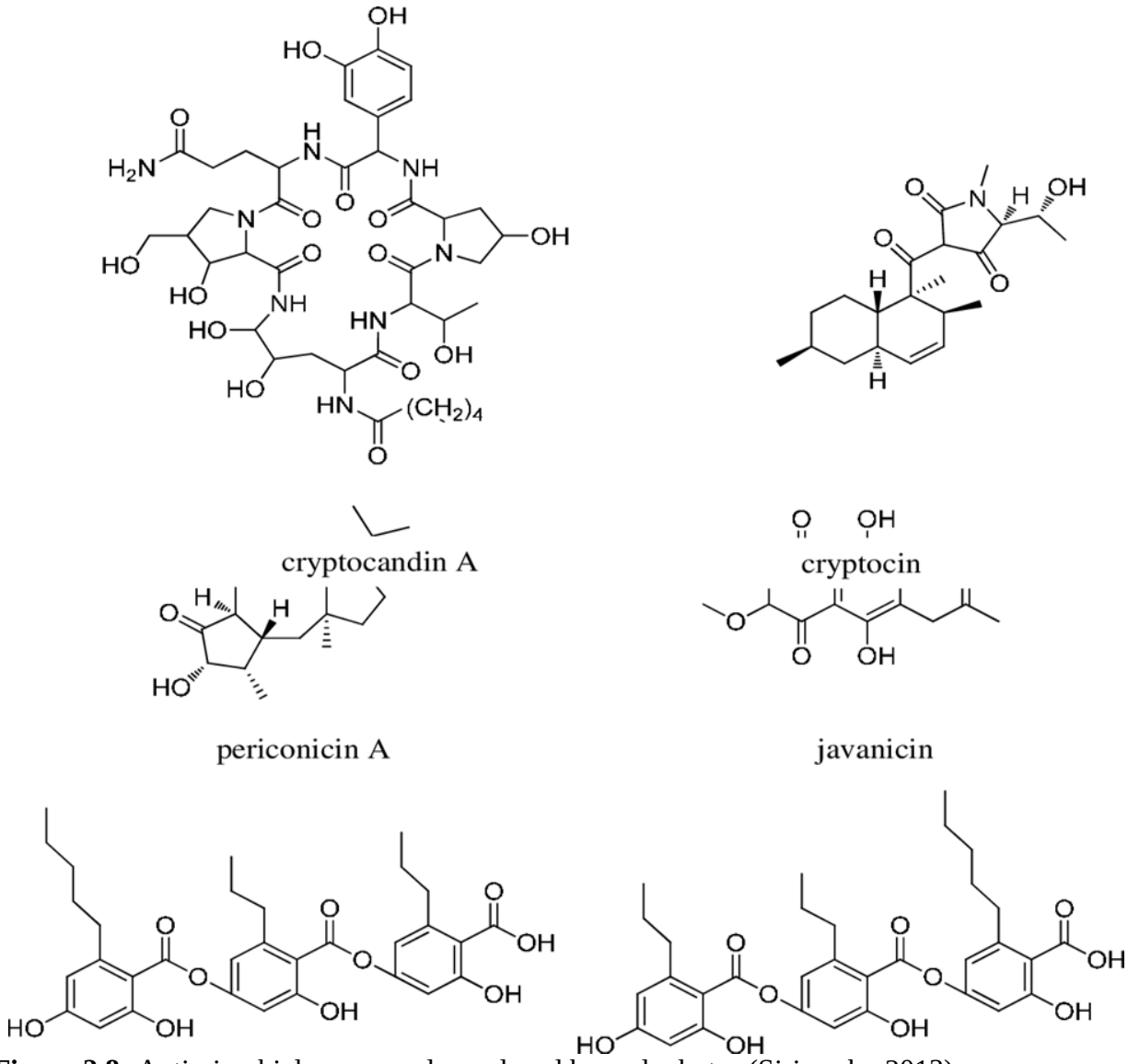


Figure 2.8: Antimicrobial compounds produced by endophytes (Siriwach., 2013)

2.8 SECONDARY METABOLITES

Plants are capable of producing unlimited organic compounds that are of a diverse range. These substances are traditionally referred to as secondary metabolites. They are often distributed among limited taxonomic groups within the plant kingdom. The primary metabolites such as phytosterols, acyl lipids, nucleotides, amino acids, and organic acids, are found in all plants and perform metabolic roles that are essential and usually evident (Croteau, 2000).

Secondary metabolites are known to be compounds that are of significance in the relation of the plant with its environment, adaptation and defence, unlike primary metabolites that do not influence in the maintenance of the plant's life processes. However, a wide variety of secondary metabolites of higher plants are synthesised from amino acids, lipids and carbohydrates which are primary metabolites. Often when plants are exposed to stress metabolites accumulate.

Some of the secondary metabolites and their biological activities are not yet known. Despite having unknown or obscure functions, they do however, have remarkable significance to humankind by the broad range they have shown of useful antibiotics and pharmaceutical activities as well as less desirable immunomodulatory and toxic activities (Khan, 2007). Due to the exhibited bioactivity, and the fact that many secondary metabolites can be regarded as promising leads for drug development efforts, Penicillin (a β-lactam antibiotic) and lovastatin (a cholesterol-lowering drug) are some examples of pharmaceutical significance and industrial impact associated with the application of secondary metabolites (Boruta, 2018; Hyde *et al*., 2019). Plant secondary metabolites are a distinct source of flavours and additives. They also add to the colour, taste and specific odours of plants.

Types of plant secondary metabolites

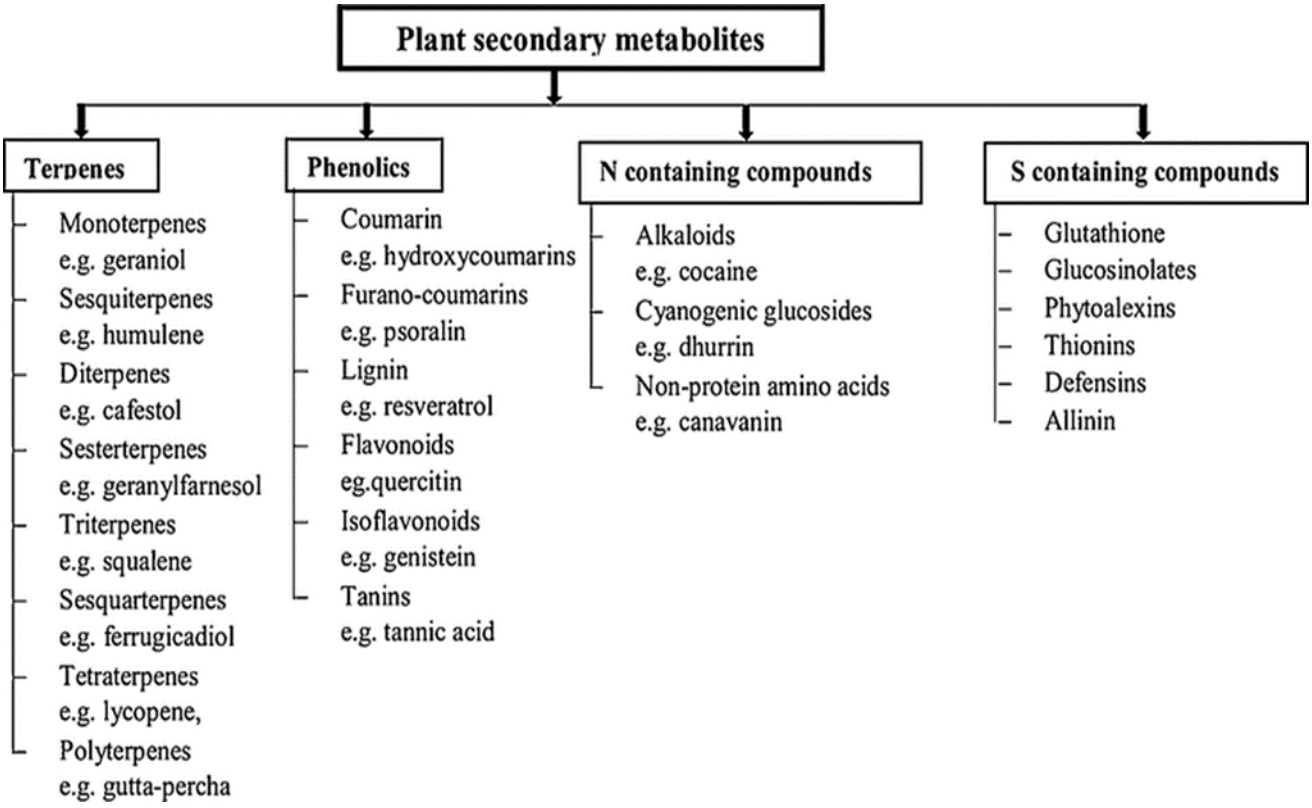
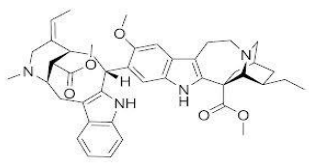
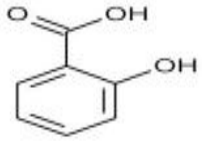
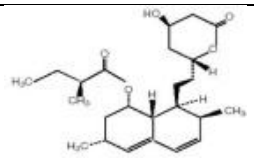
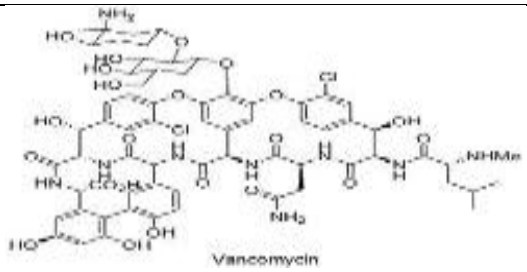


Figure 2.9: Types of secondary metabolites

2.8.1 SECONDARY METABOLITES RECOVERED FROM ENDOPHYTES

The ability of fungal endophytes to defend their host plant from pathogenic microorganisms and resist a systemic relationship in the host plant is now being implemented as the new method for controlling plant diseases. The association of the plant and endophyte regulate the fabrication of secondary metabolites in the host plant. The isolated metabolites from fungal endophytes present a broad spectrum of pharmacological properties including antifungal activity, antiviral, anticancer and antibacterial (Uzma *et al*., 2018).

Table 2.4: Secondary metabolites recovered from endophytic fungi

Secondary metabolite	Chemical structure	Endophytic fungi	Application
Penicillia		*Penicillium* sp.	Pain control
Salicliyc		*Salix* sp (willow)	Analgesic anti inflammatory
Lovastatin		*Aspergillus terrus*	Lowering of cholesterol
Vanomycin		*Nocardia orientalis*	Antibiotics

2.10 CONCLUSION

In the last century, the use of fungi to produce substances of significant commercial value has increased rapidly. The exploitation of fungi by humans is an old phenomenon. The fermentation of alcohol was one of the earliest known examples of humans using the biochemical activities of

fungi, the use of yeast to leaven bread also dates back to biblical times. The production of alcohol, biomass and the production of therapeutic compounds, as well as the production of simple organic compounds, are still the main areas where fungi are still used.

The beginning of industrial mycology was probably marked by the sulphate process developed for the production of glycerol by yeast fermentation, which was used during World War I. However, the greatest expansion in the industry took place when the submerging culture techniques were used in penicillin fermentation.

CHAPTER THREE

ISOLATION, MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF ENDOPHYTIC FUNGI FROM CANCER BUSH

ABSTRACT

Objective: The primary objective in this chapter was to determine the biodiversity of endophytic fungi isolated from the medicinal plant *S. frutescens* using conventional and molecular techniques.

Method: In the present study a total of 10 *S. frutescens* plants were purchased from Mountain Herb Estate Nursery in Pretoria, South Africa (S 25˚43.459' E 027˚57.914'). The leaves of the plants were analyzed for the presence of endophytic fungi. Initially, leave segments were surface sterilized using 2% (v/v) sodium hypochlorite and ethanol then rinsed with distilled water to remove trace residues. The leaves were placed on potato dextrose agar (PDA) and after seven days the isolates were purified. The identities of the purified fungal isolates were determined using a combination of morphological features followed by amplification of the internal transcribe spacer (ITS), specifically ITS1 and ITS4 sequences. In addition, molecular phylogenetic analysis using nuclear ribosomal DNA sequences was performed to establish the evolutionary relationships of the fungal isolates.

Results: A total of fifty-one (51) fungal endophytes were isolated, identified and classified into their respective genera. The predominant genus found was *Penicillium* (25%), followed by the *Mucor* (12%), *Alternaria* (10%) and *Coniochateta* (10%) genera. The endophytes were further classified to species-level based on sequences of known identities obtained from the GenBank database. With the sequence data, a phylogenetic tree was constructed and t isolates placed into four clusters and six sub-clusters with a large proportion (88%) belonging to the class Ascomycota.

Conclusion: To the best of our knowledge, the current study is the first report on the biodiversity of endophytic fungi from *S. frutescens* and these results suggest that *S. frutescens* can harbour diverse endophytic communities with important biologically active components.

Keywords *Sutherlandia frutescens*, Endophytic fungi, Biodiversity, ITS sequence

3.1 INTRODUCTION

The selection of a host plant is very important when working with endophytic fungi. Endophytes are ubiquitous in the plant kingdom and have been isolated from a broad spectrum of hosts. According to fungal studies, approximately1 million fungal endophytes are known to exist (Huang *et al*., 2008). These are microorganisms that asymptomatically inhabit tissues of different kinds of plants such as herbaceous plants, grasses, algae and trees. Bioactive compounds have been isolated and characterized directly from medicinal plants. However, the discovery of the ability of endophytes to produce the same bioactive compounds as their host plant has resulted to a shift, from plants to the fungi in pursuit of new drug sources (Nisa *et al*., 2018). Endophytes represent an extensive diversity of microbes that have evolved by adapting to special and unfavorable environments, presenting them as an important aspect of research in exploring new drugs for agricultural, medical and industrial applications (Santos *et al*., 2015).

The occurrence of different fungal communities varies within different host plants as well as the different portions (roots, stems and leaves) within the same plants (Yin Lu *et al*., 2012). Proper identification of fungal species is vital for both fundamental (ecological and, taxonomic grouping) and applied (genomic and bioprospecting purposes) studies. This is based on the fact that fungi are morphologically, metabolically, ecologically and phylogenetically diverse, and their diversity

offers some advantages to the host plant as well as their potential to produce unique metabolites. They produce various bioactive compounds (Tibpromma *et al*., 2018), and that makes them valuable for the discovery of alternative bioactive compounds with pharmaceutical and industrial applications (Verma *et al.*, 2017).

In this study the diversity of endophytic fungi isolated from *S. frutescens* was assessed (based on the presence of discernible spores and reproductive structures) (Visagie *et al*., 2014) and molecular techniques.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Table 3.1 illustrate the list of culture media used in the current study as well as the composition of media and preparation.

Table 3.1: Media preparations

Media Name	Constituents	Preparation procedure
Potato Dextrose Agar PDA	Typical formulation grams/ liter Potato Extract 4.0 Dextrose 20.0 Agar No.1 15.0	• 39 grams of the powder was weighed and dispersed in 1 liter of deionized water. • The solution was soaked for 10 minutes, swirled and autoclaved at 121°C for 15 minutes. • The agar was allowed to cool down and poured into plates.
Malt Extract Broth (M6409)	Malt extract Maltose Yeast extract and dextrose	• 15 grams of the powder was weighed and dispersed in 1 liter of distilled water. • The solution was boiled to dissolve the medium completely. • Sterilized by autoclaving for 15 minutes at 121°C.
70% ethanol	300mL (or 3/10) distilled water 700mL (or 7/10) absolute ethanol	• Ethanol was added to distilled water.
Sodium hypochlorite dilution	143mL Distilled water 375mL of 3.5% sodium hypochlorite	• 3.5% of Sodium hypochlorite was converted to a 2.0% by adding 375 ml of sodium hypochlorite into a 500 ml bottle and the volume was adjusted to 500 ml with distilled water.

All chemicals used in this study were purchased from Merck-Biolabs, Gauteng, South Africa.

3.2.2 METHODS

3.2.2.1 Sampling site and sample collection

Sutherlandia frutescens (cancer bush) is on the Red List of South African Plants (SANBI). This explains why healthy *S. frutescens* plants were purchased from Mountain Herb Estate located in Kameeldrift-West, Pretoria, South Africa (S 25°43.459' E 27°57.914). Collection of the plant was done following strict ethical principles from National Environmental Management Act (Act 107 of 1998) that is associated with Environmental Impact Assessments (EIA) regulations. The plants were carefully selected using random sampling based on the availability and the absence of disease symptoms. A total of 10 plants were successfully collected (Figure 3.1) couriered to the

Antimicrobial Resistance and Phage Biocontrol Research Laboratory in the Department of Microbiology, North-west University. Samples were processed within 48 hrs upon arrival in the laboratory.

Figure 3.1: Plant samples of *Sutherlandia frutescens* (cancer bush)

3.2.2.2 Isolation of endophytic fungi

The leaves of *S. frutescens* were randomly selected and harvested from the plants and washed thoroughly under running water to remove dust and debris. The leaves were sterilized according to Araújo *et al*. (2001). Accordingly, the leaves were surface disinfected with 70% (v/v) ethanol for 1 min and later disinfected with 2% (v/v) sodium hypochlorite (NaClO) solution for 2 mins. Afterwards, the leaf samples were rinsed with 70% (v/v) ethanol for 20 secs and finally rinsed twice with sterile distilled water The samples were then blotted with sterile filter paper to remove excess moisture. Finally, five segments from each leaf sample was aseptically placed on a nutrient-poor media [Water Agar (WA)], and a nutrient-rich media [Potato Dextrose Agar (PDA)]. The plates were then incubated aerobically at 25 °C for 7–10 days.

3.2.2.3 Fungal purification

Pure fungal colonies were obtained by purifying the isolates repeatedly through sub-culturing on sterile PDA. This was done by inoculating a single spore or a piece of mycelium on to fresh PDA. The inoculated plates were placed in an autoclave at 25°C for 7-10 days after which agar plates with pure isolates were used for further identification.

3.2.2.4 Morphological Identification

All isolates were deposited, preserved, maintained and stored in the National Collections of Fungi, Agricultural Research Council, Plant Protection Research Institute, Biosystematics, South Africa (ARC, PPRI). Each isolate was assigned a unique PPRI accession number issued by ARC, PPRI. Both microscopic and reproductive structures were used for the identification of fungal isolates by conventional means. All fungal isolates were subjected to morphological investigation using a light microscope with a digital imaging system to observe the special structure and nature of hyphae. Microscopic slides were prepared according to specific to specific protocols (Papagianni, 2004) and microscopic structures captured.

3.2.2.5 Molecular Identification

3.2.2.5.1 DNA extraction

Deoxyribonucleic acid (DNA) extraction was done on all investigated fungal isolates. Nearly 100 mg of the fungal mycelia was aseptically scraped and used for DNA extraction. The DNA was extracted using Zymo Research Mini Plant Kit following the manufacturer's instructions (Zymo Research, Hilden, Germany).

Fresh fungal cells were added into a ZR BashingBeadTM Lysis Tube (0.1 mm & 0.5 mm) and suspended in 750 µL of BashingBeadTM Buffer. Cells were manually pulverised in the mixture using a plastic pulveriser until homogeneous (the pulveriser was sterilised by dipping it into 70 % ethanol and passing over the flame). The homogeneous mixture was centrifuged (TOMOS MultiStar21, TOMOS Life Science Group, Singapore) for one minute at 10 000 xg. From the tube, the supernatant (400 µL) was transferred onto Zymo-SpinTM III-F Filter which was placed in a collection tube and subsequently centrifuged for one minute at 8 000 xg. The Genomic Lysis Buffer (1 200 µL) was added to the filtrate and 800 µL of the mixture transferred onto Zymo-SpinTM IIC column then placed in a collection tube. This was centrifuged for one minute at 10 000 xg. The remaining filtrate was added again onto the same column and centrifuged under the same conditions. The Zymo-SpinTM IIC Column was placed in a clean collection tube, thereafter, 200 µL of DNA Pre-Wash Buffer was added onto the column then centrifuged for a minute at 10 000 xg. Using the same column, 500 µL of g-DNA Wash Buffer was added and centrifuged under the sameconditions. The column was transferred to a new centrifuge tube. Subsequently, 100 µL of DNA elution buffer was added directly onto the column matrix and then centrifuged for 30 seconds at 10 000 xg to elute the DNA onto the centrifuge tube. The DNA was used for downstream applications, including polymerase chain reaction (PCR).

3.2.2.5.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed to amplify internal transcribed spacer (ITS) regions for all investigative fungal isolates. The amplification was done using oligonucleotide primer pairs ITS1 and ITS4 in table 3.2 adapted from Khorasani (2013). The final volume of the PCR mixtures was 20 µL. Each tube comprised of 10µL of 10X Master-mix, 0.5 µL of each primer (ITS1 and ITS4), 1µL of diluted DNA and 8µL of dH_2O. Amplifications cycles were set with an

initial denaturation at 94 ºC for 10 mins, 30 cycles denaturation at 94 ºC for 30 seconds, annealing at 50-52 ºC, elongation at 72 ºC for 45 seconds and a final elongation at 72 ºC for 7 mins. The PCR amplicons were then stored at 4 ºC until electrophoresis.

Table 3.2: Primer sequences used for PCR amplification of the ITS gene sequences

Primer[1]	Sequence	Reference
ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	Manganyi *et al*. (2018)
ITS4	5′-TCCTCCGCTTATTGATATGC-3′	

3.2.2.5.3 Gel electrophoresis

All PCR amplicons were separated by electrophoresis on a 2% (w/v) agarose gel (Sambrook *et al.*, 1989). Electrophoresis was conducted in a horizontal Pharmacia Biotech equipment system (model Hoefer HE 99X, Amersham Pharmacia biotech, Sweden) for 1 h at 75 V, 250 mA using 1X TAE buffer. A 100 bp DNA molecular weight marker (Thermo Fisher, Waltham, Massachusetts, USA) was included in each gel to confirm the sizes of the amplicons. Gels were stained in ethidium bromide (0.1 µg/mL). A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture images using Gene Snap (Version 6.00.22) software.

3.2.2.5.4 Sequencing analysis of PCR amplicons and data analysis

PCR amplicons were sequenced at Inqaba Biotec, Pretoria, South Africa. The BioEdit was used for editing and aligning both forward and reverse sequences, to construct consensus sequences. The identities of the isolates were confirmed using a Blast Search with National Centre for Biotechnology Information (NCBI) Search Tool: (http://blast.ncbi.nlm.nih.gov/Blast.cgi), *Fusarium* MLST and MycoBank databases.

3.2.2.5.5 Phylogenetic analysis

The sequences were edited and subjected to BLAST search to verify the identity, and taxonomic classification in relation to sequence similarity and phylogenetic interpretation. The investigated sequences were then aligned with other similar sequences extracted from GenBank using the ClustalX, BioEdit and MEGA program.

3.2.2.6 Statistic analysis

Colonization rate (CR) was calculated as the total number of segments incubated divided by the total number of plant tissue segments

$$\text{Colonization frequency (CF)} = \frac{\text{Number of segments colonized by the fungi}}{\text{Total number of segments observed}} \text{ X } 100$$

Isolation rate (IR) was the total number of segments incubated divided by the number of endophytic fungi isolated from plant segments

$$\text{Isolation Rate (IR)} = \frac{\text{Number of isolates obtained from tissue segments}}{\text{Total number of segments}} \text{ X } 100$$

Relative frequency (RF) was calculated by the number of isolates designated types of a strain isolated from tissue blocks/number of total tissue blocks) × 100%.

$$\text{Diversity /Relative frequency (RF)} = \frac{\text{Number of isolates of a species}}{\text{Total number of isolates}} \text{ X } 100$$

3.3 RESULTS

3.3.1 Morphological identification of endophytic fungi

In the current study, fifty-one fungal endophytes were successfully isolated 475 leaf fragments from 10 *Sutherlandia* plants and identified using morphological features. All of the isolates produced moderately fast growing colonies on PDA which were observed to be green, gray, brown, yellow, white and pink in color, as illustrated in Table 3.1. The colonization frequency from this study was 80% and the isolation rate was 11%.

Figure 3.2 and Table 3.3, show differences in morphological features of the fungi isolated. Results revealed the fungi varied in terms of color of the colonies, the nature of hyphae, and presence of special structures.

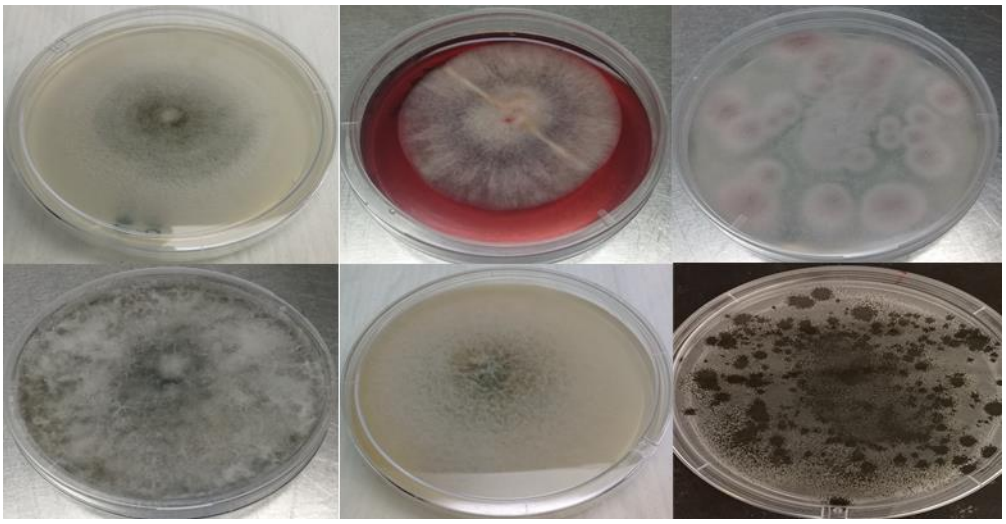


Figure 3.2: Macroscopic characteristics of endophytic fungi on PDA plates isolated from leaves of *S. frutescens*

Table 3.3: Macroscopic features to identified endophytic fungi

Sample ID	Probable genus	Microscopic Characteristics		Microscopic Characteristics	
		Colony Color		Nature of Hyphae	Presence of special structure
		Top	Bottom		
CB 001	unknown	Gray white	Black	Septate	Conidia produced on Conidiophore
CB 002	Unknown	Yellow with white	Cream white	Unknown	Unknown
CB 003	Unknown	Yellow	Yellow	Unknown	Unknown
CB 004	Unknown	Cotton white	Cream white	Unknown	Unknown
CB 005	Unknown	Gray	Red/ maroon	Unknown	Unknown
CB 006	*Curvularia*	Gray green	Black	Unknown	Conidiophore giving rise to Conidium
CB 007	Unknown	Cream white to yellowish	Cream white to yellowish	Unknown	Unknown
CB 008	*Rhizopus*	White	White	Aseptate	Sporangiophore with Sporanguim
CB 009	Unknown	Yellowish with fur	Light yellow	Unknown	Unknown
CB 010	Unknown	Cotton white	Cream white	Unknown	Unknown
CB 011	*Aspergillus*	Dusty pink	Light brown	Aseptate	Foot cell from which the conidiophore produced theconidia
CB 012	Unknown	Fluffy white	Gray yellowish	Unknown	Unknown
CB 013	Unknown	White	Brown to yellowish	Unknown	Unknown
CB 014	Unknown	Yellow	Yellow	Unknown	Unknown
CB 015	*Cladosporium*	Green	Dark green	Septate	Conidia produced on Conidiophore
CB 016	Unknown	Yellow white	Yellow	Unknown	Unknown
CB 017	Unknown	White	Yellow	Unknown	Unknown
CB 018	*Acremonium*	Yellow	Yellow	Septate	Conidiophores with conidiogenous cell and conidia
CB 019	Unknown	White gray	White green	Unknown	Unknown
CB 020	*Cladosporium*	Dark green	Black	Septate	Conidia produced on Conidiophore
CB 021	Unknown	Light gray white	Brown	Unknown	Unknown
CB 022	*Alternaria*	Green white	Black	Septate	Foot cell from which the conidiophore produced the conidia

CB 023	*Alternaria*	Green grayish	Black	Septate	Foot cell from which the conidiophore produced the conidia
CB024	Unknown	Cream white	Cream white	Unknown	Unknown
CB025	*Alternaria*	Light gray white	White gray	Septate	Foot cell from which the conidiophore produced the conidia
CB026	Unknown	Green	Black	Unknown	Unknown
CB027	*Rhizopus*	Light gray white fur	Light gray	Aseptate	Rhizoid stolon columnal
CB028	Unknown	Green	Green	Unknown	Unknown
CB029	*Rhizopus*	Light gray	Cream white	Aseptate	Rhizoid stolon columnal
CB030	*Penicillium*	Green	Green	Septate	Foot cell gave rise to conidiophore
CB031	Unknown	Purple pink	Brown	Unknown	Unknown
CB032	Unknown	Green	Green	Septate	Unknown
CB033	*Penicillium*	Green	Green	Septate	Foot cell gave rise to conidiophore.
CB034	*Penicillium*	Green	Green	Septate	Foot cell gave rise to conidiophore.
CB035	*Bipolaris*	Green	green	Septate	Unknown
CB036	Unknown	White yellowish	Yellow	Unknown	Unknown
CB037	*Penicillium*	Green	Green	Septate	Foot cell give rise to conidiophore.
CB038	*Penicillium*	Green	Green	Septate	Foot cell give rise to conidiophore.
CB039	Unknown	Yellow	Yellow	Septate	Foot cell on the macroconidia
CB040	*Alternaria*	Dark brown + white fur	Black	Septate	Foot cell from which the conidiophore produced the conidia
CB041	*Bipolaris*	Green with white fur	Black	Septate	Unknown
CB042	*Alternaria*	Black with yellow edges	Black	Septate	Foot cell from which the conidiophore produced the conidia
CB043	Unknown	Green with white fur	White	Unknown	Unknown
CB044	Unknown	Green with white edges	White	Unknown	Unknown
CB045	Unknown	Green brownish	Black	Unknown	Unknown
CB046	*Penicillium*	Green white edges	White	Septate	Foot cell gave rise to conidiophore
CB047	*Penicillium*	Green white edges	White	Septate	Foot cell gave rise to conidiophore

CB048	Unknown	Dark green	Black	Unknown	Unknown
CB049	Unknown	Cotton white	Cream white	Unknown	Unknown
CB050	*Aspergillus*	Black (yellow edges)	Cream white	Septate	Foot cell present
CB51B	*Rhizopus*	Fluffy white	White	Aseptate	Rhizoid stolon columnal

3.3.2 Molecular identification

A total of 51 presumptive fungal isolates were subjected to DNA extraction. The presence of DNA was confirmed by performing gel electrophoresis on a 1% (w/v) agarose gel with a 100 bp DNA ladder (Meck-Biolab, UK, London). The ITS gene sequences were amplified in all the isolates and the band size of 600 bp was obtained. Figure 3.3 indicates a representative image of the ITS gene sequences amplified from the isolates. The resulting sequences were cleaned, blast and aligned with closely related known sequences in GenBank. Based on their ITS sequences, fungal isolates were identified and then classified into 17 genera. The predominant genus was *Penicillium* followed by *Alternaria*, *Mucor*, and *Coniochateta*. The isolates were identified to species level based from identities in GenBank.

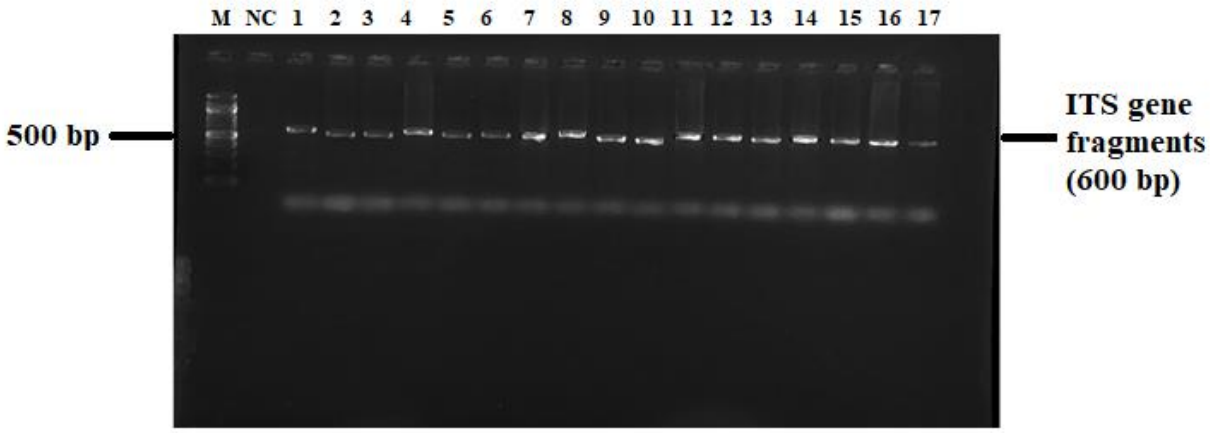


Figure 3.3: Image of 2% (w/v) agarose gel picture of the ITS fragments amplified from fungal isolates. Lane M= 100 bp Molecular weight marker; lane NC= Negative control (No template DNA reaction; Lanes 1-17 = ITS gene fragments amplified from fungal isolates in the study.

The identity of isolates according to BLAST search and the percentage similarity to previously deposited sequences are displayed in Table 3.4. Results showed that 33(65%) of the isolates had 90% - 100% similarity to the sequences in GenBank, and 12 (24%) of the isolates ranged between 62% -87%. However, isolates CB027 and CB043 had the lowest sequence similarity percentages of 21% and 32% to previously deposited sequences in GenBank and were respectively identified as *Mucor ctenidius* and *Penicillium brevicompactum*.

Table 3.4: Morphological identification, GenBank accession numbers and their top BLAST match sequences of the fungal isolates

Sutherlandia frutesence				
Sample ID	**Closest related species**	**GenBank Best Blast match**		
		Accession No.	Percentage coverage	Max Identity
CB001	*Penicillium thomii*	NR_077159.1	62	91.31%
CB002	*Arthrinium marii*	NR_166043.1	99	99.04%
CB003	*Chaetomium globosum*	MT341778.1	100	98.78%
CB004	*Pseudothielavia arxii*	NR_165589.1	99	95.64%
CB005	*Arcopilus cupreus*	MH861590.1	99	97.85%
CB006	*Pseudopithomyce palmicola*	MN788110.1	100	99.51%
CB007	*Chaetomium globosum*	MT341778.1	100	93.25%
CB008	*Mucor circinelloides*	AY243943.1	94	93.34%
CB009	*Chaetomium graminiforme*	MH861772.1	90	84.35%
CB010	*Arthrinium phaeospermum*	KC253945.1	96	98.73%
CB011	*Purpureocillium lilacinum*	MT453285.1	93	99.47%
CB012	*Kalmusia italica*	MG751297.1	75	99.27%
CB013	*Coniochaeta hoffmannii*	NR_167688.1	98	98.99%
CB014	*Coniochaeta hoffmannii*	MG491499.1	98	98.99%
CB015	*Penicillium glabrum*	NR_163530.1	96	85.97%
CB016	*Coniochaeta hoffmannii*	NR_167688.1	100	98.98%
CB017	*Chaetomium globosum*	MK773578.1	99	93.07%
CB018	*Coniochaeta hoffmannii*	NR_167688.1	99	99.49%
CB019	*Mucor* sp.	MW789352.1	80	98.75%
CB020	*Mucor* sp.	MW789352.1	73	97.66%
CB021	*Alternaria angustiovoidea*	MH861939.1	96	86.72%
CB022	*Alternaria angustiovoidea*	MH861939.1	90	92.66%
CB023	*Alternaria tenuissima*	MT671460.1	92	96.52%

CB024	*Chaetomium globosum*	MK611833.1	90	86.21%
CB025	*Alternaria tenuissima*	MH374277.1	97	95.71%
CB026	*Cladosporium* sp.	MT103034.1	65	74.16%
CB027	*Mucor ctenidius*	NR_168144.1	21	93.80%
CB028	*Cladosporium cladosporioides*	KP065743.1	100	99.46%
CB029	*Mucor* sp.	MW789352.1	100	95.61%
CB030	*Penicillium glabrum*	MN251036.1	100	90.67%
CB031	*Cadophora fastigiata*	MF077223.1	100	99.35%
CB032	*Exophiala* sp.	AB488490.1	99	99.05%
CB033	*Penicillium glabrum*	MK910051.1	87	99.14%
CB034	*Penicillium glabrum*	MK910051.1	100	98.45%
CB035	*Exophiala* sp.	AB488490.1	99	98.43%
CB036	*Cadophora malorum*	MF494620.1	66	98.37%
CB037	*Penicillium glabrum*	MK910051.1	99	98.28%
CB038	*Penicillium glabrum*	MK910045.1	79	98.10%
CB039	*Coniochaeta hoffmannii*	NR_1677688.1	67	98.31%
CB040	*Alternaria* sp.	MK640595.1	100	97.91%
CB041	*Curvularia spicifera*	MH271090.1	100	100.00%
CB042	*Pithomyces chartarum*	MH860227.1	99	98.36%
CB043	*Penicillium brevicompactum*	MT558924.1	32	78.97%
CB044	*Penicillium brevicompactum*	MN577353.1	100	97.77%
CB045	*Penicillium brevicompactum*	KR704880.1	99	98.12%
CB046	*Penicillium brevicompactum*	KR704880.1	100	98.13%
CB047	*Penicillium brevicompactum*	NR_121299.1	98	83.71%
CB048	*Penicillium kongii*	MT558920.1	74	93.47%
CB049	*Simplicillium* sp.	MN788113.1	77	99.48%

CB050	*Aspergillus brasiliensis*	KT378129.1	99	98.17%
CB051	*Mucor* sp.	MW789352.1	76	99.84%

3.3.3 Diversity of cultured endophytic fungi associated with *S. frutescens*

Fifty-one endophytic fungi with diverse colony morphologies were isolated from the leaves of *S. frutescens*. The identities of these endophytic fungi was confirmed by PCR sequencing of internal transcribed spacer regions (Table 3.4) and by phylogenetic analysis (Figure 3.5). The dominating isolates among the fungal isolates from *S. frutescens* leaves were *Penicillium* (25%), followed by *Mucor* (12%), whereas *Alternaria* and *Chaetomium* represented only 10% of the fungal community (Figure 3.4). Additionally, the molecular data showed the presence of isolates that belong to other genera viz: *Cadopora* (4%), *Aspergillu*s (2%), and *Cladosporium* (4%).

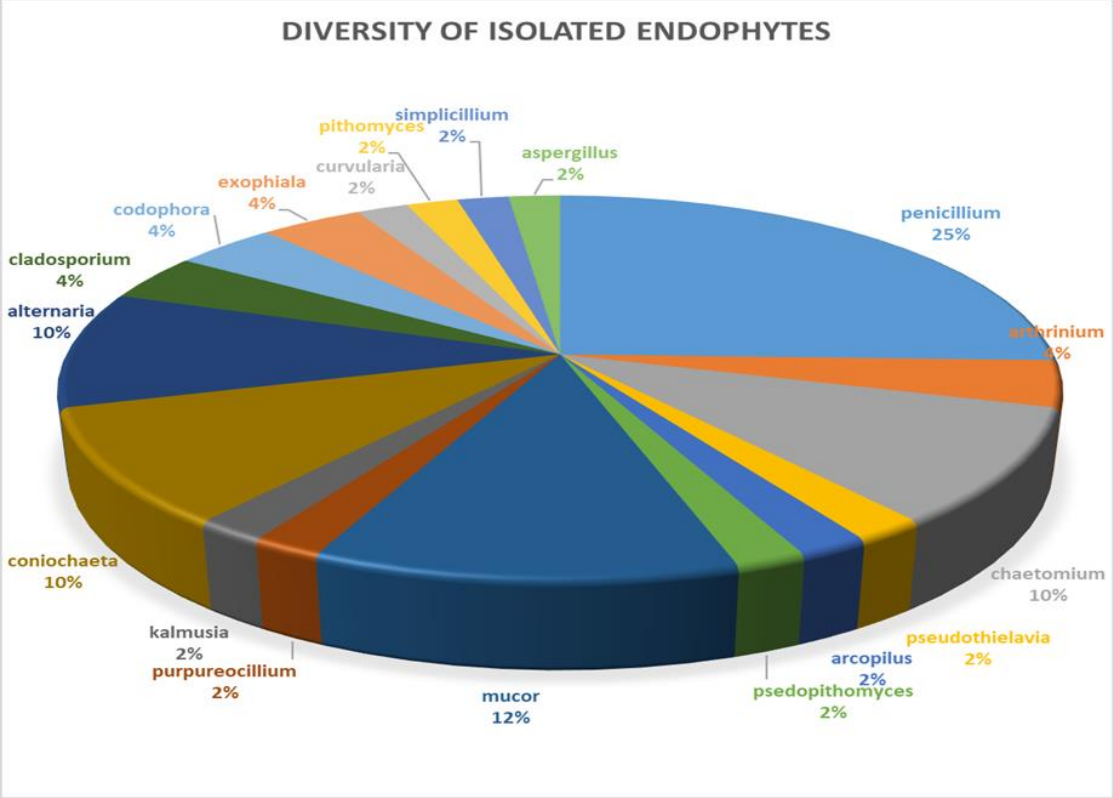


Figure 3.4: Diversity of isolated endophytic fungi from the medicinal plant *Sutherlandia frutescens*

3.3.4 Phylogenetic Relationship

The evolutionary history was inferred using the minimum evolutionary method and the optimal tree is shown in Figure 3.3. The evolutionary distances were computed using the maximum composite likelihood method and are in the number of base substitutions per site. The tree was searched using the Close-Neighbor-Interchange (CNI) algorithm. The neighbor-joining algorithm was used for the initial tree. This analysis involved 51 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1265 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

The dominant (88%) isolates belong to the class Ascomycota. Upon analysis, the phylogenetic tree for association of the different fungal isolates, 4 clusters and 6 sub-clusters were obtained. Clusters 1 and 2 contained strains belonging to the division Ascomycota, while clusters 3 and 4 comprised those belonging to both Ascomycota and Zygomycota. The *Mucor* genera was identified in different clusters (sub-clusters 3b and 4a).

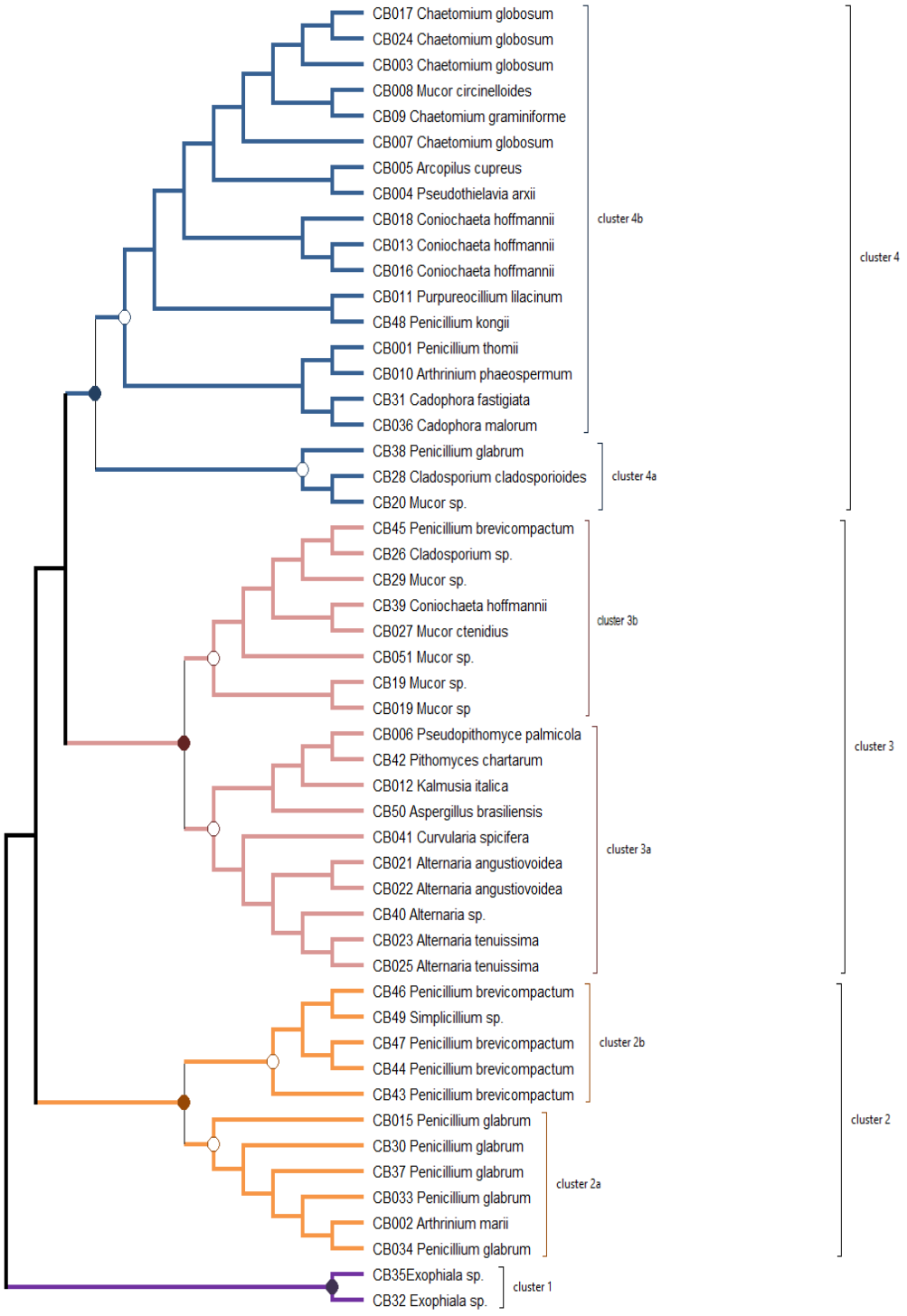


Figure 3.5: Phylogenetic tree constructed by minimum evolution method using ITS sequences of 51 fungal strains.

3.4 DISCUSSION

The primary objective in this chapter was to determine the biodiversity of endophytic fungi isolated from the medicinal plant *S. frutescens* using morphological and molecular techniques.

Based on the findings, the medicinal plant *S. frutescens* harbored endophytic fungi, with a total of 51 isolates obtained from 475 leaf fragments. Fungi are complex organisms with different structures, at different periods of their life cycle, with different forms of growth on the surface and different properties of the growth medium and physical environment. The spore type, the morphology of spores and the spore-bearing structure are key features in fungal identification. In this study fungi varied in terms of color of the colonies, the nature of hyphae, and presence of special structures and this was very central in identifying the fungal strains. Similar findings have been reported by Reddy *et al*., (2010).

The colonization frequency was 80% and the isolation rate was 11%. The lower isolation rate may have resulted from the small size of the *Sutherlandia frutescens* (cancer bush), which directly limited the space available for colonization by endophytes, even though Maheswari and Rajagopal (2013) had the opinion that leaf tissue may have high colonization of endophytes because of their anatomical structure and supply of nutrient elements on which the endophytes depend on. All isolates were identified through morphological and molecular techniques and the predominating genus was *Penicillium* followed by the genus *Alternaria*, *Mucor* and *Coniochateta*.

Their evolutionary relationship of these fungal isolates was established using phylogenetic analysis and interpretation revealed that the dominating scientific classification in the phylogenetic tree is Ascomycota with 88% of the isolates. These results are similar to those of Park *et al.*, (2017) as

well as those of Manganyi *et al*., (2018). The phylogenetic tree has 4 clusters and 6 sub-clusters, cluster 1 and 2 have strains belonging to the division Ascomycota, cluster 3 and 4 has strains belonging to both Ascomycota and Zygomycota division. The *Mucor* genera is found in different clusters, sub-cluster 3b and 4a. Endophytic fungi isolated from the leaves of a medicinal plant *S. frutescens* resulted in a broad spectrum of diversity, dominated by *Penicillium* (25%), followed by *Mucor* (12%), whereas *Alternaria* and *Chaetomium* represent 10% of the community. Additionally, the molecular data showed the presence of isolates that belong to other genera *Cadopora* (4%), *Aspergillus* (2%), *Cladosporium* (4%). Kim *et al*., (2014) reported comparable results when researching cultivatable endophytic fungi isolated from the roots of coastal plants and Manganyi *et al*., (2018) had similar results while studying the biodiversity and antibacterial screening of endophytic fungi isolated from *Pelargonium sidoides.* Endophytes have a positive role in plant tolerance to abiotic stress and plant growth. For example, the association of *Penicilium* endophytes with plants helps plants to resist salinity stress and to improve plant growth (Khan *et al*., 2011). In Ascomycota, the majority of endophytes isolated endophytes was *Penicillium*, which is usually a sporophyte, but also a plant symbiont playing a role in nutrient absorption, defense rfesponse against pathogens, resistance to harsh environmental conditions (Hossain *et al*., 2007 and Khan *et al*., 2008).

3.5 CONCLUSION

To the best of our knowledge, this is the first report on the biodiversity of endophytic fungi recovered from *S. frutescens* and these findings revealed that the plant possesses diverse endophytic fungi. Understanding the endophytic fungi present in *S. frutescens* through direct isolation techniques coupled with morphological and molecular identification is important as this broadens knowledge of the distribution of these microorganisms in the plant as potential sources

for drug discovery. More importantly, these findings provide hope for the search of alternative antibacterial agents from fungi that are harbored by medicinal plants. It is therefore expected that *S. frutescens* may serve as a potential source for the isolation of fungal endophytes with potential pharmaceutical properties. Further characterization of the isolates for antimicrobial (antibacterial and antifungal) properties is of great importance to support this assertion.

CHAPTER FOUR

ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC FUNGI ISOLATED FROM *Sutherlandia frutescens*

Abstract

Objective: The objective of this study was to determine the antibacterial activity of endophytic fungi isolated from *S. frutescens* leaves on some pathogenic environmental and control bacterial strains.

Method: In total, fifty-one (51) endophytic fungi isolated from *S. frutescens* were used to determine their potential to produce bioactive antibacterial agents. After fermentation, the extractswere screened for antibacterial activity using the following control [*Listeria monocytogenes* (ATCC 19115), *Bacillus cereus* (ATCC 10876), *Enterococcus faecium* (ATCC 700221), *Salmonella enterica* (MG663463), *Enterococcus faecalis* (ATCC 29212), and *Enterococcus gallinarum* (ATCC 700425)] and environmental [*Listeria monocytogenes*, *Mannheimia haemolytica*, *Salmonella enterica*, and *Escherichia coli* O177] bacterial isolates using the disc diffusion and agar plug assay techniques. In selecting these bacterial isolates, both Gram-positive and Gram-negative bacteria were considered.

Results: Of the 51 endophytic fungi screened for their potential to produce antibacterial secondary metabolites, 26 (51%) produced bioactive compounds that were active onat least one or more the microorganisms. *Chaetomium globosum* (CB03) and *Penicillium glabrum* produced the most active filtrates and inhibited the growth of three bacteria strains tested. Sixteen (16) fungal extracts inhibited the growth of the environmental isolate of *Salmonella enterica*. An extract from (*Penicillium glabrum*) exhibited a large zone of inhibition (18.3 mm) against *Listeria monocytogenes*. Based on the agar plug assay, 8 (15%) endophytic fungi inhibited the growth of 5

(*Mannheimia haemolytica*, *Salmonella enterica*, *Salmonella enterica*, *Enterococcus faecalis*, and *E. coli* 0177) different (Gram-positive and Gram-negative) bacteria isolates respectively. *Coniochaeta hoffmannii* (CB013) according to our results was the most active endophyte as it produced an extract that inhibited the growth of two bacteria (*Enterococcus faecalis* and *E. coli*). The growth of *Salmonella enterica* (control), *E. faecalis* and *E. coli*, were each inhibited by two different endophytes. The largest zone of inhibition (21.0 mm) was produced by an extract from *Coniochaeta hoffmannii* (CB016) and this was against an *E. coli* O17 environmental isolate.

Conclusion: These results confirm that endophytes from *S. frutescens* produce secondary metabolites that exhibit effective antibacterial activities against resistant bacterial strains and thus these fungi may potentially serve as a source for new or alternative antimicrobial agents. Further characterizations of the extracts to identify the active components is of great importance.

Keywords Endophytic fungi, Antibacterial activity, *Sutherlandia frutescens*, Secondary metabolites

4.1 INTRODUCTION

After the discovery of almost all important groups of antibiotics in the 1960s, aimed at addressing public health complications, these antibacterial agents are now known to pose significant danger to humans especially due to the increasing rise in microbial resistance to these antibiotics (Balouri *et al*., 2016). The health care system has been facing a major public health challenge caused by antimicrobial resistance (Jindal *et al*., 2015). Moreover, the development of microbial resistance to antibiotics is due to different molecular mechanisms such as modification of drugs and prevention of access to drug targets (Elisha *et al*., 2017) just to mention a few. In addition, factors such as rampant and inappropriate use of antibiotics also increase the potential to develop and

increase the spread of multidrug resistant strains. The impact of multidrug resistant strains is more serious in communities with large proportions of immuno-compromised patients but also worse in areas where poor hygiene and limited access to diagnostic facilities exist. These justify the need to search for alternative antibacterial agents especially from endophytic fungi isolated from medicinal plants (Santos *et al*., 2015).

There is evidence to suggest that research on natural products still considers plants as a significant source of biologically active compounds and this has increased the interest studies on plants. Traditionally, many plants have been used for healing purposes while both extracts from plants and endophytes have displayed various pharmacological properties (Gómez and Luiz, 2018). Moreover, it is also evident that the biological activity exhibit by plants may also be associated with secondary metabolites produced by some fungi harboured by the plants. The fungi protect the host plant by providing resistance mechanisms from pathogenic invasion via producing secondary metabolites that have antagonistic activity, thus leading to endophytic fungi being considered for drug development as they are a reservoir of active metabolites (Marcellano *et al*., 2017).

The ability of plants to synthesize aromatic secondary metabolites is limitless. Flavonoids, phenols, and quinolones are some of the significant subclasses of these compounds. These compounds exhibit antimicrobial properties and defense mechanisms against pathogenic microorganisms to the benefit of the plant (Das *et al*., 2010). Natural products from plants therefore can treat bacterial infections and they have been demonstrated to be highly efficient, in addition to their diverse drug base (Fernebro, 2011). It is in this light that this study was conducted to

determine the antibacterial properties of endophytic fungi isolated from *S. frutescens* leaves making use of some bacterial species.

4.2 MATERIALS AND METHODS

4.2.1 Materials

4.2.1.1 Pathogenic bacterial strains

The pathogenic bacterial strains used in this study were purchased from American Type Culture Collection (ATCC) and some were environmental strains. Table 4.1 shows a list of the pathogenic bacterial strains used in this study.

Table 4.1: List of bacteria used to test the antibacterial activity of extracts of endophytic fungi isolated from *S. frutescens*.

Bacteria		**Source**
Listeria monocytogenes (ATCC 19115)	Control	American Type Culture Collection
Listeria monocytogenes	Environmental	Water
Bacillus cereus (ATCC 10876)	Control	American Type Culture Collection
Mannheimia haemolytica	Control	American Type Culture Collection
Enterococcus faecium (ATCC 700221)	Control	American Type Culture Collection
Salmonella enterica (MG663463)	Control	American Type Culture Collection
Salmonella enterica	Environmental	Cattle
Enterococcus faecalis (ATCC 29212)	Control	American Type Culture Collection
Enterococcus gallinurium (ATCC 700425)	Control	American Type Culture Collection
E. coli O177	Environmental	Cattle

4.2.2.2 Endophytic fungi isolated from cancer bush

Fifty-one (n=51) endophytic fungi were successfully isolated from healthy leaves of *S. frutescens*. Morphological and molecular identifications were performed using the internal transcribe spacer

(ITS) region as elaborated in Section 3.2.5. Pure cultures were preserved at the Agricultural Research Council (ARC, Mycology).

4.2.2 Methods

4.2.2.1 Antibacterial assays of endophytic fungi

Agar plug diffusion assay

The isolated endophytic fungi from *S. frutescens* were subjected to preliminary screening through agar plug diffusion method. Endophytic fungi were cultured on PDA for seven days at room temperature. The test bacteria were cultured on Mueller-Hinton Agar (MHA) and incubate at 37°C for 24 hours. Then, agar plugs with a diameter of approximately 6 mm were cut using the back of sterile yellow tips from the PDA plate of actively growing endophytic fungi and were transferred to MHA containing the test bacteria. These plates were sealed and kept in a refrigerator at 4°C for 12 hours for diffusion of metabolites. Afterwards, the plates were incubated at 37°C for 24 hours to enable the growth of test microorganisms. After incubation, the diameter of the zone of inhibition was measured using a ruler. The experiment was carried out in triplicates (Marcellano *et al*., 2017)

4.2.3.2 Disc Diffusion Assay

Endophytic fungi were made to go through a fermentation process in order to produce the secondary metabolites. Each of the fungal isolates were placed into a 50mL of malt extract broth (Merck, Darmstadt, Germany) enclosed in 250ml of Erlenmeyer flasks. A rotary shaker (Labcon FSVE-Spo8, Gauteng, South Africa) containing the flasks was set at 150rpm and fungal isolates incubated (Labcon FSVE-Spo8, South Africa) for 5 days at 25 ^{0}C. The culture broth was filtered

through a 0.45µm PALL Sterile Acrodisc Syringe Filter (Separation, South Africa) (Manganyi *et al*., 2019).

The antibacterial activity assays of the fungal extract (secondary metabolites) were evaluated using the agar disc diffusion method (Ahmad *et al.*, 2013). The disc was prepared by punching Whatman No.3 filter paper (Separations, South Africa, Johannesburg) and sterilized twice using an autoclave to ensure that they are completely free of microbial contaminants. Bacterial cultures of test strains were sub-cultured on Nutrient broth and incubated aerobically at 37 ℃ for 24 hours. The bacterial suspension was prepared at a concentration of $1x10^7$ cells/mL. A bacterial lawn was formed by spread-plating aliquots of 100 micro-liter of bacterial suspensions on Muller Hinton agar. The discs were soaked in fungal extracts for 10 minutes and placed on the inoculated agar plates using sterile inoculating needles. The inoculated plates were incubated aerobically at 37 ^{0}C for 24 hours. Biological activity was determined based on the presence of a zone of inhibition whose diameter which was measured in millimeters (mm). This experiment was carried out in triplicates.

4.2.4 Statistical analysis

The antibacterial activity was calculated using the formula given $\mu = \frac{\mu_1+\mu_2}{N}$ where μ = mean, μ_1 and μ_2 = the measured diameter of the zone of inhibition and N = the number of plates representing a certain isolate. The standard deviation for each isolate was calculated using the formula;

$$\sigma = \sqrt{\frac{\sum(x_1-\mu)^2 + (x_2-\mu)^2}{N}}$$

The standard deviation is represented by the = σ, $x_{1 \text{ and } 2}$= the measured diameter of the zone of inhibition of each plate, μ = mean, and *N* is the number of plates.

4.3 RESULTS

4.3.1 Antibacterial activity (agar plug diffusion Assay)

The ability of endophytes to inhibit growth of the bacteria isolates was tested following agar plug assay. A total of 8 (15%) isolates of endophytic fungi inhibited the growth of five (5) different bacteria as shown in Table 4.2. According to the findings, *C. hoffmannii* (CB013) was the most active endophyte found, inhibiting the growth of two bacteria isolates (*E. faecalis* and *E. coli*). *Salmonella enterica* (control), *E. faecalis* and *E. coli* were each inhibited by extracts from two different endophytes; *M. haemolytica* was inhibited by *Arcopilus* while *S. enterica* was inhibited by *Cadophora*, conversely, the other five bacteria were resistant to all fungal endophytes. Largest zones of bacteria growth inhibition were obtained when extract from *C. hoffmannii* (CB016) was tested against *E. coli* O177 (21.0 mm), followed by *C. hoffmannii* (CB013) against *E. coli* (20.6 mm) and *E. faecalis* (15.0 mm), as well as *C. globosum* (CB014) against *E. faecalis* (14.0 mm).

4.3.2 Antibacterial activity (Disc diffusion Assay)

Fifty-one secondary metabolites that were successfully extracted from endophytic fungi in Chapter Three were assessed for their potential to inhibit the growth of bacteria using the disk diffusion method. The proportion of endophytes that displayed antimicrobial activity against the bacterial isolates following the disk diffusion assay is displayed in Figure 4.1. A total of 26 (51%) endophytic fungi produced bioactive secondary metabolites that exhibited antimicrobial activity against at least one or more tested microorganisms (Table 4.3). Accordingly, *C. globosum* (CB03) and *Penicillium glabrum* produced the most active extracts that inhibited the growth of three

bacteria strains. The environmental *Salmonella* strain was the most sensitive bacteria as its growth was inhibited by sixteen (16) extracts from different fungal endophytes. *L. monocytogenes, B. cereus* and *E. faecium,* were on the other hand, resistant to all fungal endophytes. The largest zone of inhibition was observed on *L. monocytogenes* (18.3 mm), followed by *M. haemolytica* (16.3 mm) and this was exhibited by an extract from *P. glabrum*. More to that, the extract from *Pseudothielavia arxii* exhibited activity (12.0 mm) against *L. monocytogenes* while the growth of *M. haemolytica* was inhibited by *Alternaria tenuissima* (12.0 mm), meanwhile *Salmonella enterica* (12.3 mm) growth was inhibited by an extract from *P. brevicompactum*.

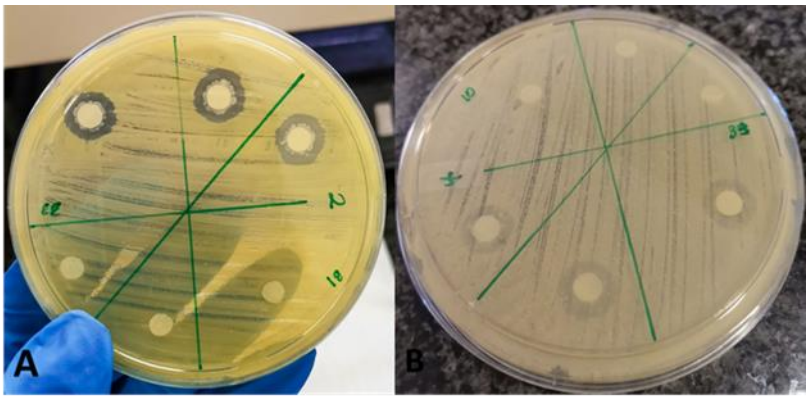


Figure 4.1: Representative image of some of the antibacterial *Penicillium glabrum* against *Listeria monocytogenes* (A) and (B) is *Penicillium glabrum* against *Mannheimia haemolytica*

4.3.3 Antibacterial activity (Agar plug assay)

Table 4.2: The activity displayed by agar plugs of endophytic fungi isolated from *S. frutescens* against pathogenic bacterial strains

Probable name	Zone of inhibition (mm)									
	Listeria monocytogenes	*Listeria monocytogenes*	*Bacillus cereus*	*Mannheimia haemolytica*	*Enterococcus faecium*	*Salmonella enterica*	*Salmonella enterica*	*Enterococcus faecalis*	*Enterococcus gallinurium*	*E. coli 0177*
Penicillium	-	-	-	-	-	-	-	-	-	-
Arthrinium	-	-	-	-	-	-	-	-	-	-
Chaetomium	-	-	-	-	-	-	-	-	-	-
Pseudothielavia	-	-	-	-	-	-	-	-	-	-
Arcopilus	-	-	-	9.0±1.7	-	-	-	-	-	-
pseudopithomyce	-	-	-	-	-	9.0±0.0	-	-	-	-
Chaetomium	-	-	-	-	-	-	-	-	-	-
Mucor	-	-	-	-	-	9.6±1.7	-	-	-	-
Chaetomium	-	-	-	-	-	-	-	-	-	-
Arthrium	-	-	-	-	-	-	-	-	-	-
Purpureocillium	-	-	-	-	-	-	-	-	-	-
Kalmusia	-	-	-	-	-	-	-	-	-	-
Coniochaeta	-	-	-	-	-	-	-	15.0±2.2	-	20.6±2.0
Coniochaeta	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Coniochaeta	-	-	-	-	-	-	-	-	-	21.0±1.6
Chaetomium	-	-	-	-	-	-	-	-	-	-
Coniochaeta	-	-	-	-	-	-	-	-	-	-
Mucor	-	-	-	-	-	-	-	-	-	-
Mucor	-	-	-	-	-	-	-	-	-	-
Alternaria	-	-	-	-	-	-	-	-	-	-
Alternaria	-	-	-	-	-	-	-	-	-	-
Alternaria	-	-	-	-	-	-	-	-	-	-
Chaetomium	-	-	-	-	-	-	-	14.0±1.6	-	-
Alternaria	-	-	-	-	-	-	-	-	-	-
Cladosporium	-	-	-	-	-	-	-	-	-	-
Mucor	-	-	-	-	-	-	-	-	-	-
Cladosporium	-	-	-	-	-	-	-	-	-	-

Mucor	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Cadophora	-	-	-	-	-	-	13.6±3.4	-	-	-
Exophiala	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Exophiala	-	-	-	-	-	-	-	-	-	-
Cadophora	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Coniochaeta	-	-	-	-	-	-	-	-	-	-
Alternaria	-	-	-	-	-	-	-	-	-	-
Curvularia	-	-	-	-	-	-	-	-	-	-
Pithomyces	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Simplicillium	-	-	-	-	-	-	-	-	-	-
Aspergillus	-	-	-	-	-	-	-	-	-	-
Mucor	-	-	-	-	-	-	-	-	-	-

-:no activity +: slight activity(5-10mm) ++: good activity (11-19mm) +++: very good activity (20mm)

Mean diameter zone of inhibition ± S.D. (n=3)

Agar plug diameter was 6mm

Table 4.3: The activity displayed by fermented endophytic extracts from *S. frutescens* against pathogenic bacterial strains

Probable name	Zone of inhibition (mm)									
	Listeria monocytogenes	*Listeria monocytogenes*	*Bacillus cereus*	*Mannheimia haemolytica*	*Enterococcus faecium*	*Salmonella enterica*	*Salmonella enterica*	*Enterococcus faecalis*	*Enterococcus gallinurium*	*E. coli 0177*
Penicillium	-	-	-	-	-	-	-	-	-	-
Arthrinium	-	7.0±0.0	-	-	-	-	-	-	-	-
Chaetomium	-	9.0±0.0	-	-	-	-	7.0±0.0	-	9.7±0.6	-
Pseudothielavia	-	12.0±0.8	-	-	-	-	-	-	-	-
Arcopilus	-	-	-	-	-	-	-	-	-	-
Pseudopithomyce	-	-	-	-	-	-	-	-	-	-
Chaetomium	-	-	-	-	-	-	-	-	8.7±0.3	-
Mucor	-	11.0±2.2	-	-	-	-	9.0±0.0	-	-	-
Chaetomium	-	-	-	-	-	-	9.0±0.3	-	-	-
Arthrinium	-	-	-	-	-	-	9.0±0.0	-	-	-
Pupureocillium	-	-	-	-	-	-	-	-	-	-
Kalmusia	-	-	-	-	-	-	7.0±0.8	-	8.3±0.6	-
Coniochaeta	-	-	-	-	-	-	10.6±1.8	-	-	-
Coniochaeta	-	-	-	-	-	-	7.0±0.8	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Coniochaeta	-	-	-	-	-	-	-	-	-	-
Chaetomium	-	-	-	-	-	-	-	-	-	-
Coniochaeta	-	-	-	-	-	-	-	-	-	-
Mucor	-	-	-	-	-	-	-	-	-	-
Mucor	-	-	-	-	-	-	-	-	-	-
Alternaria	-	-	-	-	-	-	7.3±1.0	-	9.0±0.0	-
Alternaria	-	-	-	7.0±0.8	-	-	-	-	-	-
Alternaria	-	-	-	12.0±2.2	-	-	-	-	-	-
Chaetomium	-	-	-	-	-	-	10.0±0.0	-	-	-
Alternaria	-	-	-	-	-	-	-	-	-	-
Cladosporium	-	-	-	-	-	-	-	-	-	-
Mucor	-	-	-	-	-	-	-	-	-	-
Cladopsporium	-	-	-	-	-	-	-	-	-	-
Mucor	-	-	-	-	-	-	-	-	-	-
Penicillium	-	9.0±0.0	-	-	-	-	-	-	-	-

Cadophora	-	-	-	-	-	-	10.6±1.8	-	-	-
Exophiala	-	-	-	-	-	-	-	-	-	-
Penicillium	-	18.3±0.5	-	16.3±1.3	-	-	9.3±0.6	-	-	-
Penicillium	-	-	-	-	-	-	7.0±0.0	-	-	-
Exophiala	-	-	-	-	-	-	-	-	-	-
Cadophora	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Coniochaeta	-	-	-	-	-	-	9.0±1.7	-	-	-
Alternaria	-	-	-	-	-	-	-	-	-	-
Curvularia	-	-	-	-	-	-	10.0±4.6	-	-	-
Pithomyces	-	-	-	-	-	-	8.0±0.8	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	8.0±0.0	-	-	10.0±4.6
Penicillium	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	8.3±0.6	-	-
Penicillium	-	-	-	-	-	12.3±2.5	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-
Simplicillium	-	7.0±0.8	-	-	-	-	-	-	-	-
Aspregillus	-	-	-	-	-	9.0±0.0	-	-	-	-
Mucor	-	-	-	-	-	-	-	-	-	-

-:no activity +: slight activity(5-10mm) ++: good activity (11-19mm) +++: very good activity (20mm)

Mean diameter zone of inhibition ± S.D. (n=3)

Agar disk diameter is 5mm

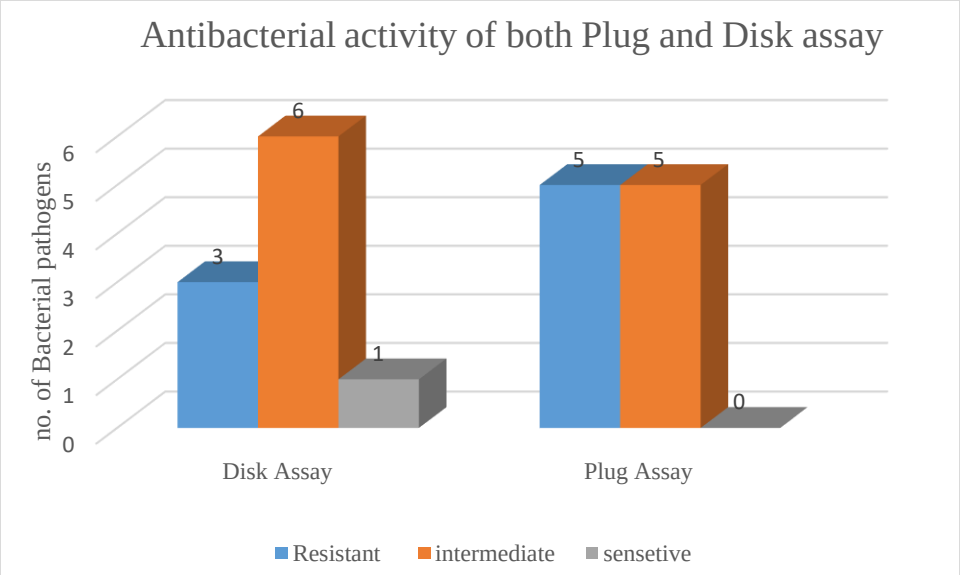


Figure 4.2: The antibacterial activity of both plug and disk assay. Intermediate = inhibited by 1-8 fungal extracts. Sensitive= inhibited by 9-51 fungal extracts.

On a comparative basis, Figure 4.2 is a representation of the activity of the pathogenic bacteria against the fungal extracts used in the study on both techniques. The environmental *S. enterica* isolate was the most sensitive bacterial pathogen based on the disk assay and as it was inhibited by over 16 fungal extracts. In addition, 6 bacterial isolates exhibited intermediate activity as they were inhibited by 1 or up to 7 fungal extracts. Three of the bacteria were resistant as none of the fungal extracts were able to inhibit their growth. On the contrary, in the case of the agar plug assay none of the bacteria was sensitive to extracts from the endophytes. However, 5bacterial isolates displayed intermediate growth activity being inhibited by one or up to two fungal extracts. Moreover, 5bacteria isolates (*Listeria monocytogenes, Listeria monocytogenes, Bacillus cereus, Enterococcus faecium* and *Enterococcus gallinurium*) were resistant to the tested fungal extracts.

4.4 DISCUSSION

Endophytic fungi isolated from *Sutherlandia frutescens* were subjected to preliminary antibacterial screening by agar plug method, a qualitative assay only used to determine the presence of antibacterial substances secreted by the fungi following the agar plug assay agar plugs. The results showed that some fungal isolates exhibited antibacterial activity. Endophytic fungi are known to secrete extracellular secondary metabolites into the fermentative medium. To quantify the antibacterial activity of secondary metabolites, disc diffusion assay was performed as recommended by many researchers (Marcellano *et al*., 2017 Ynalvez *et al*., 2018 Handayani *et al*., 2021). The results demonstrated the size of the inhibition zone which reflects the susceptibility level of the test bacteria.

Penicillium, *Chaetomium* and *Alternaria* showed substantial antibacterial activity against various bacteria tested, and this is in agreement with (Kharwar *et al*., 2011). Other researchers also state that endophytic fungi *Penicillium* sp. has antimicrobial activity against *Salmonella* and *L. monocytogenes* (Handayani *et al*., 2017). *Alternaria* sp. displayed activity against both gram-positive and gram negative bacteria activity that which was also reported by Gunasekaran *et al*., (2017).

The difference in bacterial response was possibly due to the nature of the bacterial species. Extract showed greater activity against the growth of Gram-positive than that of Gram-negative bacteria. These findings agree with those of other researchers who reported that a majority of fungal extracts were more active in inhibiting the growth of Gram-positive than that of gram-negative bacteria (Marcellano *et al*., 2017). The difference in the cell envelope composition of Gram- positive and Gram-negative bacteria could be the reason for these findings.

Gram-negative bacteria are more resistant to the penetration of antibacterial agents (Zhao *et al*., 2020), because their cell envelope is surrounded by a thin peptidoglycan cell wall, in which it's also surrounded by an outer membrane consisting of the lipoprotein, phospholipids outer membrane and lipopolysaccharides. Consequently, these lead to the prevention of cell membrane permeability and also delays the movement of foreign substances into the cell. However, these characteristics are absent in the cell envelope of Gram-positive bacteria because of the absence of the outer membrane but is surrounded by layers of peptidoglycan, which makes them more susceptible to antibacterial agents as they absorb antibiotics easily (Swoboda *et al*., 2010). Marcellano *et al.* (2017) reported that endophytic fungi show activity in solid media but not when subjected to fermentation, however that was not the case in this study, fermentation (disk assay) of extracts ensured the antibacterial activity more than what was noted when following the agar plug assay without fermentation performed on the extracts.

4.5 CONCLUSION

Antimicrobial resistance has become a serious global health concern and consequently immediate solutions are needed to solve this challenge. The increased resistance to drugs by infectious pathogens as well as undesirable effects of certain chemotherapeutic agents indicates that there is an urgent need in search for novel and effective bioactive compounds which is what this study aimed at achieving. It has been observed that much of the wealth of microbial biodiversity with novel biochemistry and secondary metabolite production resides in plant tissues. The activity displayed by endophytic fungi on pathogenic bacteria shows there could be a promising alternative solutions to antibiotic resistance, using natural products from plants such as *Sutherlandia frutescens*.

CHAPTER FIVE

ANTIFUNGAL ACTIVITY OF ENDOPHYTIC FUNGI ISOLATED FROM *Sutherlandia frutescens*

Abstract

Objective: Endophytic fungi live inside the host plant tissue without causing any disease or harm to the plant. This chapter aimed to study the antifungal activity of endophytic fungi isolated from native *S. frutescens* against some plant pathogenic fungi.

Method: In all, 51 endophytic fungi were tested for their antifungal activity against some plant pathogenic fungi, in which dual culture and culture filtrate assays were carried out. Temperature, pH and salt tolerance conditions were maintained to study stress resistance of the endophytic fungi.

Results: In the dual assay, 84% of the endophytes were active against one or more of the pathogens. *Mucor*, *Penicillium* and *Aspergillus brasiliensis* exhibited a broad spectrum of antifungal activity for three or all pathogenic fungi of plants tested in this assay (Table 5.1). Endophytes *Exophiala sp.* and *Coniochaeta hoffmannii* showed no activity at all against the pathogens. *Collectortrichum gleosporioides* (12517) was the most sensitive in this study, considering that it was inhibited by fourteen (14) extracts of endophytic fungi with a measured growth of above5 mm. Conversely, *Botrytis cinerea* (13071) was resistant sinceonly six (6) endophytic fungi inhibited its growth with a diameter of 5 mm while the remaining 45 endophytic fungi measured 0 to 3mm.

The overall activity displayed by filtrate assay was78%. The culture filtrate of the endophytic fungi CB011 (*Purpureocillium lilacinum*) exhibited a broad range of antifungal activity against all the pathogens. *Mucor* and *Penicillium* species that exhibited broad spectrum activity in dual culture

assay did not present similar activity in filtrate assayrather they were least active against two or more pathogens with moderate growth against others pathogens. The most sensitive pathogen was *Borytis cinerea*, 43% of the fungal extracts managed to inhibit its growth, while *Fusarium oxysporum* was the most resistant demonstrating significant growth on 45% of the fungal extracts. On the temperature stress tolerance test, growth of all fungal isolates tested grew at 25°C. Under salty conditions, maximum growth was observed at 3% concentration with 90% growth rate, while for the pH test, a majority of fungal pathogens grew at pH 5 with 44% growth rate recorded.

Conclusion: Findings from the study indicates that the findings could pave a way for new therapeutic agents in the management of fungal plant pathogens and microbial resistance in agriculture.

Keywords: *Sutherlandia frutescens*, Antifungal activity, Pathogenic fungi, Diversity

5.1 INTRODUCTION

Plant pathogens are microorganisms that include fungi, nematodes, bacteria, and viruses that can cause diseases or damages to plants. Among these pathogens, fungi are the main pathogen and cause many diseases. In doing so, they reduce productivity of numerous economically important crops in the field. Usually, soil is considered as one of the important inoculum sources for these microbial species including fungi. Aside from field fungi, several fungi have been found to induce post-harvest spoilage of food, some of which areassociated with a decrease in nutrients and safety quality of food (Chang *et al*., 2008; Al-Ani, 2018; Khan and Sharma, 2020).

The identification of fungal pathogenesis not only enables us to understand better how fungal pathogens infect the host plant but also supplies good information for administering of plant diseases, involving new prevention strategies, inhibition of fungal development, or delay. The vast loss experienced in the yield and quality of field crops is caused by fungal plant pathogens which

also affect the fruit and other palatable parts of the plant, and with climate change and increase in population which serve as a threat to arable land and this is an important issue the economy, plant and human health (Yang *et al.*, 2016).

There is a need to develop novel control strategies against plant pathogens, endophytic fungi can be an interesting alternative means of biocontrol (Poveda *et al*., 2020). Although the control of seed-borne fungi can be attained by the incorporation of synthetic chemical fungicides, due to pesticide toxicity that method cannot be applied to grains. Better alternatives such as plant based pesticides and plant metabolites are known to have a minimum environmental impact and danger to consumers unlike the use of synthetic pesticides (Satish *et al.*, 2007). Natural products have great potential as novel fungicide sources for controlling pathogenic fungi (Chang *et al.*, 2008). Fungal infections have aroused much interest over the last years because of their involvement in several human diseases (Di Mambro *et al.*, 2019)

Endophytes perform as a defensive bio resource, with an application ability that inspires researchers to look more into the mechanism by which it protects the associated host. The capacity of endophytes to act as biocontrol agents is the important contribution they have on agriculture because they act against a wide range of microbial pathogens, nematodes, insects and pets. Most significantly, fungal endophytes facilitate induced systemic resistance in plants which is a vital mechanism for disease management and plant protection (Patshangba, 2017). Furthermore, the ability of endophytes to produce bioactive compounds plays a significant role as a biocontrol against plant pathogens.

5.2 MATERIALS AND METHODS

5.2.1 Plant pathogenic fungi

In this chapter, the tested plant fungal pathogens listed in Table 5.1 were purchased from Agricultural research council (ARC), Plant Protection Research Institute (PPRI), located in Pretoria, South Africa.

Table 5.1: Pathogen fungi with their origin and accession number

PPRI no.	Fungal species	Host/substrate	Locality
13071	*Botrytis cinerea*	Chrysanthemum flower	Gauteng, Tarlton
2929	*Fusarium oxysporum*	Wheat	Free state
10139	*Fusarium graminearum*	Maize	North West
12517	*Collectotrichum gleosporioides*	Papaya	Nelspruit Mpumalanga,

5.2.2 Antifungal activity of endophytic fungi against pathogenic fungi

5.2.2.1 Dual culture method

The preliminary assay was conducted to check the antifungal activity of the endophytic fungi against selected pathogenic fungi using the dual culture method. Potato Dextrose Agar (PDA, Merck Biolab, Gauteng, South Africa) media was used to perform a 2-point inoculation of 6mm discs of the endophyte and at the other side of the agar plate the pathogen was inoculated (figure 5.1). The plates were inoculated for 5-8 days at 25±10℃. Hindrance of the pathogen's growth in the direction of endophytic fungus would suggest a hostile activity (Luo *et al*., 2015).

5.2.2.2 Extraction of secondary metabolites from the endophytic fungi

Endophytic fungi were fermented to induce them to produce the secondary metabolites. Each of the fungal isolates was placed in a 50mL of malt extract broth (Merck, Darmstadt, Germany) in a 250 ml Erlenmeyer flask. Rotary shaker (Labcon FSVE-Spo8, Gauteng, South Africa)

containing the culture flasks was set at 150rpm and fungal isolates were incubated for 5 days at 25 0C. The culture broth was filtered through a 0.45µm PALL Sterile Acrodisc Syringe Filter (Separations, South Africa, Johannesburg) (Manganyi *et al*., 2019).

5.2.2.3 Testing culture filtrates of endophytic fungi against pathogenic fungi of plants

Fungal extracts were subjected to antifungal screening against four selected pathogenic fungi. Thirty (30) mL of PDA was poured into a sterilized 90 mm petri dish and supplemented with 2 mL of the fungal extracts (Section 3.2.2). PDA only was then poured as a control. Upon solidification, plant pathogens were inoculated at the center of the plate, then incubated at room temperature for 7 days and then the growth was measured by mycelial growth inhibition and calculated according to the formula of Pandey *et al*. 2015).

5.2.3 DETERMINATION OF STRESS TOLERANCE

5.2.3.1 Temperature

Potato Dextrose Agar was prepared according to the manufacturer's instructions. Fungal isolates were inoculated on the PDA plated and incubated at 4, 25, and 37ºC (Potshangbam *et al*., 2017).

5.2.3.2 Salt

Potato Dextrose Agar was prepared according to the manufacturer's instructions and was supplemented with sodium chloride (3, 10 &50%). The endophytic fungi under investigation were plated on the supplemented PDA. Subsequently, plates were incubated at 25 ºC for 2 weeks, (Potshangbam *et al.*, 2017).

5.2.2.3 pH

Malt extract broth was prepared and used in this experiment. The pH assay was conducted at various levels (pH 2, 5, 12). The pH of malt extract broth was adjusted using hydrochloric acid (HCl) to pH 2, and 5 as well as sodium hydroxide (NaOH) to a pH 12. The study was conducted in triplicates for reproducibility and reliability. Approximately 2-3 pieces of fungal isolates were inoculated in the adjusted pH solution inside 50mL conical centrifuge tubes and incubated for 2 weeks. Colony measurement was taken at an optical density of 600 using spectrophotometer (Potshangbam *et al.*, 2017).

5.2.4 Statistical analysis

The activity of the cultural filtrates was calculated using the formula given $\mu = \mu_1 + \mu_2/N$ where μ = mean, μ_1 and μ_2 = the measured diameter and *N* = the number of plates representing a certain isolate. The standard deviation for each isolate was calculated with the formula

$$\sigma = \sqrt{\frac{\sum(x_1 - \mu)^2 + (x_2 - \mu)^2}{N}}$$

The standard deviation is represented by the = σ, $x_{1\text{ and }2}$= the measured diameter of each plate, μ = mean, and *N* is the number of plates. These calculations were used to explain how diameters of the isolate (plate) spread out from the mean average or expected diameter. A lower standard deviation means the diameter of the zone of inhibition is close to the mean. A higher standard deviation means the diameter of the zone of inhibition is spread out.

5.3 RESULTS

5.3.1 Diversity of endophytic fungal isolates with some antifungal activity using dual culture test

A dual assay was used to determine the antifungal activity of the selected endophytic fungi; this is shown in Figure 5.1. From a total of 51) tested endophytic fungi, 84% of them exhibited inhibitory

activity against one or more of the tested pathogens (Table 5.2). *Mucor*, *Penicillium* and *Aspergillus brasiliensis* exhibited broad antifungal activity spectra for three or all pathogenic fungi of plants tested in this study (Table 5.1). Endophytes *Exophiala sp.* (CB032 and CB035) and *Coniochaeta hoffmannii* (CB039) susceptible against all pathogens. Conversely, *Collectortrichum gleosporioides* (12517) was the most sensitive plant pathogen in the study, withthe growth of fourteen (14) endophytic fungi inhibited 5 mm in diameter. As found, the resistance of *Borytis cinerea* (13071) only six (6) endophytic fungi gave an inhibition of T>5mm while over 34 gave 0 to 3mm inhibition. As found, the resistance of *Botrytis cinerea* (13071) was noted wherein only six (6) endophytic fungi were able to inhibit its growth with a diameter of 5 mm while the remaining 45 endophytic fungi measured 0 to 3mm.

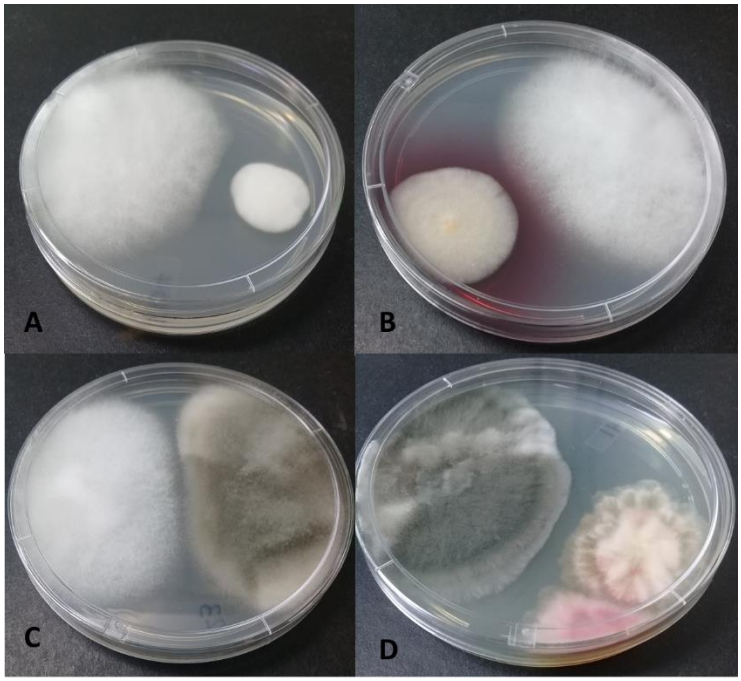


Figure 5.1: Endophytic fungi from *Sutherlandia frutescens* showing activity in dual culture against fungal pathogens (A) *Pseudothielavia arxii* against *Fusarium oxysporum, (B) Arcopilus cupreus* against *Collectortrichum gleosporioides (C) Alternaria tenuissima* against *Fusarium oxysporum, (D) Curvularia spicifera* against *Fusarium graminearum*

Table 5.2: Identification of endophytic fungi with antifungal activity against some plant pathogenic fungi of plant established following the dual culture technique

codes	Identified as	Potential antifungal activity			
		2929	13071	10139	12517
CB001	*Penicillium thomii*	+++	++	+++	+++
CB002	*Arthrinium marii*	++	+	++	+
CB003	*Chaetomium globosum*	+	+	+	+
CB004	*Pseudothielavia arxii*	+	-	+	+
CB005	*Arcopilus cupreus*	+	+	+	++
CB006	*Pseudopithomyce palmicola*	+	+	+	++
CB007	*Chaetomium graminiforme*	+	+	+	+
CB008	*Mucor circinelloides*	+++	++	+++	+++
CB009	*Chaetomium graminiforme*	+	+	++	++
CB010	*Arthrinium phaeospermum*	-	+	+	-
CB011	*Purpureocillium lilacinum*	+	+	+++	+
CB012	*Kalmusia italic*	+	+	++	++
CB013	*Coniochaeta hoffmannii*	++	+	+	+
CB014	*Coniochaeta hoffmannii*	-	+	+	+
CB015	*Penicillium glabrum*	+++	++	++	+++
CB016	*Coniochaeta hoffmannii*	-	+	-	-
CB017	*Chaetomium globosum*	-	+	+	+
CB018	*Coniochaeta hoffmannii*	+	+	+	+
CB019	*Mucor* sp.	++	+++	+++	+++
CB020	*Mucor* sp.	++	+++	+++	+++
CB021	*Alternaria angustiovoidea*	+	++	++	++
CB022	*Alternaria angustiovoidea*	++	+	++	+
CB023	*Alternaria tenuissima*	++	+	++	+
CB024	*Chaetomium globosum*	+	+	+	++
CB025	*Alternaria tenuissima*	+	+	++	++

CB026	*Cladosporium globosum*	+	+	+	+
CB027	*Mucor ctenidius*	+++	+++	+++	+++
CB028	*Cladosporium cladosporioides*	+	+	+	+
CB029	*Mucor* sp.	+++	+++	+++	+++
CB030	*Penicillium glabrum*	+++	++	+++	+++
CB031	*Cadophora fastigiata*	+	-	+	+
CB032	*Exophiala* sp.	-	-	-	-
CB033	*Penicillium glabrum*	+++	++	++	+++
CB034	*Penicillium glabrum*	+++	++	++	+++
CB035	*Exophiala* sp.	-	-	-	-
CB036	*Cadophora malorum*	+	+	+	+
CB037	*Penicillium glabrum*	+++	++	++	+++
CB038	*Penicillium glabrum*	+++	++	++	+++
CB039	*Coniochaeta hoffmannii*	-	-	+	-
CB040	*Alternaria* sp.	++	+	++	++
CB041	*Curvularia spicifera*	++	+	++	++
CB042	*Pithomyces chartarum*	++	+	++	+
CB043	*Penicillium brevicompactum*	+++	+++	+++	+++
CB044	*Penicillium brevicompactum*	+++	+++	+++	+++
CB045	*Penicillium brevicompactum*	+++	+	+++	+++
CB046	*Penicillium brevicompactum*	+++	-	+	+
CB047	*Penicillium brevicompactum*	+++	+++	+++	+++
CB048	*Penicillium kongii*	+++	-	+++	++
CB049	*Simplicillium* sp.	+	+	+	+
CB050	*Aspergillus brasiliensis*	+++	++	+++	+++
CB051	*Mucor* sp.	+++	+++	++	+++

[a]Width of growth inhibition zone T= 0 mm; -, 0<T≤ 3mm; +, 3< T ≤5 ++, T>5mm +++ (measuring from the endophyte to the pathogen)
[b]2929, *Fusarium oxysporum*; 13071, *Borytis cinerea* 10139 *Fusarium graminearum*, 12517 *Collectortrichum gleosporioides*

The diversity of antifungal activity, 28% of the endophytes gave high activity, while 22% gave moderate activity, 39% slight activity and 11% of the endophytes had no activity (Figure 5.2).

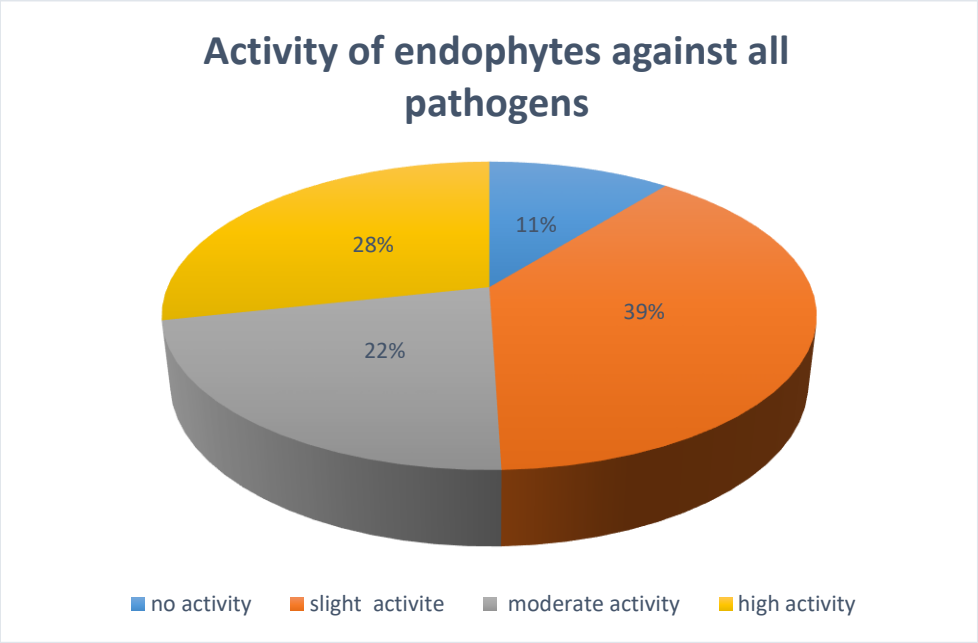


Figure 5.2: The overall activity displayed on dual culture assay by endophytes isolated from *S. frutescens* against all pathogens selected for this study

5.3.2 Diversity of endophytic fungi isolates with some antifungal activity using filtrate culture (secondary metabolites) test

Table 5.3 presents data on the antifungal effect of the selected endophytic fungi against some pathogenic fungi established following the filtrate culture assay. Out of a total of 51 tested endophytic fungi, 40 (78%) showed inhibitory activity against the growth of one or more fungal pathogens (Table 5.1). The cultural filtrate of the endophytic fungi CB011 (*Purpureocillium lilacinum*) exhibited a broad range of antifungal activity against all the pathogens. *Mucor* and

Penicillium species that exhibited broad activity in dual culture assay did not present the same activity in this culture, instead were least active against two or more pathogens with moderate growth exhibited by other pathogens. Amongst the filtrates tested, 40% were highly active against the growth of *Borytis cinerea* (most sensitive pathogen), 14% highly active against *Fusarium graminearum*, 6% active *Fusarium oxysporum* (most resistant).

Table 5.3: Identification of endophytic fungi and activity against pathogenic fungi of plants based on the filtrate culture technique

Codes	Growth inhibition			
	A	B red	C	D
CB001	3.75±0.35	2.24±3.39	3.15±0.25	1.45±0.35
CB002	7.95±0.75	2.95±0.25	6.00±0.10	4.00±1.10
CB003	6.00±0.70	6.10±1.00	5.60±0.00	4.95±0.05
CB004	2.50±1.60	6.40±0.50	0.00±0.00	0.00±0.00
CB005	6.20±1.10	3.60±2.20	4.85±1.65	2.30±0.20
CB006	0.00±0.00	2.35±0.25	2.40±0.30	2.70±1.00
CB007	3.75±0.35	7.35±0.45	6.10±0.20	5.80±0.10
CB008	4.90±1.30	1.05±0.05	2.35±0.75	1.60±0.50
CB009	8.70±0.00	5.90±0.30	6.50±0.40	5.45±0.15
CB010	1.20±0.85	1.80±0.30	5.40±0.10	4.65±1.25
CB011	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CB012	8.70±0.00	5.00±0.10	7.20±0.10	5.40±0.20
CB013	0.00±0.00	0.70±0.70	6.20±0.20	5.45±0.35
CB014	3.40±1.10	2.60±0.50	3.85±0.35	2.65±0.25
CB015	0.00±0.00	0.00±0.00	1.35±0.75	0.00±0.00
CB016	0.00±0.00	3.05±0.32	6.15±0.15	3.75±0.15
CB017	2.40±0.30	3.05±0.05	7.60±0.20	1.90±0.20
CB018	0.00±0.00	2.25±0.25	4.95±0.05	2.40±0.30
CB019	0.00±0.00	1.20±0.20	2.45±0.05	0.00±0.00
CB020	0.00±0.00	2.55±0.05	7.00±0.10	4.50±0.10
CB021	0.00±0.00	2.90±0.10	7.05±0.15	4.20±0.20
CB022	0.00±0.00	2.10±0.10	2.55±0.35	5.10±0.20
CB023	0.00±0.00	2.85±0.05	7.20±0.10	5.40±0.16
CB024	0.00±0.00	0.00±0.00	7.10±0.10	0.00±0.00
CB025	0.00±0.00	3.10±0.10	7.55±0.35	5.50±0.90
CB026	1.10±0.10	3.45±0.25	7.30±0.30	3.50±0.30
CB027	0.00±0.00	0.00±0.00	7.60±0.00	5.50±0.50
CB028	0.00±0.00	0.00±0.00	7.00±0.10	2.50±0.10

CB029	8.70±0.00	8.70±0.00	6.30±0.60	5.50±0.50
CB030	8.30±0.40	8.70±0.00	8.30±0.40	5.55±0.45
CB031	6.05±2.68	8.70±0.00	7.00±0.10	3.10±0.10
CB032	0.00±0.00	2.65±0.55	4.25±0.15	4.35±0.15
CB033	3.05±0.05	2.15±0.05	3.55±0.35	2.95±0.15
CB034	0.00±0.00	2.70±0.30	7.50±0.10	3.05±0.15
CB035	0.00±0.00	3.45±0.35	2.15±0.05	3.55±0.05
CB036	3.65±0.05	3.30±0.10	5.30±0.20	5.25±0.35
CB037	5.65±0.05	5.90±0.80	3.70±0.10	4.70±0.30
CB038	2.05±0.15	0.00±0.00	0.40±0.28	3.05±0.05
CB039	6.40±0.10	5.75±0.55	5.50±0.10	3.60±0.20
CB040	2.20±1.00	8.70±0.00	7.60±0.00	4.50±0.50
CB041	0.00±0.00	7.85±0.85	7.60±0.00	0.00±0.00
CB042	8.70±0.00	4.70±0.20	7.10±0.00	4.35±0.05
CB043	8.30±0.40	8.70±0.00	7.25±0.15	3.05±0.15
CB044	0.00±0.00	8.70±0.00	7.50±0.10	3.70±0.30
CB045	8.70±0.00	5.95±0.45	6.75±0.15	4.10±0.10
CB046	8.70±0.00	7.00±0.10	7.70±0.10	3.10±0.10
CB047	8.70±0.00	6.00±0.90	7.45±0.05	4.50±0.40
CB048	8.70±0.00	8.30±0.40	7.70±0.10	4.75±0.35
CB049	8.70±0.00	8.70±0.00	7.15±0.34	0.00±0.00
CB050	7.05±0.15	2.25±0.15	6.25±0.15	5.10±0.20
CB051	8.70±0.00	6.60±1.30	7.15±0.05	4.40±0.30

[a]13071(A): *Borytis cinerea;* 10139(B): *Fusarium graminearum;* 2929(C): *Fusarium oxysporum*; 12517 (D): *Collectortrichum gleosporioides*
Mean diameter of zone of inhibition ± S.D. (n=3)
[b] 0.99±0.00 to 0.00±0.00 = most active filtrate, 8.70±0.20 = least active filtrate

The results presented in Figure 5.3 revealed that the endophytic fungal extracts from medicinal plants have potential antifungal activity. The filtrates were able to show some activity against the pathogens at varying levels with 18% of the filtrates each being most active, while 22, 19 and 23%, respectively, showed moderate, slight and least activity on growth of the tested pathogens.

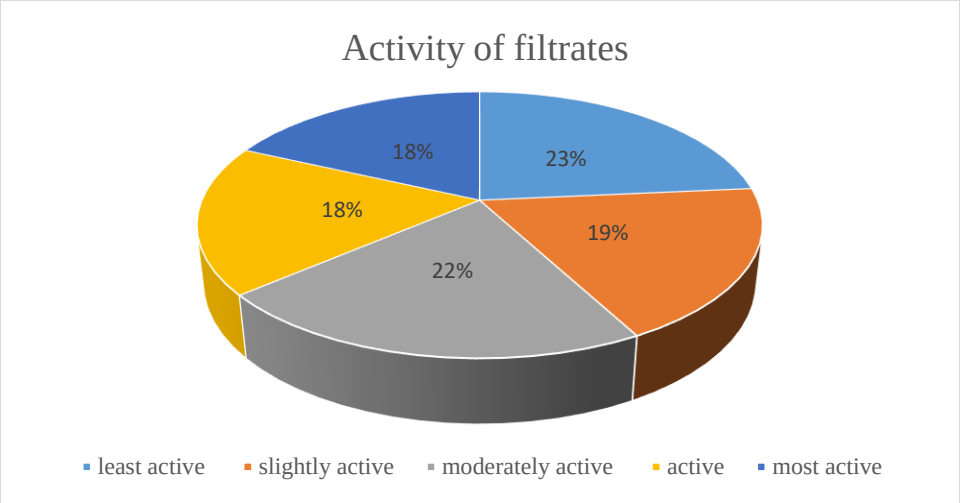


Figure 5.3: The overall activity displayed on filtrate culture assay by extracts retrieved from *S. frutescens* against pathogens selected for this study.

5.3.4 STRESS TOLERANCE ASSAY

In this section, we assessed the ability of isolated endophytes to thrive under adverse stressful conditions such as temperature, salt concentration and pH.

5.3.4.1 Temperature

To assess the growth potential of endophytic fungi isolated from *Sutherlandia frutescens* under stressful conditions, temperature in varying degrees

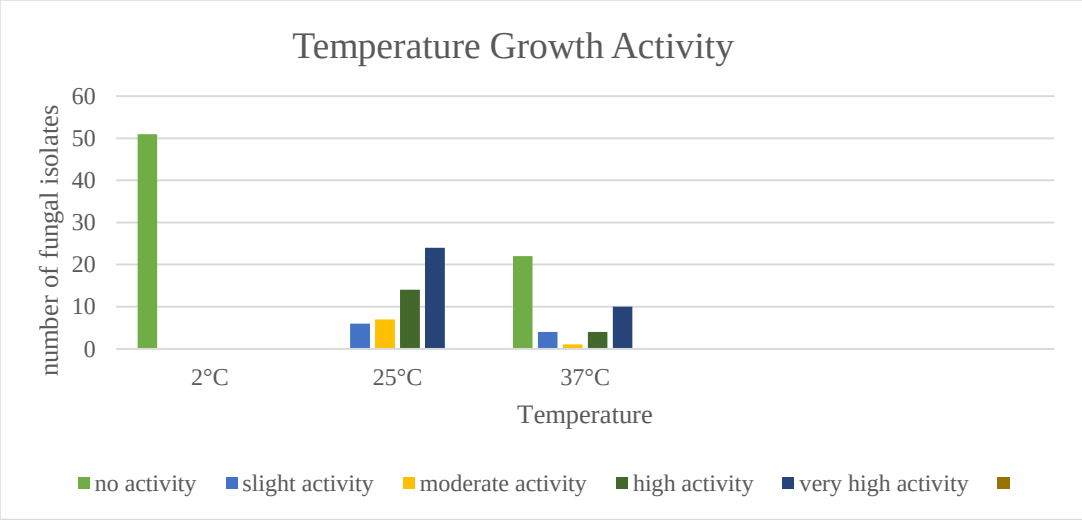


Figure 5.4: The effect of temperature on the growth of endophytic fungi isolated from *Sutherlandia frutescens* leaves. Bar graph of temperature growth activity. (-) no activity (0.00-19.0mm), (±) tinge activity (20.0-30.0mm), (+) moderate activity (31.00-49.0mm), (++) high activity (50.0-69.0mm), (+++) very high activity (70.0- 87.0mm)

As found, none of the fungal isolates produced any colony as their growth was completely suppressed at 2 ℃, however at 25℃ all the fungal isolates were capable of growing in various levels. At 37 ℃, no activity was displayed by 50% of the fungal isolates, while others grew to varying levels with 7% of isolates which gave slight activity, moderate activity shown by 1%, high activity recorded for 7% of the isolates, and lastly 19% of the isolates having very high activity.

5.3.4.2 Salt

To determine the growth potential of endophytes in this study under stress conditions, salt (NaCl) concentration in the growth medium was varied and at the end of the study period, colony diameter for each isolate was measured. Results obtained (Fig 5.5) revealed that the fungal isolates had extreme growth at NaCl concentration of 3% (w/v) wherein 90% of the endophytic isolates produced colonies.

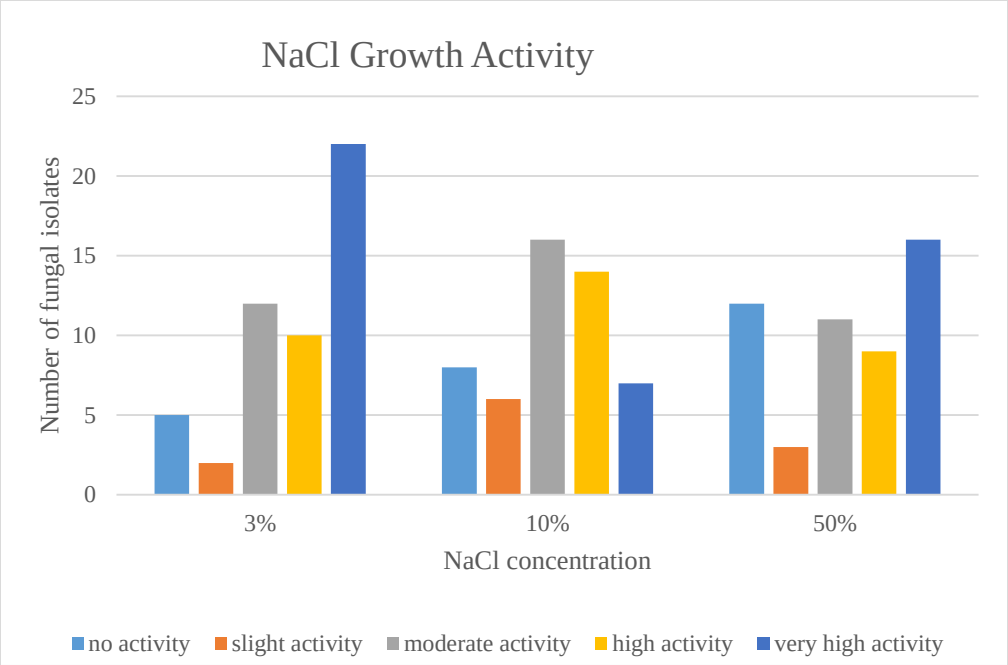


Figure 5.5: Bar graph of salt test growth activity. (-) no activity (0.00-19.0mm), (±) tinge activity (20.0-30.0mm), (+) moderate activity (31.00-49.0mm), (++) high activity (50.0-69.0mm), (+++) very high activity (70.0- 87.0mm)

The NaCl concentration was increased to 10% and 84% of the isolates showed growth, lastly the concentration was increased to 50% and a maximum of 76% of the isolates showed growth.

5.3.4.3 pH

In this study, the effects of pH on growth of fungal endophytes was determine and colony diameter of each fungal isolate is presented in (Fig 5.6), it was found that the fungal isolates tested grew the most at pH5 with a percentage of 44% followed by 35% growth at pH of 12 and lastly, 21% for pH of 2.

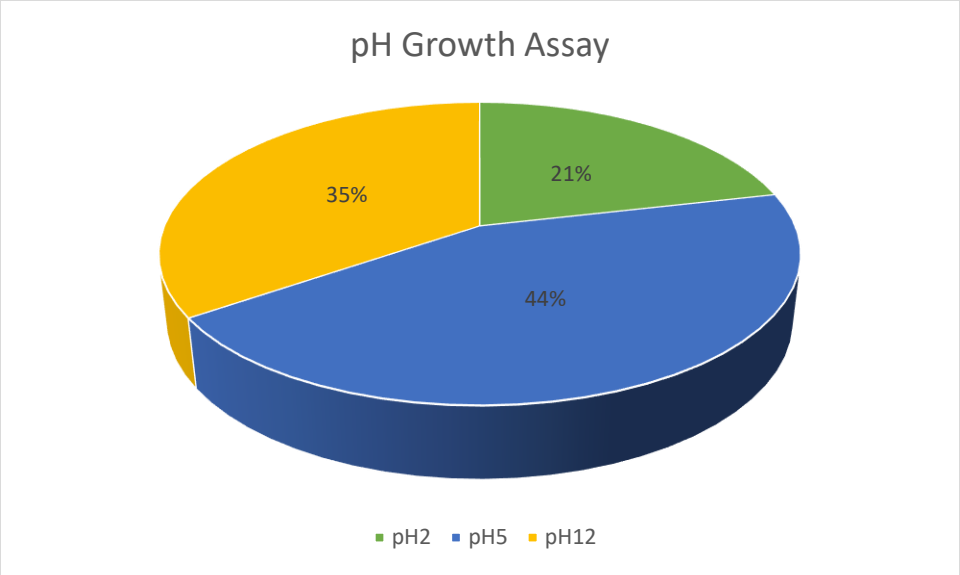


Figure 5.6: Pie chart showing the effect of pH on the endophytes isolated from S. frutescens in growth percentage

5.4 DISCUSSION

Previous studies reported that endophytes isolated from medicinal plants produce a wealth of bioactive compounds which have shown antifungal activity which proves the exceptional potential of antifungal agents and fungicide (Suraddkar *et al*., 2017; Yu *et al*., 2018; Nurhaida *et al*., 2020; Sishuba *et al*., 2021;). Numerous interesting metabolites that are biologically active which are obtained from endophytic fungi of medicinal plants have been investigated (Kaul *et al*., 2012). In our study, the dual culture method and filtrate culture method were used to evaluate the antimicrobial activity of fifty-one *S. frutescens* strains against plant pathogenic fungi. The activity displayed by the isolated strains was 84% and 78% for dual and filtrate culture assay, where they were active against one or more pathogenic fungi, other studies such attest to the ability of isolated strains to display activity against one or more plant pathogens (Gong and Guo., 2009; Luo *et al*., 2015). It was proposed that the assay method used has a significant impact on the percentage of antibiotic-producing strains, as well as the species (Shnit-Orland and Kushmaro., 2019). In dual

culture assay, *Mucor*, *Penicillium* species and *Aspergillus brasiliensis* exhibited a broad antifungal activity spectrum for three or all pathogenic fungi of plants tested in this assay. While in the filtrate culture method *Mucor* and *Penicillium* species which exhibited broad activity in dual culture assay did not present the same activity in this culture rather they were least active for two or more pathogens and showed moderate growth for others. The mycelial growth inhibition assay showed an overall activity that is higher than that of fermentation broths, the same was also observed with (Sishuba *et al*., 2021).

In this study, the effects of temperature, NaCl concentration and pH on the growth of fungal endophytes were also determined. As noted, some endophytes that were isolated can resist stress (salt, temperature and pH) at varying levels. The fungal community is responsible for various ecosystem services together with agricultural and industrial products. Nonetheless, the functioning of these fungal species and execution of their services is dependent on their ability to grow under different stress conditions which result from different abiotic factors such as temperature, pH, environments of low nutritional status, low water activity etc. (Rangel *et al*., 2018).

Many endophytes have been investigated and found to protect a diverse range of host plants against adverse climatic conditions and enhance their growth along the process (Aishwarya *et al*., 2017). Fungal isolates were tested for temperature tolerance but none of them grew at 2°C, however at 25°C all the fungal isolates tested grew to various levels. As the temperature increased to 37°C, only 50% of the fungal isolates were able to grow. Gopane *et al*, (2021) also studied the community diversity and stress tolerance of culturable endophytic fungi from black seed (*Nigella sativa* L.). The ideal growth conditions of fungi are temperature, ranging from 25-30 °C, high moist humid environment and pH from 3 to 7. Temperature is considered one of the significant abiotic factors

in modulating ecological processes. The thermal sensitivity of fungi can alter their cellular and molecular biology, behavior, distribution, ecophysiology and abundance. It is important to understand the thermal sensitivity profile and study the effect of thermal performance (Voyles *et al*., 2017).

The fungal isolates showed maximal growth at a NaCl concentration of 3% wherein 90% of the tested endophytic isolates showed high growth potential. The potential of the tested isolates however, reduced from 90 to 76% as NaCl concentration was increased to 50%. Salinity is one of the major environmental stresses affecting crop production worldwide. The salt effects on plants include osmotic stress, ion toxicity, nutrient imbalance and deficiencies, resulting in membrane damage, changes in metabolic processes, oxidative stress and genotoxicity (Vitti *et al*., 2015). One of the factors endophytic fungi is known for is its exogenous secretion of phytohormones and alleviation of salinity stress. These phytohormones producing endophytes can affect the production of secondary metabolites like flavonoids and also assist the plant to tolerate/ avoid stress, under extreme environmental conditions (Khan *et al*., 2012).

Ripa *et al*., (2019) also found that at 25 °C, most isolates grew but growth was suppressed at temperature below 5 °C and in addition to their findings, fungal isolates were able to grow on a medium with NaCl at high concentration of 10%. The fungal isolates grew the most in pH of 5 with a percentage of 44% followed by 35% growth at pH of 12 and lastly 21% for pH of 2. This supports that some fungi are able to grow at both acidic and alkaline conditions. pH is an influential environmental factor in the growth of microbes. The majority of fungi prefer a low-pH medium (Yang *et al*., 2008). The growth of fungi is favoured by acidic pH values and that increases the

importance and dominance of fungi. However, various fungi can grow over a wide pH range from extreme acidity to alkaline conditions (Rangel *et al*., 2018).

5.5 CONCLUSION

Generally, the findings reported in this study indicates that a majority of the fungal isolates are an important source of antifungal compounds that may provide renewable sources of useful antifungal drugs against pathogenic fungi. Naturally grown plants are exposed to several physico-chemical stresses influenced by the geographical location and type of soil. In this study, it's shown how the endophytes present in plants used herein were confirmed to be biologically active against a wide range of pathogenic fungi and equally showed to have resistance towards varying pH, temperature and salt levels to a certain degree that may likely be beneficial to the host plant. Endophytic fungi found herein are untapped territories filled with effective, novel bioactive compounds to control fungal pathogens responsible for the high crop losses. In conclusion, these endophytic fungi have the potential to be used as potential natural biocontrol agents in the agricultural, medical as well as pharmaceutical industries. This is probably the first report on the antifungal activity of endophytic fungi isolated from *S. frutescens* plants.

CHAPTER SIX
GENERAL DISCUSSION AND CONCLUSION

6.1 GENERAL DISCUSSION AND CONCLUSION

6.1.1 DISCUSSION

There is an increased interest in the study of endophytes as potential source of biocontrol agents for use in the agricultural, medicinal and pharmaceutical industries and various medicinal plants have been exploited in this regard. In this study, the biodiversity and antimicrobial activity of endophytic fungi isolated from *S. frutescens* were investigated with the plant used for the first time for this purpose. Antimicrobial resistance is an issue of global concern given the challenges on therapeutic processes involved. The findings of this study revealed that 51 fungal endophytes were isolated from cancer bush leaves and identified based on morphological identification using macroscopic and microscopic features, coupled with molecular identification ITS specific PCR analysis.

In addition, study demonstrated that these endophytes have the potential to produce bioactive secondary metabolites with significant antibacterial and antifungal activities as extracts from this plant were able to inhibit microbial growth. Cancer bush is used for medicinal purposes in South Africa and thus the therapeutic potential of extracts from this plant may be combination of the metabolites from the plant as well as those produced by endophytes that it harbours. Further investigations on its exact components in the metabolites may open doors for drug development studies. The low antimicrobial activities displayed by some fungal extracts may be as a result of

low quantities of active ingredients. However, it is suggested that these extracts should be purified to improve their potency (Fabry *et al*., 1998).

Given the knowledge that endophytic fungi can protect the plant host against pathogens and pets, it is expected that these endophytes be favored by natural selection as they produce the same defensive chemicals as their host plants (Saikkonen *et al*., 2004). A second antimicrobial test was then done to test the antifungal activity where plant fungal pathogens were tested against endophytic fungi and their extracts. The results revealed that a majority of the fungal isolates could be an important source of antifungal compounds that may serve as a renewable source for useful antifungal drugs. The stress tolerance of these endophytes was determined and a large proportion were able to withstand harsh/ unusual conditions. This is very important given concerns surrounding global warming.

Endophytic fungi play a vital role in the biosynthesis of new bioactive compounds and thus they can be utilized for the discovery of untapped bioactive compounds with pharmacological potential. The interaction of endophytic fungi and their host plants occurs through complex chemical systems, which are associated with their survival in different ecological niches. It therefore means that understanding these interactions is critical in the realization of the processes through which these compounds can be harvested.

6.1.2 CONCLUSION

In conclusion *S. frutescens* harbours a diverse group of endophytic fungi with varied biological activities and investigations of this nature that to the best of our knowledge, is the first report on

the isolation and identification of endophytes from *S. frutescens* (cancer bush) as well as the antibacterial and antifungal properties of the secondary metabolites should be encouraged.

6.2 LIMITATIONS OF THE STUDY AND FUTURE WORK

All the aims and objectives outlined in section 1.3 (Chapter One) were achieved. However, this study had some limitations.

6.2.1 Limitations of the study

Different locations and host plants contribute to the diversity of endophytic fungi, however in this study sampling was only limited to one place because of the availability of the selected medicinal plant and limited funds. *S. frutescens* plants do not grow in all locations of South Africa. The isolation was mainly based on the leaves of the plant and they are the ones mostly used. Another shortfall was the small size of the leaves of cancer bush resulting a smaller surface area making isolation a little bit challenging. The interactive effect of salt, pH and temperature could not be established in this study due to the large number of isolates tested in addition to limited funding available for the study.

6.2.2 Recommendations and Future directions

- A comprehensive study needs to be taken using the same samples to explore other biological properties such as antiviral, antioxidant, etc.
- Tagging of other gene regions and phylogenetic analysis with the same genus and species as a molecular technique to determine and confirm the novel endophytes.
- The antimicrobial activities and growth enhancement may be improved by combining the endophytic fungi extracts.

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