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Endophytic fungi hosted by medicinal plants may potentially exhibit antimicrobial activity

Zainub B. Aboobaker



A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Pharmacy

Johannesburg, 2018

Declaration

I, Zainub Aboobaker, hereby declare that the work contained in this dissertation is my own original work, with all other sources of information acknowledged by means of a complete reference list. This dissertation is being submitted for the degree of Master of Pharmacy, at the University of the Witwatersrand, Johannesburg. This work has not been submitted for any other degree or examination at this or any other university.

…ZAboobaker….

Z. B. Aboobaker

The …26th….day of ……October……, 2018.

Dedication

I dedicate this dissertation:

To my husband, Nadeem Cajee, who has always encouraged me in my endeavours to achieve success. You have been my strength and support in difficult times.

To my mother, Mariam Aboobaker, your persistent efforts to motivate me in achieving academic excellence have not gone unnoticed. You have had an unwavering determination to drive me towards realizing my goals.

To my farther, Ahmed Aboobaker, your constant prayers for my success and achievement has given me inner strength and determination to achieve my goals.

To my four sisters and brother, I am so thankful to have been blessed with siblings that are my best friends. I am forever in your debt.

Thank you for your unwavering faith and confidence in my abilities.

Publications and presentations arising from this study

Conference presentations (oral)

Zainub B. Aboobaker, Sandy F. van Vuuren, Alvaro M. Viljoen, Sushil K. Chaudhary, Weiyang Chen, Pedro W. Crous. Endophytic fungi and their role in antimicrobial activity. Indigenous Plant Use Forum (IPUF) Conference, Batter Boys Guest House, Pretoria, South Africa. 10^{th}– 12^{th} July, 2017. (refer to Appendix A for abstract).

Zainub B. Aboobaker, Sandy F. van Vuuren, Alvaro M. Viljoen, Sushil K. Chaudhary, Weiyang Chen, Pedro W. Crous. School of Therapeutic Sciences 2017 Research Day, University of the Witwatersrand (Faculty of Health Sciences), Johannesburg, South Africa. 12^{th} September, 2017. (refer to Appendix B for abstract).

ZB Aboobaker, SF van Vuuren, AM Viljoen, PW Crous. South African endophytes- potential antimicrobial agents. The 65th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research. 2-6 September, Basel, Switzerland, 2017, podium presentation. (refer to appendix C for abstract)

Papers for publication

Zainub B. Aboobaker, Sandy F. van Vuuren, Alvaro M. Viljoen, Sushil K. Chaudhary, Weiyang Chen, Pedro W. Crous. 2018. Endophytic fungi isolated from *Pelargonium sidoides* DC: Antimicrobial interaction and isolation of a bioactive compound. Submitted 2018 to the South African Journal of Botany. (refer to Appendix D for abstract).

Abstract

Endophytes are micro-organisms that colonize plant tissue internally. They are characterized by the feature that they do not cause any harm to the host plant. In recent years, endophytes have generated great interest around the globe as they produce a wide array of functional metabolites. Endophytes have been found in every plant species examined to date and have been recognized as potential sources of novel antimicrobials. The antimicrobial potential of endophytes isolated from South African medicinal plants has, however, been poorly explored, and there is a lack of scientific investigations on the potential of South African endophytes. A large knowledge gap exists in this area of study considering the high floristic diversity in the country. Thus, this study set out to investigate the antimicrobial activity of endophytes isolated from the roots of three popular South African medicinal plant species *Pelargonium sidoides* DC, *Hypoxis hemerocallidea* Fisch., C.A.Mey. & Avé-Lall, and *Gunnera perpensa* L. The study further aimed to isolate and identify secondary metabolites from the endophyte exhibiting promising antimicrobial activity. The hypothesis formulated aims to determine that medicinal plants used as an anti-infective may not only rely on the medicinal plant alone but also on the residing endophytes in combination.

Fungal cultures were isolated from root material of *P. sidoides*, *H. hemerocallidea* and *G. perpensa*. Endophyte extracts were prepared and tested independently and in combination with the host plant (aqueous and organic extracts) using the broth micro-dilution technique to determine antimicrobial activity. Minimum inhibitory concentrations (MIC) were determined against four pathogens, Gram-positive (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212) and Gram-negative (*Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 27853) bacteria. The Fractional inhibitory concentration index (FICI) was used to determine if synergistic interactions were apparent during combination studies. Preparatory high performance liquid chromatography-mass spectrometry (prep HPLC-MS) was used to fractionate the bioactive crude extracts, and secondary metabolites were isolated and identified using ultra performance liquid chromatography-mass spectrometry (UPLC-MS) and nuclear magnetic resonance (NMR). Identification of endophytes displaying the most noteworthy antimicrobial activity was

undertaken by polymerase chain reaction (PCR) amplification and deoxyribonucleic acid (DNA) sequence analysis.

A total of 19 endophytic fungi were isolated from the *P. sidoides* root. One of these fungal endophyte isolates (PS8) was identified as *Penicillium skrjabinii* and the ethyl acetate crude extract of this isolate exhibited antimicrobial activity at MIC values 0.03 mg/mL and 0.06 mg/mL (batch 1) and 0.03 mg/mL and 0.13 mg/mL (batch 2) against *S. aureus* and *E. coli* respectively. Bioassay-guided isolation was carried out and the major compound, dibutyl phthalate was isolated from the most active fraction. Interactions between *P. sidoides* endophytes and the host plant displayed higher synergy when compared to *H. hemerocallidea* and *G. perpensa*. A total of 144 combinations were tested from *P. sidoides* with 14.58% displaying synergy and 8.33% displaying antagonism, the remainder 77.03% were either additive/indifferent. One combination PS17 and organic extract displayed synergy (FICI value of 0.09). Six endophytes were isolated from *H. hemerocallidea*, and one endophyte (H4) displayed noteworthy activity at 0.25 mg/mL against *S. aureus*. There was a synergistic interaction between the endophyte H1 and aqueous extract (FICI 0.50) while H1 and organic extract displayed synergy at a FIC of 0.38, both these interactions inhibited *E. coli* at 1.00 mg/mL. From a total of 48 combinations tested, there were no antagonistic interactions. Five fungal endophytes were isolated from *G. perpensa*. An interesting isolate (GP5) inhibited *S. aureus* at 0.50 mg/mL when tested independently, and also displayed synergistic activity with the aqueous plant extract against *S. aureus* and *E. coil* at 0.25 mg/mL and 0.50 mg/mL respectively.

In summary a number of endophyte extracts displayed a synergistic interaction with the host plant extracts, proving the positive influence of endophytes on the antimicrobial activity of plants. This is the first study to identify *P. skrjabinii* as an endophyte from *P. sidoides* that produces the compound dibutyl phthalate. It is also the first report documenting endophytes isolated from *Pelargonium sidoides, H. hemerocallidea,* and *Gunnera perpensa* root material. Lastly, it is the first report that provides some insight into the combined antimicrobial efficacy of endophytes with the host plant providing evidence of synergy. This study has highlighted the importance of endophytes as a potential source of antimicrobials and has demonstrated their ability to exert a positive effect on plant extracts against pathogenic micro-organisms.

Acknowledgements

In The Name of God, The Most Beneficent, The Most Merciful

First I would like to give thanks to my creator for all the countless blessings he has enveloped me in, one of which is, guiding me through the path of knowledge.

To my supervisor, Professor Sandy van Vuuren, it has been an absolute honour and privilege working with you. Observing your success and determination has left me in awe, and has allowed me to set a higher standard for myself. Your constant support through the journey of attaining this master's degree has made these last two years a memorable experience. Should we ever feel our endeavours have to lead us astray; we need only re-orientate ourselves back to true north, to you Professor Sandy van Vuuren−our compass rose.

To my co-supervisor Professor Alvaro Viljoen, it has been a privilege to work with you. Thank you for lending your insurmountable wisdom and knowledge to this project. You have been a great help during the last two years.

To Dr W. Cheng and S. Chaudhary, from Tshwane University of Technology, Thank you for all the assistance with prep-HPLC and UPLC analysis.

To Professor V. Maharaj, from the University of Pretoria. Thank you for your assistance with Nuclear Magnetic Resonance (NMR) spectroscopy

To Professor Pedro Crous, from the fungal biodiversity institute in Westerdijk, Netherlands. Thank you for identifying important fungal isolates for this project.

To the staff at the University of Witwatersrand, Department of Pharmacy and Pharmacology, particularly Phumzile Madondo, thank you for all your assistance in the laboratory.

I would like to thank the National Research Foundation (NRF) for financial assistance (scholarship) during the two year period of my thesis, and also many thanks to the University of Witwatersrand for the merit award funding provided during the second year of the master's degree.

I would like to thank the Wits Faculty Research Committee (FRC) for the provision of partial funding for the research running costs for the year 2017.

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Abbreviations

AIDS - Acquired Immune Deficiency Syndrome

ADD - Additive interacting

ANT - Antagonistic interaction

Aq - aqueous plant extract

ATCC - American Type Culture Collection

CFU - Colony forming units

$CDCL_3$ - Deuterated chloroform

CLSI - Clinical and Laboratory Standards Institute

DD - Disc diffusion

Dd - Doublet of doublet

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

Dm - Dichloromethane

eV - Electron volt

FIC - Fractional inhibitory concentration

FICI - Fractional inhibitory concentration index

G - Gram

g/L - Grams per litre

^1H - Proton

Hrs - Hours

Hz - Hertz

INT - *p*-Iodonitrotetrazolium violet

IND - indifferent interaction

ITS - Internal Transcriber Spacer

J - Coupling constant

LC-MS - Liquid Chromatography Mass Spectrometry

mM - Multiplet

min - Minutes

MIC - Minimum inhibitory concentration

MEB - Malt Extract broth

mg - Milligram

MHz - Megahertz

mL - Millilitres

mm - Millimetre

mol/L - Moles per litre

MS - Mass Spectrometer

m/z - Mass to charge ratio

MRSA - Methicillin resistant *Staphylococcus aureus*

N_2 - Nitrogen

n/a - Not active

ND - Not determined

n/t - Not tested

NMR - Nuclear Magnetic Resonance

NCBI - National Centre for Biotechnology Information

Org - Organic plant extract

PCR - Polymerase chain reaction

PDA - Potato Dextrose agar

pH - Potential hydrogen

ppm - Parts per million

Prep HPLC - Preparatory High Performance Liquid Chromatography

QDa - Quadrupole dalton

Rpm - Revolutions per minute

SANBI - South African National Biodiversity Institute

SDB - Sabouraud Dextrose broth

Sec - Seconds

SYN - Synergistic interaction

t - Triplet

TSA - Tryptone Soya agar

TSB - Tryptone Soya broth

UPLC - Ultra Performance Liquid Chromatography

UTI's - Urinary tract infections

V - Volts

VRE - Vancomycin resistant Enterococci

WHO - World Health Organisation

w/v - Weight per volume

µl - Micro-litre

µm - Micro-meter

°C - Degrees celsius

δ (delta)

% - Percent

Chapter 1
Introduction

1.1. Endophytes

The term endophyte refers to fungi or bacteria, which live asymptomatically and unobtrusively inside living plant tissue for all or part of their life cycle (Petrini, 1991; Wilson, 1995). Epiphytic bacteria and fungi colonize plant surfaces. In contrast to epiphytic bacteria and fungi, endophytes are contained entirely within plant tissues and their association with the host may be described as mutualistic (Clay, 1990). The birth of endophyte research was initiated as early as the nineteenth century; however, research in this field flourished only much later when taxol, the anti-cancer compound was initially isolated from the Pacific yew tree (*Taxus brevifolia*). It was later isolated from a fungal endophyte from the same plant (Stierle *et al*., 1993a; Compant *et al*., 2012; Hardoim *et al*., 2015; Saikkonen *et al*., 2016). Endophytes build a microbial community within the plant, and it is this complex interaction between the microbiome and host plant that is of much interest, yet poorly studied (Hardoim *et al*., 2015; Compant *et al*., 2016).

1.2. Fungal endophytes

Before a fungus can be named an endophyte, it ought to show that its hyphae are in the living tissue of the host. By definition, an endophytic fungus lives in the mycelial form in metabolic association with the living plant for at least some part of the plants life cycle (Kaul *et al*., 2012). Both plants and endophytes produce secondary metabolites. Endophytes are producers of growth promoting secondary metabolites, insects and pest repellents, act as antimicrobials against plant pathogens, and are protectors in stress conditions, these micro-organisms assist the plant in growth and diversification (Ryan *et al*., 2008; Staniek *et al*., 2008; Rai *et al*., 2014a, 2014b). The Glomeromycota, Ascomycota, and Basidiomycota are divisions within the kingdom of fungi that make up 91% of endophytic fungi that colonize plants. Glomeromyceta make up 40% of endophytic fungi that colonize plants, while Ascomycetes, Basidiomycetes, and Zygomycetes make up 31%, 20%, and 0.1% respectively. The rest of

the endophytic fungi belong to an unidentified phylum (Figure 1.1) (Hardoim *et al*., 2015). Glomeromycota is known as arbuscular mycorrhizal fungi and have symbiotic relationships with land plants, they are mostly found in roots and soil (Jeffries *et al*., 2003). Ascomycetes are a class of fungi that is characterised by spore formation in cylindrical sacs. Most mould and yeasts form part of this group of fungi. Some genera of this class are known to produce antibacterial secondary products e.g. *Phomopsis* spp. and *Pestalotiopsis* spp. (Deshmukh *et al*., 2015). Basidiomycota are characterized by their club-shaped end cell bodies (basidia) responsible for sexual reproduction. Mushrooms belong to this group of fungi and are sometimes responsible for wood decay (Veneault-Fourrey *et al*., 2013).

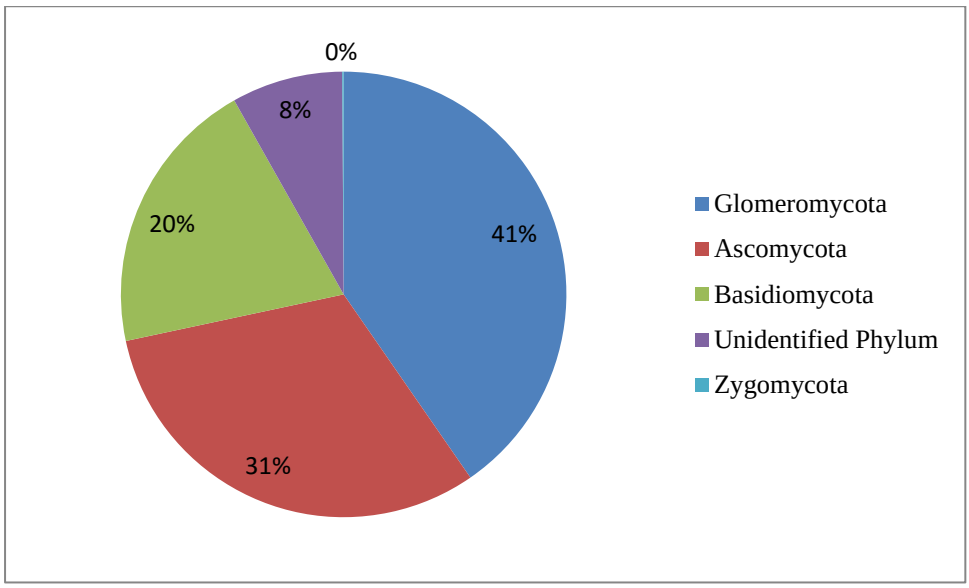


Figure 1.1: Endophyte divisions that colonize plants internally

1.3. Natural product research and endophytes

Natural products are compounds and substances that are naturally acquired through the process of metabolism or as a by-product of plants, micro-organisms and animals (Strobel and Daisy, 2003). The earliest record of medicinal plants dates back to 2600 BC when plant-derived natural products were used therapeutically in Mesopotamia (modern day Iran) (Borchardt, 2002). Ancient Greeks, Egyptians and Chinese civilizations have used plants as a remedy for many ailments for centuries. Every country has local flora that is explored and used for medicinal purposes. International travel has led to the interaction between different cultures and thus resulted in the emergence of pharmacopoeias for each culture (Kaul *et al*.,

2012). Thus, for decades natural product research has focused on plants with therapeutic relevance.

The utilization of natural products for therapeutic properties is a consistently developing market and millions of people in developed and developing countries of the globe are using natural products (most commonly medicinal plants) as a way to treat infections (World Health Organization., 2005). South Africa is no exemption to this and because of the wide variety of southern African plant species and the country's rich African heritage of traditional healing practices, there have been numerous well-cited publications with respect to ethnobotany and antimicrobial properties (van Vuuren *et al*., 2008; van Vuuren and Holl, 2017).

In spite of the number of publications dedicated to natural products and antimicrobial activity, very little recognition has been given to endophytes that may harbour antimicrobial activity. Fungal endophytes were not considered as a potential repository for natural products until paclitaxel (Taxol®), the world's first multi-billion dollar antitumor drug, was isolated from *Taxomyces andreanae,* an endophytic species, in 1993 (Stierle *et al*., 1993b).

1.4. Importance of endophytic fungi in the pharmaceutical industry

The importance of fungi in the production of bioactive secondary metabolites emerged when Sir Alexander Fleming made the discovery of penicillin from *Penicillium notatum* more than 80 years ago (Nisa *et al*., 2015). The discovery of penicillin led to the uncovering of Cephalosporin C (primary precursor), which was isolated from *Cephalosporium* spp. Cephalosporin C was later chemically modified to produce a range of semi-synthetic cephalosporin antimicrobials (Newton and Abraham, 1955; Higgens *et al*., 1974; Gaurav *et al*., 2012). Different fungal strains have paved the way for natural product research, with the focus on antimicrobials. A few endophytic fungal isolates, such as *Aspergillus* spp., *Penicillium* spp., and *Phoma* spp. have been investigated for their biological applications; including their antimicrobial activity. The *Penicillium* spp. often colonizes plant tissue internally and has gained increasing interest since the isolation of the antibiotic Penicillin. This endophytic fungal species is not only found in medicinal plants but also in marine algae. *Penicillium chrysogenum* isolated from the marine red algae produced an antimicrobial compound conidiogenone B, which exhibits potent activity against methicillin resistant

Staphylococcus aureus (MRSA), *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Staphylococcus epidermidis* (Li *et al*., 2010; Gao *et al*., 2011).

1.5. Interaction of endophytes with host plant

The concept of synergy is not only ubiquitously encountered in in nature but also considered as a possible hypothesis for the growing complexity of the evolutionary process. Synergy is defined as a cumulative effect produced due to interactions by different forces, particles, elements and individuals in a given context (Biavatti, 2009). In terms of pharmacology, a therapeutic approach using more than one drug to target a disease may be more effective than using a single drug with a single target. Combination therapy has been successful since different drugs have different modes of action and therefore when used simultaneously these drugs attack the target more successfully (Csermely *et al*., 2005). Penicillin is a good example of an antibiotic that shows synergy when in combination with aminoglycosides. These antimicrobials show synergy due to their different mode of action when attacking bacterial cells (Kohanski *et al*., 2010). The synergy between antibiotics is an effective way to combat bacterial strains that cause resistance e.g. amoxicillin and clavulanic acid are used in combination to combat beta-lactamase resistant bacteria. Even though clavulanic acid has weak antibacterial activity, it has a strong affinity for inhibiting the beta-lactamase enzyme responsible for resistance to beta-lactam antimicrobials, thus the strong antibacterial activity of amoxicillin and the strong affinity of clavulanic acid to beta-lactamase enzyme results in an accumulative effect (Matsuura *et al*., 1980). A recent study by Zin *et al*. (2017), evaluated the possible synergistic interactions between eight compounds isolated from an endophytic fungus *Eurotium chevalieri* with commercial antimicrobials. Emodin was found to exhibit synergistic activity with oxacillin against MRSA. Phytotherapeutic practitioners instinctively believe that the "*total extract works better than an equivalent dose of an isolated substance"* (Nelson and Kursar, 1999).

It has, however, never been explored that the entirety of a plant extract could include the secondary metabolites produced by the endophytic fungi within the plant. Since both plants and endophytes have biosynthetic pathways that affect each other during the life cycle of the plant, plant and endophytic extracts should be tested in combination to elucidate if the interaction between the latter is that of synergy, additive, non-interactive or antagonistic effects. While endophytes have globally been well studied (Zhang *et al*., 2008; Hussain *et al*.,

2014; Pinheiro *et al.*, 2017), the interaction with the host has been neglected. This has been highlighted in a recent editorial by Compant *et al*. (2016), who emphasised the need for future directives in this research domain. Furthermore, the interaction between endophytes originating either from the same host or different hosts may reveal an increase in bioactive compounds which may have a synergistic effect. Some studies documenting this for the control of insect pests and plant pathogens are found in the very recent review by Jaber and Ownley (2017). A South African study on the host-endophyte-pest interactions of endophyte *Fusarium oxysporum* was found to be antagonistic to *Radopholus similis* in banana (Athman, 2006). Endophytes grow within the host for a reason and isolation of the endophyte should not be the endpoint of research. If one is undertaking research from an antimicrobial perspective, the combination of endophyte with host/hosts (when combined plants are used in therapy) or even between endophytes should be a prominent factor to study.

1.6. Relevance of endophytes to South African medicinal plants

Antimicrobial activity of endophytic fungi has been studied in countries such as Indonesia, Brazil and China, to name a few regions (Wang *et al*., 2007; de Siqueira *et al*., 2011; Astuti *et al*., 2014). However, there is a lack of research in South Africa on this topic. The research group at the Faculty of Natural and Agricultural Sciences, University of Pretoria have led the way in terms of endophyte research in South Africa. Most of this endophyte research has focused on genetics, taxonomic diversity, and aspects other than antimicrobial properties (Denman *et al*., 2003; Kemler *et al*., 2013; Pavlic-Zupanc *et al*., 2015). Very few endophyte studies on South African species have had an antimicrobial focus (Table 1.1). A very recent study by Manganyi *et al*. (2018) investigated the biodiversity of fungal endophytes from *P. sidoides*, as well as the antibacterial activity of these endophytes against the pathogen *E. coli.* A study by Human *et al*. (2016) was undertaken to isolate antibiotic producing actinomycetes from infructescences of *Protea repens* and *Protea neriifolia.* The focus of this study was to describe the role of endophytes within the plant species. Endophytic bacteria were isolated and identified from *Monsonia burkeana* and eight other medicinal plants in South Africa. The focus of the study was to catalogue bacterial endophytes in medicinal plants (Nnzeru *et al*., 2017). A large volume of South African endophyte research has focused on the Botryosphaeriaceae, which is a fungal endophyte genus that is often associated with woody plants. This genus is mostly responsible for the latent pathogenesis of *Eucalyptus grandis* and *Syzygium cordatum*, Furthermore this fungal species has a wide range of hosts (Smith *et al*.,

1996; Pillay *et al*., 2013) (Table 1.1). Serdanil *et al*., (1998), examined the role of endophytes in the crop loss of apples. A study on bacterial endophytes from sugarcane demonstrated the ability to inhibit the growth of two important fungal pathogens of sugarcane (van Antwerpen *et al*., 2002). Most of these studies have focused on identifying the latent plant pathogens and biological control measures to prevent crop loss. The Proteaceae has gained considerable attention since it is of much importance in the cut-flower industry (Denman and Crous, 1998). Furthermore, Taylor *et al.* (2001), has researched *P. repens*, *P. nitida*, and *P. neriifolia* extensively, and the role of endophytes in the proliferation and survival of the plant. While much of the scientific research on endophytes in South Africa has focused on biological control of crop loss, and most medicinal plant research has focused on plant extract antimicrobial *in vitro* testing (van Vuuren, 2008; van Vuuren and Holl, 2017). There is clearly a void and a latent research opportunity on medicinal plant endophytes and their antimicrobial uses.

Many South African ethnobotanical leads have focused on the traditional and historical use of plant material. Informal traders collect and sell medicinal plants and this is one of the main sources for African medicines. Interestingly, some of this plant material at the trade markets is favoured if containing visible fungal cultures. There is the belief that this enhances the efficacy of the medicinal plant (personal communication, informal traders, Faraday Muthi Market, Johannesburg). It is possible that the therapeutic efficacy of these sought-after medicinal samples is a combination of endophyte and medicinal plant.

Table 1.1: Endophyte host plant studies in South Africa.

Host plant	**Endophyte isolate**	**Relevance of study**	**References**
Triticum aestivum L.	*Alternaria* spp., *Epicoccum* spp., *Fusarium* spp., *Phoma* spp.,	Occurrence of fungal endophytes	Crous *et al*., 1995
Eucalyptus grandis W.Hill., *Eucalyptus nitens* Maiden.	*Botryosphaeria dothidea*	Latent pathogen	Smith *et al*., 1996
Malus domestica Baumg. (Apples)	*Alternaria spp*.	Core rot of apples	Serdanil *et al*., 1998
Amaranthus hybridus L.	*Alternaria tenuissima*	Plant composition of endophytic fungi	Blodgett *et al*., 2000
Protea neriifolia R.Br., *Protea nitida* Mill., *Protea repens* Thunb.	*Botryosphaeria protea*	Pathogenesis	Taylor *et al*., 2001
Saccharum officinarum L. (Sugarcane)	*Burkholderia spp.*	Assessing stalk rot	van Antwerpen *et al*., 2002
Amaranthus hybridus L.	*Alternaria* spp.	Recovery and frequency of fungal endophytes in plant parts	Blodgett *et al.*, 2007
Leucadendron xanthoconus K.Schum., *Mimetes cucullatus* (L.) R.Br., *Protea lepidocarpodendron* L.	*Botryosphaeriaceae* spp.	Latent pathogens	Marincowitz *et al*., 2008

Host plant	Endophyte isolate	Relevance of study	References
Persea americana Mill.	26 fungal species	Biological control	Hakizimana *et al*., 2010
Fadogia homblei De Wild., *Pavetta harborii* S.Moore., *Vangueria pygmaea* Schltr., *Vangueria thamnus* (Robyns) Lantz.,	*Burkholderia* spp.	*Gousiekte* (quick disease)	Verstraete *et al*., 2011
Eucalyptus grandis W.Hill., *Syzygium cordatum* Hochst.	*Botryosphaeriaceae* spp.	Latent pathogens	Pillay *et al*., 2013
Vangueria infausta Burch., *Vangueria macrocalyx* Sond., *Vangueria madagascariensis* J.F.Gmel.	*Burkholderia* spp.	*Gousiekte* (quick disease)	Stanton *et al*., 2013
Acacia karroo Hayne., *Celtis Africana* Burm.f., *Gymnosporia buxifolia* (L.) Szyszyl., *Searsia lancea* (L.f.) F.A.Barkley.	*Botryosphaeriaceae* spp.	Latent pathogens	Jami *et al*., 2014
Avicennia marina (Forssk.) Vierh., *Bruguiera gymnorrhiza* (L.) Lam.	*Lasiodiplodia avicenniae*	Latent pathogens	Osorio *et al*., 2017
Monsonia burkeana Planch. ex Harv. & Sond.	*Bacillus* spp.	Cataloguing endophytes in medicinal plants	Nnzeru *et al*., 2017

*Gousiekte – the term refers to fatal heart disease in livestock induced by ingestion of certain plants.

1.7. Importance of plant roots as a reservoir for endophytes

Root exudates yield a variety or chemicals released by the root into the soil. These exudates serve as a means to control the surrounding environment in a way that benefits the plant. Plant roots experience biological interactions below ground level that are more complex than the interactions occurring above the soil surface (McCully, 1999). These interactions are propelled forward by root exudates. Antibacterial and antifungals released by the root of a plant are a few of the many secondary metabolite activities released from the plant root to control soil pathogens in the vicinity (Nardi *et al*., 2000; Wu *et al*., 2001). Endophytes play a significant role in the production of these antimicrobials (Tontou *et al*., 2016). The root not only has to regulate the possible threats from soil microbes but also has to compete for survival against neighboring plants. Certain chemicals released from the root can also be detrimental to surrounding plants (Flores *et al*., 1999; Inderjit and Nishimura, 1999). The role of the plant root is often compared to the significance of the gut in the human body. Both the root and the gut are considered primary organs in communication and signal exchange with the rest of the plant body and human body respectively (Sekirov *et al*., 2010; Mitter *et al*., 2013). Endophytes within the root have to compete for their place in the plant microbiome, as the root outsources micro-organisms from the surrounding rhizosphere depending on root exudates released from the root, as opposed to endophytes within the seeds, corms and tubers which seem to be inherited between plant generations (Compant *et al*., 2010; Philippot *et al*., 2013; Hardoim *et al*., 2015; Mitter *et al*., 2016).

Research on fungal endophytes have focused on vertically-transmitted fungi that colonize the above-ground parts of grasses systemically (Kuldau and Bacon, 2008), however, horizontally

transmitted fungal endophytes that belong mostly to the ascomycetes have not been given considerable attention (Arnold and Lutzoni, 2007). The horizontal modes of transmission is characterized by the transfer of fungi via air-born spores that colonize the leaves of a plant, while vertically transmitted fungal endophytes is a systemic process that occurs by means of transfer of fungi from maternal plant to offspring via seed and rhizome (Rodriguez *et al*, 2009).

The plant root has a reservoir of taxonomically diverse endophytes that play a major role in these plant processes (Compant *et al*., 2016). Root endophytes are known for their potential to promote plant growth and also to increase tolerance to stress. A good example is the root fungal endophyte *Piriformospora indica*, which was isolated from different host plants, such as, *Hordeum vulgare* and *Arabidopsis thaliana* to name a few (Deshmukh *et al*., 2015). This root endophyte was studied further by Ngwene *et al.* (2016), and it was proposed that *P. indica* promoted plant growth successfully by the ability to lower pH of the surrounding environment, and in turn solubilize inorganic phosphate for uptake by the host plant. The later gives insight into the vast capabilities of endophytic root fungi.

1.8. Plant selection for study

The Fynbos biome in the Western Cape harbours diverse plant species, with over 6000 plant species being endemic to South Africa. Furthermore, South Africa harbours approximately 3000 plant species that are used for traditional medicine (van Wyk and Gericke, 2000). There is emerging evidence that a significant number of these plant species may be a repository for unique endophytic micro-organisms (Cowan *et al*., 2013). In addition, many South African plant species harbour antimicrobial activity (van Vuuren, 2008; van Vuuren and Holl, 2017). The rationale for choosing plants was guided by medicinal plant roots used traditionally in South Africa to treat various infections (Table 1.2). Another important criterion that narrowed down the search was to focus on traditional plants used for antimicrobial therapy, but has displayed poor *in vitro* activity when tested using minimum inhibitory concentration (MIC) assays. There are about 38 plant species that are of commercial interest in South Africa. Three of these plant species are *Hypoxis hemerocallidea* Fisch. & C.A. Mey., *Pelargonium sidoides* DC., and *Gunnera perpensa* L. (Cunningham, 1988; Mander, 1998).

Table 1.2: Examples of South African medicinal plant roots used traditionally to treat infections.

Medicinal plant roots	Family	Antimicrobial uses	References
Achyranthes aspera L.	Amaranthaceae	abscess, boils	Hutchings *et al*., 1996
Adansonia digitata L.	Bombacaceae	Diarrhoea, fever, Haemoptysis	Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; van Wyk *et al*., 1997; van Wyk and Gericke, 2000; van Wyk and Wink, 2004
Boophone disticha Herb.	Amaryllidaceae	Boils, wounds	Rood, 1994
Carpobrotus edulis (L.) L.Bolus.	Aizoaceae	Gargle for mouth and throat infections, tuberculosis, wounds	Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; van Wyk *et al*., 1997; van Wyk and Gericke, 2000
Clivia miniata (Lindl.) Bosse.	Amaryllidaceae	Urinary complaints, wounds	Bryant, 1966; Hutchings *et al*., 1996
Centella asiatica (L.) Urb.	Apiaceae	Acne, wounds	Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; van Wyk *et al*., 1997; van Wyk and Gericke, 2000; van Wyk and Wink, 2004
Euclea natalensis A.DC.	Ebenaceae	Bronchitis, chest ailment, Urinary tract infections, toothache	Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; van Wyk and Gericke, 2000
Eucomis autumnalis (Mill.) Chit.	Hyacinthaceae	Boils, diarrhoea, fever, syphilis, urinary diseases	Roberts, 1990; Rood, 1994; Hutchings *et al*., 1996
Gunnera perpensa L.	Gunneraceae	STI's, UTI's, wounds	(Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; van Wyk et al., 1997; van Wyk and Gericke, 2000)
Hypoxis hemerocallidea Fisch.&Ave-Lall.	Hypoxidaceae	Tuberculosis, UTI's, wounds	van Wyk *et al*., 1997; van Wyk and Gericke, 2000
Merwilla natalensis (Planch.) Speta.	Hyacinthaceae	Ointment for wounds	van Wyk *et al*., 1997
Pelargonium sidoides DC.	Geraniaceae	Bronchitis, diarrhoea, rheumatic fever, tuberculosis	Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; van Wyk *et al*., 1997; van Wyk and Gericke, 2000; van Wyk and Wink, 2004
Scadoxus puniceus (L.) Friis & Nordal.	Amaryllidaceae	Skin ulcers and wound	Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; van Wyk and Gericke, 2000
Securidaca longepedunculata Fresen.	Polygalaceae	Cough, chest complains, toothache, wounds and sores	Watt and Breyer-Brandwijk, 1962; Bryant, 1966; Roberts, 1990; Rood, 1994; Hutchings *et al*., 1996
Senna italica Mill.	Leguminosae	Burns, wounds	Hutchings *et al*., 1996
Siphonochilus aethiopicus (Schweinf.) B.L.Burtt	Zingiberaceae	Colds, cough, influenza	Watt and Breyer-Brandwijk, 1962; van Wyk *et al*., 1997; van Wyk and Gericke, 2000
Solanum hermannii Dunal.	Solanaceae	Boils, wounds	Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996
Solanum incanum Ruiz & Pav.	Solanaceae	Furuncles, wounds	Hutchings *et al*., 1996; von Koenen, 1996

Medicinal plant roots	Family	Antimicrobial uses	References
Tulbhagia violacea Harv.	Alliaceae	Colds, fever, tuberculosis	Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; van Wyk *et al*., 1997; van Wyk and Gericke, 2000
Urginea sanguinea Schinz.	Hyacinthaceae	Venereal diseases	Foukaridis *et al*., 1995
Warburgia salutaris (Bertol.f.) Chiov.	Canellaceae	Coughs, colds, influenza, toothache, gastric ulcer, STI's	(Watt and Breyer-Brandwijk, 1962a; Hutchings et al., 1996)
Xysmalobium undulatum (L.) W.T.Aiton	Asclepiadaceae	Diarrhoea, wound-healing	Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; van Wyk *et al*., 1997; van Wyk and Gericke, 2000; van Wyk and Wink, 2004

1.8.1. *Pelargonium sidoides*

The roots of the South African plant *P. sidoides* (Umckaloabo), are widely used by traditional healers for respiratory tract infections and is marketed in Germany as a cough syrup for children (Zschocke *et al*., 2000). Furthermore, it is marketed in South Africa as Flugon® and Linctagon®. A literature search by van Wyk (2008), displayed that there was a total of 68 citations of scientific publications of *P. sidoides* from the year 1990-2008, and 12 "hits' on cited patents. Even though this plant is commercialised and used traditionally as an anti-infective, *in vitro* studies on *P. sidoides* show poor antimicrobial activity of plant crude extracts with concentrations ranging from 5.00–7.50 mg/mL, when tested against micro-organisms responsible for respiratory tract infections (Kayser and Kolodziej, 1997). Even though a great deal of pharmacological research has been conducted on *P. sidoides* further studies is still required to fully demonstrate the biological principles and mode of action underlying the therapeutic capacity (Moyo and van Staden, 2014).

1.8.2. *Hypoxis hemerocallidea*

Hypoxis hemerocallidea (African potato) is a plant widely used by traditional healers in South Africa for its therapeutic effects in treating burns and urinary tract infections (UTI's) (van Wyk *et al*., 1997). The corms are also used to make a weak infusion for treatment of tuberculosis and cancer (van Wyk and Gericke, 2000). *H. hemerocallidea* research was initiated before 1990. It resulted in a total of 52 citations for scientific publications and 19 cited patents. The rise in ethnopharmacological studies on this indigenous medicinal plant is indicative of its therapeutic importance (van Wyk, 2008) The results from *in vitro*

antimicrobial testing vary between studies. One study shows strong activity (0.31 mg/mL) against *S. aureus* (Katerere and Eloff, 2008), while another study displays weak activity against *S. aureus* (1.56 mg/mL) (Ndhlala *et al*., 2013).

1.8.3. *Gunnera perpensa*

Gunnera perpensa root, also known as river pumpkin, is mainly used topically for wound healing and psoriasis (Watt and Breyer-Brandwijk, 1962). *In vitro* antimicrobial studies of the plant root extract inhibited *S. aureus* at a concentration of 0.50 mg/mL, which is considered noteworthy activity for a plant extract (Mabona *et al*., 2013). A study by Steenkamp *et al*. (2004) displayed moderate inhibitory activity against *S. aureus* (1.00 mg/mL) and poor inhibitory activity against *E.coli* (4.00 mg/mL). This *in vitro* activity is conflicting ranging from moderate to noteworthy, leading to the assumption that the antimicrobial activity in *G. perpensa, P. sidoides* and *H. hemerocallidea* may possibly be attributed to other indirect factors such as the endophytes within the plants or synergistic interactions between host plant and endophytes.

1.9. Global studies on fungal endophytes, particularly with emphasis on antimicrobial activity

Resistance to antimicrobials, not only bacterial but also of fungal origin is increasing, with new problems emerging, thus, further complicating and rendering treatment of critical infections is more difficult. It is necessary to research and develop new antimicrobial agents for these serious infections (Levy, 2005). Many endophytic fungal isolates have been investigated for their antimicrobial activity (Ramos *et al*., 2010; Liang *et al*., 2012; Powthong *et al*., 2012; Hussain *et al*., 2014). The *Alternaria* spp., *Collectotrichum* spp., and *Phomopsis* spp. are endophytic fungi that have repeatedly been found to colonize medicinal plants and display antibacterial activity (Wang *et al*., 2007; Huang *et al*., 2008; Ding *et al*., 2010; de Siqueira *et al*., 2011; Desale and Bodhankar, 2013). A comprehensive review compiled by Deshmukh *et al.* (2015) summarised over 200 antimicrobial compounds isolated from endophytes, which comprised of 39 different fungal genera and more than 84 plants, from the year 1995 to 2014. Antibacterial activity exhibited by these compounds ranged from 37 ng/mL–256 µg/mL against various common human pathogens (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* etc.), resistant

pathogens (MRSA, vancomycin resistant *Enterococcus faecalis* (VRE) etc.) and a variety of plant pathogens (*Xanthomonas oryzae*, *Clavibacter michiganesis* and *Erwinia amylovora* etc.) (Deshmukh *et al*., 2015). There has been a rise in research in this field of study in the last two decades. According to a literature search on ScienceDirect, between the year 1996-1997, there was only one study published on fungal endophytes and antimicrobial activity, however, a decade later there has been a steady increase in publications on this topic. Some recent studies are included in Table 1.3., which provides an idea of the progress made globally in this field of study. In spite of this, there are over 300 000 plant species and each one hosts at least one or more endophyte (Strobel and Daisy, 2003), therefore the surface has only been scratched in terms of endophytic fungi and further investigations.

1.10. Chemical diversity of antimicrobial metabolites isolated from endophytes

The 20th century brought about considerable attention to bioactive secondary metabolites from fungal endophytes. Publications grew from as little as 18 articles during the year 2000–2001 to 113 articles during 2008–2009 (Aly *et al*., 2010). According to a literature search, between the years 2015 to 2017 several novel antimicrobial compounds have been isolated. Secondary metabolites are organic compounds that promote growth, development, and reproduction of an organism indirectly. The absence of secondary metabolites will not induce immediate death of a plant but will hinder the organisms' survival and aesthetics (Croteau *et al*., 2000; Dixon, 2001; Ibáñez *et al*., 2010).

Fungi can produce compounds for their own benefit, for example, to eradicate bacteria and other competitors in a host plant. The plant may produce these metabolites to alter the growth of fungi within their tissue before and after colonization (Ludwig-Müller, 2015). The biosynthetic pathways in plants are very complex. However, the syntheses of many secondary metabolites are a result of biotic and abiotic stressors (Ludwig-Müller, 2015). The secondary metabolites obtained from endophytes belong to diverse structural groups such as isocoumarins, xanthones, terpenoids, phenols, tetralones, steroids, benzopyranones (Schulz *et al*., 2002), some of which exhibit antimicrobial activity. Many bioactive compounds have been isolated from endophytic fungi (Suryanarayanan *et al*., 2009). The relationship between plant and endophyte is an aspect that requires more intensive research; however, it is very interesting to acknowledge that different plants can host the same endophytic fungal strain.

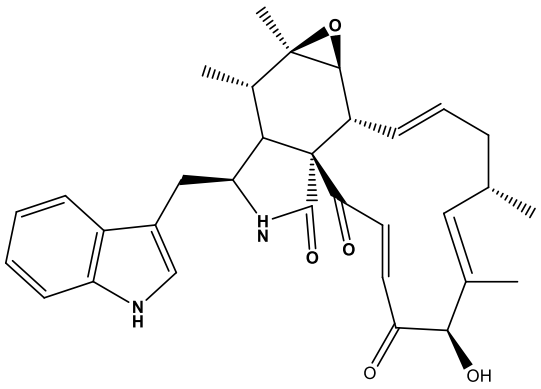
This fungal strain can produce the same antimicrobial metabolite in different plants e.g. chaetotglobosin A & C (cytochalasans) (Figure 1.2) were produced by the fungi *Chaetomium globosum* from two different unrelated host plants, namely *Ginkgo biloba* and *Nymphaea nouchali* (Qin *et al*., 2009; Dissanayake *et al*., 2016). Monocerin an isocoumarin derived compound (Figure 1.2) has been isolated from different fungal species and different plant species, this leads to the assumption that certain metabolites are not restricted to a fungal species or plant species, but rather produced as a response to biotic and abiotic stress factors. Another example is the antibacterial terpenoid, helvolic acid (Zhang *et al*., 2008; Zhao *et al*., 2010; Zhang *et al*., 2012; Pinheiro *et al*., 2017). It is here that the importance of endophyte isolation extends to isolation and identification of bioactive molecules. To isolate bioactive compounds, investigations often employ the use of preparatory high performance liquid chromatography (prep-HPLC), which allows for crude extracts to be fractionated according to the retention time of compounds. Liquid chromatography-mass spectrometry (LC-MS) provides the molecular mass, compound formula and fragmentation pattern of active compound, which is often used for identification. Furthermore, spectroscopy techniques like Nuclear Magnetic Resonance (NMR) is also used globally for identification of active compounds (de Felício *et al*., 2015; Perveen *et al*., 2017). There is the belief that endophytes pose promising alternatives to synthetic chemistry (Compant *et al.,* 2016).

Table 1.3: Chemical diversity of antimicrobial compounds isolated from endophytic fungi from the year 2015-2017.

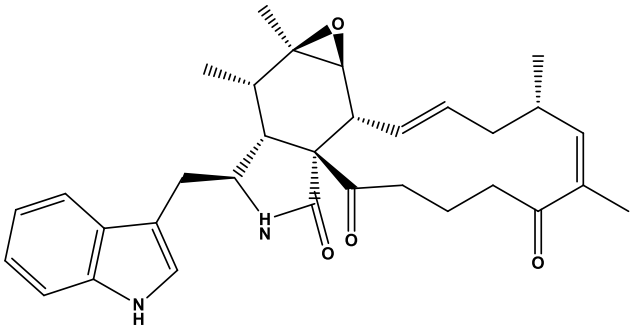
Endophytic fungi	**Host plant**	**Antimicrobial compound isolated**	**References**
Chaetomium globosum	*Nymphaea nouchali* Burm.f.	Chaetoglobosins A,C	Dissanayake *et al*., 2016
Cladosporium spp.	*Rauwolfia serpentina* (L.) Benth. ex Kurz.	Anhydrofusarubin, methyl ester of Fusarubin	Khan *et al*., 2016
Epicoccum nigrum	*Entada abyssinica* Steud.	Beauvericin, Indole-3-carboxylic acid	Dzoyem *et al*., 2017
Epicoccum nigrum	*Ferula sumbul* Hook.f.	2-methyl-3-nonyl Prodiginine, Di-(2-ethylhexyl) phthalate (DEHP), Preaustinoid A	Perveen *et al*., 2017
Eurotium chevalieri	Mangrove tree	Emodin	Zin *et al*., 2017
Exserohilum rostratum	*Bauhinia guianensis* Aubl.	Monocerin	Pinheiro *et al*., 2017
Fusarium spp.	Unknown plant	Colletorin B, Colletochlorin B, Llicicolin B, 4,5-Dihydroascochlorin, 4,5-Dihydrodechloroascochlorin	Hussain *et al*., 2015a
Fusarium chlamydosporium	*Anvillea garcinii* (Burm.f.) DC.	**Fusarithioamide A**	Ibrahim *et al*., 2016
Fusarium equseti	*Terminalia pallida* Brandis.	(E)-3- (2, 3- Dihydroxyphenyl) acrylic acid	Venkateswarulu *et al*., 2017
Leptosphaerulina spp.	Mangrove tree	Diaportheins B, **Leptosnaphthoic acid A, Leptospyranonaphthazarin A**	Cui *et al*., 2017
Microdiplodia spp.	*Quercus serrata* Thunb.	**3-epi-Phomadecalin D, 13-Hydroxylmacrophorin A**	Shiono *et al*., 2015

Endophytic fungi	Host plant	Antimicrobial compound isolated	References
Nemania serpens	*Vitis vinifera* L.	**Nemanifuranone A**	Ibrahim *et al*., 2017
Neopestalotiopsis spp.	unknown plant	**Neopestalotin B**	Zhao *et al*., 2015
Penicillium brocae	*Avicennia marina* (Forssk.) Vierh.	Pyranonigrin A, **F**	Meng *et al*., 2015
Pestalotiopsis spp.	*Enhalus acoroides* Rich. ex Chatin.	Aspergillumarins A, B	Arunpanichlert *et al*., 2015
Penicillium spp.	*Gastrodia elata* Blume.	**Austin, Dihydroxyneogrifolic acid, (S)-18,19-Dihydroxyneogrifolin, Preaustinoid A, Preaustinoid D,**	Duan *et al*., 2016
Phoma spp.	*Kleinia neriifolia* Haw.	Atrovenetinone, Sclerodine, Sclerodione	Hussain *et al*., 2015b
Scleroderma UFSMSc1	*Eucalyptus grandis* W.Hill.	**Sclerodols A and B**	Morandini *et al*., 2016

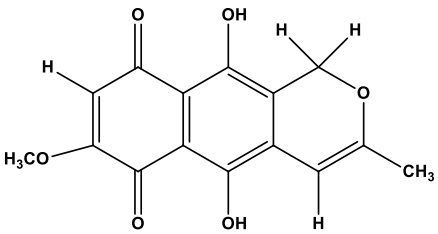
*compounds highlighted in bold represent novel compounds.



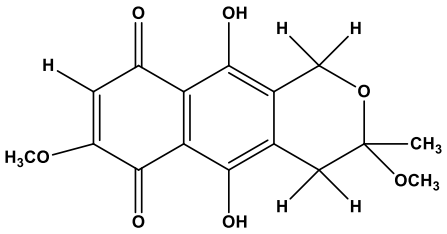
Chaetoglobosin A



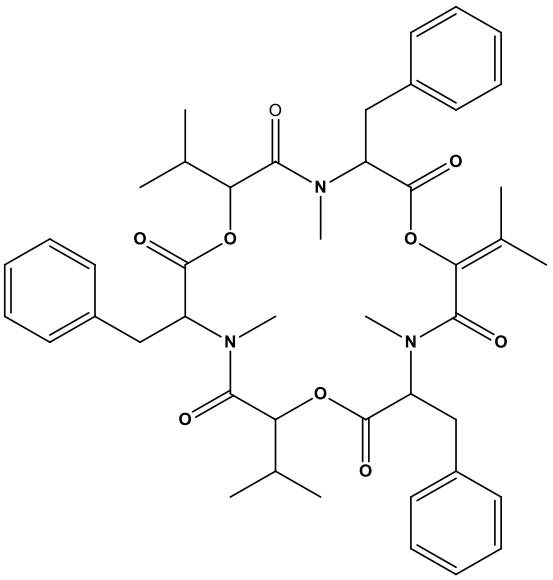
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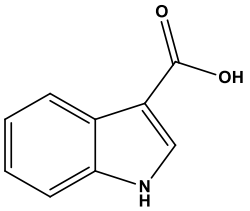
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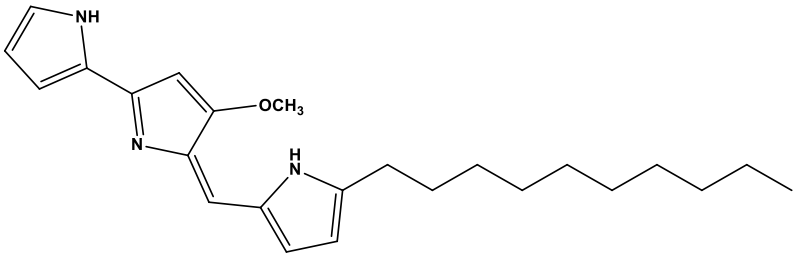
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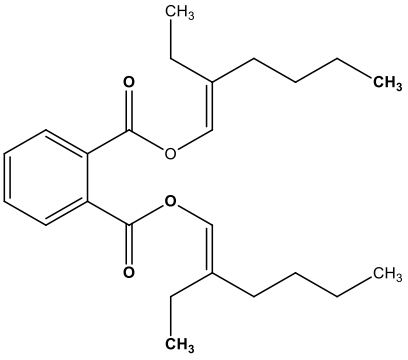
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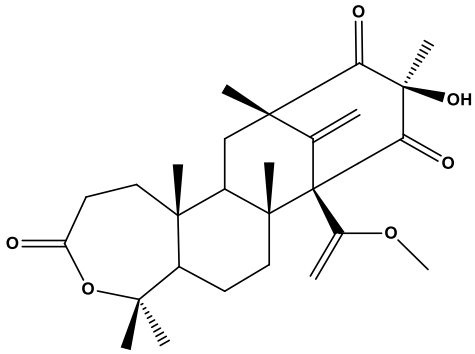
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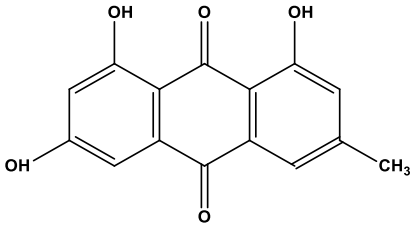
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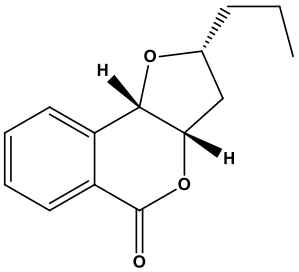
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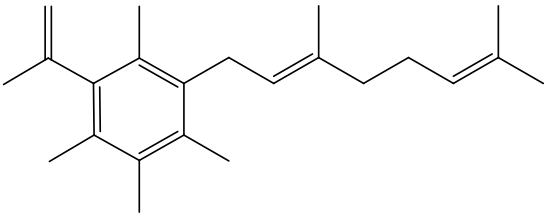
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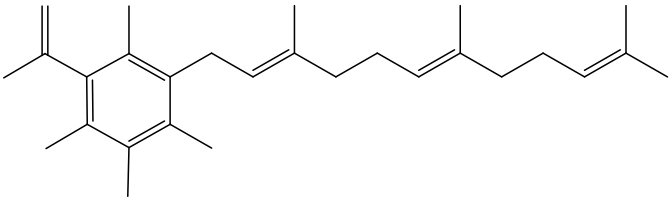
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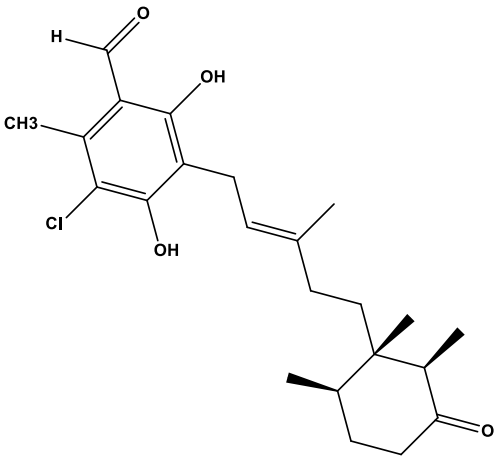
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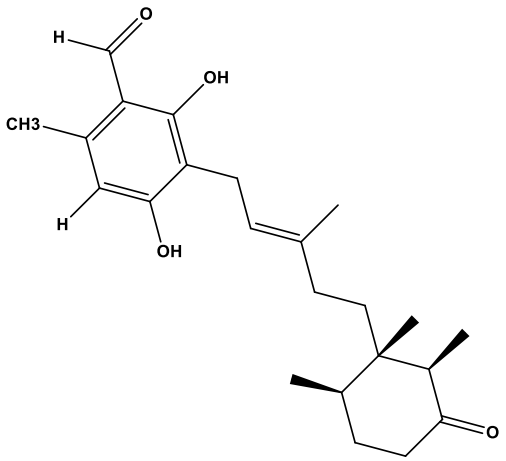
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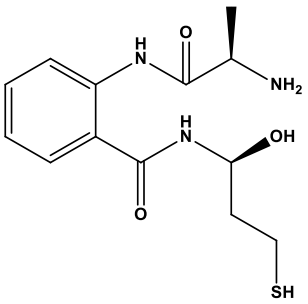
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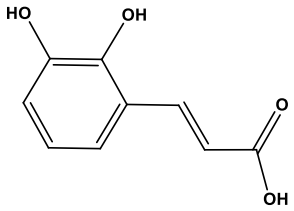
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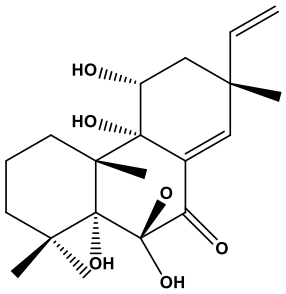
4, 5-Dihydrodechloroascochlorin



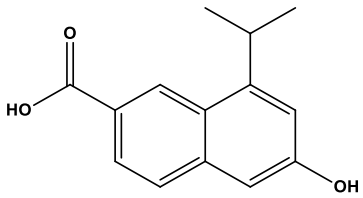
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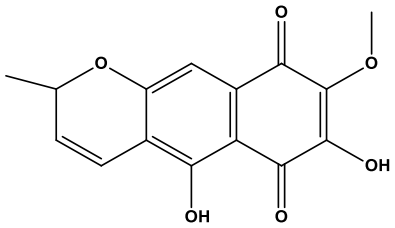
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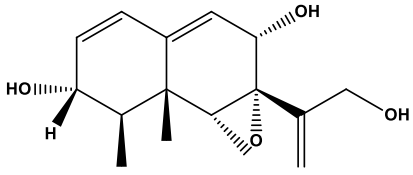
Diaportheins B



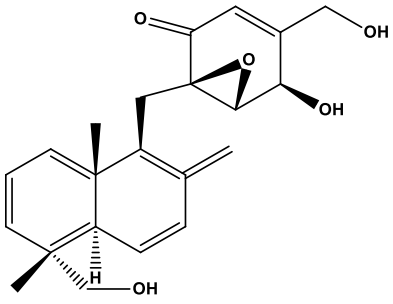
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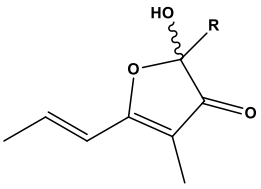
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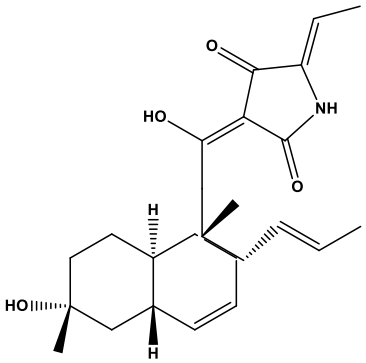
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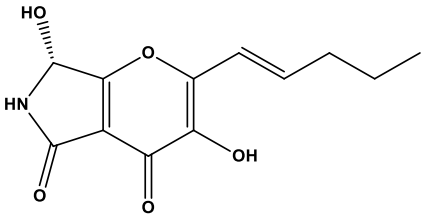
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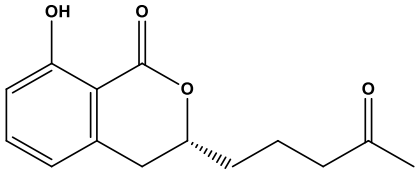
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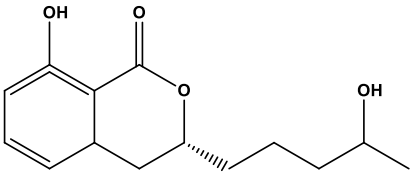
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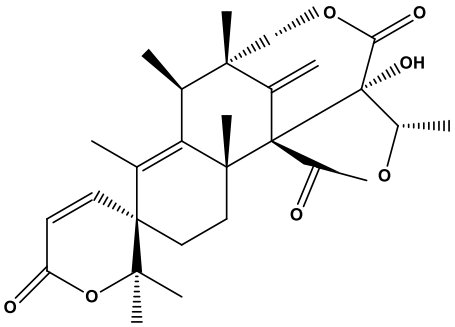
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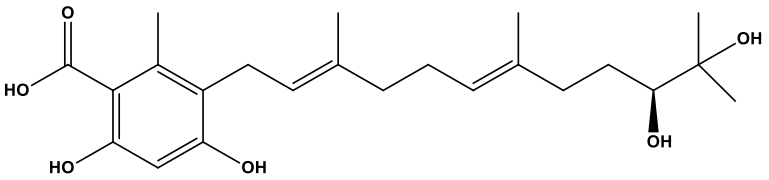
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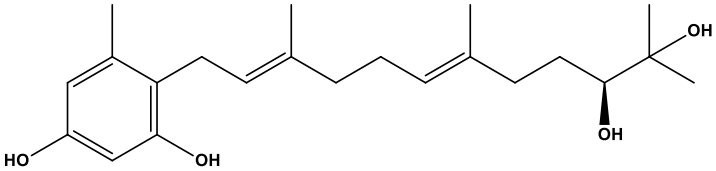
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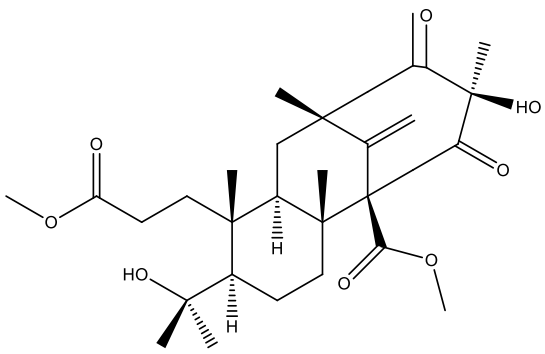
Austin



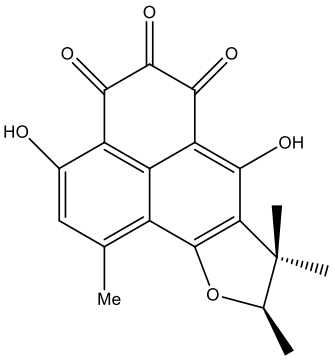
Dihydroxyneogrifolic acid



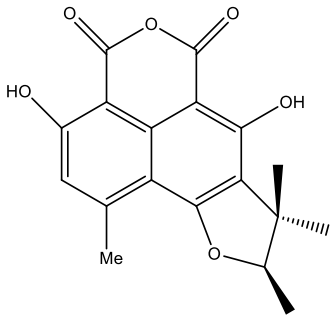
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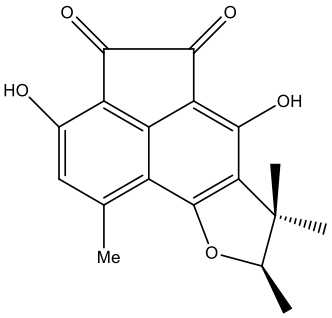
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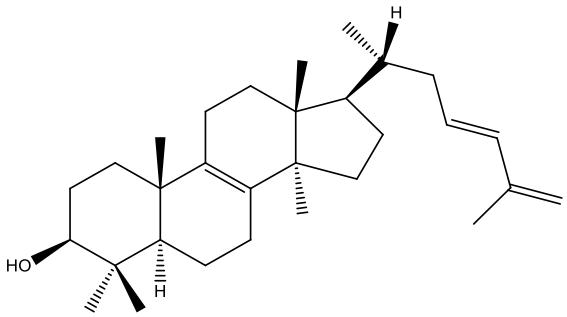
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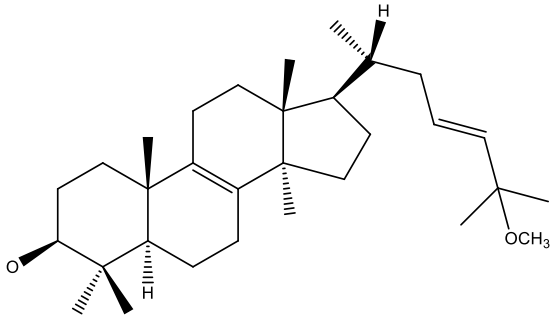
Sclerodin



Sclerodione



Sclerodol A



Sclerodol B

Figure 1.2: Chemical structures of antimicrobial compounds isolated from different endophytic fungi. (Refer to Table 1.3 for references for the compounds).

1.11. Identification of the diverse endophytic microbiome isolated from plants

A combination of factors such as environmental, genetic and phenotypic aspects influence endophytic microbiomes. These either work with the host plant or act separately and are dynamically influenced by plant-endophyte association (Compant *et al*., 2016). All of these factors will play a role for the emerging endophyte. Fungal endophyte communities vary between plant part, the season of harvest and geographical source (Giauque and Hawkes, 2016; Martins *et al*., 2016). Molecular phylogeny of seasonally collected endophytes can reveal a number of genera as observed by Wani *et al*. (2016). It is of much importance for investigators to include taxonomy and molecular phylogeny of fungi as their objectives during an antimicrobial study of fungal endophytes. The identification process begins with a region of deoxyribonucleic acid (DNA) internal transcriber spacer (ITS), which is amplified or copied by polymerase chain reaction (PCR) techniques. The amplified DNA sequence (nucleotide chain) is then studied using an automated sequencer to determine taxonomy and molecular phylogeny of fungal endophyte (Jeewon *et al*., 2013). As of 2014, there were a total of 8,439 DNA sequences from endophytic fungi retrieved from the National Centre for Biotechnology Information (NCBI) nucleotide data base. This data base has made it possible to categorise endophytic fungi into different divisions e.g. Ascomycota, Basidiomycota etc. (Figure 1.1) (Hardoim *et al*., 2015). It is imperative to grow the fungal nucleotide database so that researches can correctly characterise and identify unknown fungal species (Smith *et al*., 2008). The South African National Collection of Fungi Institute was established in 1905. A century later approximately 60 000 specimens of fungi were housed at the institute (Rong and Baxter, 2006). Professor Pedro Crous is one of the few researchers that have made a contribution to taxonomy and phylogeny in South Africa (Victor *et al*., 2013). Crous *et al*. (1995), identified fungal endophytes in the plant *Triticum aestivum* for the purpose of documenting their occurrence in the plant, Crous *et al*. (2006), stated that South Africa has approximately 171 000 fungal species associated with plants; however, only 780 new fungal species were documented. Furthermore, Victor *et al*. (2013) pointed out that South Africa has a major gap in the documenting and identification of South African fungi. Studies which focus on isolation and identification of fungal endophytes with antimicrobial properties will be of great value therapeutically and to the South African National Collection of fungi.

1.12. Aim and objectives of the study

The aim of this study was to establish the antimicrobial activity of endophytes within three South African medicinal plant roots species.

Objectives

- Sourcing of medicinal plants and isolation of endophytic fungi from the roots.
- Fermentation and ethyl acetate extraction of endophytic fungal cultures.
- Residue preparation of plant root material using organic and aqueous solvents for extraction of plants.
- Determination of the antimicrobial activity of fungal extracts and plant extracts independently using the MIC assay.
- Determination of the antimicrobial activity of fungal extracts and plant extracts in combination using the MIC assay.
- Isolation and identification of secondary metabolites using Preparatory high performance liquid chromatography-mass spectrometry (Prep HPLC-MS), Ultra performance liquid chromatography-mass spectrometry (UPLC-MS) and Nuclear magnetic resonance (NMR) techniques.
- Identification of fungal strains that show antimicrobial activity using PCR amplification and DNA sequence analysis.

1.13. Summary of study design

An overview of the work plan is provided in Figure 1.3.

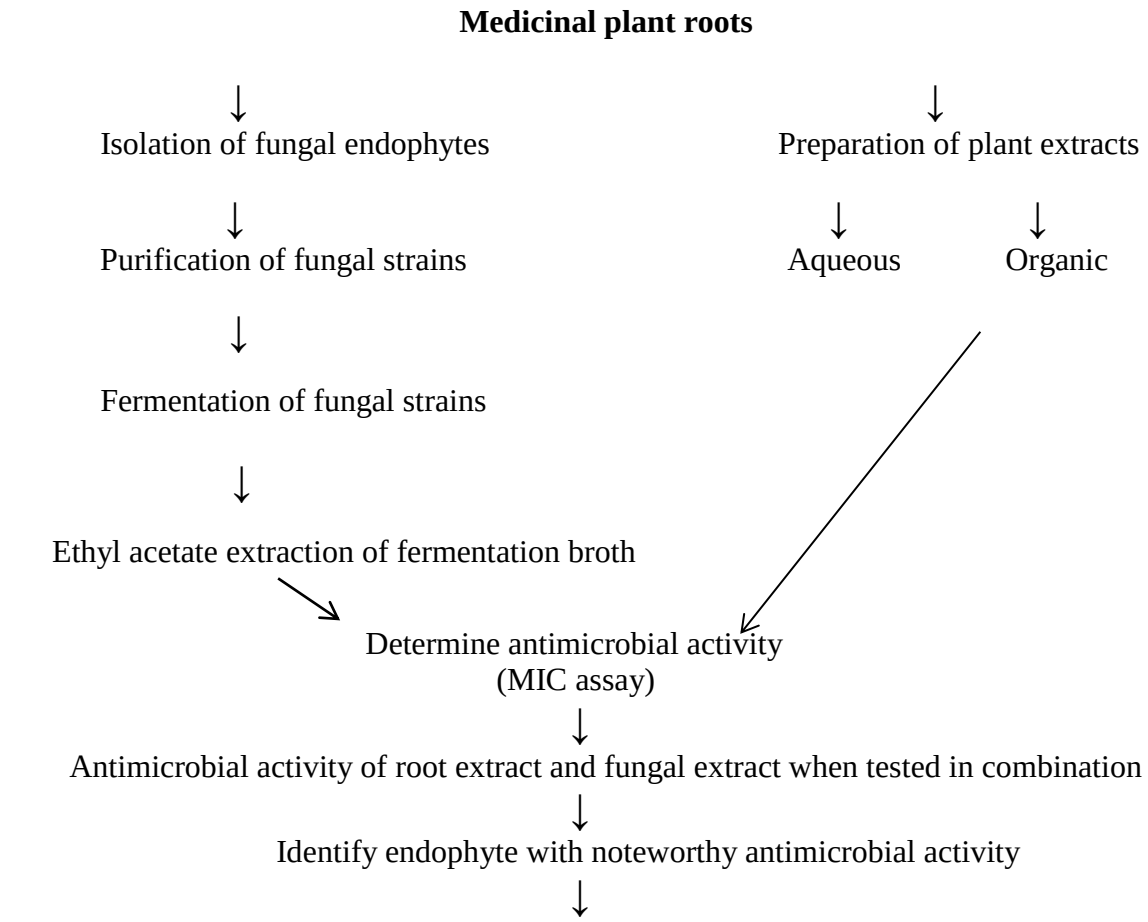


Figure 1.3: Schematic representation showing a summary of the study design

Chapter 2

Materials and Methods

2.1. Sourcing of medicinal plants

The plants selected for this study were undertaken based on their popularity as medicinal plant roots and corms used in South Africa. Other criteria which narrowed down the selection for this study were plants that show weak antimicrobial activity or no antimicrobial activity during MIC testing of the plant extracts. The hypothesis was that during conventional MIC screening as indicated in well cited paper by Eloff (1998), the endophyte viability is compromised and because these may be the source of potential activity, it might explain the poor *in vitro* efficacy encountered in conventional MIC screening assays. *Gunnera perpensa* (voucher number: KSK 0017) and *Hypoxis hemerocallidea* (voucher number KSKP 0041) were collected from the Maseru District of Lesotho under the permission of the respective curators. Plant identification for the latter was confirmed by Mr Khotso Kobisi (botanist) and Mr Moretloa Polaki (Herbarium Curator). The rootstock of *P. sidoides* (Voucher number SVVAV 244) was collected from the Eastern Cape during the months of January and June (the year 2016) and identified and donated by Mr Ulrich Feiter (Parceval Pty Ltd) (Table 2.1).

Table 2.1: South African medicinal plant roots used for the study of endophytes.

Plant name	Family	Author	Voucher number	Locality
Pelargonium sidoides	Geraniaceae	De Candolle, Augustin Pyramus	SVVAV 244	Eastern Cape
Hypoxis hemerocallidea	Hypoxidaceae	Fisch., C.A.Mey. & Avé-Lall	KSKP 0041	NUL Botanical garden, Roma
Gunnera perpensa	Gunneraceae	Linné, Carl von	KSK 0017	Molimo Nthuse, on the way to Likhahlaneng

2.2. Method development

2.2.1. Surface sterilization of roots and isolation of endophytic fungi

A pilot study was conducted to assist in method development. *Pelargonium sidoides* root was used during the pilot study. The root of the plant was sterilized aseptically (Ramos *et al.,* 2010). Tryptone Soya agar (TSA) (Oxoid) plates were placed in the left and right corner of the laminar flow unit (Esco) during the sterilization procedure. The TSA (Oxoid) plates were incubated for 24 hrs at 37 ºC. This control was to ensure that the environment was sterile. Greenfield *et al*. (2015) in a study conducted on surface sterilization of plants in relation to fungal endophyte isolation, during the year 2014, revealed the surface sterilization process per plant sample usually ranges between 4−6 mins (Higgins *et al*., 2014; Jaber and Salem, 2014; Terhonen *et al.,* 2014). Sterilization of roots was carried out by testing two potential sterilization protocols. The plant part was rinsed under running tap water for 5 min, to remove soil. The first surface sterilization procedure was adopted from Fisher *et al.* (1992). The root was sequentially immersed in 50 mL of 75% (v/v) ethanol (ACE) for 2 min, 3.5% sodium hypochlorite (Jik Industries Ltd.) for 3 min, then another 1 min in 75% (v/v) ethanol. The second alternate procedure involved immersion of the plant part in 70% (v/v) ethanol for one min and then transferred to a bottle of 3.5% sodium hypochlorite (Jik industries Ltd.) for five min (Liang *et al.*, 2012). Both procedures required roots to be immersed in 60 mL of sterile distilled water for 1 min and this process was repeated three times. Water should always cover the entire plant part. The root was air dried for +/- 10 minutes in a sterile environment. The roots were then cut aseptically into 0.50 x 0.50 cm^2 segments (Liang *et al*., 2012). These segments were plated on Potato Dextrose agar (PDA) (Oxoid) supplemented with an antibiotic to inhibit antimicrobial contamination (Figure 2.1). Potato dextrose agar (PDA) (Oxoid) was supplemented with different antimicrobials to determine the appropriate antimicrobial to inhibit endophytic bacterial growth from plant root (Table 2.2).

Table 2.2: Antibiotics used for supplementation of Potato dextrose agar in different investigations.

Antibiotics	**References**
Chloromycetin (100 mg/L)	Sun *et al*., 2013
Streptomycin (30 μg/mL)	Astuti *et al*., 2014
Chlortetracycline10 mg , Penicillin G 100 mg and 50 mg Streptomycin-sulphate per liter	Athman, 2006
Penicillin G (30 mg/L) and Streptomycin sulfate (30 mg/L)	Ramos *et al*., 2010

The first batch of agar was made with ciprofloxacin (Sigma-Aldrich) (30 mg/L), another batch was made with penicillin G (30 mg/L) and the last batch was made with a combination of penicillin G (Sigma-Aldrich) and streptomycin sulphate (30 mg/L) (Sigma-Aldrich). *Staphylococcus epidermidis* (ATCC 12228) were streaked onto antibiotic infused agar to ensure that each antibiotic was active. This control ensured that all antibiotic infused agar plates were appropriate. These plates were incubated for 37 ºC for 24 hrs. After the sterilization procedure, roots were placed on agar infused antibiotic plates. The plates were then incubated at room temperature in a biohazard fume hood (BSC No. II) (Labotec) for 10 days. A control of the water washings from the surface sterilized samples were streaked onto antibiotic-free PDA (Oxoid) and incubated under the same conditions for 10 days (Ramos *et al*., 2010). This control was conducted to ensure no epiphytic micro-organisms remain on root after the sterilization process. The samples exhibited emerging fungi four days after placed onto agar. Emergent fungi were isolated and inoculated onto fresh PDA (Oxoid) antibiotic-free medium and incubated at room temperature for seven days (Ramos *et al*., 2010). Roots had endophytic bacteria pooled at the bottom of root on agar plates that were infused with penicillin G (30 mg/mL) alone, however, both ciprofloxacin and the combination of penicillin G and streptomycin sulphate plates inhibited the growth of endophytic bacteria, allowing for the successful isolation of fungal endophytes.

Isolation of fungi

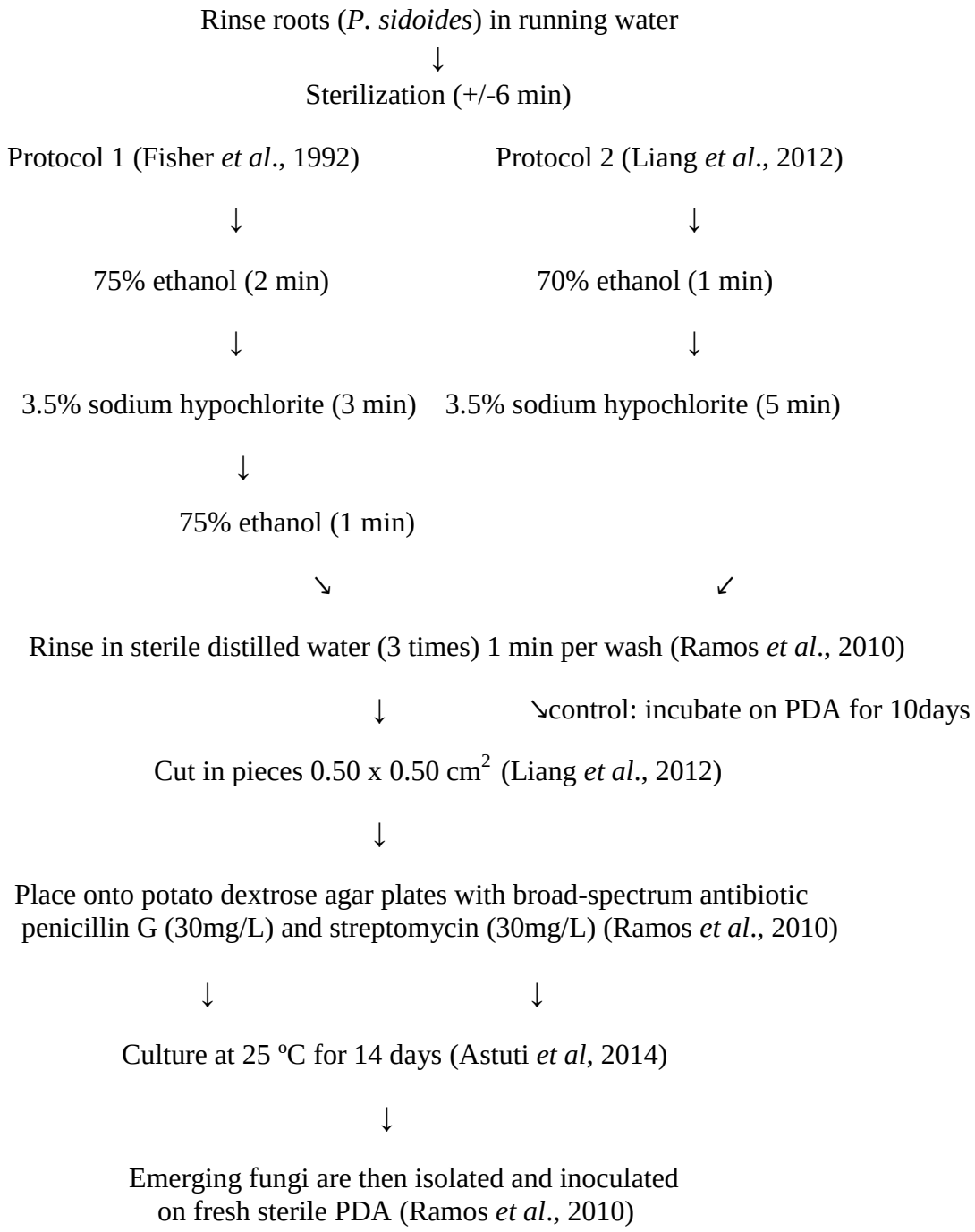


Figure 2.1: Sterilization of plant root and isolation of endophytic fungi.

The fungi strains were purified by repeated streaking of fungi on antibiotic-free PDA until fungal strains were pure. A mucoid, smooth opaque substance was observed after seven days on the control PDA plate that was streaked with distilled water from water washings of root during sterilization protocol (protocol one); however, there was no presence of mould. This

mucoid substance was streaked onto two plates, the first agar plate was infused with penicillin G (Sigma-Aldrich) and streptomycin sulphate (30mg/L) (Sigma-Aldrich) and another PDA plate received a nystatin disc. The mucoid substance did not grow on the antibiotic infused agar plate. Furthermore, the nystatin disc did not produce a zone of inhibition, this confirmed that mucoid substance is of bacterial nature and not yeast in origin. The presence of bacteria confirms that sterilization procedure was not adequate for epiphytic bacteria but was adequate for sterilization of epiphytic fungi. Samples that displayed the presence of epiphytic bacteria in control plates were discarded. Endophytic fungi that originated from the root were transferred with a sterile loop onto new anti-biotic free agar (PDA) (Oxoid). Fungi were sub-cultured by transferring strains onto agar repeatedly until strains were separated from each other and purified.

2.2.2. Fermentation process and secondary metabolite production

Each isolate was inoculated in 200 mL of Sabouraud Dextrose broth (SDB) (Oxoid) at room temperature. This procedure was carried out under a biohazard fume hood (BSC No. II) (Labotec) for reasons of personal safety and to prevent contamination of broth. After seven days of fermentation 5 mLs of fermented broth was removed and transferred to a sterile test tube and this process was repeated for each fungal strain. Fermentation liquid was streaked onto a PDA (Oxoid) plate to determine the purity of the strain during the fermentation process. The fermentation process was undertaken for a period of 14 days in a shaking incubator (Labcon) (Astuti *et al.* 2014) (Figure 2.2). The fermentation process took place at 160 rpm and 25 ºC +/- 2 ºC. In this initial fermentation process residue production varied from one fungi strain to the next with the largest residue production of 100 mg from 200 mL fermentation broth and the least yielded 29 mg, which was the smallest yield of residue from the fermentation broth.



Figure 2.2: Fermentation broth after a period of 14 days

2.2.3. Ethyl acetate extraction of fermentation broth and mycelia

Once a significant amount of fungi had grown, the mycelia were separated from culture broth with sterile Whatman® filter paper. The instruments used for the extraction procedure were sterilized. The filtrate and ethyl acetate was mixed for 10 min in a shaking incubator at 240 rpm and then left to stand for a further five min until the two clear immiscible layers form (Figure 2.3). The upper layer of ethyl acetate containing the extracted secondary metabolites was separated using a separating funnel (Bhardwaj *et al*., 2015). The filtrate was further extracted with equal amounts of ethyl acetate three times, and the residue was produced by means of evaporation of ethyl acetate. The fungal balls were removed off the filter paper with a sterile spatula and immersed in ethyl acetate (Associated Chemical Enterprises) (Raju and Victoria, 2015). This process with fungal balls immersion in ethyl acetate was repeated at 48 hrs and then seven days to determine which duration delivers a higher yield of the residue (Desale and Bodhankar, 2013). After immersion in ethyl acetate, fungal balls were filtered again to separate fungi from ethyl acetate (ACE). Immersion of fungal balls in ethyl acetate for 4 hrs did not yield any residue; however, duration of seven days was more successful. Ethyl acetate (ACE) was allowed to air dry. The residue was mixed with dimethyl sulphoxide (Sigma-Aldrich) and stored at 4 ºC before antimicrobial testing (Bhardwaj *et al*., 2015) (Figure 2.4).

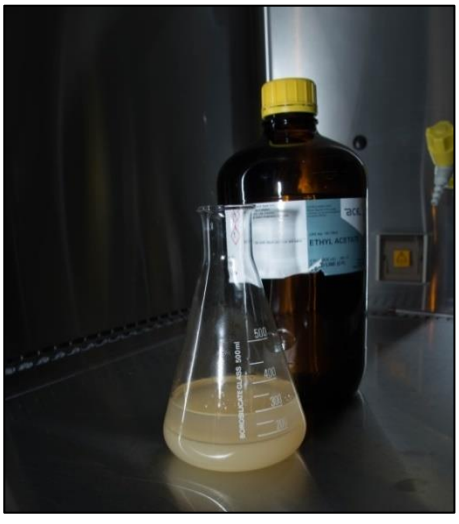


Figure 2.3: Extraction process - flask displays two clear immiscible layers due to different polarities of solvent and filtrate mixed.

Fermentation and ethyl acetate extraction of endophytic culture broth and mycelia

Remove hyphal tip of emerging fungi (Powthong *et al*., 2012)

↓

Place onto sterile PDA incubate at 26 ºC for 4−14 days and check (Casella *et al*., 2013)

↓

Pure culture

↓

Strains are separately inoculated in 200 mL SDB in Erlenmeyer Flasks at 160 rpm at 25 °C (Astuti *et al*., 2014)

↓

Mycelia are separated from culture broth with Whatman® filter paper

↓

Mycelia are immersed in ethyl acetate

↓ ↓

4 hrs (Raju and Victoria., 2015) 7 days (Desale and Bodhankar, 2013)

↓

Upper layer of ethyl acetate containing the extracted compounds is separated is from fungal balls by using Whatman® filter paper

↓

The extract was concentrated by removing the solvents under reduced pressure at 35−40 °C with a rotary evaporation then weighed (Bhardwaj *et al*., 2015)

↓

Residue is mixed with Dimethyl sulphoxide (DMSO) and stored at 4 °C before antimicrobial testing (Bhardwaj *et al*., 2015)

Figure 2.4: Schematic representation of fermentation process of endophytic fungi and extraction of secondary metabolites from mycelia (pilot study).

2.2.4. Antimicrobial testing using disc diffusion assay (pilot study)

The antimicrobial activities of the fermented fungal cultures were initially tested by qualitative biological analysis in triplicate, using the disc diffusion (DD) technique. The micro-organisms selected comprised of the pathogenic strains Gram-negative *Escherichia*

coli (ATCC 8739) and Gram-positive *Staphylococcus aureus* (ATCC 25923). These use of micro-organisms required an ethics waiver from the Human Research Ethics Committee (Medical) which was attained (Appendix D). The bacterial cultures were grown in Tryptone Soya broth (TSB) (Oxoid) for 24 hrs at 37 °C, and adjusted to an appropriate inoculum concentration of approximately 1 $x10^6$ colony forming units per millilitre (CFU/mL). An amount of 0.10 mL was spread onto sterile PDA (Oxoid). Discs of sterile filter paper Whatman® no. 4 (6 mm) inoculated with 10.00 µL of fermented fungal culture from seven different fungal strains were placed on the agar. The plates were incubated at 37 °C for 24 hrs. This method yielded no significant results as there was no zone of inhibition around the disc. The concentrated residues yielded from the fermentation broth were not used. Instead the actual fermentation broth was used, therefore this was not the preferred method for antimicrobial testing. This method did not support the combination studies of plant extract and fungal extract against bacteria, and therefore the 96 well micro-titre plates were used to determine MIC. The latter proved to be more accurate and efficient (Garcia *et al*., 2012).

2.3. Revised/refined protocol methodology

2.3.1. Sterilization of plant root and isolation of endophytic fungi

Sterilization protocol two was successful and therefore was used for subsequent sterilization of roots, with minor modifications made to the protocol. The Root was submerged in ethanol 70% (v/v) for 30 seconds after it was submerged in sodium hypochlorite. This protocol was chosen as the preferred method of sterilization as it resulted in the adequate sterilization of epiphytic micro-organisms. A control of the water washings from the surface sterilized samples were streaked onto antibiotic-free PDA (Oxoid) and incubated under the same conditions. The outside of sterilized root was also streaked onto PDA (Oxoid) with and without antibiotic. This was done in addition to water washings being streaked onto PDA (Oxoid) as a control (Figure 2.5). This step was undertaken under the laminar flow cabinet (Esco), before the root was sliced into pieces. Both these controls are put in place to ensure no epiphytic micro-organisms remain on root after the sterilization process. Emerging endophytes from roots of *P. sidoides* (a), *H. hemerocallidea* (b), and *G. perpensa* (c) were isolated and streaked onto fresh PDA (Figure 2.6).

Isolation of fungi from plant part

Rinse roots (*P. sidoides, G. perpensa, H. hemerocallidea*) in running water

↓

Sterilization (+/- 6 mins)

(Liang *et al*., 2012)

↓

70% ethanol (1 min)

↓

3 .5% sodium hypochlorite (5 min)

↓

70% ethanol (30 sec)

↓

Rinse in sterile distilled water (3 times): 1 min per wash (Ramos *et al*., 2010)

↓ ↘control: incubate on PDA for 10 days

Cut in pieces 0.5 x 0.5 cm^2 (Liang *et al*., 2012)

↓

Place onto Potato Dextrose agar plates with broad-spectrum antibiotic penicillin G (30 mg/L) and streptomycin (30 mg/L) (Ramos *et al*., 2010)

↓

Culture at 25 °C for 14-21 days (Astuti *et al*., 2014)

↓

Emerging fungi are then isolated and inoculated on fresh sterile PDA

Figure 2.5: Schematic representation of final sterilization protocol.

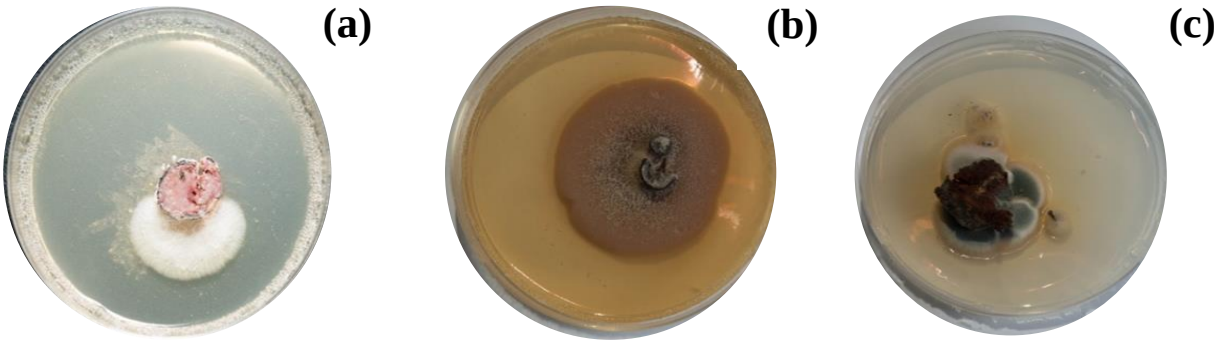


Figure 2.6: Endophytes emerging from the roots of *P. sidoides* (a), *H. hemerocallidea* (b), and *G. perpensa* root (c).

2.3.2. Fermentation of endophytic fungal strains and ethyl acetate extraction of broth

The fungal strains of roots were inoculated into modified MEB. This broth was modified to increase secondary metabolite production. The broth was transferred to a 200 mL Erlenmeyer flasks under a laminar flow unit to ensure that media remains sterile before inoculation. Different strains of endophytic fungi were separately inoculated in 200 mL of MEB at 25 ºC and 160 rpm for 14 days (Astuti *et al*., 2014). Strains that were placed in a stationary incubator (Labcon) were fermented for a period of 21 days (Powthong *et al*., 2012; Gulhane *et al*., 2016). Inoculation was done in a sterile manner by using a sterile pipette to cut out five discs (6 mm) of each strain (de Siqueira *et al*., 2011). This ensures that the fermentation procedure of endophytic fungi is standardised. To ensure that enough residues were collected after the ethyl acetate extraction procedure, each strain was grown in 2 x 200 mL broth. This allowed for an increase in residue production. During the initial pilot study, each strain was grown in 200 mL broth and this gave yield to as little as 30 mg of the residue which was considered to be inadequate for MIC Studies. Malt extract broth was the basal broth and additional energy sources were added to the broth to optimise conditions for growth of fungi during the fermentation phase. Sucrose (10 g/L) (ACE) was added as a carbon source, and yeast extract (Oxoid) added as an additional nitrogen source (5 g/L) (Mathan *et al*., 2013). Minerals added to broth included magnesium sulphate dihydrogen (ACE), potassium chloride (Saarchem), calcium chloride (Rochelle chemicals). The latter was added at a concentration of (0.5 g/L) (Agastian *et al*., 2013). Sodium chloride is also essential to optimise conditions, therefore 1.5 g/L was added to the basal broth (Kumar *et al*., 2016). To optimise growth of

fungi the pH of broth was taken after addition of minerals to basal broth. The pH was taken before broth was autoclaved to ensure that broth remains sterile before inoculation with fungi. The pH of broth was increased to six using 1 mol/L of sodium hydroxide (Sigma-Aldrich) (de Siqueira *et al*., 2011). The fungal ball extracts showed no significant antimicrobial activity alone or in combination with plant extracts during the pilot study, therefore fungal ball extracts were not prepared for antimicrobial testing of subsequent roots after the pilot study. After ethyl acetate (ACE) extraction of fermentation broth by separating funnel, the ethyl acetate was allowed to air dry (Figure 2.7). This was done to prevent loss of extract due to transfer between apparatus. Endophyte extract (residue) was produced as a result of this process and used for subsequent antimicrobial testing (Figure 2.10).

Fermentation and Ethyl acetate extraction of endophytic culture broth

Remove hyphal tip of emerging fungi (Powthong *et al*., 2012)

↓

Place onto sterile PDA incubate at 25 ºC for 3 to 14 days and check

↓

Pure culture

↓

Strains are separately inoculated in 200 mL MEB in Erlenmeyer Flasks at 25 ºC

↓ ↓

160 rpm 14 days (Astuti *et al*., 2014) Stationary conditions 21 days (Bhardwaj *et al*., 2015)

↓

Mycelia are separated from culture broth with Whatman® filter paper

↓

Broth is extracted thrice with equal volume of ethyl acetate (Astuti *et al*., 2014)

↓

The filtered filtrate was then air dried (Raju and Victoria., 2015)

↓

Extract was dissolved in DMSO before antimicrobial testing and stored at 4 ºC (Bhardwaj *et al*., 2015)

Figure 2.7: Schematic representation of final protocol of ethyl acetate extraction.

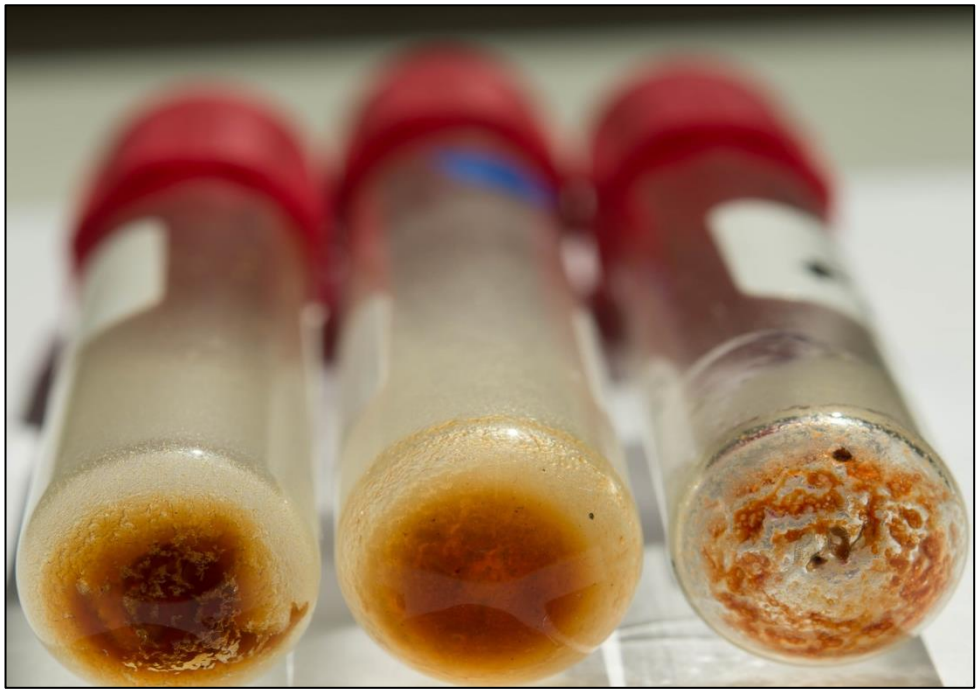


Figure 2.8: Endophyte residue produced after ethyl acetate evaporation.

2.4. Potential hydrogen (pH) of fungal broth

The pH is a significant factor when optimizing conditions for the growth of secondary metabolites during the fermentation phase. Research indicates that fungi show promising production of antimicrobial secondary metabolites between pH 5.5-8.5 (Thongwai and Kunopakarn, 2007). A pH of 7 is considered neutral; however, there are certain strains of fungi that require a slightly acidic medium and are considered acidophilic isolates (Mathan *et al*., 2013). Every batch of broth was corrected to pH 6 to ensure conditions were standardised. The broth was autoclaved after pH was corrected therefore ensuring sterility.

2.5. Preparation of plant extract

2.5.1. Organic extract preparation

Extractions of plant material require extraction of the majority of the soluble metabolites from plants, leaving behind mostly insoluble structural components of the plant e.g. cellulose. Solvents that extract polar and non-polar compounds are utilized during the extraction process to ensure that this extraction process is successful in the production of residue for phytochemical investigation (Liu, 2008). Organic extracts were prepared for all selected

plants. Plant material was allowed to air-dry at room temperature. A high speed Fritsch Pulverisette grinder (Labotec) was then used to grind plant material into a fine powder. The ground plant material (approximately 20 g per 250 mL) was submerged in a solvent system consisting of methanol and dichloromethane, which were combined in equal ratios (1:1). Methanol ensured the extraction of non-polar compounds, whereas dichloromethane ensured the extraction of polar compounds within the plant material. The ground plant material, submerged in the solvent system was left for 24 hrs at 37 ºC, in a platform shaker/incubator (Labcon), to allow for the extraction. After evaporation, the organic extracts (yield: 3.18%) were stored at room temperature until further analysis (Hübsch *et al*., 2014a).

2.5.2. Aqueous extract preparation

The aqueous extracts were prepared by immersion of macerated plant material in sterile distilled water, which was rotated in a platform shaker/incubator for 24 hrs at 25 ºC. The liquid was then filtered using Whatman® No. 1 filter paper and the filtrate stored at -80 ºC before lyophilisation (Virtis). Lyophilised aqueous extracts were left overnight under ultra-violet light to ensure the eradication of microbial contamination. The aqueous extracts (yield: 7.84%) were then stored in sterile, sealed plastic containers at 25 ºC and kept away from light until further investigation (Hübsch *et al*., 2014a).

2.6. Antimicrobial testing using the micro-titre dilution assay

The minimum inhibitory concentration (MIC) of all the fungal extracts and plant extracts were determined by using the micro-titre broth dilution technique (Eloff, 1998). A sterile 96 micro-titre plate was prepared by adding 100 µL of sterile TSB in each of the wells of the plate under aseptic technique. The crude extracts were added to the first row of the micro-titre plate at a starting concentration of 16 mg/mL and a volume of 100 µL. A 100 µL volume of each positive, negative and culture control was included for each micro-organism. The positive control (ciprofloxacin 0.01 mg/mL) was included to ensure microbial susceptibility. The negative control DMSO was included to determine if the solvent exhibits any antimicrobial effect. Residues were dissolved in concentrations of DMSO (Sigma-Aldrich) ranging from approximately 10% –25% depending on the solubility of the extract. Several studies advocate the use of DMSO for solubility of fungal extracts (Fernandes *et al*., 2009; Astuti *et al*., 2014; Bhardwaj *et al*., 2015). Controls for different strengths of DMSO were

carried out in the antimicrobial studies to determine the effect of the solvent on antimicrobial assay results. The culture control TSB (Oxoid) for bacteria, was included to monitor the viability of the micro-organism. The plant and fungal extracts were tested in duplicate. The micro-organisms for testing were diluted using the required medium to achieve an approximate concentration of 1 x 10^6 (CFU)/mL. These solutions were then added to all the wells at a volume of 100 µL. The solutions were also streaked onto a TSA plate to ensure that culture is pure. The micro-organisms used to test the efficacy of plant and fungal extracts ranged from Gram-positive to Gram-negative strains, namely, *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 8739), *Enterococcus faecalis* (ATCC 1883) and *Pseudomonas aeruginosa* (ATCC 27853). Each micro-titre plate was sealed with a sterile adhesive sealing film (AEC Amersham) to prevent loss of the crude extract due to evaporation when incubated. The micro-titre plates were incubated at 37 ºC for 24 hrs for bacteria. A volume of 40 µL *p*-iodonitrotetrazolium violet solution (INT) (Sigma-Aldrich) at a concentration of 0.04 mg/mL was then added to each well of the micro-titre plate. Viable micro-organisms interacted with the INT solution to produce a colour change from clear to a pink-purple. The MIC value of the extract was taken as the lowest concentration that shows no microbial growth (pink-purple colouration). Different test pathogens react differently to INT, therefore results could be read between 2-4 hrs after adding INT (results are read in relation to reference culture control).

2.7. Trouble shooting with mycelial extracts from *P. sidoides*.

Endophytic isolates (PS13), (PS15), and (PS17) from *Pelargonium sidoides* was used as a pilot study. These isolates were used to produce the crude extract from mycelia, which was separated from the fermentation broth and prepared in accordance with Figure 2.4. This was done to determine if these mycelial extracts displayed stronger activity then extracts prepared from the extraction of fermentation broth

The MIC results for PS13, PS15, and PS17 mycelial extracts and broth extracts were tested against test pathogens and most extracts displayed results ≥8.00 mg/mL, with the exception of PS17 broth extract which inhibited *S. aureus* and *E. faecalis* at 4.00 mg/mL, which is a MIC value lower than mycelial extract for the same isolate (Table 2.3). Therefore, mycelial

extracts for the remainder of isolates were not prepared. All test extracts were prepared from ethyl acetate extraction of fermentation broth.

Table 2.3: Comparison of biological activities (MIC mg/mL) of ethyl acetate broth extract and mycelial extracts from three endophytic isolates from *P. sidoides*.

Endophyte extract	Average MIC (mg/mL)	Test pathogen
PS13 (BE)	>8.00	*S. aureus*
PS13 (ME)	>8.00	
PS15 (BE)	>8.00	
PS15 (ME)	>8.00	
PS17 (BE)	4.00	
PS17 (ME)	>8.00	
PS13 (BE)	>8.00	*E. faecalis*
PS13 (ME)	>8.00	
PS15 (BE)	>8.00	
PS15 (ME)	>8.00	
PS17 (BE)	4.00	
PS17 (ME)	>8.00	
PS13 (BE)	>800	*P. aeruginosa*
PS13 (ME)	>8.00	
PS15 (BE)	>8.00	
PS15 (ME)	>8.00	
PS17 (BE)	>8.00	
PS17 (ME)	>8.00	
PS13 (BE)	>8.00	*E. coli*
PS13 (ME)	>8.00	
PS15 (BE)	>8.00	
PS15 (ME)	>8.00	
PS17 (BE)	>8.00	
PS17 (ME)	>8.00	

BE= broth extract, ME= mycelial extract

2.8. Optimization of growth conditions with the use of different media using three isolates from *Hypoxis hemerocallidea.*

Three different isolates from *H. hemerocallidea* (H2, H3 and H5) were grown in Malt Extract broth (MEB) and MIC results (Table 2.4) were compared to results from isolates grown in Sabouraud Dextrose broth (SDB). The method followed for the production of extracts was in

accordance with Figure 2.11 and fermentation was carried out at shaking conditions (160 rmp) and 14 days. This was carried out to determine if the different media resulted in a difference in inhibitory activity against the test pathogens.

The endophyte strain H3 grown in MEB inhibited *S. aureus* at 1.00 mg/mL while H3 grown in SDB inhibited *S. aureus* at 4.00 mg/mL. Similar results were noticed for endophyte H5 (MEB), which displayed stronger activity at 2.00 mg/mL against *E. faecalis* as opposed to H5 (SDB) which inhibited *E. faecalis* at 4.00 mg/mL. The endophyte strain H2 in (SDB) is the only endophyte that displayed antimicrobial activity against *E. coli* at 2.00 mg/mL, and the same endophyte displayed poorer activity when inhibited *E. coli* at 4.00 mg/mL. In summary, MEB demonstrated beneficial effect on the antimicrobial results presented with H3 against *S. aureus* and H5 against *E. faecalis*. The other results display that extracts produced in both MEB and SDB have displayed the same MIC values, the difference is negligible, and therefore neither media broth choice makes a noteworthy difference in antimicrobial activity. Furthermore, growing the isolates in different media did not potentiate the production of secondary metabolites that yielded noteworthy MIC results. However, there are many variables additionally to media that can be used to enhance growth and production of secondary metabolites e.g. temperature, stationary or shaking conditions, humidity etc. (Merlin *et al*., 2013; Kumar *et al*., 2016; Shen *et al*., 2016). These conditions are considered once an antimicrobial compound is isolated and the latter is used to determine the correct variable manipulation for increased production of the specific compound of interest. Stationary and shaking conditions were considered and tested during the production of extract for endophyte PS8 to determine which condition supported the production of antimicrobial compounds.

Table 2.4: A comparison of the effect that media has on the antimicrobial activity of endophytes isolated from *H. hemerocallidea* against test pathogens using MIC assays (mg/mL).

Endophyte extracts	*S. aureus*	Positive control	*E. faecalis*	Positive control	*P. aeruginosa*	Positive control	*E. coli*	Positive control
	MIC	(µg/mL)	MIC	(µg/mL)	MIC	(µg/mL)	MIC	(µg/mL)
H2 (MEB)	4.00	1.25	3.00	0.08	2.00	2.50	**4.00**	1.25
H2 (SDB)	4.00	5.00	2.00	0.08	2.00	1.25	**2.00**	5.00
H3 (MEB)	**1.00**	1.25	2.00	0.08	2.00	2.50	1.00	1.25
H3 (SDB)	**4.00**	2.50	2.00	0.08	2.00	5.00	2.00	2.50
H5 (MEB)	4.00	1.25	**2.00**	0.08	2.00	2.50	4.00	1.25
H5 (SDB)	4.00	1.25	**4.00**	0.08	4.00	2.50	4.00	1.25

SDB= Sabouraud Dextrose broth, MEB= Malt Extract broth, Positive control= Ciprofloxacin, **bold font**= most significant data

2.9. Antimicrobial activity of plant and fungal extract in combination using the micro-titre dilution assay

The 96 micro-titre plate was used to test the interaction between plant and endophyte extract. The same method was used as in Section 2.6; however, a volume of 50 µL of each crude extract was added to the same well (first row of micro-titre plate) at a starting concentration of 16 mg/mL.

Interactions between the combinations of plant samples and endophytic fungal extracts were evaluated using the sum of the fractional inhibitory concentration index (FICI). The FICI is calculated using the following equation, where (a) represents the plant extract and (b) represents the endophytic fungal extract (van Vuuren and Viljoen, 2011):

$$FIC^{(i)} = \frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}}$$

$$FIC^{(ii)} = \frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}}$$

The FIC index was then calculated using the following equation: $FICI = FIC^{(i)} + FIC^{(ii)}$. The interactions were classified as being synergistic if the FICI is (<0.5), additive (0.5 – 1.0), indifferent (>1.0 - ≤0.4) or antagonistic (>0.4) (van Vuuren and Viljoen, 2011).

2.10. Fractionation of crude extract using Prep HPLC-MS

The fractionation of crude extract was performed on a Waters Chromatographic system equipped with a Waters Photodiode Array (PDA) (2998) and Mass Spectrometer (MS) detector (Waters, Milford, MA, USA). The extract (50 mg) was diluted to 2.0 mL with methanol, and the solution was filtered through a 0.22 µm membrane (Millipore). To achieve chromatograms with better resolution in short analysis time, the chromatographic conditions were optimised. Separation was achieved on an XBridge Prep C_{18}column (19 x 250 mm, i.d., 5 µm particle size, Waters) maintained at 40 °C. The mobile phase consisted of 0.10% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 20 mL/min; a gradient elution was as follows: The initial ratio was 90% A: 10% B, keeping for 1 min, changed to 10% A: 90% B in 7 min, changed to 100% B in 4 min, and back to initial ratio in 0.50 min. The total running time was 14 min. The injection volume was 200 µL. Data were collected by chromatographic software MassLynx 4.1^{TM} (Waters, USA). The Prep HPLC system was interfaced with a Quadrupole Dalton (QDa) mass spectrometer. Positive ion mode was selected. The probe temperature was set at 600 °C. The source temperature was 120 °C. The capillary and cone voltages were set to 800 and 10 V, respectively. The eluents were collected every 1 min in a tube using a fraction collector. All fractions subsequently combined and concentrated to give residues, which were analysed by UPLC-MS (Pudumo *et al*., 2018).

2.11. Isolation of marker compounds using Prep HPLC-MS

Isolation of bioactive compounds was performed on a Waters Chromatographic system with Waters PDA (2998) and MS detector (Waters, Milford, MA, USA). The extract (80 mg) was diluted to 2.00 mL with methanol, and the solution was passed through a 0.22 µm membrane filter (Millipore). To achieve chromatograms with better resolution in short analysis time, the chromatographic conditions were optimised. Separation was achieved on an XBridge Prep C_{18} column (19 x 250 mm, i.d., 5 µm particle size, Waters) maintained at 40 °C. The mobile phase consisted of 0.10% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 20 mL/min. The crude extract was eluted isocratically at an initial ratio of 90% A: 10% B, keeping over 1 min. Gradient elution from 10% A: 90% B in 7 min, to 100% B in 4 min, the solvents were then returned to the starting conditions and held for 0.50 min to re-

equilibrate. The total run time was 14 min. the injection volume was 200 µL. Data were collected by chromatographic software MassLynx 4.1^{TM} (Waters, USA). The Prep HPLC system was interfaced with a QDa mass spectrometer. Positive ion mode was selected. The probe temperature was set at 600 °C. The source temperature was 120 °C. The capillary and cone voltages were set to 800 and 10 V, respectively. Data were collected between 100 and 75 *m/z*. The eluents were fractionated into 200 drops/tube (about 2.0 mL) using a fraction collector. The target compounds were collected in various fractions, subsequently combined, and concentrated to give residues, which were analysed by UPLC-MS (Pudumo *et al*., 2018).

2.12. UPLC-MS

Chromatographic separation was performed on a Waters Acquity Ultra Performance Liquid Chromatographic system with PDA detector (Waters, Milford, MA, USA). Separation was achieved on an Acquity UPLC BEH C_{18} column (150 mm x 2.1 mm, i.d., 1.7 µm particle size, Waters) maintained at 40 °C. Some preliminary runs was performed prior to setting the chromatographic conditions to obtain chromatograms with better resolution and short analysis time. The mobile phase consisted of 0.10% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.30 mL/min. the gradient elution was executed as follow: initial ratio was 95% A: = 5% B, keeping for 1 min, changed to 5% A: 5% B in 9 min, to 90% A = 10% B in 5 min, keeping for 1 min and back to initial ratio in 0.50 min, equilibrium the system for 1.50 min. The total rung time was 18 min. The injection volume was 5.0 µL (full-loop injection). The positive and negative ion modes were examined and the positive ion mode provided results with more information and higher sensitivity. Thus, the mass spectrometer was operated in a positive ion electrospray mode and nitrogen (N_2) was used as desolvation gas. Data were acquired between 80 and 1200 m/z. the following parameters were set: capillary voltages were 3500 V; sampling cone voltages as 45 V; extraction cone was 4; source temperature was 100 °C; desolvation temperature was 45 °C; desolvation gas 45 L/hr (Pudumo *et al*., 2018). Chromatographic software MassLynx 4.1^{TM} was used to process and obtain all the chromatographic data (Figure 2.9).

2.13. Nuclear magnetic resonance

The active fraction (0.9 mg) was purified using an Agilent 1260 Infinity HPLC equipped with a with a Prontosil bond C18 5µm column (4.0 mm id × 125mm length). Agilent G1315D

PDA detector, set at both 240 nm and 260 nm was used for detection during HPLC analysis. The mobile phase consisted of water with 0.1% formic acid (solvent D) and acetonitrile with 0.1% formic acid (solvent B) at a flow rate of 0.5 mL/min. The gradient elution was executed as follows: initial ratio was 5% B: 95% D, changed to 50 % B: 50% D in 10 min, to 90% B: 10% D in 8 min, maintained at 90% B: 10% D for 6 min, changed to 100 % B: 0% D in 2 min, maintained at 100% B: 0% D for 5 min and back to initial ratio in 2 min, and the system was equilibrated for 2 min. The total running time was 35 min. The injection volume was 40 µL.

Trapping was done with the Bruker Prospect II SPE interface, equipped with polymer-based SPE cartridges (Spark Hysphere^{TM}). A Knauer K120 pump was used to introduce water at a flow rate of 1.5 mL/min during trapping of peaks into SPE cartridges. Two major peaks from the active fraction were trapped or collected from the HPLC into the SPE cartridges at retention times of 20.00 to 20.40 min and 20.80 to 21.20 min. The trapped samples in the cartridges were dried with nitrogen for 58 min. Bruker Sample Pro Tube Transfer sample handler was employed to automatically transfer the trapped compounds from SPE cartridges into 3 mm NMR tubes using CD_3CN or $CDCl_3$. The system was driven by HyStar software coupled with Sample Pro control software for eluting trapped samples into NMR tubes. Chromatographic software HyStar post processing was used to process and obtain all the chromatographic data. NMR data was acquired on a Bruker AVANCE III^{TM} HD 500 MHZ NMR Spectrometer equipped with prodigy probe.

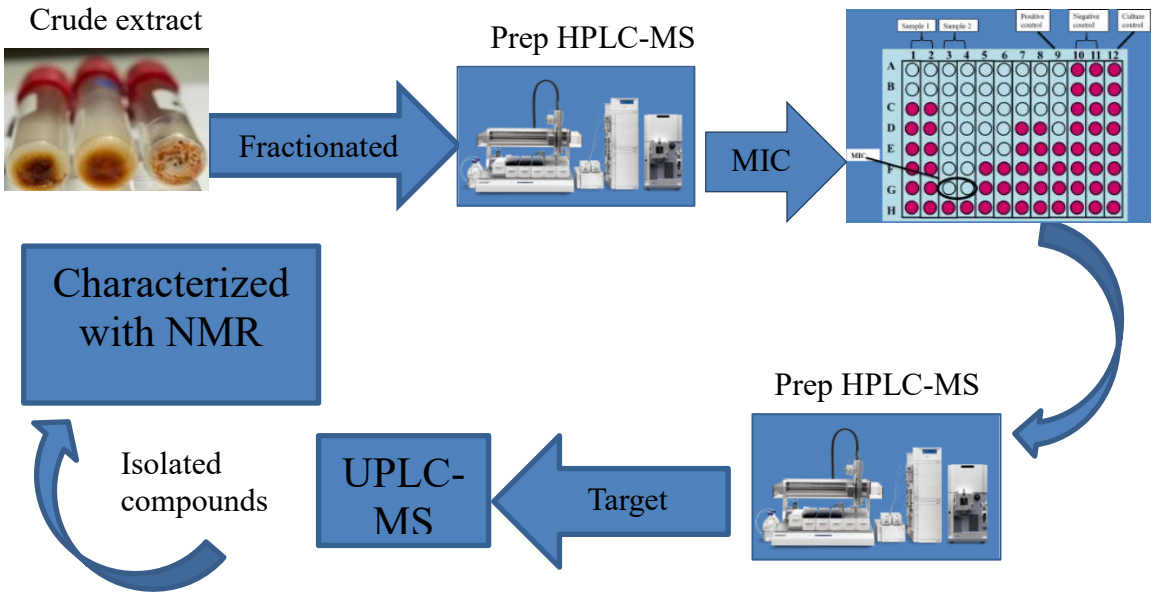


Figure 2.9: Summary of techniques used for isolation and identification of secondary metabolites.

2.14. PCR amplification and DNA sequence analysis

Genomic DNA was isolated from mycelium scraped from the colony surface using the Wizard® Genomic DNA purification Kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. Two loci were amplified and sequenced as described by Visagie *et al*. (2014, 2015); namely the internal transcribed spacer rDNA area (ITS) and the partial beta-tubulin (*BenA*) gene. It is more accurate to use *BenA* gene as a secondary barcode in addition to ITS as a DNA barcode for identification, since there are certain limitations with ITS as a species marker for *Penicillium* species (Skouboe *et al*., 1999; Seifert *et al*., 2007; Schoch *et al*., 2012). The ribosomal ITS region and β-tubulin (*BenA*) genes of the fungal endophyte were amplified using universal primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) (White *et al*., 1990).The primers used for amplification of β- tubulin (*BenA*) gene for forward direction and reverse direction were Bt2a (GGT AAC CAA ATC GGT GCT GCT TTC) and Bt2b (ACC CTC AGT GTA GTG ACC CTT GGC G) respectively (Glass and Donaldson, 1995). The PCR conditions used for both ITS and *BenA* were the standard thermal cycle: initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 45 s, annealing at 55 °C for 45 s, elongating at 72 °C for 60 s, and a final elongation at 72 °C for 7 min (Visagie *et al*., 2014). The ITS and *BenA* sequences isolated from the endophyte strain were submitted to NCBIs GenBank nucleotide database for a megablast search.

Chapter 3

Pelargonium sidoides



(www.plantlust.com)

3.1. Introduction

Pelargonium sidoides DC. is also known as "Umckaloabo", belongs to the Geraniaceae family. It is an indigenous South African plant found in the Eastern Cape Province and other coastline regions of South Africa. This plant is characterized by its dark reddish-purple flowers that occur abundantly between spring and summer. The long stalked leaves are heart-shaped, velvety in texture and possess a slightly aromatic scent. The plant roots are used traditionally to treat different ailments (van Wyk *et al*., 1997; van Wyk and Wink, 2004; Gurib-Fakim *et al*., 2010). Traditional healers use this plant mainly for stomach ailments. The roots are used traditionally in a form of a decoction which is taken orally, to treat diarrhoea and dysentery in adults (Watt and Breyer-Brandwijk, 1962; Brendler and van Wyk, 2008; Kolodziej, 2011). Antibacterial traditional uses of the plant also include topical use for acne (Lewu *et al*., 2007). This plant was introduced in Europe during the late 1890s for the treatment of tuberculosis (Brendler and van Wyk, 2008; Gurib-Fakim et al., 2010). *Pelargonim sidoides* DC is commercialised in Germany (EPs® 7630) and South Africa (Linctagon®) as a phytopharmaceutical for respiratory tract infections (Zschocke et al., 2000; Brendler and van Wyk, 2008). Due to the formulation of EPs® 7630 (Umckaloabo®) in

Germany, a lot of clinical and scientific research has been conducted on *P. sidoides* ranging from randomised, double-blind, placebo-controlled clinical trials, to *in vivo* and *in vitro* testing (Gericke, 2011; Moyo and van Staden, 2014). The pharmacological activity of extracts from the plant has been partly attributed to coumarins, flavonoids, gallic acid-derivatives, hydroxycinnamic and phenolic acid-derivatives (Kayser and Kolodziej, 1995; Brendler and van Wyk, 2008; Colling *et al*., 2010; Kolodziej, 2011). Umckaloabo® was approved in Germany for the treatment of acute bronchitis due to results of randomized double-blind clinical studies that support the use as a phytopharmaceutical (Matthys and Funk, 2008; Kamin *et al*., 2012). It is interesting to note, however, that in spite of the use to treat infections, the *in vitro* minimum inhibitory concentration (MIC) assays of the crude plant root displayed moderate to poor antibacterial activity against test pathogens. Kayser and Kolodziej (1997) reported that MIC values range from 5.00–7.50 mg/mL when extracts were tested against micro-organisms responsible for respiratory tract infections (*Staphylococcus aureus*, beta-hemolytic *Streptococcus* 1451, *Streptococcus pneumoniae*, *Escherichia coli*, *Haemophilus influenza*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus mirabilis*). While Uslu *et al*. (2009) investigated Umckaloabo, which is a herbal medication that contains *P. sidoides* extracts, against 192 strains of Gram-positive and Gram-negative bacteria (including *Staphylococcus* spp, *Streptococcus* spp, *Moraxella catarrhalis*, *Neisseria* spp, and *Haemophilus influenzae* activity for 24 strains) where activity range from 0.20–1.60 mg/mL. Therefore, further *in vitro* studies were conducted to demonstrate other modes of action of active compounds. One such study displayed that *P. sidoides* immunomodulatory activity may account for the antibacterial activity through stimulation of host macrophage activity. It was also proposed by Conrad and Frank (2008), that a possible mode of action of compounds could be through phagocytosis and host-bacteria interactions, which result in bacteria anti-adhesion to human epithelial cells. A very informative observation was noted by Mativandlela *et al*., (2007), where it was proposed that bioactivity was as a result of a synergistic interaction between chemical constituents within *P. sidoides* as opposed to bioactivity being attributed to a single constituent. Therapeutic activity of natural products can often be attributed to the synergistic interaction between phytochemical compounds, whereby separation of chemical compounds in an extract can result in diminished bioactivity (Li and Vederas, 2009; Wagner and Ulrich-Merzenich, 2009). Even though extensive research has been conducted on this commercialised South African plant, there has been no investigation into the endophytes of the plant to elucidate the role of individual endophytes, as well as, the possible synergistic interactions between the host plant and endophytes against

human pathogens. Therefore the aim of this chapter is to ascertain if the antimicrobial activity in *P. sidoides* may possibly be attributed to other indirect factors such as the endophytes within the plants or synergistic interaction between host plant and endophyte.

3.2. Summary of methods

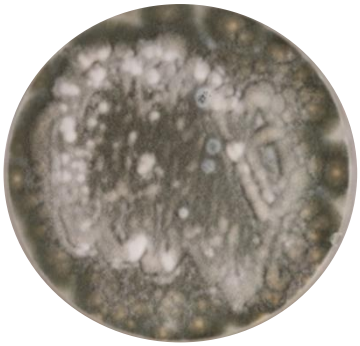
The detailed methodology can be found in Chapter 2 (Section 2.3.2), however, brief trouble shooting specific to *P. sidoides* is provided here. From a total of 19 fungal extracts tested against micro-organisms, one fungal extract (PS8) displayed noteworthy activity against the Gram-positive and Gram-negative bacteria. This fungal strain was regrown in MEB at a pH of 6 and a volume of 1000 mL. The fermentation method adapted from Chapter 2, Figure 2.9 was used for the upscale production of crude extract. The first batch was regrown under shaking conditions (160 rpm) for a period of 14 days. However, under shaking conditions antimicrobial metabolites were not produced as observed by the poor MIC results against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 8739). Therefore, it was deduced that the fungal isolate (PS8) grown under stationary conditions for a period of 21 days is crucial for the production of antimicrobial secondary metabolites. A total of 120 mg of crude extract was obtained and processed using different chemistry techniques (Chapter 2, Sections 2.10−2.13)

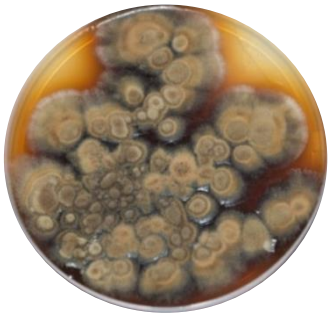
3.3. Results and discussion

A total of 19 strains of endophytic fungi were isolated from the root of *P. sidoides*. These isolates differed from each other in colour, texture and topography; these macroscopic differences made them distinguishable (Table 3.1.)

Table 3.1: Endophytes isolated from *P. sidoides* with allocated codes and macroscopic descriptions.

Code	**Description**	**Endophyte image**
PS1	Colonies are circular and raised. They are brown in the centre and off-white on the periphery. Colour diffused into agar. Cotton texture.	
PS2	Dense colonies are convex and white with cotton texture. Fungal endophyte fills the plate.	
PS3	Colonies are raised slightly and white in colour. The reverse is pale brown, and velvety texture.	
PS4	Colonies are flat irregular and dark brown. Colour diffuses into agar. It has a creamy leathery texture.	

Code	Description	Endophyte image
PS5	Colonies are filamentous and white. The reverse colour is orange. The elevation of colonies in the centre is umbonated.	
PS6	Colonies are raised, filamentous, and beige. with a cottony texture and umbonated centre.	
PS7	Colony is flat, and grey, with a velvety texture.	
PS8	Dense colonies are raised and centre of colony is yellow-beige and periphery is olive green. Also colonies are overlaid with a white colour and a cotton texture.	

Code	Description	Endophyte image
PS9	Colonies are flat and irregular in form with a turquoise colour and powdery texture. Margins are undulate.	
PS10	Colonies are raised and off-white in colour. Reverse is pale-brown with a cotton texture.	
PS11	Colonies are rugose, khaki on the inside and dark brown on the periphery with a velvety texture and umbonate centre.	
PS12	Colonies are flat and have a rhizoid form. They are translucent in colour and have a brown umbonate elevation in the centre. The reverse is brown in colour.	

Code	Description	Endophyte image
PS13	Colonies are raised with a brown undertone and overlaid with a white cotton appearance. Isolate fills the plate.	
PS14	Colonies are flat and white or pale pink with a velvety appearance.	
PS15	Colonies are raised and irregular with a brown undertone and a white cotton appearance.	
PS16	Punctiform colonies are circular and white with a yellow umbonate centre.	

Code	Description	Endophyte image
PS17	Colonies are raised, filamentous and white, with a cotton texture	
PS18	Colonies are raised, filamentous, and white with a yellow florescent undertone (reverse) and a cotton texture.	
PS19	Colonies are raised and circular with a reddish-purple colour and creamy texture. Colour diffuses into agar.	

3.3.1. Antimicrobial activities of *P. sidoides* plant and endophytic extracts

Plant and endophyte extracts were measured based on criteria in accordance with a study by (Ríos and Recio, 2005), natural product extracts that exhibit MIC values < 1.00 mg/mL should be considered worthy of attention. *Pelargonium sidoides* plant and endophyte extracts were measured based on these criteria.

Pelargonium sidoides and organic extracts exhibited weak activity against test pathogens ranging from 1.0 – 4.0 mg/mL (Figure 3.1−3.4). These results are supported by *in vitro*

antimicrobial test of plant extracts carried out by Kayser and Kolodziej, (1997) in which methanol crude extracts demonstrate poor activity ranging between 5.00–7.50 mg/mL, however, extracts partitioned using ethyl acetate, water and butanol displayed higher antimicrobial activity ranging from 0.60–1.20 mg/mL against respiratory causing pathogens. While Moyo *et al*. (2013) displayed methanol extracts having promising activity against *S. aureus* at 0.68 mg/mL. It has been proposed by Janecki *et al*., (2011) that antimicrobial properties of *P. sidoides* can be attributed to the interference of the plant extract with adhesion of bacteria (*Streptococcus pyogenes*) to human epithelial cells, while, investigations by Neugebauer *et al*., (2005) proposed that the stimulation of the mucociliary beat frequency of nasal epithelial cells (defence mechanism) could justify the use of this plant root for respiratory tract infections. What has not been proposed, however, is that the antimicrobial properties of *P. sidoides* could be enhanced by the secondary metabolites produced by endophytes within the plant.

All 18 crude extracts derived from endophytic fungi were screened for antibacterial activity against four different bacterial strains. The endophyte fungal extracts were tested alone and in combination with plant extract, and the MIC values can be found in Figure 3.1–3.4. Interestingly, one fungal strain PS8 from *P. sidoides* showed noteworthy activity against *S. aureus* (ATCC 25923), *E. faecalis* (ATCC 1883), and *E. coli* (ATCC 8739) at 0.03 mg/mL, 0.50 mg/mL, and 0.06 mg/mL respectively. Endophyte PS8 underwent a repeated fermentation–extraction process to produce a larger quantity of crude extract for chemical analysis. No parameters were changed. The second quantity of ethyl acetate crude extract inhibited *S. aureus* (ATCC 25923) and *E.coli* (ATCC 8739) again at 0.03 mg/mL and 0.125 mg/mL respectively, therefore, confirming that this fungal strain (PS8) could repeatedly produce the same antimicrobial secondary metabolites. Isolate PS18 was another endophyte that displayed moderate activity at 1.00 mg/mL against *E. coli.* The culture control samples for MEB did not show antimicrobial activity in all biological evaluations, therefore demonstrating that MEB did not have any effect on the results. A very recent study by Manganyi *et al*. (2018), documented the diversity of endophytic fungi from *P. sidoides.* This study also carried out preliminary *in vitro* screening on the antibacterial activity of endophytic fungi against *E. coli*. This study demonstrated that an *Aspergillus* sp. isolated from *P. sidoides* had the strongest activity with an inhibition zone of 11 mm, while

Penicillium expansum exhibited a zone of inhibition of 5 mm against *E. coli* (Manganyi *et al*., 2018).

The endophyte PS19 was an isolate that proved to be fastidious and growth in fermentation broth was negligible. This isolate was grown in both MEB and SDB to establish growth, however, neither broth used was successful in promoting a good yield of fungal growth, therefore MIC results were excluded for this isolate. It was interesting to note the colour of the culture was dark purple in colour, which reflected the colour of flowers and roots of *P. sidoides* plant, which is described as dark reddish-purple (van Wyk and Gericke, 2000; van Wyk and Wink, 2004). Possibly the fungal culture requires some nutrient from the *P. sidoides* root from which it was isolated and hence future recommendations to culture the endophyte with plant extract is recommended. The culture controls (not included in Figures 3.1-3.4 due to scale) responded as expected. All cultures were susceptible to the ciprofloxacin positive control. All solvent controls did not show susceptibility and culture controls were viable and served as a comparator when reading MIC results.

3.3.2. Antimicrobial activity of *P. sidoides* and endophytic extracts in combination

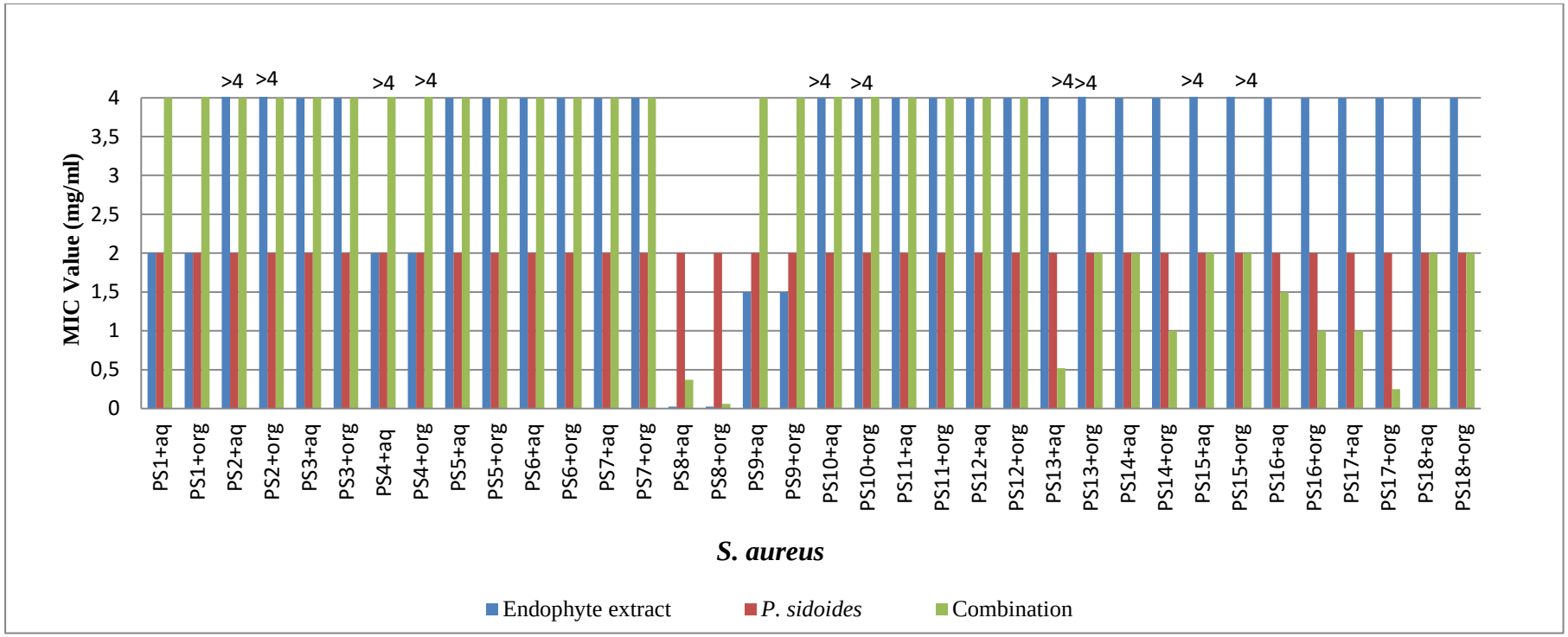
When evaluating the host interaction with endophytes 14.58% of combinations exhibited synergistic activity against all four test pathogens (Gram-positive and Gram-negative bacteria) (Figures 3.1-3.4) and summary (Table 3.2). Synergistic interactions varied between endophyte isolate as well as pathogen studied, but the most frequently encountered synergy was for the Gram-positive strains. Some endophytes (PS15 against *E. faecalis*, PS16 and PS18 against *S. aureus* and PS18 against *E. coli*) demonstrated synergy with both the organic and aqueous extracts of the host plant. Interestingly, isolate PS6, PS16, and PS18 displayed synergy against *P. aeruginosa* (ATCC 27853) when in combination with aqueous plant extract.

Endophyte isolates PS13 and PS17, when tested in combination with *P. sidoides* extracts displayed noteworthy activity (0.52 and 0.25 mg/mL) against *S. aureus* (ATCC 25923) while PS18 displayed noteworthy activity (0.50 mg/mL) in combination against *E. coli* (ATCC 8739). The endophyte isolate PS17 showed the lowest FICI value, as low as 0.09 (Table 3.2). Certain FICI values for plant extract: endophyte combinations could not be calculated due to certain plants or fungal extracts displaying individual results at ≥ 8.00 mg/mL, therefore,

interactions (PS13+aq and org, PS15+aq and org, PS16+aq, PS18+aq) should be examined on histograms within Figures 3.1-3.4, which demonstrates combination results having a much lower MIC value than either the endophyte and/or plant extract. These samples are represented by the symbol * in Table 3.2.

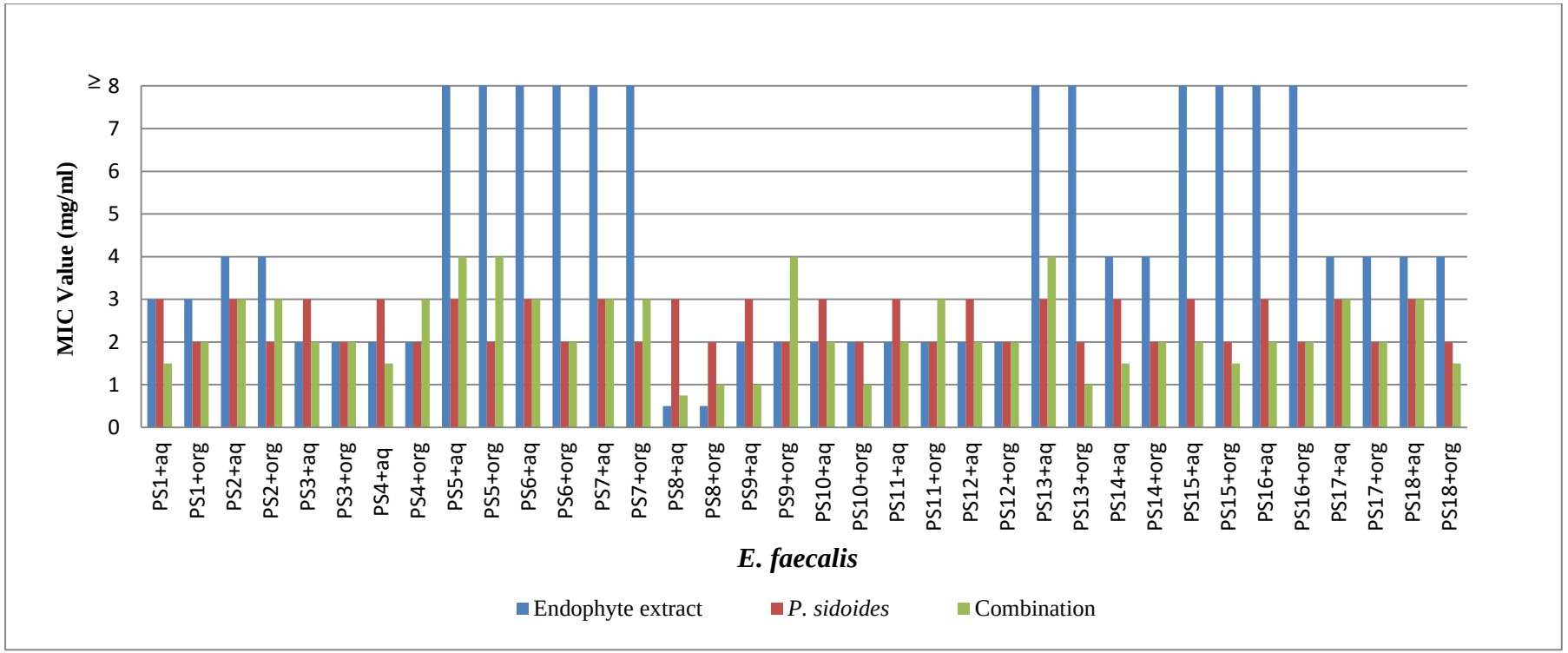
There was a small percentage of combinations (8.33%) where the endophyte displayed an antagonistic interaction with the plant aqueous and organic extracts. This was observed against *S. aureus* (PS1, PS4, and PS9) and against *E. coli* (PS10, PS11, and PS12). Antagonistic interactions were also observed for PS1, PS4 and PS12 with the organic extract of *P. sidoides* when tested against *P. aeruginosa* (Figure 3.1, 3.3 and 3.4). The remainder of 77.08% of combinations displayed either an additive or non-interactive interaction against the test pathogens (Figure 3.5).

The *in vitro* MIC assays proposes that selected synergistic interactions between plant and endophyte improve the antimicrobial activity of the plant. Many antimicrobial combination studies have focused on plant-plant interaction or plant and commercial antibiotic interactions (Hübsch *et al*., 2014b; Naidoo *et al*., 2013). One recent study by Zin *et al*. (2017) investigated the interactions of fungal endophytes with commercial antimicrobials. Unfortunately, there are no similar studies of interactions between endophyte and host plant with which to compare and thus makes this study novel. It has never been proposed that the entirety of a plant extract could include the secondary metabolites produced by the endophytic fungi within the plant.



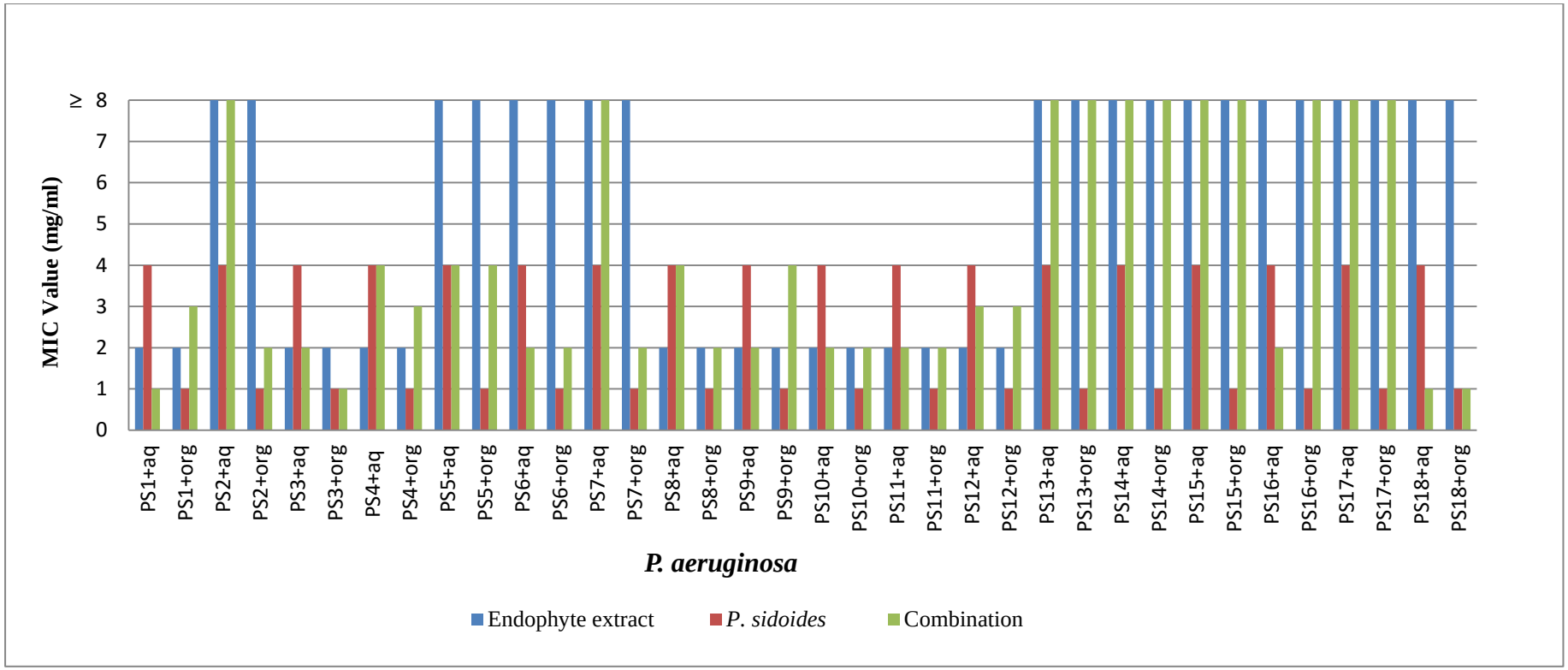
aq= aqueous extract, org= organic extract

Figure 3.1: Antimicrobial activities of *P. sidoides* endophyte extracts, and combinations against *S. aureus*.



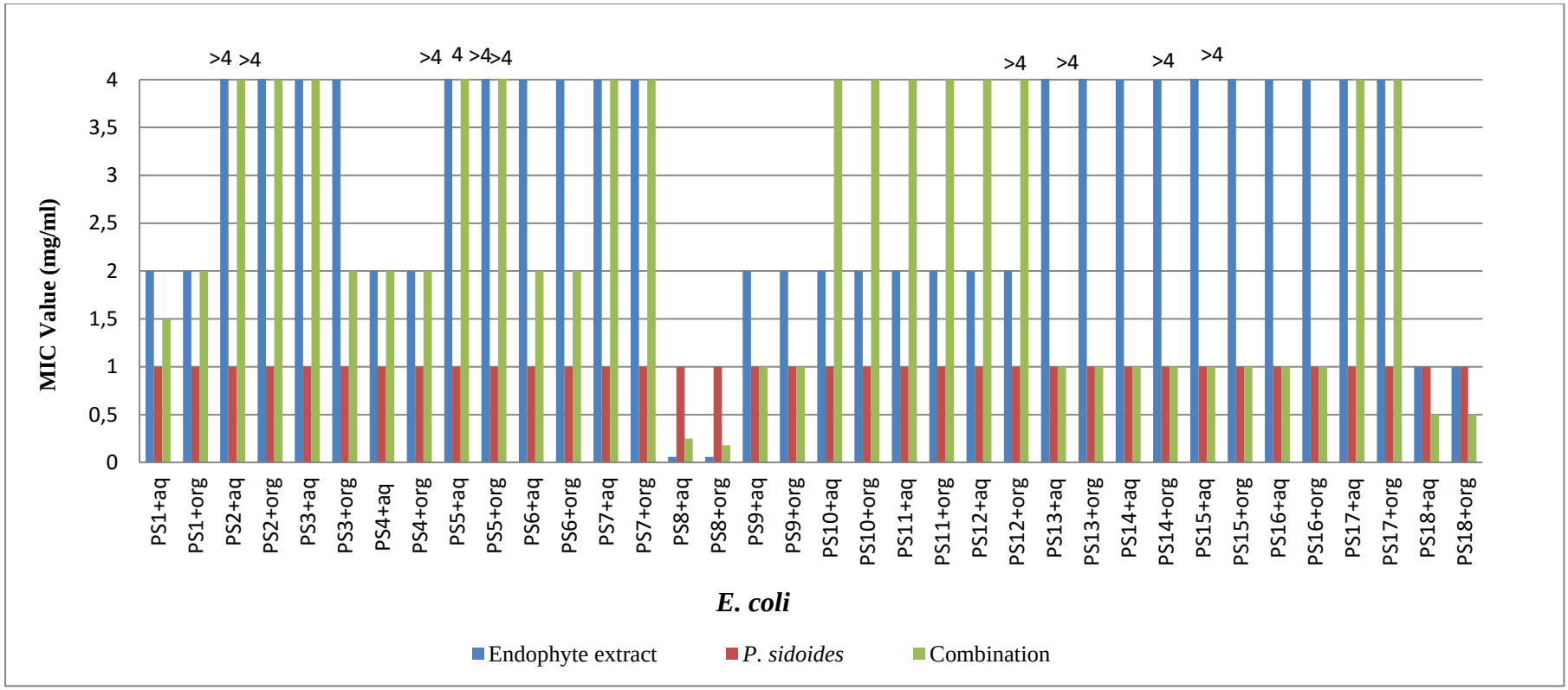
aq= aqueous extract, org= organic extract

Figure 3.2: Antimicrobial activities of *P. sidoides*, endophyte extracts, and combinations against *E. faecalis*.



aq= aqueous extract, org= organic extract

Figure 3.3: Antimicrobial activities of *P. sidoides*, endophyte extracts, and combinations against *P. aeruginosa*.



aq= aqueous extract, org= organic extract

Figure 3.4: Antimicrobial activities of *P. sidoides*, endophytes extracts, and combinations against *E. coli*.

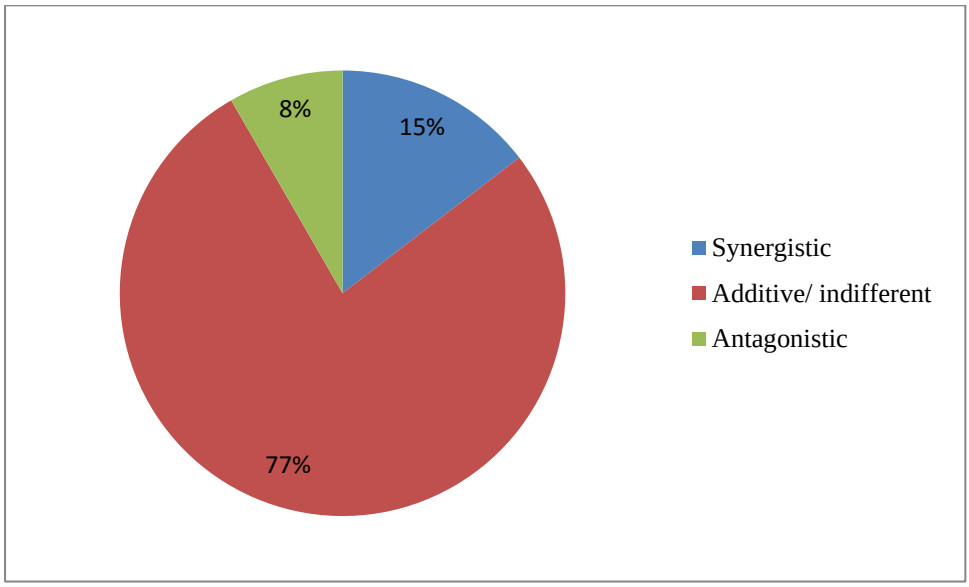


Figure 3.5: Summary of the antimicrobial activities (MIC mg/mL) of the synergistic combinations between *P. sidoides* and endophyte extracts (a total of 144 combinations tested).

Table 3.2: Summary of the antimicrobial activities (MIC mg/mL) of the synergistic combinations between *P. sidoides* and endophyte extracts.

Sample	Combination MIC (mg/mL)	FICI	Test pathogen	Positive control Ciprofloxacin (µg/mL)	Negative control DMSO
PS13+aq	**0.52**	*			
PS14+org	1.00	0.37			
PS16+aq	1.50	0.56	*S. aureus*	2.81	4.00
PS16+org	1.00	0.37			
PS17+aq	1.00	0.37			
PS17+org	**0.25**	0.09			
PS13+org	1.00	*			
PS14+aq	1.50	0.44			
PS15+aq	2.00	*			
PS15+org	1.50	*			
PS16+aq	2.00	*			
PS18+org	1.50	0.56	*E. faecalis*	0.08	2.00
PS1+aq	1.50	0.50			
PS4+aq	1.50	0.62			

Sample	Combination MIC (mg/mL)	FICI	Test pathogen	Positive control Ciprofloxacin (μg/mL)	Negative control DMSO
PS9+aq	1.00	0.42			
PS10+org	1.00	0.50	*E. faecalis*	0.08	2.00
PS16+aq	2.00	*			
PS18+aq	1.00	*	*P. aeruginosa*	2.50	2.00
PS1+aq	1.00	0.38			
PS18+aq	0.50	0.50	*E. coli*	1.67	3.50
PS18+org	0.50	0.50			

*the asterisk represents results that cannot be calculated using FICI formula, figures 1- 4, can be used to tentatively determine the interaction between combinations. aq= aqueous plant extract, org= organic plant extract, **Bold font**= most significant data.

3.3.3. Identification of endophytes

The endophytic fungal isolate PS8 was identified as *Penicillium skrjabinii* using polymerase chain reaction amplification of DNA sequences and further phylogenetic analysis based on its ITS and *BenA* sequences. Based on a megablast search against the NCBIs GenBank nucleotide database, the ITS sequence of PS8 was identical to *P. skrjabinii* (strain CV85, GenBank JX091432.1) and differed with 1 nucleotide from *P. skrjabinii* (culture NRRL 13055, GenBank EU427287.1 and culture CBS 439.75, GenBank GU981576). Likewise, the *BenA* sequence differed in one nucleotide from *P. skrjabinii* (strain CV85, GenBank JX091529.1) and five nucleotides from *P. skrjabinii* (culture NRRL 13055, GenBank EU427271.1 and culture CBS 439.75, GenBank GU981626).

Penicillium skrjabinii is a fungus that is classified as part of the phylum division of Ascomycota and Trichocomaceae family. It was first isolated from soil in Russia during 1971 (Shmotina and Golovleva, 1975). Later studies demonstrate that it can be categorised as an endophyte, since it was isolated from stem and roots of Chinese medicinal plant *Kadsura angustifolia* (Huang *et al*., 2015). Furthermore, another very recent investigation isolated *P. skrjabinii* from the rhizosphere soil of the Chinese medicinal plant *Pulsatilla chinensis* (Feng *et al*., 2018). The latter study isolated 18 compounds from this fungus some of which displayed cytotoxic and antimicrobial activity. Visagie *et al.*, (2015) isolated several *Penicillium* spp. From three fynbos sites in South Africa these strains, which included *P. skrjabinii*, were isolated from samples which included soil, air, and also the plant *Protea*

infructescence. A recent study by Manganyi *et al*. (2018), displayed 23% of endophytic fungi (133 isolates) from roots and leaves of *P. sidoides* belong to the genus *Penicillium.* The latter and present study substantiates that *Penicillium* isolates from *P. sidoides* display antibacterial activity against pathogenic bacteria.

3.3.4. Bioassay guided isolation

The ethyl acetate crude extract of the most active endophyte strain *P. skrjabinii* was fractionated using prep-HPLC into 11 fractions. These fractions were tested for antimicrobial activity against a Gram-positive (*S. aureus* ATCC 25923) and a Gram-negative *E. coli* (ATCC 8739) strain. Fraction 10 (Figure 3.6) demonstrated the most active fraction with MIC values of 0.01 mg/mL and 0.06 mg/mL for *S. aureus* (ATCC 25923) and *E. coli* (ATCC 8739) respectively (Table 3.3). According to Rios and Recio (2005), natural products compounds that show antimicrobial activity at less than 0.10 mg/mL should be considered worthy of attention.

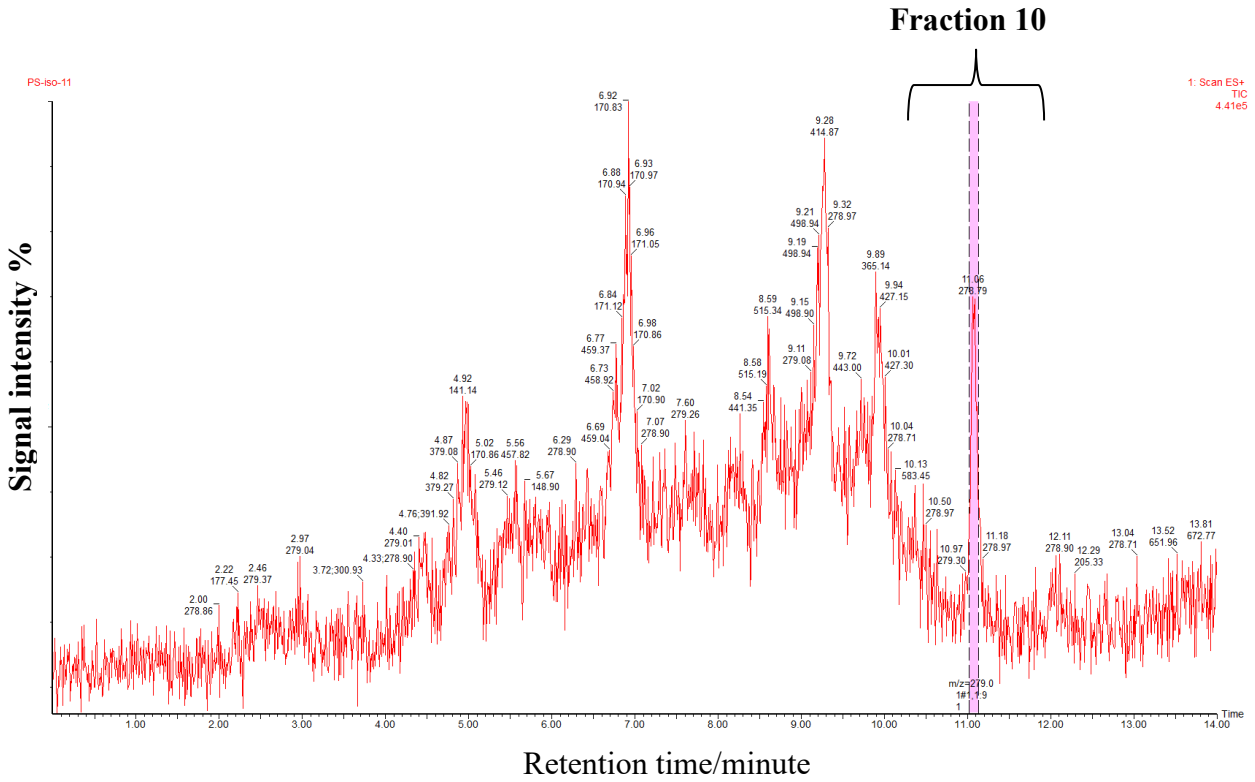


Figure 3.6: Molecular mass of compounds at different retention times, which highlight fraction ten peaks.

Table 3.3: Antimicrobial activity (mean MIC in mg/mL) of fractions from the crude extract of endophytic isolate *P. skrjabinii.*

Fractions	*S. aureus* MIC (mg/mL)	*E. coli* MIC (mg/mL)
1	0.50	0.50
2	0.50	0.50
3	0.50	0.50
4	0.50	0.50
5	0.25	0.50
6	0.50	0.50
7	0.50	0.38
8	0.50	0.50
9	0.50	0.25
10	**0.01**	**0.06**
11	0.50	0.25
PS8 Crude Extract	0.03	0.13
Negative control	0.50	0.50
Positive control	1.25 µg/mL	1.25 µg/mL

Bold font= most significant data

The ESI positive mode mass spectrometry chromatogram, fraction 10 (0.9 mg) (yellow viscous liquid) comprised of two compounds. The first peak (compound 1) displayed a molecular ion peak at 219.11 *m/z* and retention time of 14.32 min. The latter had a calculated molecular formula of $C_{13}H_{16}O_3$. The second peak (compound 2) which was a major peak showed a molecular ion peak at [M+H] 148.02 *m/z* and retention time of 14.98 (Figure 3.7). According to the nitrogen rule, when the mass spectrometer is run in ESI positive mode and the molecular iron peak is an even number e.g. 148.02 *m/z* it is indicative of the absence of a nitrogen atom or an even number of nitrogen atoms. While an odd number molecular mass e.g. 219.11 *m/z* is indicative of the presence of an odd number of nitrogen atoms (Barwick *et al*., 2006). It is possible that compound 2 does not have any nitrogen atoms.

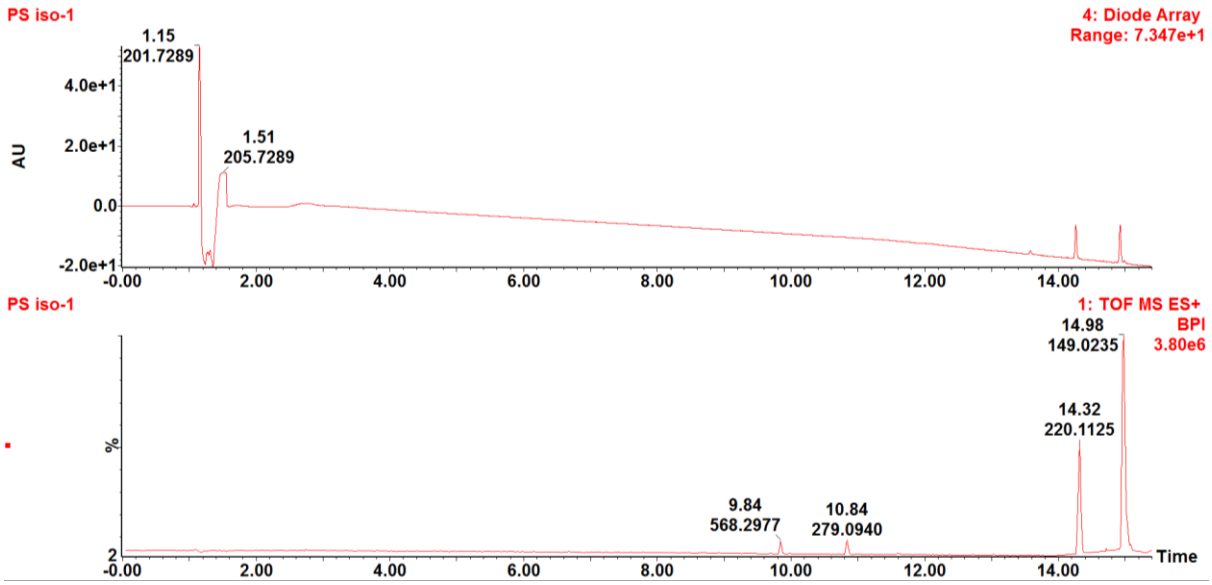


Figure 3.7: UV diode array and MS of bioactive compounds from fraction ten.

3.3.5. Characterization of most active compounds

Fraction 10 (0.9 mg) was obtained in low amounts as a result conventional chromatography could not be used to obtain sufficient quantities of the major compound for structure elucidation using NMR. The fraction was purified using a High Performance Liquid Chromatography-Mass Spectrometry-Solid Phase Trapping-Nuclear Magnetic Resonance (HPLC-MS-SPT-NMR). The first compound was collected between retention time 20.00 and 20.40 min and the second major compound between 20.80 and 21.20 min, both trapped separately onto the SPE. Due to the insufficient quantities for the first minor compounds, the NMR was of poor quality for structure elucidation purposes. According Prep-HPLC data, the light yellow oily liquid correlates with compound formula $C_{16}H_{23}O_4$. The ^{1}H NMR of the major compound when compared to that in literature (Khatiwora *et al*., 2012) confirmed it as dibutyl phthalate (Figure 3.8). The ^{1}H NMR spectrum of dibutyl phthalate shown in Table 3.4 (500 MHz, $CDCl_3$., *J* (coupling constant) measured in Hz- Hertz) displayed an up-field triplet at δ 0.99 (6 H, *J* = 7.42 Hz) for C5′ and C5″ methyl protons, the multiplets at δ 1.47 (4 H,) were assigned to the C4′ and C4″ methylene protons, while multiplets for the C3′ and C″ methylene protons were at δ 1.74 (4 H). A downfield triplet was observed for C2′ and C″ methylene protons at δ 4.33 (4 H, *J* = 6.74 Hz) indicating that these are oxygen bearing carbon atoms. Two sets of apparent doublet of doublets were observed for aromatic protons

C-3 and C- 4) and C-2 and C-5 at δ 7.55 (2 H, *J* = 9.06 Hz, 3.30 Hz) and δ 7.74 (2 H, *J* = 9.06 Hz, 3.33 Hz), respectively.

Dibutyl phthalate is characterised as a plasticizer. It is mostly used in polyvinyl chloride (PVC), which in turn is used for the production of children's toys and blood bags. Furthermore it is also used as an additive in nail lacquers (Blount *et al*., 2000; Kopelovich *et al*., 2015). It has also shown efficacy in treating human ectoparasite dermodicidosis (Yuan *et al*., 2000). Phthalates are considered an endocrine disruptor. While initially it was thought that the compound was a contaminant, upon further investigation it was found that phthalate compounds may also be a bioactive natural products that are produced by fungi, bacteria, and plants. These compounds have been shown to exhibit cytotoxic, anti-oxidant, antiviral and antimicrobial activity (El-Naggar, 1997; Habib and Karim, 2009; El-Sayed *et al*., 2015). Furthermore, animal studies have also displayed teratogenicity, and toxicity to male testicular organs (Gray and Beamand, 1984; Ema *et al*., 1993; Czernych *et al.*, 2017). Dibutyl phthalate is a compound that displayed antimicrobial activity, however Lehmann *et al*. (2004) reports that sub-chronic/ chronic exposure to dibutyl phthalate 0.3 mg/kg/day can be hazardous.

Fungi can produce compounds for their own benefit, for example, to eradicate bacteria and other competitors in a host plant. The plant may produce these metabolites to alter the growth of fungi within tissue before and after colonization (Ludwig-Müller, 2015). The biosynthetic pathways in plants are very complex. However, the syntheses of many secondary metabolites are a result of biotic and abiotic stressors. This compound could have been produced by *P. skrjabinii* as a means for the fungus to protect itself from biotic and abiotic stressors. It has been documented that *Penicillium janthinellum* is another *Penicillium* fungal isolate that produced a biologically active phthalate compound. Some studies highlight the production of phthalates by *Streptomyces* spp. (El-Naggar, 1997; Smaoui et al., 2012). Roy *et al*. (2006) isolated dibutyl phthalate from *Streptomyces albidoflavus*. In this study, dibutyl phthalate exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria ranging between 50–120 µg/mL, therefore substantiating the results of antimicrobial activity exhibited by the active fraction in this study. A very recent study (Manganyi *et al*., 2018), it was suggested that characterizing metabolites should be undertaken in future endophyte studies.

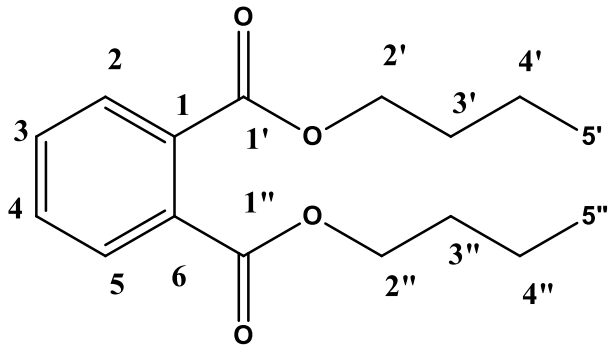


Figure 3.8: Dibutyl phthalate isolate from *P. skrjabinii*.

Table 3.4: ^{1}H-NMR of isolated compound (dibutyl phthalate) ($CDCL_{3,}$ at 500 MHz)

Atom number	**^{1}H chemical shift (ppm) and multiplicity**	**Published chemical shift and multiplicity (**Khatiwora *et al*., 2012**)**
H5' and H5"	0.99 (t, J=7.42 Hz, 6 H)	0.98 (t, J= 5.00Hz, 6 H)
H4' and H4"	1.47 (m, 4 H)	1.47 (m, 4 H)
H3' and H3"	1.74 (m, 4 H)	1.74 (m, 4 H)
H2' and H2"	4.33 (t, 6.74 Hz, 4 H)	4.33 (t, J= 5.00Hz, 4 H)
H3 and H4	7.55 (dd, 9.06 Hz, 3.30 Hz, 2 H)	7.56 (dd, J= 10.00, 2.00Hz, 2 H)
H2 and H5	7.74 (dd, 9.06 Hz, 3.33 Hz, 2 H)	7.74 (dd, J= 10.00, 2.00Hz, 2 H)

3.4. Summary

- This is the first study to explore the antimicrobial interactions between the South African medicinal plant root species of *P. sidoides* and associated endophytes.
- From a total of 19 endophytes tested in combination with aqueous and organic plant extracts 14.58% of combinations displayed synergistic activity while 8.33% of combinations displayed antagonistic activity against test pathogens.
- Endophyte isolate PS18 when in combination with *P. sidoides* aqueous and organic extracts demonstrated synergy against three of the four test pathogens (*E. faecalis*, *E. coli* and *P. aeruginosa*).
- DNA analysis of most active endophyte isolate was identified as *P. skrjabinii,* which produced an antimicrobial compound dibutyl phthalate.

- It can be concluded that *P. sidoides* has a greater antimicrobial potential when used in combination with selected endophytic isolates and this demonstrates for the first time how the antimicrobial efficacy of a plant can be enhanced by including associated endophytes.

Chapter 4

Hypoxis hemerocallidea



(Bassey *et al*., 2014)



(www.herbgarden.co.za)

4.1. Introduction

Hypoxis hemerocallidea (Fisch. & C.A. Mey.) is a medicinal plant that belongs to the Hypoxidaceae family. Star-like yellow leaves and a rootstock that looks like a potato are characteristic for this plant (van Wyk *et al*., 1997). It is distributed through various provinces in South Africa, namely, Mpumalanga, KwaZulu-Natal, Eastern Cape, Gauteng and Limpopo (Singh, 2000, 2007; Drewes *et al*., 2008). Extracts have been used since the 1960's, and it is referred to as the 'miracle muthi' or 'wonder potato' (Drewes and Khan, 2004). The tuberous rootstock (corms) are used medicinally by the Sotho's and Zulus in South Africa to treat different ailments (van Wyk and Gericke, 2000), and is therefore amongst the top ten most sold medicinal plants in South Africa. Additionally it is also exported to Europe (Dold and Cocks, 2002; Mulholland and Drewes, 2004). Decoctions are applied topically to treat burns, skin wounds, and also given to weak children as a tonic (Watt and Breyer-Brandwijk, 1962) Traditional healers use the plant to treat diseases such as urinary tract infections (UTI's), sexually transmitted infections and testicular tumours (Grierson and Afolayan, 1999; Louw *et al*., 2002). It is also used traditionally for benign prostatic hypertrophy and phytochemical studies on crude extracts attribute this activity to sitosterols within the plant (Bruneton,

1995). A series of investigations displayed that the anti-oxidant and antitumour activity was attributed to the compound rooperol (van der Merwe *et al*., 1993; Albrecht *et al*., 1995b; Dietzsch *et al*., 1999). Due to the clinical and scientific studies on rooperol and many patents on the compound rooperol and its derivatives, the extract has been commercialised in Germany as Harzol® for the treatment of prostate cancer (Pegel, 1979; Tyler, 1986; Albrecht *et al*., 1995a; Nair *et al*., 2007). Further *in vivo* studies on the aqueous plant extracts demonstrated anti-inflammatory and antidiabetic activity (Liebenberg, 1969; Ojewole, 2006; Laporta *et al*., 2007). However, *in vitro* antimicrobial studies which included testing of aqueous and organic extracts against both Gram-positive and Gram-negative pathogens demonstrate results which vary from poor to noteworthy activity (Steenkamp *et al*., 2006; Ncube *et al*., 2012; Ndhlala *et al*., 2013). A study by Katerere and Eloff (2008), displayed *H. hemerocallidea* acetone extract with MIC values as low as 0.31 mg/mL against *S. aureus* while the same study displayed poor activity when testing both acetone and ethanol extracts against *E. coli* and *P. aeruginosa* at 2.50 mg/mL and > 5.00 mg/mL respectively. Ndhlala *et al*. (2013), tested *H. hemerocallidea* aqueous and organic extracts against *S. aureus*, *Bacillus subtilis*, *E. coli* and *Klebsiella pneumoniae*. The only interesting activity noticed during this study was inhibition of *K. pneumoniae* at 0.78 mg/mL when testing *H. hemerocallidea* aqueous extract. Organic extracts displayed poor activity against all four organisms. Another study conducted by Laporta *et al*. (2007) highlighted the poor activity of the *H. hemerocallidea* extract against *S. aureus* and *E. coli* at 3.00 mg/mL (+/- 0.01) and 4.00 mg/mL (+/-0.01) (Table 4.1). Even though *H. hemerocallidea* is a popular commercial medicinal plant in South Africa that has been investigated by means of exploring the plant extract by *in vivo* and *in vitro* studies, endophytes within the plant have not been isolated and investigated for their biological activity. It is a possibility that antibacterial activity exhibited by *H. hemerocallidea* can be attributed to secondary metabolites produced by endophytic fungi within the plant (Kaul *et al*., 2012; Zhang *et al*., 2006). The antibacterial activity could also be due to synergistic interactions between the endophyte and plant extracts.

Table 4.1: Summary of MIC results (mg/mL) of *H. hemerocallidea* extracts when tested against various pathogens.

Plant sample	Gram-positive			Gram-negative			References
	B. subtilis	***E. faecalis***	***S. aureus***	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	
H. hemerocallidea extract	n/t	n/t	3.00	4.00	n/t	n/t	Laporta *et al*., 2007
	n/t	0.63-1.25	0.31 - 0.63	2.50	n/t	>5.00	Katerere and Eloff, 2008
	0.20->12.50	n/t	0.39 - 12.50	0.78 - 12.50	0.39 - 3.13	n/t	Ncube *et al*., 2011
	1.56	n/t	1.56	1.56 - 3.13	0.78 - 3.13	n/t	Ndhlala *et al*., 2013

n/t= not tested

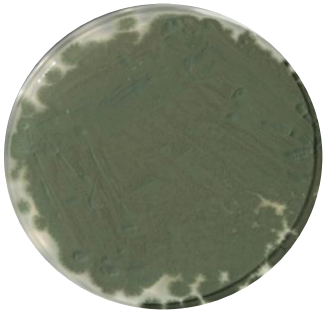
4.2. Results and discussion

4.2.1. Endophytes isolated from *H. hemerocallidea*

A total of six strains of endophytic fungi were isolated from the root of *H. hemerocallidea*. Each strain isolated from the root differed in colour and texture (Table 4.2).

Table 4.2: Endophytes isolated from *H. hemerocallidea* with allocated codes and macroscopic descriptions.

Code	**Description**	**Endophyte image**
H1	Colonies were verrucose and raised. Dark green in colour. After two weeks of culturing the isolate would darken in colour to blackish-green. Margins irregular.	
H2	Dense colonies are raised and colour is white to off-white or slightly brownish. Fluffy cotton wool appearance is noticed.	

Code	Description	Endophyte image
H3	Turquoise (blue-green) in colour with a chalk-like powder texture and a curled margin.	
H4	Colonies are irregular and dark green in colour, with rust underdone. This isolate appears to have a creamy glistening appearance.	
H5	Circular punctate colonies. White and yellow in colour and has a cotton appearance with entire margins.	
H6	Colonies are raised and irregular with a deep beige colour and a blue outline. Texture is granular.	

4.2.2. Antimicrobial activities of *H. hemerocallidea* and endophytic extracts

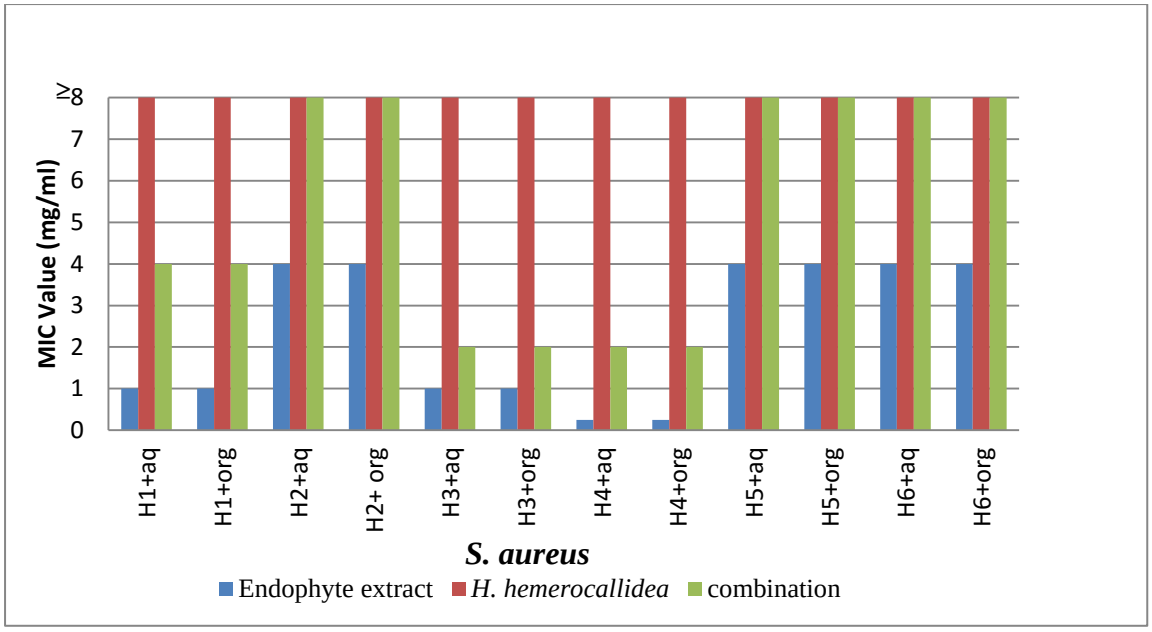
Six crude extracts derived from the endophytic fungi were screened for antibacterial activity against four test pathogens (Figure 4.1−4.4). These extracts were tested alone and in combination with the crude root plant extract. Measurement of noteworthy results for natural products can be deduced based on publication by Ríos and Recio (2005) (Chapter 3, Section 3.3.1).

The aqueous and organic plant extracts of *H. hemerocallidea* exhibited poor activity against the standard ATCC bacterial strains with values ranging from 2.00–≥ 8.00 mg/mL. These weak results are substantiated by MIC results of plant extracts in a study by Ndhlala *et al*. (2013), which displayed MIC results of plant extracts against *S. aureus* and *E. coli* at 1.56 mg/mL and 3.13 mg/mL respectively. A study by Ncube *et al*. (2011) displayed seasonal variations where activity ranged from poor to noteworthy. *Hypoxis hemerocallidea* exhibited poor activity against *S. aureus* at 12.5 mg/mL during spring, while noteworthy activity was noticed from aqueous extract during summer (0.39 mg/mL), autumn (0.78 mg/mL), and winter (0.78 mg/mL). Aqueous extracts collected during spring and winter displayed poor-moderate activity against *E. coli* with values as high as 12.5 mg/mL and values as low as 1.56 mg/mL respectively, while extracts collected during summer and autumn months displayed more consistent noteworthy activity and inhibited *E. coli* at a concentration of 0.78 mg/mL. Overall plant corms exhibited poor antimicrobial activity during the spring season.

The endophyte isolate H1 displayed activity at 1.00 mg/mL against *S. aureus* and *E. faecalis*, while isolate H3 displayed inhibitory activity against *S. aureus* and *E. coli* at 1.00 mg/mL. Furthermore, H4 was the only strain that exhibited noteworthy activity against *S. aureus* (ATCC 25923) at 0.25 mg/mL (Figure 4.1, 4.2, 4.4), However, endophytic fungi from *H. hemerocallidea* have not been isolated previously. With the MIC results depicting isolate H4 exhibiting activity against *S. aureus* at 0.25 mg/mL. It is probable that more or less the antimicrobial properties exhibited by the plant against *S. aureus* may be due to endophyte H4. Endophytes are surely a component within the plant that contributes to antibacterial activity in addition to other compounds such as rooperol and lectin-like proteins (Gaidamashvili and van Staden, 2002; Laporta *et al*., 2007).

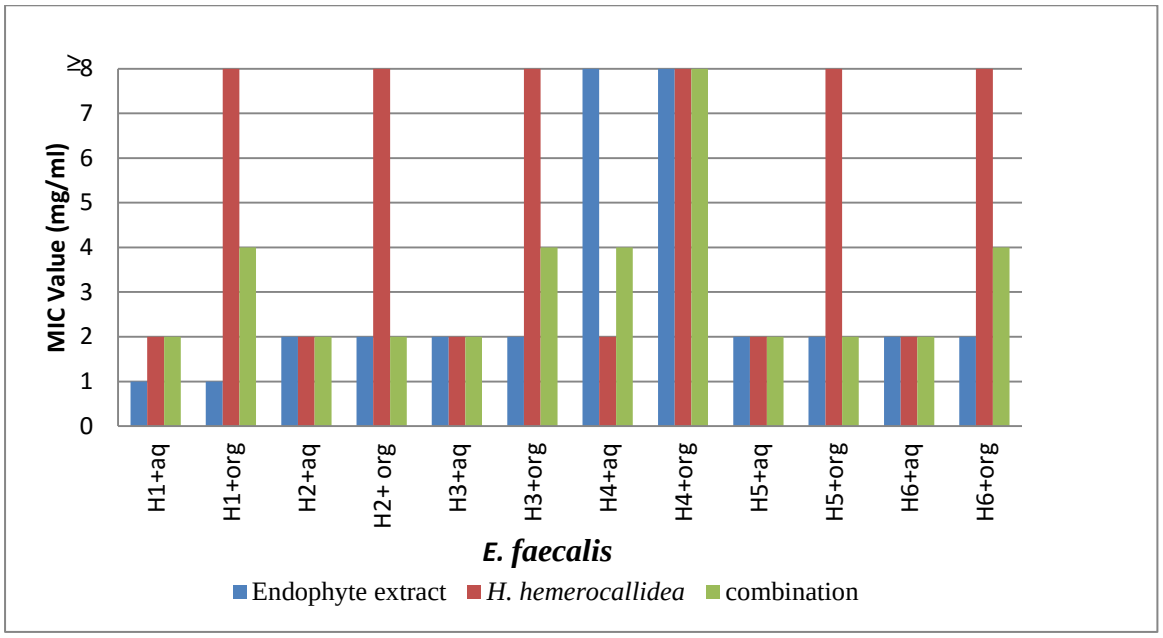
4.2.3. Antimicrobial activity of plant and endophytic fungal extracts in combination

No antagonism was observed against any of the combinations, while the majority of combinations (96%) were either additive or indifferent (Figure 4.5). In some combinations (H2, H3, H5 and H6 against *E. faecalis*) the endophyte combinations with aqueous extracts were equally as effective as the individual samples. Endophyte H1 exhibited synergistic activity with both the aqueous and organic plant extract against *E. coli* (ATCC 8739) at a fractional inhibitory concentration index value of 0.50 and 0.38. The aqueous and organic plant extracts inhibited *E. coli* at 2.00 mg/mL and 4.00 mg/mL respectively, while the combination of extracts inhibited antibacterial activity against *E. coli* at 1.00 mg/mL. Even though *H. hemerocallidea* is used to treat UTI's, some *in vitro* MIC studies display poor activity when plant extracts are tested against *E. coli* at values as high as 2.50 and 3.13 mg/mL (Katerere and Eloff, 2008; Ndhlala *et al*., 2013). Therefore, synergistic activity between plant and endophyte extracts could possibly provide a plausible option for the treatment of UTI's. The FICI values could not be calculated for extracts tested against *S. aureus* and *P. aeruginosa* due to values for individual plant or endophyte extracts being ≥ 8.00 mg/mL thus Figures 4.1−4.4 can be viewed to deduce if other combinations were either, additive/indifferent or antagonistic. Some FICI values for *E. faecalis* and *E. coli* can be calculated and these values are displayed in Table 4.3. The results of synergy depicted in this investigation cannot be compared to other similar studies since the interaction between combinations of a medicinal plant extract and endophytic extracts have not been investigated before locally and internationally.



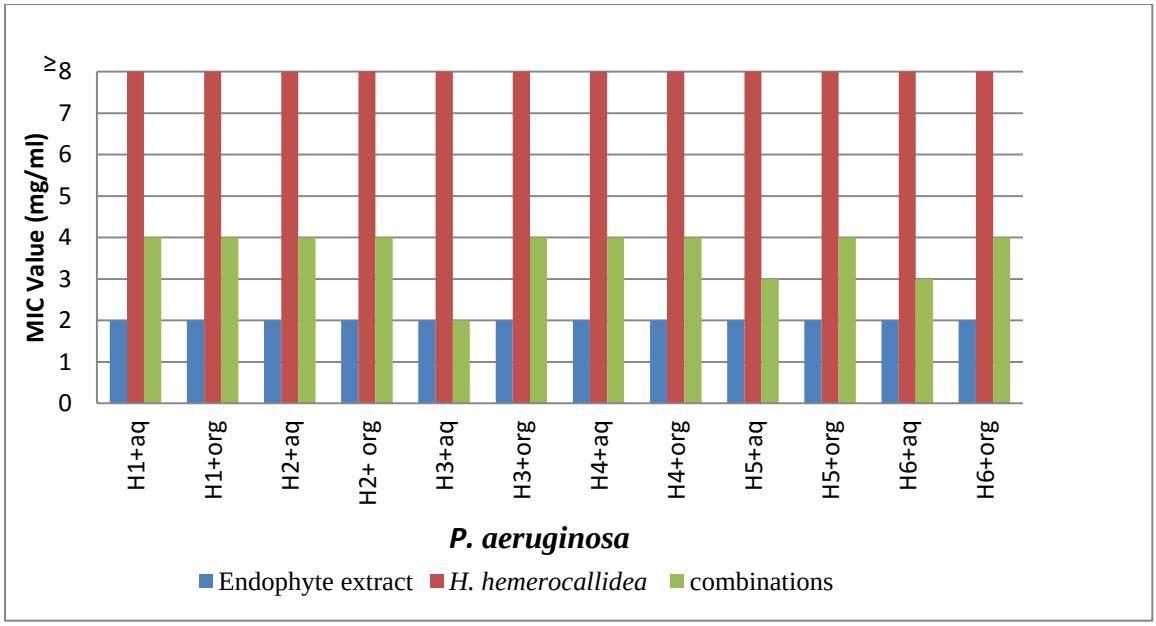
aq= aqueous extract, org= organic extract

Figure 4.1: Antimicrobial activities of *H. hemerocallidea*, endophyte extracts, and combinations against *S. aureus*.



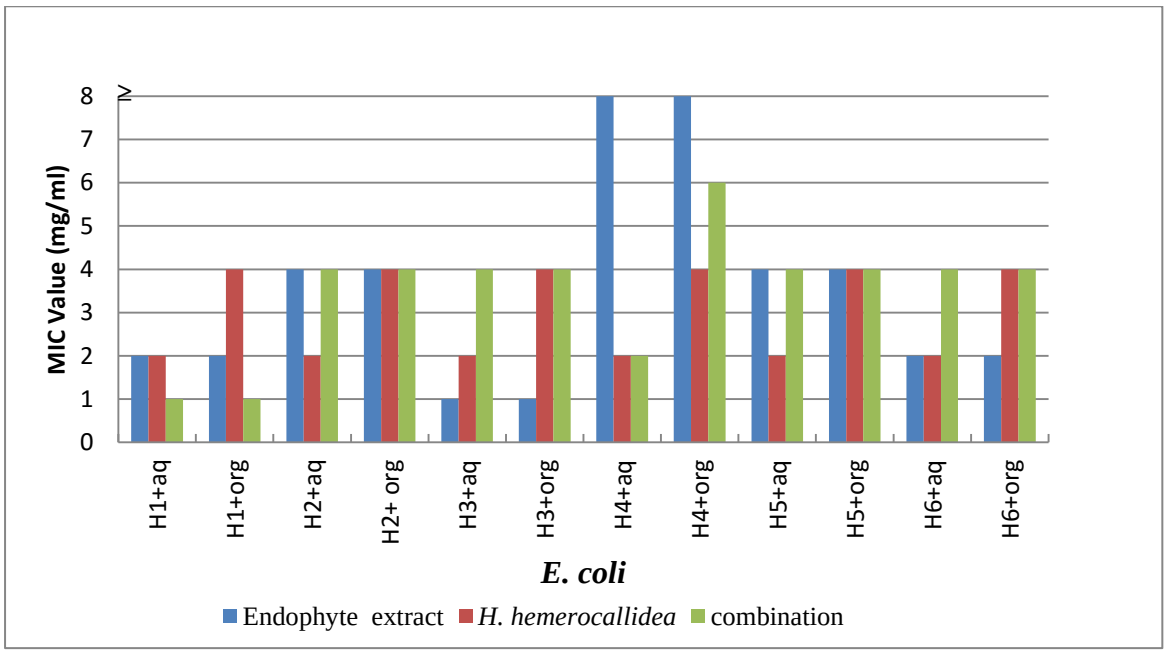
aq= aqueous extract, org= organic extract

Figure 4.2: Antimicrobial activity of *H. hemerocallidea*, endophyte extracts, and combinations against *E. faecalis*.



aq= aqueous extract, org= organic extract

Figure 4.3: Antimicrobial activities of *H. hemerocallidea*, endophyte extracts, and combinations against *P. aeruginosa*.



aq= aqueous extract, org= organic extract

Figure 4.4: Antimicrobial activities of *H. hemerocallidea*, endophyte extracts, and combinations against *E. coli*.

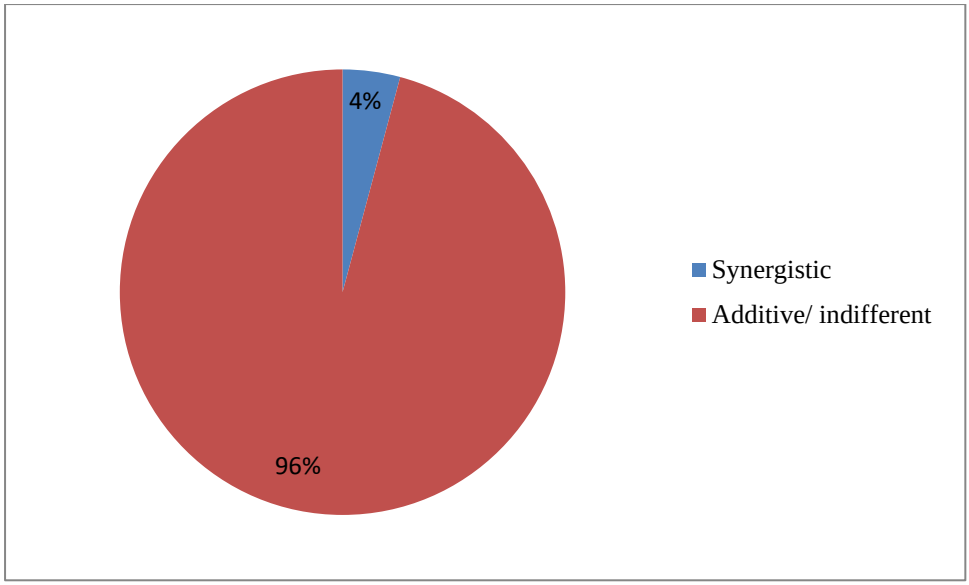


Figure 4.5: A summary of the interactions between *H. hemerocallidea* and isolated endophytes (total of 48 combinations tested).

Table 4.3: Summary of the FICI values calculated for endophyte and *H. hemerocallidea* extracts in combination when tested against *E. faecalis* and *E. coli*.

Sample	**FICI** ***E. faecalis***	**INT**	**FICI** ***E.coli***	**INT**
H11+aq	1.50	IND	0.50	SYN
H1+org	*	*	0.38	SYN
H2+aq	1.00	ADD	1.50	IND
H2+org	*	*	1.00	ADD
H3+aq	1.00	ADD	3.00	IND
H3+org	*	*	2.50	IND
H4+aq	*	*	*	*
H4+org	*	*	*	*
H5+aq	1.00	ADD	1.50	IND
H5+org	*	*	1.00	ADD
H6+aq	1.00	ADD	3.00	IND
H6+org	*	*	1.50	IND

4.2.4. Bioassay-guided isolation of active compounds

The H4 crude extract was fractionated into 11 fractions for possible isolation of the active compounds. These 11 fractions were tested against both Gram-positive and Gram-negative pathogens following the method in Chapter 2, Section 2.6. Fraction 7 and 8 displayed activity against *S. aureus* at 0.25 mg/mL (Table 4.4). However, at this point in the investigation, the crude extract for isolate H4 was depleted. The process of fermentation broth and ethyl acetate extraction of broth was re-visited to produce more extract for further chemical isolation studies. However, production of a new batch of extract displayed poor antibacterial activity of 1.00 mg/mL against *S. aureus*. The new batch of the extract was further studied using LC-MS. The fingerprints of both new (Figure 4.6) and old batch (Figure 4.7) of H4 extract were compared. The chromatogram indicate that the extracts differ chemically, thus demonstrating the active compounds produced by the endophyte during the initial fermentation-extraction procedure were not produced by the endophyte when this isolate was re-grown. Therefore isolation and characterisation of active compounds could not be confirmed. Kusari and Spiteller. (2011), have proposed that repeated sub-culturing of an active endophyte can result in loss of desired compounds and this is elaborated in Chapter 6, Section 6.2.

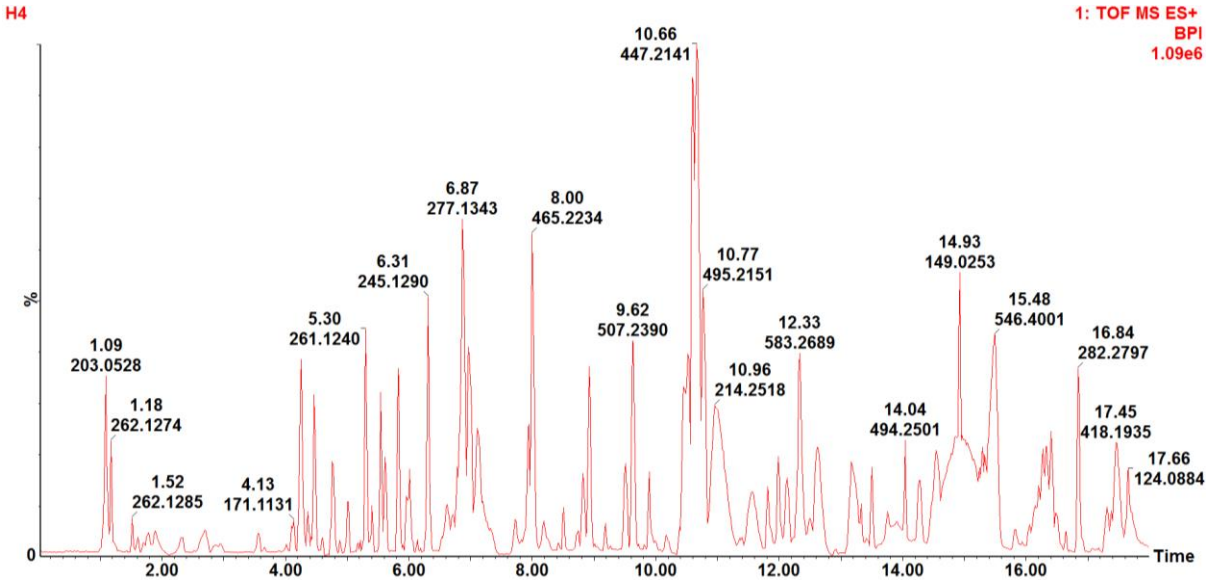


Figure 4.6: LC-MS chromatogram depicting molecular mass of compounds within crude extract produced by isolate H4 during the first round of fermentation process.

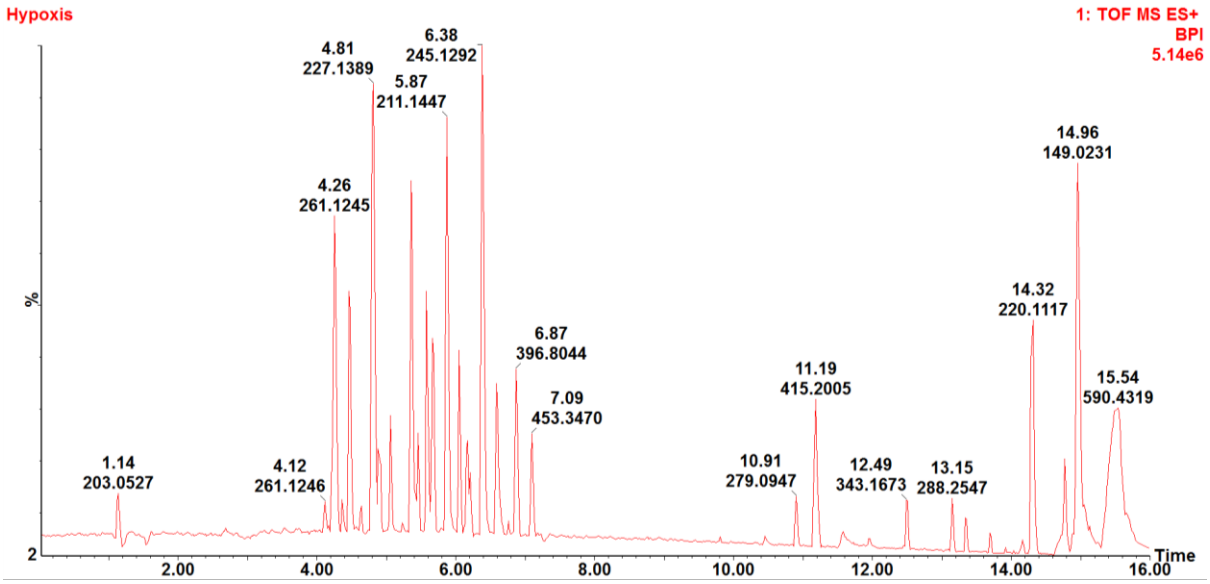


Figure 4.7: LC-MS chromatogram depicting molecular mass of compounds within crude extract produced by isolate H4 during the second round of fermentation process.

Table 4.4: Antimicrobial activity (mean MIC in mg/mL) of fractions from the crude extract of endophytic isolate H4 against *S. aureus*.

Fractions	***S. aureus*** **MIC (mg/mL)**
1	2.00
2	1.00
3	2.00
4	2.00
5	2.00
6	2.00
7	**0.25**
8	**0.25**
9	0.38
10	0.50
11	0.50
Crude extract	0.25
Positive control	1.25 µg/mL

*Values in bold depict active fractions

4.3. Summary

- This is the first study to explore the antimicrobial interactions between a South African medicinal plant species *H. hemerocallidea* and associated endophytes
- From a total of six endophyte isolates, one isolate H4 displayed noteworthy activity against *S. aureus* at 0.25 mg/mL.
- The endophyte H1 inhibited *S. aureus* and *E. faecalis* at 1.00 mg/mL, while isolate H3 inhibited *S. aureus* and *E. coli* at 1.00 mg/mL.
- There was one endophyte H1 that displayed synergy in combination with aqueous (FICI 0.50) and organic (FICI 0.38) *H. hemerocallidea* extract against *E. coli* at 1.00 mg/mL. Synergistic interactions between plant and endophyte could enhance the effectiveness of the plant when used or the treatment of UTI's.
- The remainder of combinations were additive/ indifferent, while none of the combinations were antagonistic.

Chapter 5

Gunnera perpensa



(www.bihrmann.com)

5.1. Introduction

Gunnera perpensa L. is a popular medicinal plant used in South Africa and belongs to the Gunneraceae family. This plant is also known as river pumpkin due to its characteristic large rounded leaves and its abundance near rivers (Bryant, 1909; van Wyk and Gericke, 2000). The fleshy pink roots are used by traditional healers for the treatment of female infertility and for the relief of menstrual cramps (van Wyk *et al*., 1997). Decoctions of the roots are also used as an antibacterial for the dressing of wounds and psoriasis (Watt and Breyer-Brandwijk, 1962). Furthermore, root decoctions are also used to treat sexually transmitted infections such as gonorrhoea and syphilis, as well as, urinary tract infections (UTI's) (Buwa and van Staden, 2006). This plant is also used in veterinary science for the purpose of speeding up the labour process and expulsion of the uterus, as well as applied topically to animals to protect them from tick bites and parasites (Pujol, 1990). *In vivo* studies carried out by Khan *et al*. (2004), displayed direct contractility of isolated uterine smooth muscle from rats, while a study by Brookes and Dutton (2007) confirmed that *G. perpensa* plant extracts possess utero-active components, therefore confirming the traditional use of this plant during pregnancy. *Gunnera perpensa* extracts display antimutagenic, anticarcinogenic and antihaemorrhagic activity in addition to antiseptic properties (Do Khac *et al*., 1990; Loarca-Piña *et al*., 1996; Buwa and van Staden, 2006). *Gunnera perpensa* root extracts display

conflicting antimicrobial results when tested with different MIC assays. These *in vitro* results range from poor to noteworthy activity against Gram-positive and Gram-negative bacteria. A study by Nkomo and Kambizi (2009) displayed noteworthy activity of methanol extract against *Bacillus cereus* at 0.50 mg/mL, while the aqueous extract displayed poor activity at 4.00 mg/mL (Drewes *et al*., 2005). A study by Drewes *et al*. (2005) displayed *G. perpensa* aqueous extract having poor activity at 6.40 mg/mL against *Klebsiella pneumoniae*, while Buwa and van Staden (2006) demonstrated that the aqueous extract exhibited activity against *K. pneumoniae* at 0.78 mg/mL, which is considerably more promising activity than the later. The same conflicting results which ranged from poor to noteworthy were noticed for methanol extracts when tested against *E. coli*. A study by Nkomo and Kambizi (2009) displayed noteworthy activity at 0.50 mg/mL against *E. coli*. In a study by McGaw *et al*. (2005), poor activity at a value as high as 6.25 mg/mL was observed against *E. coli*. Many studies propose that extraction solvents of the plant have an effect on the antibacterial activity exhibited. One such study carried out by McGaw *et al*. (2000) displayed noteworthy activity exhibited by aqueous extracts against *S. aureus* at 0.78 mg/mL, while the ethanol extract displayed poor results inhibiting *S. aureus* at 3.13 mg/mL (Table 5.1). Nkomo and Kambizi (2009) also reported that methanol extracts displayed greater antibacterial activity then the aqueous counterparts. McGaw *et al*. (2005), proposed that the antibacterial compounds are probably polar since water extracts display stronger activity than ethanol extracts. Even though *G. perpensa* is a popular South African medicinal plant the endophytes from this plant have not been investigated to date. Therefore this study focused on the isolation of *G. perpensa* endophytes to investigate antibacterial activity, and the interaction between endophytes and host plant to determine if these endophytes display antibacterial activity that is equivalent to that of plant extract or more promising activity then plant extracts.

Table 5.1: Summary of MIC assays (mg/mL) of *G. perpensa* extracts against nosocomial infections from 2004-2013.

***G. perpensa* extract (mg/mL)**	**Gram-positive**				**Gram-negative**			**References**
	B. subtilis	***B. cereus***	***E. faecalis***	***S. aureus***	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	
methanol	n/t	n/t	n/t	1.00	4.00	n/t	>4.00	Steenkamp *et al*., 2004
methanol	n/t	n/t	3.13	3.13	6.25	n/t	>12.5	McGaw *et al*., 2005
methanol	n/t	0.50	n/t	1.00	0.50	1.00	1.00	Nkomo and Kambizi, 2009
DCM: methanol	n/t	n/t	n/t	0.50	n/t	n/t	2.00	Mabona *et al*.,

G. perpensa extract (mg/mL)	Gram-positive				Gram-negative			References
	B. subtilis	***B. cereus***	***E. faecalis***	***S. aureus***	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	
								2013
aqueous	n/t	4.00	8.00	4.00	4.00	6.40	n/t	Drewes *et al*., 2005
aqueous	12,5	n/t	n/t	0.78	0.78	0.78	n/t	Buwa and van Staden, 2006
aqueous	n/t	1.00	n/t	1.00	n/a	1.00	5.00	Nkomo and Kambizi, 2009
aqueous	n/t	n/t	n/t	4.00	n/t	n/t	8.00	Mabona *et al*., 2013
acetone	n/t	n/t	4.17	2.61	12.5	n/t	>12.5	McGaw *et al*., 2005
ethanol	3.13	n/t	n/t	6.25	1.56	1.56	n/t	Buwa and van Staden, 2006

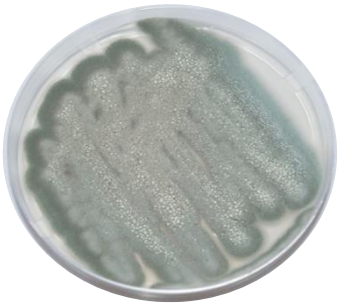
n/t= not tested, DCM= dichloromethane

5.2. Results and discussion

5.2.1. Endophytes isolated from *G. perpensa*

A total of five strains of endophytic fungi were isolated from the root of *G. perpensa*. Each isolate from the root differed in colour and texture (Table 5.2).

Table 5.2: Endophytes isolated from *G. perpensa* with allocated codes and macroscopic descriptions.

Code	**Description**	**Endophyte image**
GP1	Colonies are raised and light grey on the inside while the periphery of the colony is dark green in colour. Texture is velvety. Droplet formation is noticed on the top of hyphae.	
GP2	Colonies are flat and turquoise in colour (blue-green) with a powdery texture.	

Code	Description	Endophyte image
GP3	Colonies are raised and verrucose. They are yellow in colour, overlaid with an olive green mat which appears after 7 days. Texture is rubbery.	
GP4	Colonies are raised and rugose. They are blue-grey in colour on the periphery, while the inside of the colony is off-white in colour. With a velvety texture.	
GP5	Colonies are raised and irregular. They have a beige centre and an olive green periphery. With a white overlay. Texture is woolly.	

5.2.2. Antimicrobial activities of *G. perpensa* and endophytic extracts

Five crude extracts derived from the endophytic fungi were screened for antibacterial activity against four test pathogens. These extracts were tested alone and in combination with the crude root plant extract (Figure 5.1−5.4). Measurement of noteworthy results for natural products can be deduced based on the publication by Ríos and Recio (2005) (refer to Chapter 3, Section 3.3.1).

The aqueous and organic plant extracts of *G. perpensa* exhibited *in vitro* activity ranging from 0.50–≥ 8.00 mg/mL against the standard ATCC bacterial strains when tested alone.

Plant extracts displayed more promising activity against Gram-positive bacteria. The aqueous plant extract displayed noteworthy activity against *S. aureus* at 0.50 mg/mL (Figure 5.1). The noteworthy activity displayed against *S. aureus* in this study is substantiated by *in vitro* investigations of aqueous plant extracts in a study by McGaw *et al.* (2000) and Buwa and van Staden (2006), which displayed MIC results of 0.78 mg/mL against *S. aureus.* Mabona *et al*. (2013), demonstrated that DCM: methanol extracted *G. perpensa* inhibited *S. aureus* at 0.50 mg/mL. Organic *G. perpensa* extract inhibited *E. faecalis* at 1.00 mg/mL, while the aqueous extract displayed poorer activity than the organic extract and inhibited *E. faecalis* at 2.00 mg/mL. Several studies testing *G. perpensa* methanol, water, and acetone extracts against *E. faecalis* all displayed poor activity at 3.13 mg/mL, 8.00 mg/mL and 4.17 mg/mL against *E. faecalis*. *G. perpensa* extracts displayed poor activity (1.50 mg/mL–≥ 8.00 mg/mL) against the Gram-negative bacteria tested. This investigation displayed antibacterial activity against *E. coli* aqueous extracts at 1.50 mg/mL and inhibited organic extracts at 2.00 mg/mL. Most investigations that test *G. perpensa* extracts against *E. coli* demonstrate poor activity Steenkamp *et al*. (2004) and Drewes *et al*. (2005) displayed inhibitory activity at 4.00 mg/mL. Both Organic and aqueous extracts displayed poor activity against *P. aeruginosa* at 4.00 mg/mL and ≥ 8.00 mg/mL. Many *in vitro* studies display results that are consistent with the later results, MIC results for methanol and aqueous extracts tested against *P. aeruginosa* range between 1.00− >12.5 mg/mL (Table 5.3) (Steenkamp *et al*., 2004; McGaw *et al*., 2005; Nkomo and Kambizi, 2009; Mabona *et al*., 2013).

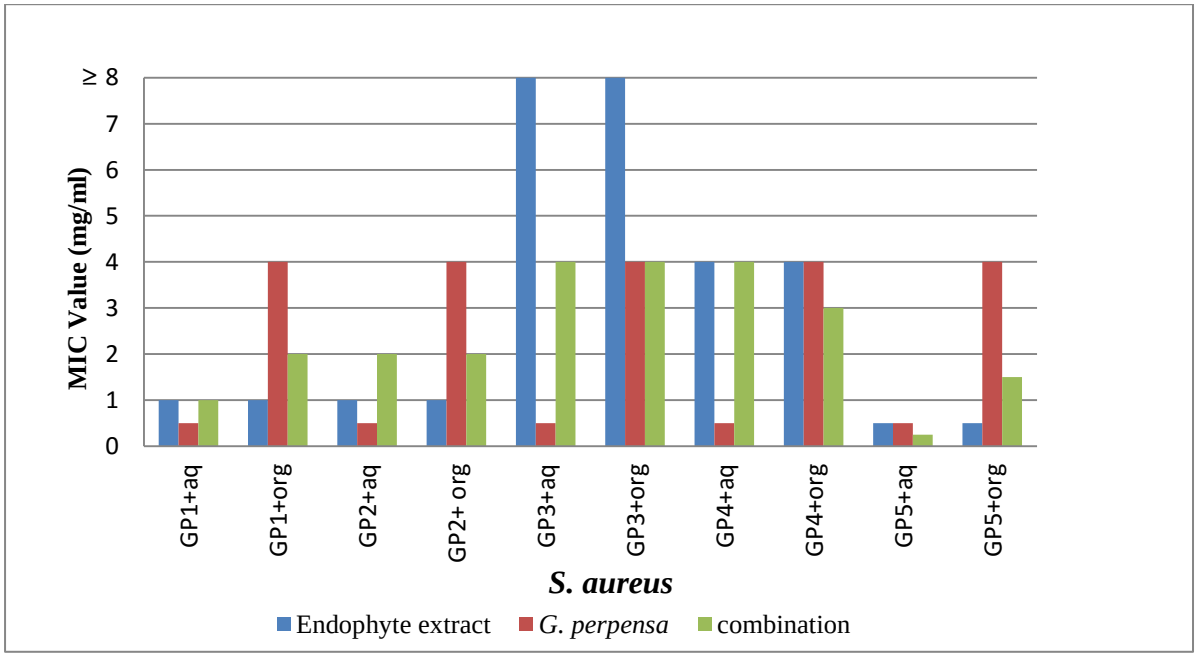
The endophyte isolate GP5 exhibited noteworthy activity at 0.50 mg/mL against *S. aureus*, while isolate GP1 and GP2 inhibited *S. aureus* at 1.00 mg/mL. Furthermore, GP5 and GP1 displayed moderate activity at 1.00 mg/mL against *E. coli*. Endophyte isolates did not show any promising activity against *E. faecalis* and *P. aeruginosa* (Figure 5.1−5.4). Endophytic fungi from *G. perpensa* have not been isolated previously. This study demonstrates for the first time that endophyte isolate GP5 exhibited noteworthy activity against *S. aureus* when compared to the plant extracts (Mabona *et al*., 2013).

5.2.3. Antimicrobial activity of plant and endophytic fungal extracts in combination

A total of 40 combinations were tested against four pathogens. Two combinations displayed synergistic activity. Endophyte GP5 with the aqueous extract exhibited synergistic activity

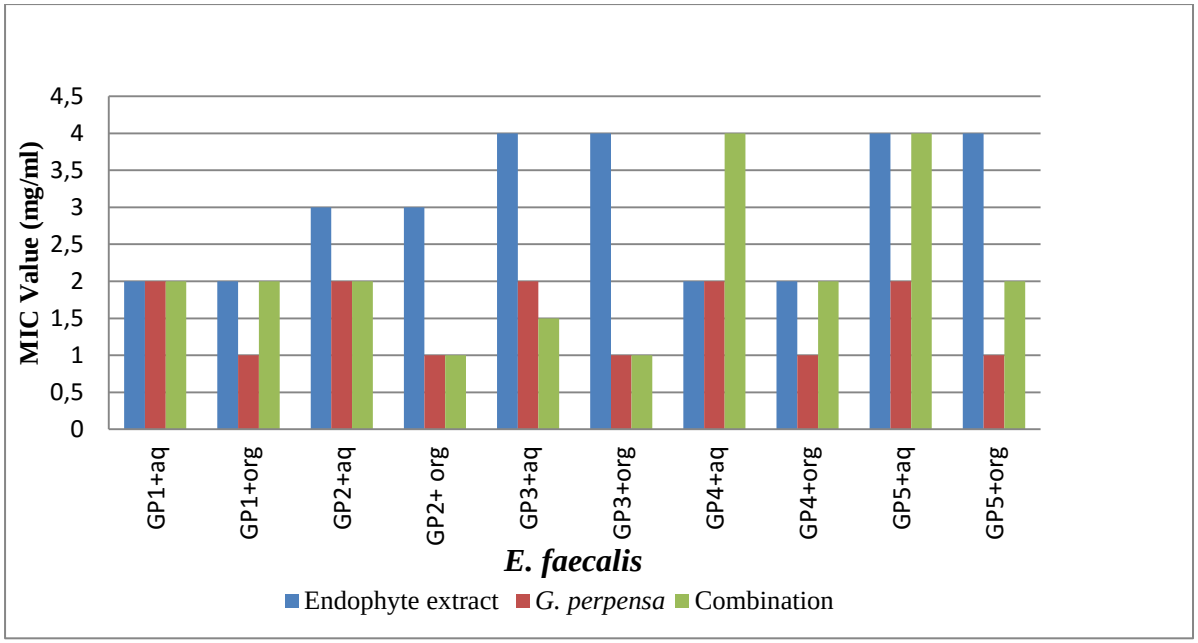
against *S. aureus* at 0.25 mg/mL and at a FICI value of 0.50 (Figure 5.1). This synergistic combination displayed noteworthy activity against *S. aureus* which is a very common nosocomial pathogen that plays a role in infectious wounds, and therefore substantiate the traditional use of this combination for wound healing (Archer, 1998).

The combination of endophyte GP5 with the aqueous extract of *G. perpensa* inhibited *E. coli* at 0.50 mg/mL and at an FICI of 0.42 (Figure 5.4). This substantiated the traditional use of the combination for the treatment of UTI's, which is predominantly caused by *E. coli* (Ronald, 2002). Figure 5.3 demonstrated that the combination of GP4 and aqueous extract displayed synergistic activity against *P. aeruginosa*, while the same combination displayed antagonism against *S. aureus* at a calculated FICI value of 4.5 (Table 5.3) Certain combinations may appear to be synergistic when making a tentative interpretation of the graph, however FICI values calculated for the following combinations GP4+org against *S. aureus*, GP3+aq against *E. faecalis*, GP4+org against *P. aeruginosa*, lend themselves to additive activity (Table 5.3). In summary the majority of combinations were additive or indifferent (Figure 5.5). As expected, the antimicrobial activities of the positive control, which was ciprofloxacin, were found to be superior to the combinations of *G. perpensa* and endophyte isolates. The positive control inhibited *S. aureus* at (1.25 µg/mL), *E. faecalis* (0.16 µg/mL), *P. aeruginosa* (0.63 µg/mL), and *E. coli* at (0.63 µg/mL).



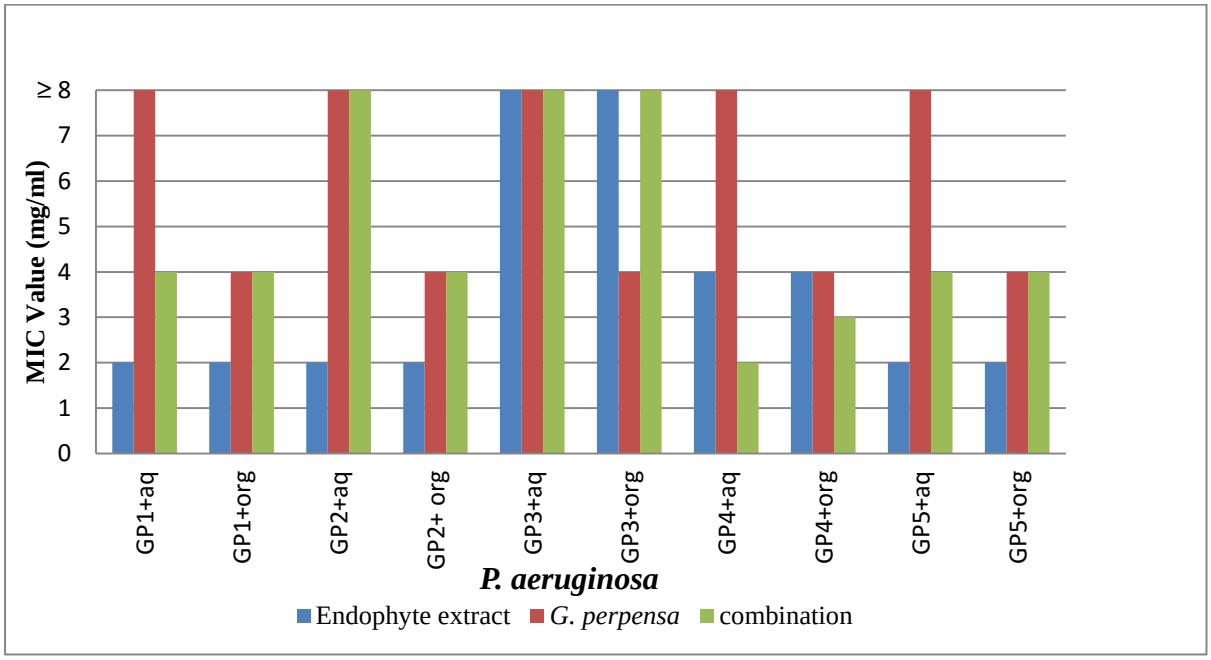
aq= aqueous extract, org= organic extract

Figure 5.1: Antimicrobial activities of *G. perpensa*, endophyte extracts, and combinations against *S. aureus*.



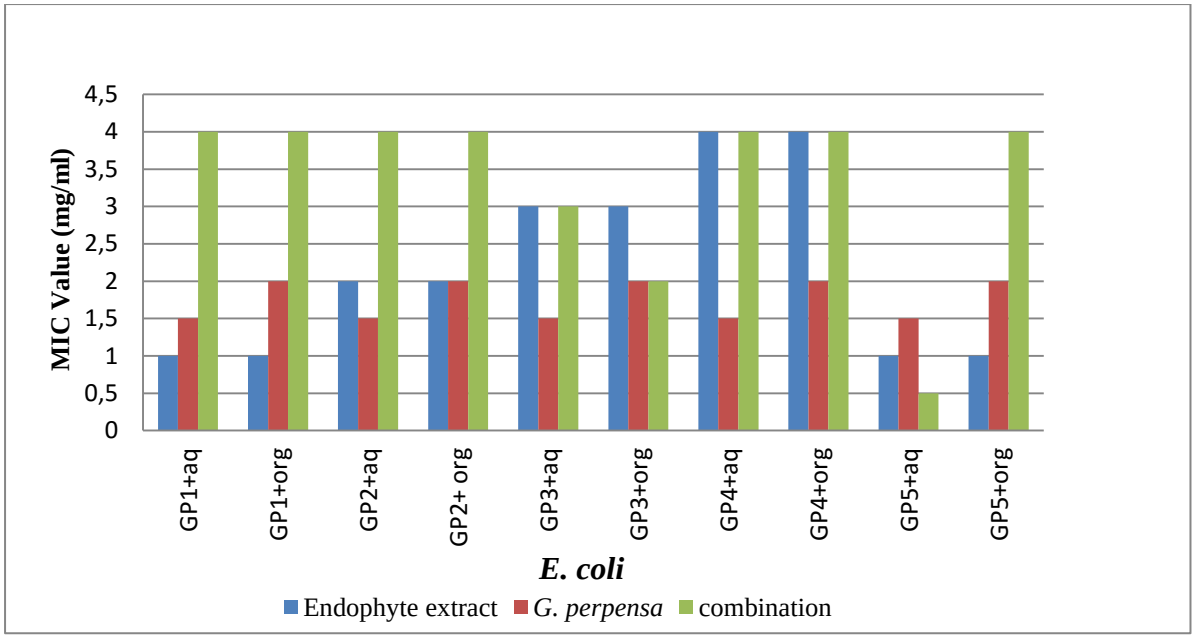
aq= aqueous extract, org= organic extract

Figure 5.2: Antimicrobial activities of *G. perpensa*, endophytes extracts, and combinations against *E. faecalis*.



aq= aqueous extract, org= organic extract

Figure 5.3: Antimicrobial activities of *G. perpensa*, endophyte extracts, and combinations against *P. aeruginosa*.



aq= aqueous extract, org= organic extract

Figure 5.4: Antimicrobial activities of *G. perpensa*, endophytes extracts, and combinations against *E. coli*.

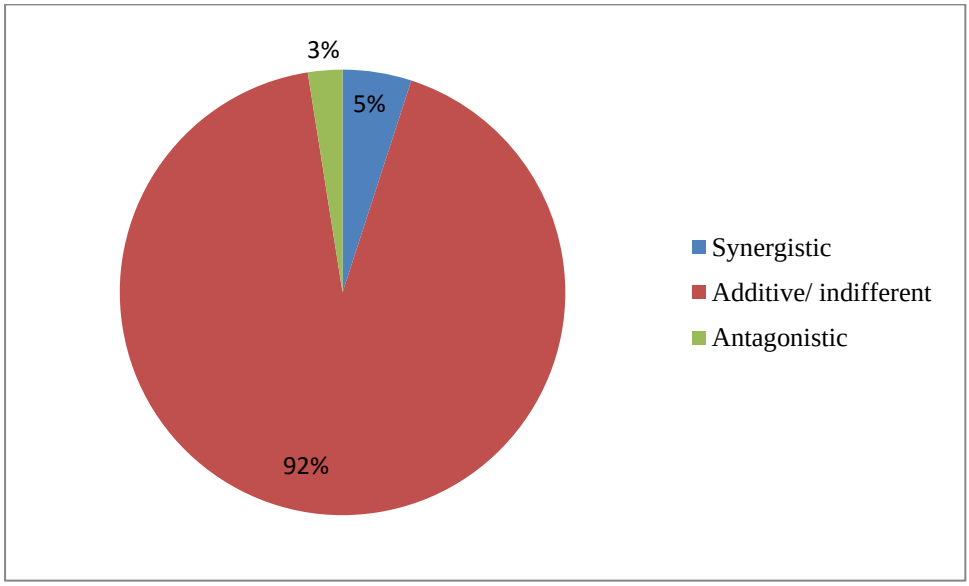


Figure 5.5: A summary of the interactions between *G. perpensa*, and isolated endophytes (total of 40 combinations tested).

Table 5.3: Summary of the FICI values calculated for endophytes and the *G. perpensa* extracts in combination.

Sample	FICI *S. aureus*	FICI *E. faecalis*	FICI *P. aeruginosa*	FICI *E. coli*
GP1+aq	1.50	1.00	*	3.33
GP1+org	1.25	1.50	1.50	3.00
GP2+aq	4.00	0.83	*	2.33
GP2+org	1.25	0.67	1.50	2.00
GP3+aq	*	0.56	*	1.50
GP3+org	*	0.63	*	0.83
GP4+aq	4.50	2.00	*	1.83
GP4+org	0.75	1.50	0.75	1.50
GP5+aq	0.50	1.50	*	0.42
GP5+org	1.69	1.25	1.5	3.00

aq= aqueous extract, org= organic extract

5.3. Summary

- This is the first study to explore the antimicrobial interactions between a popular South African medicinal plant species *G. perpensa* roots and the associated endophytes
- From a total of five endophyte isolates, one isolate GP5 displayed activity against *S. aureus* at 0.50 mg/mL when tested independently.
- Endophyte GP5 and the aqueous plant extract exhibited noteworthy synergistic activity against *S. aureus* and *E. coli* at 0.25 mg/mL and 0.50 mg/mL respectively.
- From a total of 40 combinations tested against four pathogens only one combination displayed antagonistic activity (GP4 and aqueous extract). The majority of combinations were additive or indifferent.

Chapter 6

Conclusion and future recommendations

6.1. Summary

The aim of this study was to investigate the antimicrobial activity of endophytes isolated from popular South African medicinal plant roots (*P. sidoides*, *H. hemerocallidea*, and *G. perpensa*), and also to investigate the interactions between endophytes and host plant using combination studies. The hypothesis that there is possibly synergistic activity present between certain endophytes and host plant has been demonstrated by means of combination antimicrobial *in vitro* studies between *P. sidoides*, *H. hemerocallidea* and *G. perpensa* and their respective endophytes. A total of 232 combinations were tested against four pathogens. Of these combinations, 10.78% were found to be synergistic, 5.60% were antagonistic, 25.43% were additive, and 58.19% were found to be indifferent in nature. By proving the hypothesis of this study, it is proposed that when certain traditional antimicrobial plants appear to have poor *in vitro* antimicrobial activity, the activity could be attributed to the endophyte or from both endophyte and host plant interactions. The current study varies from previous global investigations, in that no combination studies have evaluated the interaction of endophytes and host plant. The identification of synergistic interactions can be advantageous by providing new insight into medicinal plants used traditionally for infections, but that display moderate to poor *in vitro* antimicrobial activity when plant extracts are tested independently. During the study emphasis was placed on the results from synergistic interactions, however, more than 25% of interactions tested displayed additive activity which should also be considered as positive.

Even though the aim of the study was to evaluate synergistic interactions between medicinal plant and endophytes, the occurrence of antagonistic interactions cannot be ignored. With *P. sidoides* the most frequent antagonistic interaction was noticed with Gram-negative strains. Some endophytes (PS10, PS11, and PS12), when tested with both aqueous and organic extracts, displayed antagonism against *E. coli*, while PS1, PS4 and PS12 in combination with the organic extract displayed antagonism when tested against *P. aeruginosa.* Antagonism was

also observed with PS1, PS4, PS9 when tested against *S. aureus* (Figure 3.1, 3.3 and 3.4). With *G. perpensa* only one combination displayed antagonism, GP4 and aqueous extract against *S. aureus*. Antagonism did not occur with host plant and endophytes isolated from *H. hemerocallidea*. Even though antagonistic interactions were not frequently observed during testing of combinations, antagonistic activity between plant and endophyte can give us insight into the strain of fungi that work in opposition to the plant. This antagonism could possibly be explained as a means for the endophyte to survive in their habitat against biotic and abiotic factors present.

There is still much research needed in this area of study, isolation of endophytes from traditionally used medicinal plants native to South Africa, further contributions in this field will increase a chance at finding novel secondary metabolites

6.1.1. Antimicrobial activity of the individual plant and endophyte extracts

Both *P. sidoides* and *H. hemerocallidea* displayed poor antimicrobial activity against the tested pathogens (Fig 3.1−3.4 and Fig 4.1−4.4), while *G. perpensa* aqueous extract displayed noteworthy activity against *S. aureus,* which is in accordance with literature (Mabona *et al*., 2013). Nineteen endophytes were isolated from *P. sidoides* with endophyte *Penicillium skrjabinii* display antibacterial activity against *S. aureus* at 0.03 mg/mL (batch 1 & 2) and *E. coli* at 0.06 mg/mL (batch 1), and 0.13 mg/mL (batch 2) respectively . When evaluating *in vitro* antimicrobial results from a previous investigation, Kayser and Kolodziej (1997) displayed that *P. sidoides* crude extracts inhibited respiratory pathogens poorly at values ranging between 5.00−7.50 mg/mL. The crude extract from endophyte *P. skrjabinii* demonstrated higher antimicrobial effects against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria at MIC's (average of batch 1 and 2) of 0.03 and 0.09 mg/mL. This demonstrates that an endophyte isolate from *P. sidoides* exhibits greater inhibitory activity against pathogens when compared to their respective host plant extracts. This could be due to the fact that when plant extracts are prepared they are dried out, which leads to the removal of endophytes from the plant before testing.

Hypoxis hemerocallidea gave rise to six endophyte isolates. Endophyte H4 inhibited *S. aureus* at 0.25 mg/mL. This endophyte extract inhibited *S. aureus* at MIC values lower than values reported by Laporta *et al*. (2007) at 3.00 mg/mL, Katerere and Eloff (2008) at

0.31−0.63 mg/mL, Ncube *et al*., (2011) at 0.39−12.5 mg/mL, and Ndhlala *et al*., (2013) at 1.56 for plant extracts tested against *S. aureus*. While, five strains isolated from *G. Perpensa*, one isolate GP5 inhibited *S. aureus* at 0.50 mg/mL, which displayed equivalent activity when compared to MIC values of plant extract which was reported by an investigation by Mabona *et al*. (2013). All active endophytes displayed antimicrobial activity that substantiated antimicrobial uses of plant by traditional healers.

6.1.2. Endophyte and host plant combination studies

Three medicinal plant roots (*P. sidoides*, *H. hemerocallidea*, and *G. perpensa*) were tested in combination with the endophytes that were isolated from the respective plant species. When evaluating a total of 144 interactions between *P. sidoides* and endophytes, 14.58% of combinations displayed synergy against both Gram-positive and Gram-negative test pathogens. Some endophytes (PS15 against *E. faecalis*, PS18 against *E. coli*, PS16 and PS18 against *S. aureus*) displayed synergy with both the aqueous and organic extracts of the host plant. While most of the synergistic interactions between plant and endophytes originated between *P. sidoides* endophytes and host plant, *H. hemerocallidea* and *G. perpensa* only exhibited two combinations each with a synergistic interaction. The following endophytes displayed synergy from *H. hemerocallidea* and *G. perpensa*, the endophyte H1 in combination with aqueous and organic extract against *E. coli*; and GP5 and aqueous *G. perpensa* extract against *S. aureus* and *E. coli*. The notable synergy displayed between certain isolates and their host plant proves the hypothesis that certain popular traditional plants that show varying inconclusive antibacterial activity could possibly be used as an adequate antimicrobial due to synergistic activity between endophytes and there host plant.

6.1.3. Identification of endophytic fungi and antimicrobial secondary metabolites

The isolate *Penicillium skrjabinii* (Shmotina and Golovleva, 1975) showing the most promising antimicrobial activity from *P. sidoides* was identified, using PCR amplification and DNA sequence analysis. The extract produced by this isolate was chosen for further chemical characterization. The antimicrobial compound was identified as dibutyl phthalate, which is a known plasticizer that can be applied to various products ranging from detergents to lubricating oils and can be of much importance in an industrial setting (Blount *et al*., 2000).

6.2. Limitations during the study and future recommendations

When working with fungal endophytes there are several limitations. One limitation experienced during this study was the yield of extracts during the fermentation procedure which was mostly too low, and therefore the isolates that exhibited antimicrobial activity upon initial *in vitro* antimicrobial testing did not always produce the same results when these isolates were put through a second fermentation procedure. This was the case with isolate H4 and GP5 from *H. hemerocallidea* and *G. perpensa*. The temperature and incubator parameters were not changed during the second fermentation procedure; however, the extracts when tested again did not exhibit antimicrobial activity, which leads to the assumption that the antimicrobial secondary metabolites were not produced by the endophyte during the second fermentation procedure. It was proposed by Kusari and Spiteller (2011) and Kusari *et al*. (2014) that in most cases repeated sub-culturing of an endophyte results in the endophyte losing its potential to produce desired compounds due to obliteration of the biosynthesis process or potential loss of an important natural trigger. While the same limitations were experienced with the isolate *P. skrjabinii* from *P. sidoides,* it was discovered that stationary incubator conditions as opposed to shaking conditions were imperative for the production of antimicrobial compounds. It is proposed that a useful way to tackle the low yield of secondary metabolites is optimising culture conditions for bioactive endophytes (Amna *et al*., 2012; Chaichanan *et al*., 2014) or alternatively using genetic engineering technology, which can be used to identify the regulatory gene in a biosynthetic pathway to increase production of a specific secondary metabolite (Yu *et al*., 2010; Kaul *et al*., 2012). In spite of all the limitations, this is the first pilot study successfully undertaken on antimicrobial activity and interactions of fungal endophytes with host plant at the Department of Pharmacy and Pharmacology (University of Witwatersrand) and thus provides a sound basis for future studies of a similar nature.

Fungal endophytes require a lengthy period of time for growth and production of secondary metabolites during the fermentation process. This process can last anything between 10 to 21 days, which posed as a major time constraint during laboratory investigations (Ding *et al*., 2010; Powthong *et al.*, 2012; Astuti *et al*., 2014). Certain fungal strains that displayed antimicrobial activity had to be re-grown to increase the yield of extract for chemistry testing; this added an additional time constraint.

Another limitation was identification of endophytic fungi, which required PCR amplification and DNA sequence analysis to be employed, however, the skills required for identification are not available in South Africa. Therefore these isolates had to be sent to the Westerdijk fungal biodiversity institute in the Netherlands for identification. Further recommendations have been provided for future studies.

6.2.1. Cytotoxicity studies on endophyte extracts

Researchers around the globe have isolated compounds from endophytes that display antimicrobial activity; however, the challenge that is often faced is that some of these compounds may be highly toxic to humans (Yu *et al.*, 2010). Certain antimicrobials are toxic to humans .e.g. dibutyl phthalate which was isolated from *P. skrjabinii* which does not only exhibit antimicrobial activity, but has also displayed toxicity during *in vivo* studies on rats (Lehmann *et al*., 2004; Swan *et al*., 2005). An interesting *in vitro* study would be to investigate the toxicity of different ratios of endophyte extract to host plant in combination and interpret these results using isobologram. Other *in vitro* studies used to test cytotoxicity can include human cell lines to determine if certain extracts have the potential to inhibit cancer cells. After the isolation of Taxol from the endophytic fungus *Taxomyces andreanae,* many endophyte studies have focused on the isolation of anti-cancer compounds from endophytic fungi (Stierle *et al*., 1993a; Pettit *et al*., 2010; Amna *et al*., 2012; Perveen *et al*., 2017). One such example is the isolation of the novel anti-cancer compound camptothecin from endophytic fungi (Kusari *et al*., 2009; Ran *et al*., 2017). Therefore, in addition to *in vitro* antimicrobial studies, cytotoxicity studies can also be carried out on endophytic fungi. Yu *et al*. (2010) proposed that researchers can be successful in creating novel antimicrobials if the focus is on modifying chemical structures of some of these metabolites to increase the specificity of antimicrobial activity and simultaneously reduce toxicity and side effects to humans and animals.

6.2.2. Endophyte combination studies

Kusari *et al*. (2014) point out limitations in studying the interaction between endophytes and there host plant due to their intricate sophisticated metabolic networks. However, a good starting point to determine which strains of endophytic fungi are interacting with the host plant on a synergistic or antagonistic level would be to test their crude extracts in combination using broth micro-titre dilution assay. In this study a novel approach was taken with testing host plant with endophytic fungal strains, however, this approach can be applied to testing endophytic fungal isolates in combination with other endophytes from the same host plant to get insight into possible synergistic or antagonistic interactions that occur in an endophytic microbiome.

6.2.3. Endophyte co-culturing

When co-cultivating endophytes, is it a serendipitous process or structured? Co-cultivating is a concept where two or more micro-organisms are grown together in a fermentation process, which could either be solid-state or liquid state fermentation (Kusari *et al*., 2014). Fungal endophyte extracts can be tested in combinations to detect which isolates are synergistic or antagonistic. This process of eliminating endophytes that are indifferent can initiate a more structured approach to co-cultivating endophytes during the fermentation process. Since co-cultivating endophytes that are either synergistic or antagonistic could pose as biotic stimuli needed as a trigger for a biosynthetic pathway responsible for the production of a novel antimicrobial. Also, co-cultivation and multi-cultivation can promote cross-talk between organisms which is an essential process for the production of secondary metabolites (Pettit *et al*., 2010; Wang *et al*., 2015; Akone *et al*., 2016). Researches have already initiated investigations into secondary metabolite production from co-cultivation of fungal endophytes (Zhu and Lin, 2006; Li *et al*., 2011), and the study of fungal communities in conjunction with the host plant instead of separately as both communities impact each other as they reside together (Frey-Klett *et al*., 2011; Marmann *et al*., 2014). A study by *Akone et al*. (2016), displayed that co-cultivation of the endophytic fungus *Chaetomium* spp. with pathogenic bacteria, *Bacillus subtilis* which resulted in five new natural products that were not previously present with monoculturing.

6.2.4. Combating the limitations of artificial fermentation procedure

When endophytes are immersed into an artificial fermentation procedure it may result in stagnation in the production of important secondary metabolites due to a lack of natural triggers (Wang *et al.*, 2015). A proposition is for dried plant extracts to be added to the fermentation broth during the fermentation process as the presence of plant extracts could mimic the natural environment that the fungal endophyte is more familiar with. However, it would be a challenge to determine the correct weight of dried plant extract to add per millilitre of broth to attain results. Adequate controls will need to be in place to determine if the plant extract had an advantageous effect on secondary metabolite production. A recent study by Ding *et al*. (2017) pursued co-cultivating of plant seedlings with endophytic fungi to improve bioactive secondary metabolite production.

6.2.5. Optimising culture conditions for an increase yield of compounds

The endophytes that display antimicrobial activity can be further investigated as biosynthetic pathways of endophytic fungi (and) can be optimized by altering culture condition variables to support the production of important secondary metabolites, as abiotic factors play a significant role in the production of important compounds (Amna *et al*., 2012; Chaichanan *et al*., 2014; Kumar *et al*., 2016; Shen *et al*., 2016). Variables that can be altered are the pH of fermentation broth, media, temperature, humidity, agitation, minerals and nutrients. Sattelmacher (2001) made the observation that these variables are specifically defined when doing *in vitro* investigations, which differs from the endophytes exposure to abiotic factors when in its natural habitat. Therefore conducting investigations which manipulate different abiotic variables (temperature, humidity etc.) to optimize conditions for monocultures may result in more successes with increasing the yield of important compounds.

A study by Amna *et al*. (2012) investigated the effect of nutrient combinations media manipulations to increase the yield of camptothecin (an anticancer compound) production from endophytic fungi. A study by Shen *et al*. (2016) which aimed to increase hypocrelin (a photodynamic therapy compound) production from endophytic fungi manipulated several culture conditions one at a time, such as, pH, incubation time, rotary speed and mycelial age, etc. These methods proved successful in optimizing culture conditions which led to an increased yield of the active compound.

6.2.6. Increasing the diversity of endophytes isolated from a plant

Different seasons give rise to different endophyte isolates and, in turn, this alters the metabolites produced in a plant. As light intensity, temperature and moisture not only have an effect on the endophytes present but also the bioactive metabolites produced (Wearn *et al*., 2012; Kim *et al*., 2013; Nalini *et al*., 2014). Kim *et al*. (2013) proposed that different seasons also have an effect on the distribution of fungal endophytes in the host plant. While a study by Wearn *et al*. (2012) investigated herbaceous grassland plants and noticed that roots of the plants displayed more fungal diversity then leaves, and endophytes isolated during summer where more diverse than the winter season. Endophytes have been isolated from seeds, stems, roots and leaves (Arnold and Lutzoni, 2007; Nalini *et al*., 2014; Santos *et al*., 2015). Each part of a plant organ harbours complex endophytic microbiome and that is why it is imperative to isolate endophytes from different parts of medicinal plants, using ethnobotany as a guide as to which plant part is the most appropriate (Ji *et al*., 2005; Compant *et al*., 2016). It is previously stated that there is a great diversity of endophytes from plants grown in areas of great biodiversity like tropical rain forests, medicinal plants, and special habitats like near ancient land masses (Strobel and Daisy, 2003; Strobel *et al*., 2004). The latter is the rationale that was used before endophyte studies were undertaken, since South Africa is not only a biodiversity hotspot but it is also a country that is rich in cultural heritage that has strongly influenced traditional healing practices with the use of medicinal plants (Watt and Breyer-Brandwijk, 1962; Smith, 1966; van Wyk, 1996; van Wyk *et al*., 1997).

6.3. Final conclusion

The majority of the plant-endophyte combinations tested in the current study demonstrated additive/indifferent interactions. However, a few notable synergistic and antagonistic interactions were also identified, which could have a considerable impact on future studies in understanding the interaction between host plant and endophyte. It was established that endophytes have a positive impact on the antimicrobial properties of plants. This study demonstrates that the perplexities attached to the traditional use of certain plants that displayed poor antimicrobial activity *in vitro* can possibly be attributed to either endophytes that produce antimicrobial compounds or due to the synergistic antimicrobial activity with the host plant. There is a great need for more research in this field of endophytes especially since South Africa has a wealth of endemic flora to explore (van Wyk, 1996). Further research in

this field can ultimately give us hope in finding new antimicrobials that can contribute to the ever-increasing problem of antimicrobial resistance.

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Appendix A
Abstract for oral presentation at the Indigenous Plant Use Forum (IPUF) Conference - 2017

Endophytic fungi and their role in antimicrobial activity

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Abstract

Endophytes are micro-organisms that colonize plant tissue internally. They are characterized by the feature that they do not cause any harm to the host plant. In recent years fungal endophytes have generated great interest around the globe as they produce a wide array of functional metabolites. Studies have shown that endophytic fungi are producers of bioactive metabolites that can be useful in the pharmaceutical industry. The aim of this study was to investigate the antimicrobial activity of endophytes from three popular indigenous medicinal plant roots (*Pelargonium sidoides, Hypoxis hemerocallidea* and *Gunnera perpensa*). Fungal cultures were isolated from the roots and extracts were prepared and tested independently and in combination with the host plant to determine if any synergistic interactions are apparent. Antimicrobial studies were undertaken using the micro plate dilution assay against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria. A total of 19 endophytic fungi were isolated from the *Pelargonium sidoides* root. Seven fungal extracts exhibited antibacterial activity either alone or in combination with the host plant. Noteworthy results were exhibited by a fungal isolate from *Pelargonium sidoides* root with a MIC value of 31 µg/mL against *S. aureus* and 63 µg /mL against *E. coli*. *Hypoxis hemerocallidea* root gave rise to six endophytic fungi and *Gunnera perpernsa* root gave rise to five fungal strains. *Hypoxis hemerocallidea* and *Gunnera perpensa* both had one endophytic fungal strain that exhibited antibacterial activity against *S. aureus* with MIC values of 250 µg/mL and 500 µg/mL respectively. Results indicate that endophytic fungi from these South African medicinal plants could be a promising source of antimicrobial metabolites.

Appendix B
Abstract for oral presentation at the School of Therapeutic Sciences (STS) 2017 research day

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Antimicrobial medicinal properties of endophytes.

Zainub B. Aboobaker[a], Sandy F. van Vuuren[a], Alvaro M. Viljoen[b], Sushil K. Chaudhary[b], Weiyang Chen[b], Pedro W. Crous[c].

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[b]*Department of Pharmaceutical Sciences, Faculty of Sciences, Tshwane University of Technology, Private Bag X680, Pretoria 0001, South Africa.*

[c] *Westerdijk Fungal Biodiversity Institute, Netherlands*

Introduction: Studies have shown that endophytic fungi are producers of a wide array of bioactive metabolites that can be useful as antimicrobials. Endophytes, like other fungal antimicrobials (penicillin) may have potential as a novel anti-infective.

Method: The *in vitro* antimicrobial activity of endophytes from three medicinal plant roots (*Pelargonium sidoides*, *Hypoxis hemerocallidea* and *Gunnera perpensa*) were undertaken using the micro-titre plate dilution assay against *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 27853). Fungal cultures were isolated from the roots and extracts were prepared and tested independently and in combination with the host plant to determine if any synergistic interactions are apparent. Chemical diversity of the isolated endophytes was explored using prep-HPLC to fractionate bioactive compounds.

Results: A total of 19 endophytic fungi isolated from the *P. sidoides*, 14.58% displayed an antimicrobial interaction against one or more pathogens when in combination with the host plant. *Hypoxis hemerocallidea* and *Gunnera perpensa* both had one endophytic fungal strain that exhibited antibacterial activity against *S. aureus* with MIC values of 250 µg/mL and 500 µg/mL respectively. The chemistry demonstrated complexity.

Discussion: Results indicate that endophytic fungi from these South African medicinal plants could be a promising source of antimicrobial metabolites and provide some insight into the combined antimicrobial efficacy with the host plant providing evidence of synergy.

Appendix C
Abstract for oral presentation at the 65th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research- 2017

South African endophytes- potential antimicrobial agents

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Abstract

Endophytes have been found in every plant species examined to date and have been recognized as potential sources of novel antimicrobials. The antimicrobial potential of endophytes isolated from South African medicinal plants have, however, been poorly explored. The aim of this study was therefore to investigate the antimicrobial activity of endophytes isolated from the roots of three popular South African medicinal plant species (*Pelargonium sidoides*, *Hypoxis hemerocallidea* and *Gunnera perpensa*) and explore the chemical diversity of the isolated endophytes. Fungal cultures were isolated from the roots, and extracts were prepared and tested independently and in combination with the host plant to determine if any synergistic interactions are apparent. Antibacterial studies were undertaken using the minimum inhibitory concentration (MIC) assay against Gram-positive (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212) and Gram-negative (*Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 27853) bacteria. A total of 19 endophytic fungi were isolated from the *P. sidoides* root. Four fungal extracts exhibited antibacterial activity either alone or in combination with the host plant. Noteworthy results were exhibited by a fungal isolate from *Pelargonium sidoides* root with a MIC value of 30 μg/mL against *S. aureus* and 60 μg/mL against *E. coli*. Six endophytic fungi could be isolated from the roots of *H. hemerocallidea*, and five from the roots of *G. perpensa*.

Hypoxis hemerocallidea and *G. perpensa* both had one endophytic fungal strain that exhibited antibacterial activity against *S. aureus* with MIC values of 250 μg/mL and 500 μg/mL respectively. Results indicate that endophytic fungi from these South African medicinal plants yield a complex chemistry and could be a promising source of antimicrobial metabolites. Furthermore, selected species exhibit a synergistic antimicrobial role with the host plant demonstrating a unique anti-infective role for medicinal plants.

Appendix D
Abstract for publication submitted to the *South African Journal of Botany*

Endophytic fungi isolated from *Pelargonium sidoides* DC: Antimicrobial interaction and isolation of a bioactive compound.

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[a] *Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, South Africa.*

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[c] *SAMRC Herbal Drugs Research Unit, Faculty of Science, Tshwane University of Technology, Private Bag X680, Pretoria 0001, South Africa.*

[d] *Westerdijk Fungal Biodiversity Institute, Netherlands.*

[e] *Department of Chemistry, University of Pretoria, Private Bag X20, Hatfield 0028, Pretoria, South Africa.*

Abstract

The aim of this study is to investigate the antimicrobial activity of endophytes isolated from the root of *Pelargonium sidoides* DC., and to explore the antimicrobial interactions between endophytic fungi and the host plant. The hypothesis explored is that medicinal plants used as an anti-infective may not only rely on the properties of the medicinal plant alone but also on the residing endophytes in the botanical matrix. The study further aimed to isolate and identify secondary metabolites from the endophyte exhibiting promising antimicrobial activity. Fungal cultures were isolated from *P. sidoides* root material, and extracts were prepared and tested independently and in combination with the host plant to determine the interactions. Antimicrobial studies were undertaken using the minimum inhibitory concentration (MIC) assay against Gram-positive (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212) and Gram-negative (*Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 27853) bacteria. The bioactive compound was identified using High Performance Liquid Chromatography-Mass Spectrometry-Solid Phase Trapping-Nuclear Magnetic Resonance (HPLC-MS-SPT-NMR). The identification of the bioactive

endophyte was undertaken by polymerase chain reaction (PCR) amplification and deoxyribonucleic acid (DNA) sequence analysis of the internally transcribed spacer regions and intervening 5.8S nrRNA gene and partial beta-tubulin gene. The crude extract from the fungal isolate *Penicillium skrjabinii* exhibited antimicrobial activity against *S. aureus* and *E. coli* at 0.03 mg/mL and 0.09 mg/mL respectively. Bioassay-guided isolation was carried out and the major compound, dibutyl phthalate which is known to be produced by micro-organisms was isolated from the most active fraction. A number of endophytes displayed a synergistic interaction with the host plant. This is the first study reporting *P. skrjabinii* as an endophyte that produces dibutyl phthalate and further provides insight to endophytic interactions with the host plant (*Pelargonium sidoides* root).

Appendix E
Ethics clearance certificate for microbial cultures

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Tobias Health Sciences Building, 2nd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-160720-3 20/07/2016

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Prof S van Vuuren, Z Aboobaker (Student no 0701918E).**

Project title: Endophytes hosted by medicinal plants may potentially exhibit antmicrobial actiivity.

Reason: This is a laboratory study using bacterial cultures. The bacterial strains include:
Staphylococcus aureus (ATCC 25923), Enterococcus faecalis (ATCC 29212), Pseudomonas aeruginosa (ATCC 27853), Escherichia coli (ATCC 8540).

There are no human participants.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2016 -07- 20
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi.

Appendix F
Plagiarism report

Endophytic fungi hosted by medicinal plants may potentially exhibit antimicrobial activity

ORIGINALITY REPORT

16%	10%	12%	6%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

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2	Z. Hübsch, R.L. Van Zyl, I.E. Cock, S.F. Van Vuuren. "Interactive antimicrobial and toxicity profiles of conventional antimicrobials with Southern African medicinal plants", South African Journal of Botany, 2014 Publication	1%
3	Z Aboobaker, S van Vuuren, A Viljoen, P Crous. "South African endophytes- potential antimicrobial agents", Georg Thieme Verlag KG, 2017 Publication	1%
4	wiredspace.wits.ac.za Internet Source	1%
5	Ané Orchard, Maxleene Sandasi, Guy Kamatou, Alvaro Viljoen, Sandy van Vuuren. " The Antimicrobial Activity and Chemometric Modelling of 59 Commercial Essential Oils against Pathogens of Dermatological Relevance ", Chemistry & Biodiversity, 2017 Publication	<1%