Screening, Purification and Characterisation of anti-*Pseudomonas aeruginosa* Compounds Produced by Endophytic Fungi from *Kigelia africana*

by

Mamokoena Kuali

(211512973)

Submitted in fulfilment of the academic requirements for the degree of Master of Science (MSc) in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at University of KwaZulu-Natal (Westville Campus)

As the candidate's supervisors, we have approved this dissertation for submission.

Signed: Name: R. Govinden   Date:

Signed: Name: R. Moodley   Date:

ABSTRACT

The emergence of new diseases and drug resistant pathogens coupled with the side effects presented by conventional or synthetic drug use calls for the discovery of new antibiotics and chemotherapeutics. *Pseudomonas aeruginosa* is a multifaceted Gram-negative opportunistic pathogen which is responsible for ten percent of all hospital infections. This, therefore, directed the search for novel bioactive molecules with new targets from previously understudied sources.

Plants have bioactive compounds that have been used for traditional healthcare for thousands of years. In the interest of plant preservation, the focus has shifted to include the plant microbiome; interestingly, not only were the plants themselves producing the bioactive metabolites but also their associated microbiome. The focus of this study was to screen and determine the optimum time to produce anti-*Pseudomonas aeruginosa* metabolites, to purify the compounds of interest and characterise them. Forty-five endophytic fungi were grown in solid substrate fermentation on rice to produce extracts of varying ages (one week to four weeks).

Thin layer chromatography (TLC) coupled with bio-autography revealed that the anti-*P. aeruginosa* compound was produced after three weeks. Average zones of inhibition of 40.33, 20.33 and 22 mm were obtained using the Kirby-Bauer disc diffusion assay. All rows A, B, C and D show very strong activity as the MICs of the extracts were 156.5 µg/mL, 39.06 µg/mL, 78.73 µg/mL and 19.53 µg/mL for T1, T2, T3 and T4, respectively. Thin layer chromatography was conducted, and optimum separation was observed using hexane: ethyl acetate (60:40, v/v). Fractionation was carried out using a silica gel column with six different ratios of a hexane: ethyl acetate solvent system. The first round of purification resulted in twenty-seven fractions with five fractions having similar TLC profiles. These fractions were combined and subjected

to a second round of purification that gave three fractions. One fraction was observed to have good anti-*P. aeruginosa* activity and acceptable purity levels after nuclear magnetic resonance spectroscopy. Compound one, a dilactone (3a,10b-dimethyl-1,2,3,3a,5a,7,10b,10c-octahydro-5,8-dioxa-acephenanthrylene-4,9-dione, molecular formula $C_{16}H_{18}O_4$) was isolated as a white solid from the extract of the fungus *Neofusicoccum luteum*. This compound was previously isolated from the fungus *Oidiodedron griseum*. The relative configuration of the compound was confirmed by X-ray crystallography. Although the isolated compound is not novel, its ability to inhibit the growth of *P. aeruginosa* is new. This suggests that known compounds need to be screened across a wide range of pathogens and organisms to determine potential activity.

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences and School of Chemistry & Physics, University of KwaZulu-Natal (Westville Campus), Durban, South African from February 2016 to November 2018, under the supervision of Dr. R. Govinden and the co-supervision of Dr. R. Moodley, respectively. These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been named of the work of others it is duly acknowledged in the text.

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION 1- PLAGIARISM

I,………………Mamokoena Kuali……...declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or any examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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LIST OF ABBREVIATIONS

AA- Amino acid

ADPRT- ADP ribosyltransferase

BCGs- Biosynthetic gene clusters

BLAST- Basic local alignment search tool

CF- Cystic fibrosis

$CHCl_3$- Chloroform

DEPT- Distortionless enhancement by polarization transfer

DNA- deoxyribonucleic acid

EPS- extracellular polymeric substances

ESBL- extended spectrum beta lactamase-producing

EtOAc- Ethyl acetate

HMBC- Heteronuclear Multiple Bond Correlation

HPLC- High performance liquid chromatography

ITS- Internal transcribed spacer

MDR- Multi-drug resistant

MeOH- Methanol

MIC- Minimum inhibitory concentration

MRSA- multidrug resistant Staphylococcus aureus

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NA- Nutrient agar

NCBI- National Center for Biotechnology Information

NMR- Nuclear magnetic resonance

NRPs- Non-ribosomal peptide synthases

P. aeruginosa- Pseudomonas aeruginosa

PCR- Polymerase chain reaction

PDA- Potato dextrose agar

PKs- Polyketide synthases

PPP- Pentose phosphate pathway

Rf- Retention factor

SSF- Solid substrate fermentation

TCA- Tricarboxylic acid

TLC- Thin layer chromatography

UV- Ultraviolet

VRE- vancomycin resistant *Enterococcus* sp.

VREF- vancomycin resistant *Enterococcus faecium*

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

In the world today, modern medicine is being used to treat the range of diseases and infections of individuals, with varying rates of success. In addition, preventative measures such as improved sanitation, better living conditions and clean water practises are being implemented to reduce the risk of such infections. Despite these improvements, numerous disease-causing pathogenic microbes are becoming more resistant to antimicrobial chemicals. The drug development rate is slower than the rate of microbial resistance, which is proving to be challenging (Gabriel and Olubunmi, 2009). The most encountered problem when treating microbial infections is antimicrobial resistance (Zankari *et al*., 2017).

The appearance of new diseases and drug tolerant pathogens, together with the side effects caused by conventional drug use demands that new drugs be discovered. Over the past decade, increasing outbreaks caused by methicillin resistant *Staphylococcus aureus* (MRSA), *Enterococcus* sp. that are vancomycin resistant (VRE), extended spectrum beta lactamase-producing (ESBL) *Escherichia coli* and *Pseudomonas aeruginosa* have been reported (Hancock and Speert, 2000; Hirsch and Tam 2010). There is thus, an urgent need for novel antibiotics with new targets that may prove efficacious in treating infections caused by these resistant microbes. Several candidate drugs, namely, quinupristin/dalfopristin and the oxazolidinone linezolid, have been tested and are reputed to be effective in treating vancomycin resistant *Enterococcus faecium* (VREF) and skin infections caused by MRSA, *Streptococcus pyogenes* and other Gram-positive bacteria (Kraft *et al*., 2014). However, these new antibiotics fail or have little activity against Gram-negative bacteria (Wright *et al*., 2017). This is the main hurdle that needs to be overcome in order to combat the growing resistance of *P. aeruginosa.*

1.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is aerobic, Gram-negative and rod shaped. Due to its ability to cause diseases in immuno-compromised patients, it is referred to as an opportunistic pathogen. It is a versatile microbe able to tolerate low oxygen conditions, survive in nutrient deficient environments and tolerates a temperature range from 4-42°C. It is a multifaceted pathogen which is able to cause a wide range of infections and is the main cause of opportunistic nosocomial infections, causing approximately 10% of hospital infections. Chronic lung infections which lead to the death of patients with cystic fibrosis are mainly caused by this pathogen (Lutz and Lee, 2011).

The National Nosocomial Infection Surveillance done in South African hospitals over three decades showed *P. aeruginosa* to be the eighth most isolated pathogen from blood (3.4%) and the third most common cause of urinary tract infections (16.3%) (Hancock and Speert, 2000; Hirsch and Tam, 2010). The general prevalence of *P. aeruginosa* infections remained constant from 1986 to 2003. However, the incidence of resistant strains increased drastically in 2003. The rate of resistance of *P. aeruginosa* to imipemen increased by 15.9% in 2003 and by 20% to quinolones and third generation cephalosphorins (Hirsch and Tam, 2010). Likewise, a study on Intensive Care Unit patients in South African hospitals reported a notable increase in *P. aeruginosa* strains resistant to at least three out of four antibiotics (multidrug resistant, MDR) (Nathwani *et al*., 2008).

Pseudomonas aeruginosa is highly adaptive and this quality is proved by its ability to survive in the highly stressful and hostile environment found within a lung with cystic fibrosis (CF) (Minstanley *et al*., 2016). Nitrosative, osmotic and oxidative stresses are some examples of stresses found within CF infected lungs (Silva *et al*., 2010). Phenotypic analysis of isolates found within these infected lungs show an accumulation of auxotrophic mutations, conversion

of isolates from motile to sessile and the formation of mucoid colonies. The above-mentioned adaptations all provide protection to *P. aeruginosa* from antibiotics and host responses. Other adaptations the emergence of hypermutators (Minstanely *et al*., 2016). Since CF patients are usually under long treatment with antimicrobials, the fast emergence of antimicrobial resistance is frequently encountered. Therefore, screening, purifying and characterising bioactive products with anti-*P. aeruginosa* activity is important, especially in South Africa where there is a high number of immuno-compromised patients.

1.2.1 *Pseudomonas aeruginosa* biofilms

Pseudomonas aeruginosa is considered an important plant and human pathogen due to its ability to produce various virulence factors (Epps and Walker, 2006). Apart from this, the ability of *P. aeruginosa* to form biofilms on many surfaces makes antibiotic treatments ineffective and thus promotes chronic infectious diseases. Biofilm formation is a continuous cycle, where organised bacterial communities are enclosed in a matrix of extracellular polymeric substances (EPS) which holds microbial cells together and allows adherence to surfaces (Rasamiravaka *et al*., 2015). Extracellular polymeric substances are composed mainly of exopolysaccharides, extracellular DNA (eDNA), biomolecules and polypeptides that form a highly hydrated mixture that is implicated in the overall structure and architectural strength of the biofilm (Ding *et al*., 2016).

Varying biofilm phenotypes can be expressed, depending on the strain of *P. aeruginosa* and the nutritional conditions. For example, in glucose limiting conditions, the *P. aeruginosa* PAO1 biofilm life cycle can be divided into five phenotypic stages (Figure 1.1). The reversible attachment of planktonic bacterial cells onto a growth surface begins this cycle (Figure 1.1 a, Stage I), irreversible attachment of bacteria which form microcolonies in the EPS matrix

continues this process (Figure 1.1 b, Stage II). Gradually, bacterial microcolonies expand and their individual contributions lead to a more structured phenotype with noncolonised space (Figure 1.1 c, Stage III). Later, free spaces within the biofilm become filled with bacteria and covers the whole surface (Figure 1.1 d, Stage IV). Three-dimensional communities grow as observed in Figure 1.1, Stages III and IV. Figure 1.1 e, Stage V, shows the last stage which is bacterial conversion from non-motile to planktonic form, in order to spread and inhabit other surfaces (Nikolaev and Plakunov, 2007; Flemming and Wingener, 2010; Rasamiravaka *et al*., 2015).
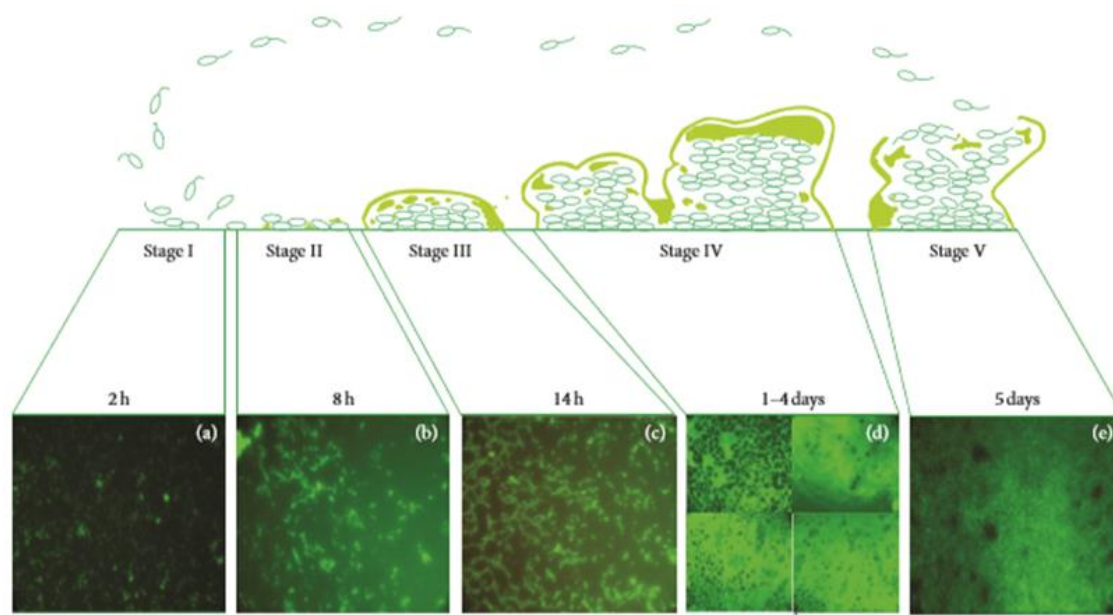
Figure 1.1: Biofilm lifestyle cycle of *P. aeruginosa* PAO1 grown in glucose minimal media. In stage I, planktonic bacteria initiate attachment to an abiotic surface, which becomes irreversible in stage II. Stage III corresponds to microcolony formation. Stage IV corresponds to biofilm maturation and growth of the three-dimensional community. Dispersion occurs in stage V and planktonic bacteria that are released from the biofilm colonise other sites (Rasamiravaka *et al*., 2015).

1.2.2 *Pseudomonas aeruginosa* **biofilm formation as a resistance mechanism**

Antimicrobial resistance is the natural response of bacteria to antibiotic exposure. It can come about by spontaneous genetic mutations, be intrinsic to a bacterium, or be linked to horizontal

gene transfer (Sherrard *et al*., 2014). *Pseudomonas aeruginosa* displays resistance to numerous antibiotics due to its ability to form biofilms. Figure 1.2 depicts the mechanisms of resistance to antibiotics which is largely due to a reduced activity of antibiotics inside the biofilm (Sherrard *et al*., 2014). It is hypothesised that antibiotics do not penetrate the biofilm (Gomes *et al*., 2012). This could be because it acts as a physical barrier which keeps antibacterial agents out of the biofilm (e.g. aminoglycosides) preventing their diffusion (Figure 1.2) (Sherrard *et al*., 2012; Gupta *et al*., 2015). This interaction also prevents the passage of antibiotics into the biofilm base, for example, in cystic fibrosis patients, steep oxygen slopes and oxygen deprived cavities form inside the cystic fibrosis mucus layer due to increased oxygen utilisation together with poor oxygen diffusion within the mucus.
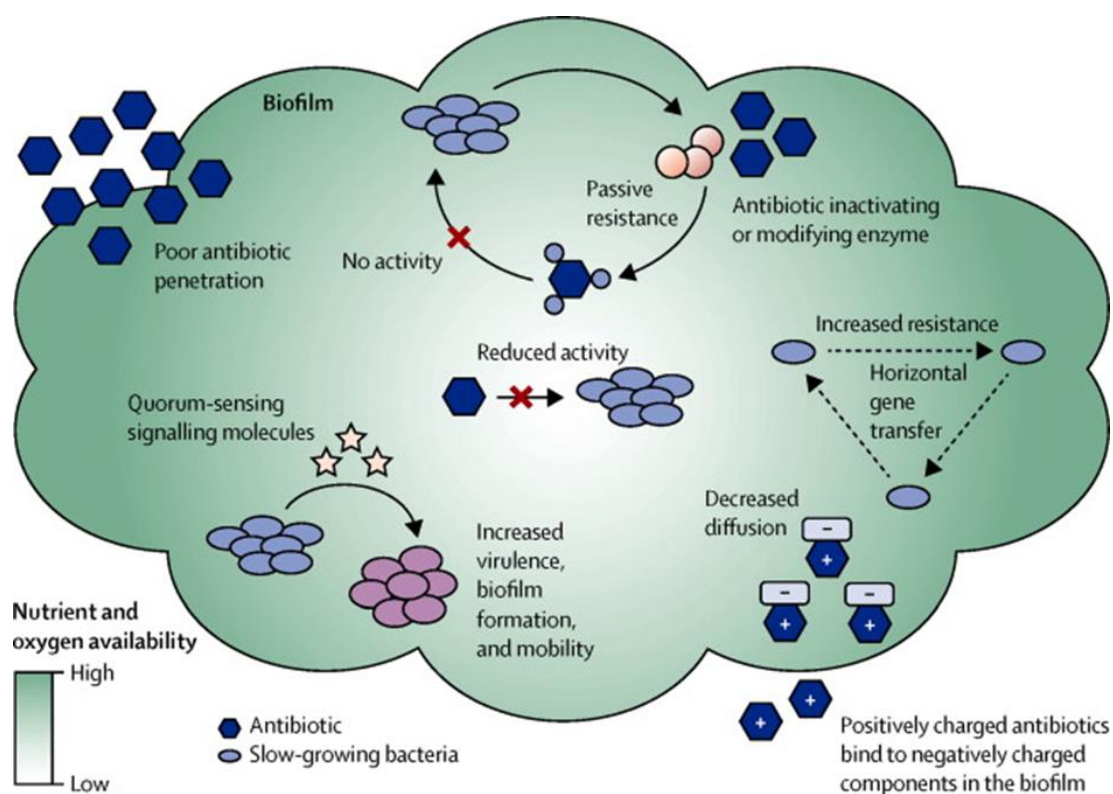
Figure 1.2: Mechanisms of resistance in multidrug resistant *P. aeruginosa* (Sherrard *et al*., 2014).

Hill and co-workers (2005) revealed aerobically grown *P. aeruginosa* to be more susceptible to β-lactam, aminoglycoside and colistin antibacterial agents than anaerobically grown *P. aeruginosa* (Hill *et al*., 2005). Furthermore, since aminoglycoside antibiotics need oxidative phosphorylation in order to enter bacterial cells, a low oxygen concentration inactivates these antibiotics (Schaible *et al*., 2012). In addition, hypoxia is reported to increase efflux pump expression in *P. aeruginosa* and this causes increased resistance to cephalosporin and penicillin antimicrobials (Schaible *et al*., 2012). Biofilm formation in cystic fibrosis is a symptom of chronic airway infection. Bacteria growing in biofilms are buried in an exopolymeric matrix (which includes exopolysaccharide, protein and DNA) that have been shown to be more resistant when compared to planktonic cells (Lutz and Lee, 2011; Sherrard *et al*., 2014). A sub population of bacteria in the biofilm may adapt a protective phenotype and become persistor cells. Figure 1.3 summarises the different types of cells found within a biofilm.
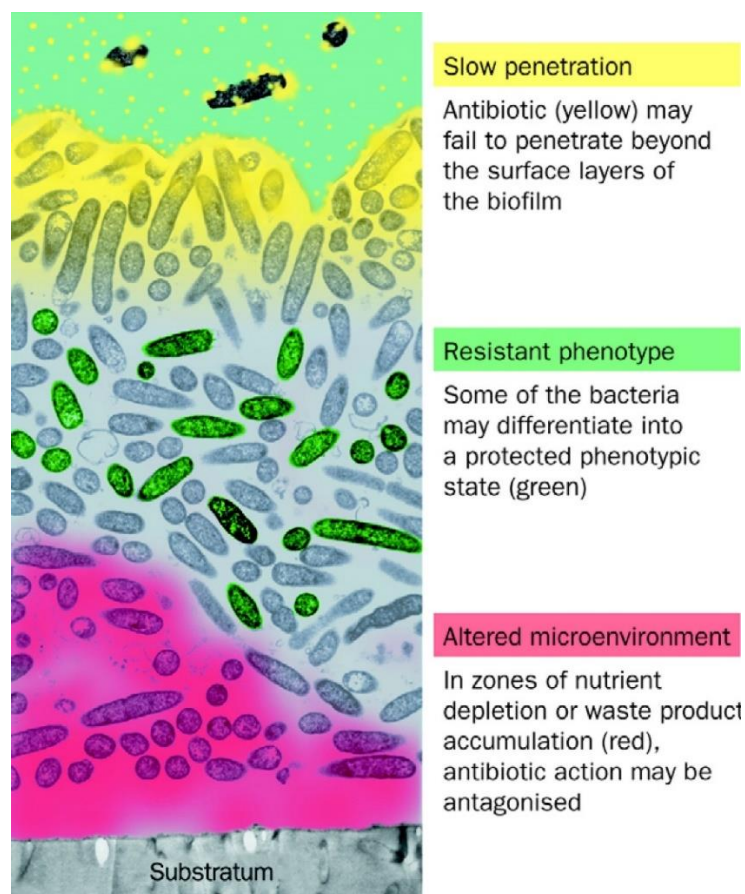
Figure 1.3: Three mechanisms of antibiotic resistance in biofilms (Stewart and Costerton, 2001).

1.2.3 *Pseudomonas aeruginosa* **virulence factors**

Pseudomonas aeruginosa has five known virulence factors: (i) flagella and type 4 pili, (ii) type 3 secretion system, (iii) quorum sensing and biofilm formation, (iv) lipopolysaccharides and (v) proteases (Figure 1.4). Each *P. aeruginosa* cell possesses several type 4 pili and a single polar flagellum that can initiate an inflammatory response. During infection, the bacterium can attach to host epithelial cells by binding its flagellum to the asialyated glycolipid asialoGM1 which can elicit a strong NFjB-mediated inflammatory response via signalling through TLR5 and a caspase-1-mediated response through the Nod-like receptor, Ipa (Mena *et al.*, 2008). Mutants which don't have flagella cannot elicit an inflammatory response. Thus, flagella are believed to be required to establish an infection. Type 4 pili are the most important attachment apparatus. Pili can also facilitate aggregation, causing the bacteria to form microcolonies on host target tissues, effectively forming a dense packing of bacteria in one location. They protect the bacteria from antibiotics and the host immune system (Williams *et al.*, 2010).

Pseudomonas aeruginosa microcolonies are highly implicated in chronic cystic fibrosis. Pilin-deficient mutants and those that do not display twitching motility, show reduced virulence in various models (Mena *et al.*, 2008). Type 3 secretion systems (T3SS) are common among several pathogenic Gram-negative bacteria as a way of injecting toxins directly into host cells. The T3SS in *P. aeruginosa* has four effectors which were identified as – ExoY, ExoS, ExoT, and ExoU – far fewer than many other well-characterised T3SS (e.g. *Salmonella enterica* SPI-1 has 13, *Shigella* sp. have 25) (Hauser, 2009). The T3SS of *P. aeruginosa* is encoded by 36 genes on five operons. The entire system is transcriptionally controlled by ExsA, a member of the AraC family of transcriptional activators (Yahr and Wolfgang, 2006). Nearly all strains express one of the two major exotoxins, exoU or exoS, but very rarely both.

ExoS is bifunctional, including both N-terminal GTPase-activating protein activity and C-terminal ADP ribosyltransferase (ADPRT) activity (Otter *et al*., 2011). Both activities are linked to the actin cytoskeletal organisation, although the ADPRT activity is understood to play a larger part in pathogenesis. ExoU is a phospholipase and is estimated to be 100 times more potent a cytotoxin than ExoS and causes rapid death of host eukaryotic cells due to loss of plasma membrane integrity consistent with necrosis (Mena *et al*., 2008). Lipid A can be sequentially bound by host cell coreceptors MD2 and CD14 leading to activation of the TLR4 to NFjB signalling pathway and triggering the production of pro-inflammatory cytokines and chemokines, inflammation, and over time endotoxic shock (Bardoel *et al*., 2011). Alkaline protease is a type 1 secreted zinc metalloprotease that is known for its degradation of host complement proteins and host fibronectin. In addition, alkaline protease has been shown to interfere with flagellin signalling through host TLR5 by degrading free flagellin monomers and thereby helping *P. aeruginosa* to avoid immune detection (Gellatly and Hancock, 2013).
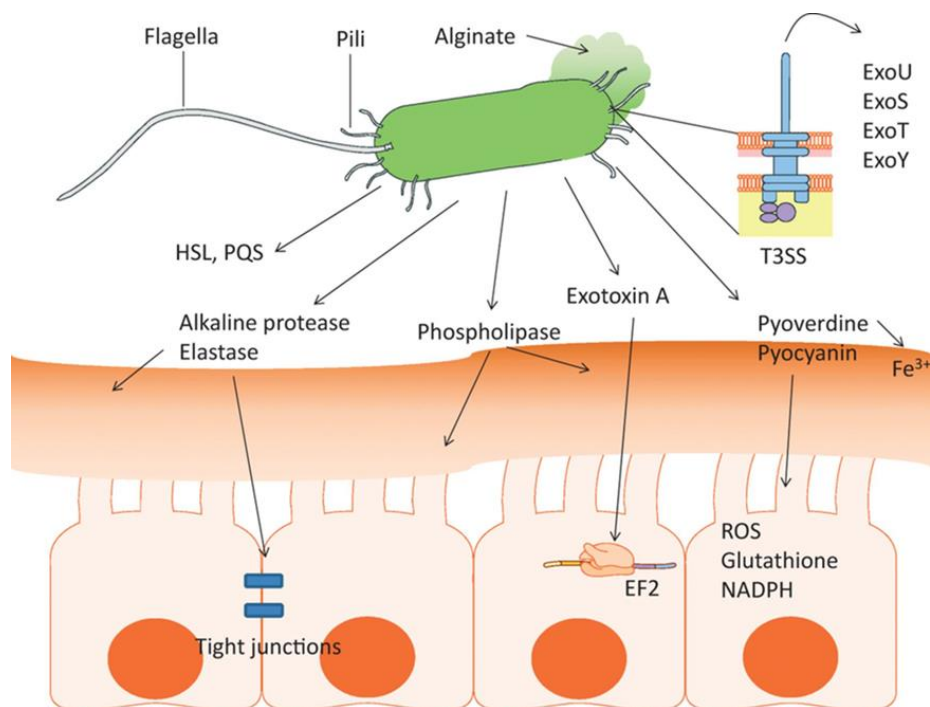
Figure 1.4: Virulence factors produced by *P. aeruginosa* (Gelattly and Hancock, 2013).

The prominence of *P. aeruginosa* as a pathogen is due to its high intrinsic resistance to antibiotics (Table 1.1), to the point that the efficacy of even the newest antibiotics is significantly altered (Lutz and Lee, 2011). This, therefore, directed the search for novel bioactive molecules from previously unexplored sources.

Table 1.1: *Pseudomonas aeruginosa* resistance mechanisms towards antibiotics (Hancock and Speert, 2000)

Class	Agents	Mechanism of action
Penicillin	Ticarcillin, Carbenicillin, Piperacillin	Depression of chromosomal β-lactam. Overexpression of the MexAB-OprM multidrug efflux pump due to a NalB mutation. Specific plasmid-mediated β-lactamases.
Cephalosporin	Ceftazidime Cefoperazone Cefepime Cefpirome	Depression of chromosomal β-lactam. Overexpression of the MexAB-OprM multidrug efflux pump due to a NalB mutation. For the fourth generation cephalosporins cefepime and cefpirome, overexpression of the MexCD-OprJ multidrug efflux pump due to an NfxB mutation.
Aminoglucoside	Gentamicin Tobramycin Amikacin	Overexpression of the MexXY efflux pump in impermeability type-resistance due to a mutation in the regulatory gene MexZ. Plasmid-mediated production of modifying enzymes.
Quinolones	Ciprofloxacin	Target site mutations in the GyrA (or sometimes the GyrB) topoisomerase subunit; Overexpression of multidrug efflux pumps due to NalB, NfxB or NfxC mutations.
Polymixin	Colistin	Outer membrane LPS changes due to PhoP/PhoQ regulatory mutations. No evidence this occurs in the clinic.
Carbepenem	Imipenem Meropenem	Loss of specific outer membrane porin channel, OprD; Reduction in levels of OprD due to an NfxC mutation that also upregulates multidrug resistance due to MexEF-OprN. For meropenem overexpression of the MexAB-OprM multidrug efflux pump due to a NalB mutation.

1.3 MEDICINAL PLANTS

Plants contain bioactive compounds which have been used for traditional healthcare for thousands of years. The ethno-pharmaceutical knowledge of Traditional Chinese medicine includes over 5000 species (Miller *et al*., 2012). Plants play a significant role in drug development and discovery, worldwide. From 1981 to 2010, 26% of new drugs were natural products and this peaked to 50% in 2010 (Newman and Cragg, 2012). Plants with antibacterial properties are of central importance as biological sources of novel active metabolites that may

provide a solution to the alarming incidence of antibiotic resistance in clinical strains. Medicinal plants offer a promising source for such drugs. These plants are reservoirs of compounds that can be used in drug development (Hassan, 2012).

Cassia fistula is a medicinal plant that produces bioactive secondary compounds, which are currently being used as dietary supplements, medicines and other useful commercial products (Kadhim *et al*., 2016). Investigations into this plant were prompted by their use in traditional medicine which has been passed down from generation to generation (Mishra and Behal, 2010). *Cassia fistula* has been reported to contain flavonoids, alkaloids, tannins, terpenes, and glucosides (Ali, 2014). These compounds are responsible for the antibacterial activity of the plant and form the basis for its use in the manufacture of antibiotics.

The combined effects of the secondary metabolites of papaya impart medicinal properties that prevent heart disease, colon cancer and strokes (Aravind *et al*., 2013). Recently, the aloe plant was discovered to possess antiseptic properties along with other properties including laxative and anti-inflammatory skin agent which clears acne and soothes eczema (Mavintha and Bidya, 2014). Plants with anti-inflammatory properties have been reported to display antimicrobial activity too.

Plant essential oils are a very diverse group of secondary metabolites that are potentially useful sources of antimicrobial compounds. Many studies have reported on the antimicrobial activity of essential oils (Soković *et al*., 2010; Moussaoui and Alaoui, 2016). According to Moreti (2004), essential oils, unlike antibiotics, consist of numerous molecules that bacteria cannot gain resistance towards. These essential oils they are known for their potent antiviral, anti-fungal, anti-inflammatory and antibacterial effects. The combination of essential oils with antibiotics may lead to new ways to treat infectious diseases. Many researchers have studied the synergistic effects resulting from the combination of antibiotics with different plant extracts

which reduced bacterial resistance to drugs (Ghaleb and Mhanna, 2008; Stefanovic and Comic, 2012; Moussaoui and Alaoui, 2016).

1.3.1 *Kigelia africana*

Taking all medicinal plants in Africa into consideration, the most widely distributed is *Kigelia africana* (Figure 1.5). *K. africana* is found in tropical regions including Central, South and West Africa (Saini *et al*., 2009; Oyedeji and Bankole-Oje, 2012). It is referred to as the sausage tree due to the big fruit that it bears (0.6 m in length and 4 kg weight) (Saini *et al*., 2009; Azu *et al*., 2010).

| A | B | C | D |

| Tree | Leaves | Flower | Fruit |
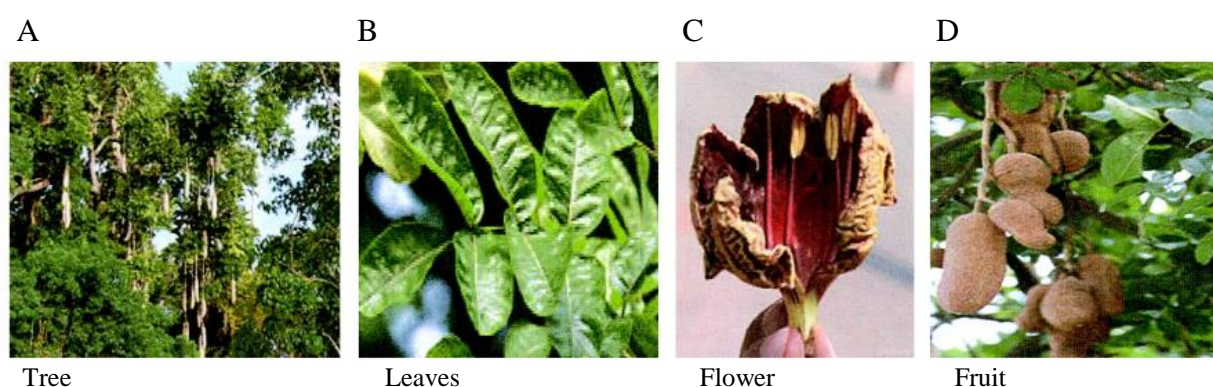
Figure 1.5: *Kigelia africana*. A, B, C, D showing the tree, leaves, flower and fruits, respectively (Saini, 2009).

All parts of the plant have been reportedly used to treat illnesses (Idris *et al*., 2013). In rural regions of Africa, various forms of the plant are believed to cure a large number of illnesses. These include topical applications for fungal infections, psoriasis, eczema and boils (Atawodi *et al*., 2017). This plant has also been administered internally for the treatment of malaria, toothache, pneumonia, ringworm, tapeworm and diabetes (Azu *et al*., 2010). During famine in Malawi, the seeds were roasted and eaten (Azu, 2013). In the Zambezi Valley, the Tonga women apply cosmetic creams of *K. africana* to their faces to maintain and ensure blemish-free faces (Saini *et al.,* 2009). Other applications of *K. africana* include a preparation of the

flowers for application on breasts of lactating females to increase the flow of milk (Atawodi *et al*., 2017*). K. africana* extracts are used to treat toothaches and sores associated with diabetes; this prompted the study of this plant for its potential to produce antibacterial compounds (Siani *et al*., 2009).

1.3.2 *Kigelia africana* **metabolites**

Several metabolites have been extracted from various parts of this plant. These include the furanone derivative 3-(2-hydroxyethyl)-5-(2-hydroxypropyl)-dihydrofuran-2-(3H)-one and four irridiods (7-hydroxy viteoid II, 7-hydroxy eucommic acid, hydroxy-10-deoxyeucommiol and 10-deoxyeucommol) (Akanni *et al.,* 2017). A novel phenylpropanoid derivative was isolated from the fruit and identified as 6-*p*-coumaroyl sucrose, as well as ten known phenylpropanoids and phenylethanoids and a flavonoid glucoside. Kigelin (Figure 1.6), the major constituent in *K. africana* was also isolated in 1971 by Govindchari *et al.* (1971).
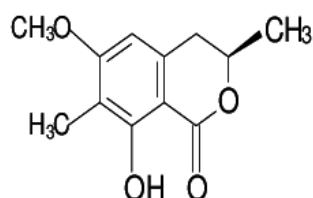
Figure 1.6: Structure of kigelin (Idris *et al*., 2013).

Kigelin is a 8-hydroxy-6,7-dimethoxy-3-methyl-3,4-dihydroisocoumarin. Other compounds such as 6-methoxymethymellein, stigmasterol, lapachol, β-sitosterol, 3-dimethyl kigelin, kigelinone, naphthaquinones, dehydro-α-lalachone and isopinnatal, isolated from various parts of the plant were found to impart antibacterial and antifungal activity (Grace *et al*., 2002; Gabriel and Olubumni 2009; Idris *et al*., 2013). Other properties of the plant include antiprotozoal and anti-amoebic activity (Oyedeji and Brankole-Oje 2012). Table 1.2 depicts

some classes of secondary metabolites isolated from *K. africana* and their biological activities based on studies by Akunyili *et al*. (1991), Kwo and Cracker (1996), Grace *et al*. (2002) and Saini *et al*. (2009).

Table 1.2: Pharmacological activities of different phytoconstituents of *Kigelia africana*

Activity	Irridiods	Naphthoquinone	Meroterpenoid naphthoquinones	Coumarin derivatives	Lignans	Sterols	Flavonoids
Anticancer	+	+	+	+	+	+	+
Anti-mollusidal	+	-	-	-	-	-	+
Against syphilis and gonorrhea	+	+	-	-	-	+	+
Antidiarrheal	+	-	-	-	-	-	+
Antiulcer	+	-	-	-	-	-	+
Antifungal	-	-	+	-	+	+	+
Antimalarial	-	-	+	-	+	-	-
Anti-inflammatory	+	+	+	-	-	+	+
Antibacterial	+	+	+	-	+	+	-
Against postpartum haemorrhage	+	-	-	+	-	+	-
Anti-Pneumonia	+	-	-	-	-	+	+

Keys: -Not active; + active

In the interest of plant preservation, the focus has shifted to include the plant microbiome. Studies have surprisingly shown that not only were the plants themselves producing the bioactive metabolites, but their associated microbiomes were as well (Jalgaonwala *et al*., 2011).

1.4 ENDOPHYTES

Investigations on the same plant species located in different geographical regions/countries have shown that they carried different active metabolites suggesting that the bioactive secondary metabolites are produced by endophytic microorganisms. Endophytes are microorganisms (including Actinomycetes, fungi and bacteria) which colonise plants without any symptoms (De Bary, 1886 cited by Sadrati *et al*., 2013; Kusari *et al*., 2013). Mutualism or asymptomatic colonisation has been attributed to a balance of antagonism between the host defence responses and the virulence factors of the endophytic microorganism by Kusari and co-workers (2013) (Figure 1.7). Figure 1.7 also shows that the presence of certain environmental factors may cause a shift from mutualism to pathogenesis.
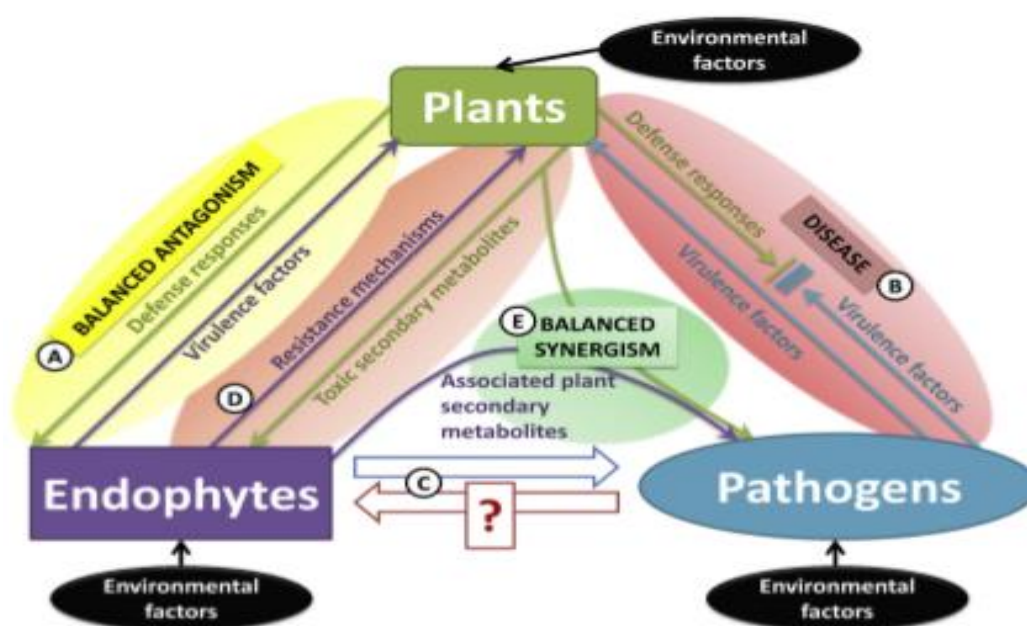
Figure 1.7: Interactions between endophyte, parasites and host plants (Kusari *et al*., 2012).

Endophytes and plants produce similar metabolites, probably due to the endophyte and plants coevolving over several millennia (Kusari *et al*., 2012). The plant provides shelter and nutrients and in return the endophytes protect the plant from biotic and abiotic stresses. An

early report of this by Webber (1981), showed that the endophytic fungus, *Phomopsis oblonga,* provided protection to elm trees against the beetle, *Physosnemum brevilineum.* This endophytic fungus reduced the spread of the causal agent of Dutch elm disease by producing toxic repellents against *P. brevilineum* (Kusari *et al.*, 2013). Figure 1.8 depicts this protection by endophytic fungi. It was later proven that endophytic fungi belonging to the family *Xylariacea* could synthesise toxic secondary metabolites that affect beetle larvae (Claydon *et al.*, 1985; Suryanarayanan, 2017).
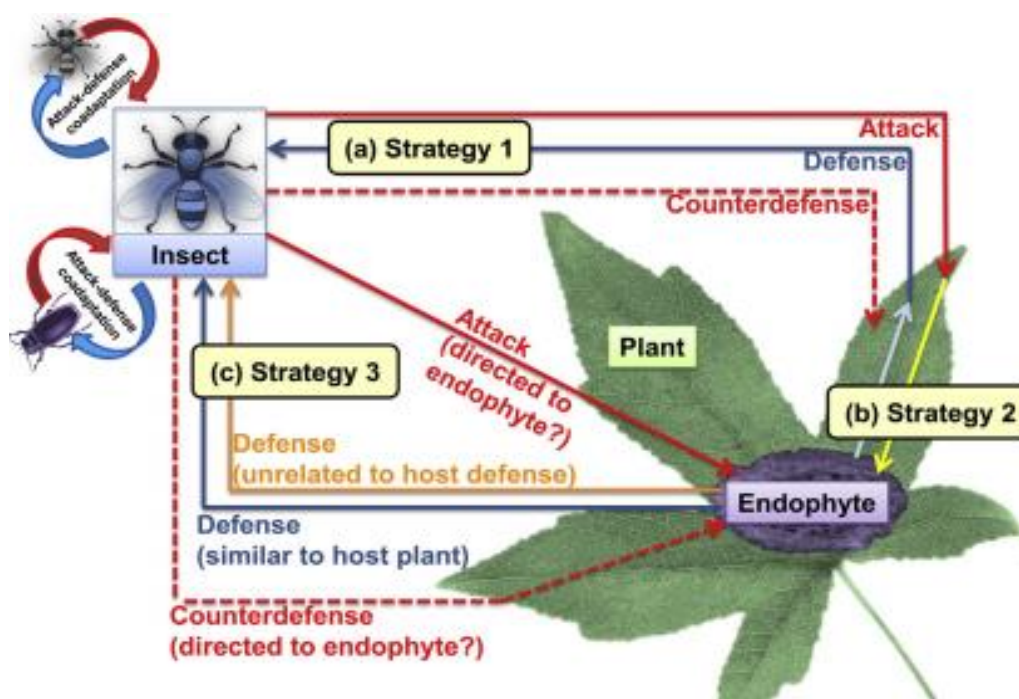
Figure 1.8: Strategies employed by endophytic fungi to protect plants against insects (Kusari *et al.*, 2013).

Due to the emergence of drug resistant superbugs, there is an ever-increasing need for obtaining new compounds which are aimed at alleviating illnesses caused by pathogens (Sadrati *et al.*, 2013). The ability of older types of antimicrobial agents to cure diseases continues to decrease. In addition, synthetic drugs threaten the environment and have serious side effects (Clay,

2014). Removal of these agents creates a gap which needs to be filled by new compounds with the ability to control human pathogens. There is an immediate need to find and produce environmentally safe drugs (Sadrati *et al*., 2013).

Research conducted by Stierle *et al*. (1993; 1995) reporting on the discovery of the endophytic fungus, *Taxomyces andreane*, which produces Taxol®, an anticancer drug, directed intense attention towards endophytic fungi (Das *et al*., 2017). This formed the basis and major motivating factor towards the study of fungal endophytic bioactive molecules. They are considered excellent reservoirs of therapeutically active compounds due to their genetic diversity (Darsih *et al*., 2017). Endophytic fungi have been found to be present in all plants studied to date and are thought to be ubiquitous in plants (Huang *et al*., 2001; Kirk *et al*., 2001; Strobel and Daisy 2003; Jalgaonwala *et al*, 2011).

It is estimated that 1.5 million endophytic fungi live within plants and lichens (Huang *et al*., 2008). The incidence of endophytes within plant tissue appears to be environmentally influenced. Arnold and Lutzoni (2007) showed that the incidence of infection by endophytes approached 100% in plants situated in tropical regions; this incidence is exponentially higher than in plants found in polar regions. Rosa *et al*. (2009) later confirmed these findings.

Most endophytic fungi belong to the phylum Ascomycota, while some belong to the phyla Basidiomycota and Zycomycota (Huang *et al*., 2001). It was shown that the presence or absence of endophytic fungi can affect plant structures and communities. Arbuscular mycorrhizal fungi grow deep into the soil facilitating better uptake of nutrients from the soil, especially the roots (Cosme *et al*., 2018). This improves plant growth and survival allowing taller and stronger plants. The presence of root endophytes. Thus, plants that grow near plants with root endophytic fungi also reap the benefits of more nutrient availability (Allen *et al.*,

2018). The whole plant community benefits. These fungi also change the internal morphology of plant tissue by forming arbuscules within the plant tissue.

Investigations into the genomes of plants and associated endophytes have revealed that they contain conserved gene clusters that have co-evolved during their long association reportedly dating back 400 million years (Chagas *et al*., 2018). These gene clusters encode novel secondary metabolites that may have potential medicinal applications (Lahn *et al*., 2017). Much work has been conducted to elucidate the genomes of several endophytic fungi. Wang and co-workers (2001) sequenced the *Pestalotiopsis fici* genome and found that it harboured numerous secondary metabolite synthesis genes: 27 polyketide synthases (PKS), 12 non-ribosomal peptide synthases (NRPS), 5 dimethylallyl tryptophan synthases, 4 putative PKS-like enzymes, 15 putative NRPS-like enzymes and 15 terpenoid synthases. These core enzymes are distributed into 74 different secondary metabolite clusters. Secondary metabolites are synthesised when precursor metabolites are limited during primary metabolism (Figure 1.9) (Nielsen and Nielsen, 2017).
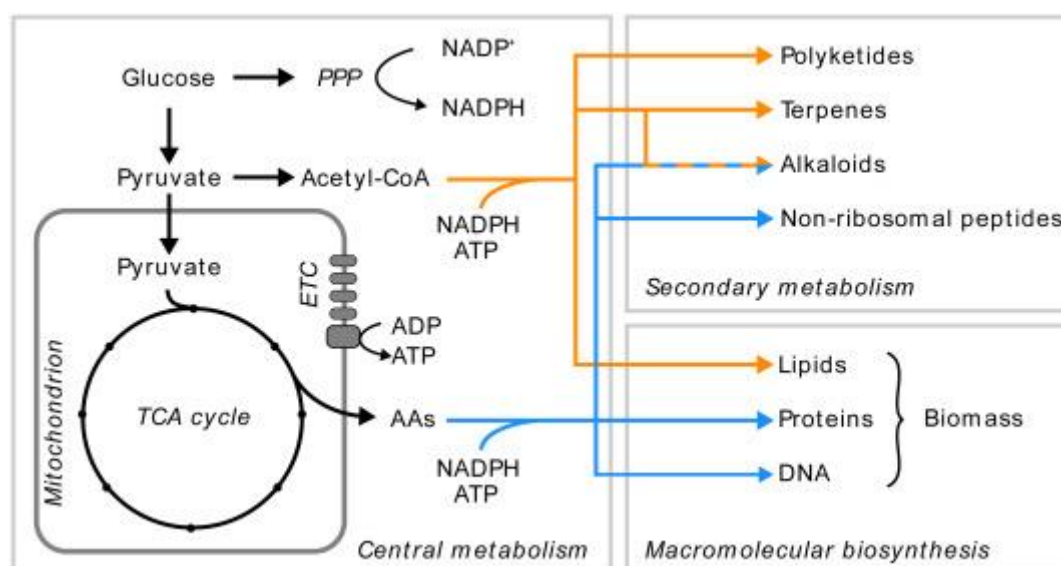
Figure 1.9: Biosynthesis of secondary metabolites from precursors of the central carbon metabolism. PPP: Pentose Phosphate Pathway. ETC: Electron Transport Chain. TCA: Tricarboxylic acid. AAs: amino acids (Nielsen and Nielsen, 2017).

These precursors are predominantly amino acids or short chain carboxylic acids, backbone enzymes like PKSs, DMATSs, NRPSs or TCs link them together. The resulting oligomers are chemically modified by enzymes which are controlled by the usual transcriptional regulators as the backbone enzymes. The characteristic trait of genes involved in the secondary metabolite pathways is that they cluster in the chromosome in biosynthetic gene clusters (BCGs) (Nielsen and Nielsen, 2017). The ability to produce secondary metabolites is common to most living organisms but it is unevenly distributed. In known antimicrobial compounds (about 22 500 in total), 45% are produced by actinomycetes, 38% by fungi and 17% are from bacteria. In addition, even with this abundance of compounds, it is estimated that only 100 are used for human therapy, with most of these being produced by actinomycetes. However, it must be acknowledged that aside from penicillin, other fungal secondary metabolites have been of pharmaceutical use; these include, but are not limited to, griseofulvin, statins and mycophenolic acid.

Natural products produced by endophytic microbes are observed to inhibit and kill a variety of harmful pathogens, including bacteria, fungi and protozoans (Darsih *et al*., 2017). These products are also known to deter herbivores (Jalgoanwala *et al*., 2011, Das *et al*., 2017). *Cryptosporiopsis quercina* is a fungus from which a unique antifungal compound named cryptocandin was extracted. This compound contains known amino acids and one new amino acid (3-hydroxy-4-hydroxy methyl proline) (Nandini *et al*., 2018). Cryptocandin has shown very efficient activity against human fungal pathogens, namely, *Trycophyton* spp. and *Candida albicans.* Antifungal activity by cryptocandin was also observed against *Botrytis cineria* and *Sclerotinia sclerontiorum* which are plant pathogens.

The study into endophytic fungi is only just beginning as only a tenth of fungal species have been isolated out of the approximately 1.5 million thought to exist. The study of the endophytes

of *K. africana* are still in the developmental stages as little is known about them. Idris *et al.* (2013) isolated endophytic fungi from *K. africana* and screened extracts produced by these fungi for antibacterial activity. The seven endophytic fungi isolated were identified to be *Aspergillus* sp., *Aspergillus flavus*, *Cladosporium* sp., *Curvularia lunata* and three unknown fungi. The *Aspergillus* sp., *Cladosporium* sp. and two of the unknown fungi displayed antibacterial activity. These results, therefore, suggest that endophytic fungi from *K. africana* have the potential to produce useful bioactive compounds with antimicrobial activity.

1.5 SEPARATION, PURIFICATION AND CHARACTERISATION OF FUNGAL BIOACTIVE EXTRACTS

Natural products, such as plant extracts, either as pure compounds or as standardized crude extracts, provide unlimited opportunities for new drug discovery because of the unmatched availability of chemical diversity (Mahmoudvand *et al.*, 2015). According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Oyebode *et al.*, 2016). A summary of the general approaches in extraction, isolation and characterisation of bioactive compound from plant extracts can be found in Figure 1.10 (Sasidharan *et al.*, 2011).
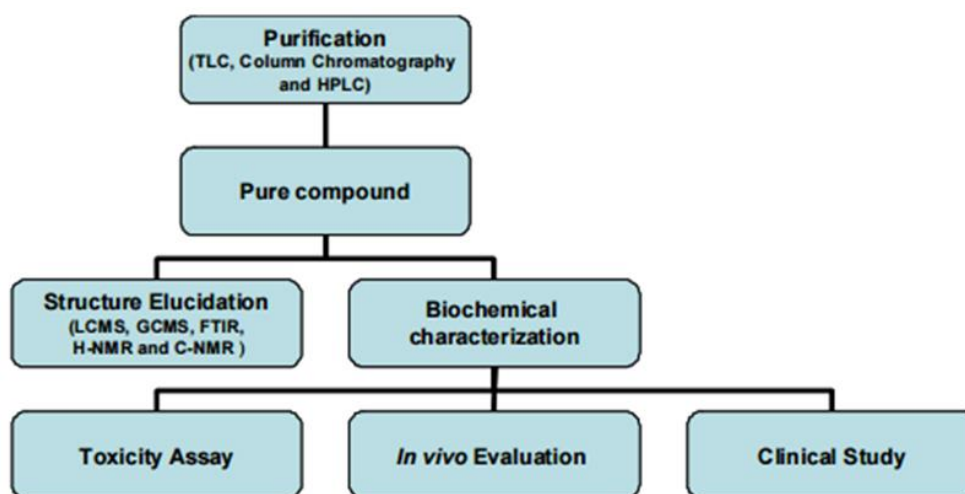
Figure 1.10: General approaches in extraction, isolation and characterisation of bioactive metabolites (Sasidharan *et al.*, 2011).

1.5.1 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is a method mainly used in forensic drug chemistry, which separates compounds of a drug mixture (Harper *et al*., 2017). Forensic laboratories use TLC to examine unknown drug mixtures. In TLC, the mobile phase is a solvent (liquid), and the stationary phase is a thin aluminium sheet coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. The mobile phase transports the solute through the stationary phase (Sasidharan *et al*., 2011). The speed at which the solute move on the TLC plate depends on the strength of the mobile phase as it dissolves the solute and migrates it up the plate (Klimek-Turek *et al*., 2016), together with the sorbent as it pulls the solute out of solution and back into the sorbent. The molecules move *via* a stop and go motion due to the solute being absorbed and desorbed repeatedly (Santiago and Strobel, 2013). A small fraction of the solute moves at a given time with each spot travelling a certain distance.

Substances that are strongly attracted to the solid layer move slowly because they spend more time in the sorbent and fast-moving compounds have a low affinity for the stationary phase and a high affinity for the mobile phase and thus spend more time in the mobile phase. Therefore, separation of compounds with varying properties is achieved by exploiting the interactions of the solutes with the mobile and stationary phases. Analysis of this assay entails calculating the Rf value and that is the distance migrated by the compound divided by the distance migrated by solvent (Kumar *et al*., 2013) (Figure 1.11). This value gives meaning to TLC as it can be compared to the Rf values of known standards.
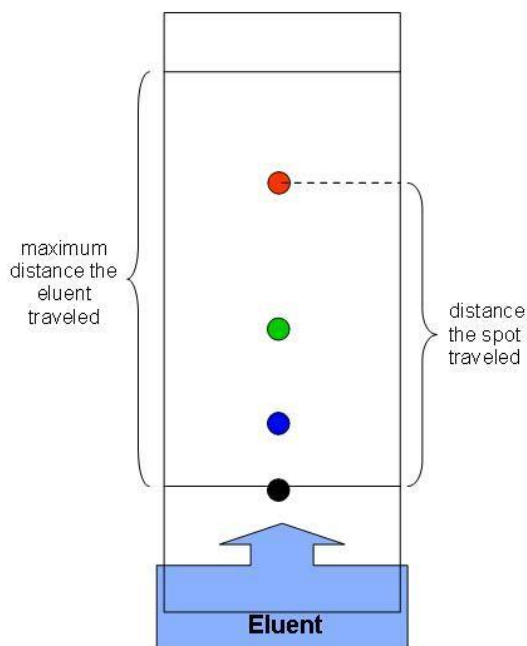
Figure 1.11: Thin layer chromatography (Kumar *et al*., 2013).

A study by Abok and Manulu in 2017, was conducted to identify phytochemical constituents of *Syzygium guineense* leaf extract. The bark and leaves of *S. guineense* are used to treat dysentery, wounds, ulcers, tuberculosis and chronic diarrhea. TLC analysis was conducted using hexane and ethyl acetate as solvents. Hundred % hexane, hexane: ethyl acetate (2:1) and hexane: ethyl acetate (3:1) were used. It was observed that 100% hexane did not resolve the contents, while hexane:ethyl acetate (2:1) and hexane: ethyl acetate (3:1) yielded three and seven bands with Rf values 0.13, 0.23, 0.84 and 0.007, 0.13, 0.23, 0.32, 0.40, 0.54, 0.81 respectively (Abok and Manulu, 2017).

1.5.2 Bio-autography

Bio-autography enables the localising of antimicrobial activities of an extract on the chromatogram, thus it supports the search for new antimicrobial agents through bioassay-guided isolation (Swapna *et al*., 2015). The advantages of the bio-autography agar overlay method includes use of small amounts of sample extract and the ability to identify the exact

component with antimicrobial activity from the crude sample (Rahalison *et al*., 1991). There are various methods to TLC bio-autography (direct, contact and agar overlay). Figure 1.12 represents direct bio-autography which entails the spraying of a 24-hour culture on a dried TLC plate, incubating at 37°C for 24 hours and spraying with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as conducted by Samrot *et al*., 2016. The resultant spots (compounds) with antibacterial activity appear clear while the compounds with no antimicrobial activity appear green. The most commonly used dyes are MTT and tetrazolium dye.
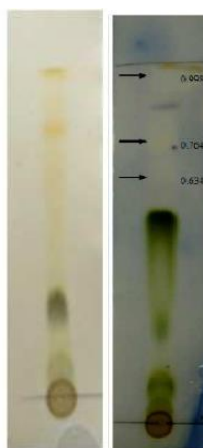
Figure 1.12: Thin layer chromatography (TLC) and TLC bioautography for antimicrobial activity in *Acacia* sp. (Samrot *et al*., 2016).

In contact bio-autography, antimicrobial compounds diffuse from a developed TLC plate to an agar plate inoculated with the test strains (Sherma, 2008; Dewanjee *et al*., 2015). The chromatogram is placed face down onto the inoculated agar layer for a specific period to enable diffusion of the compounds from the TLC plate to the agar plate. The chromatogram is removed, and the agar layer is incubated. The zones of inhibition on the agar surface, corresponding to the spots in chromatographic plates, are indicative of the antimicrobial agents. An overall view of contact bio-autography is shown in Figure 1.13. Incubation time for the

growth ranges between 16 and 24 hours but it can be reduced to 5–6 hours by 2,3,5-tetrazolium chloride (Dewanjee *et al*., 2015).
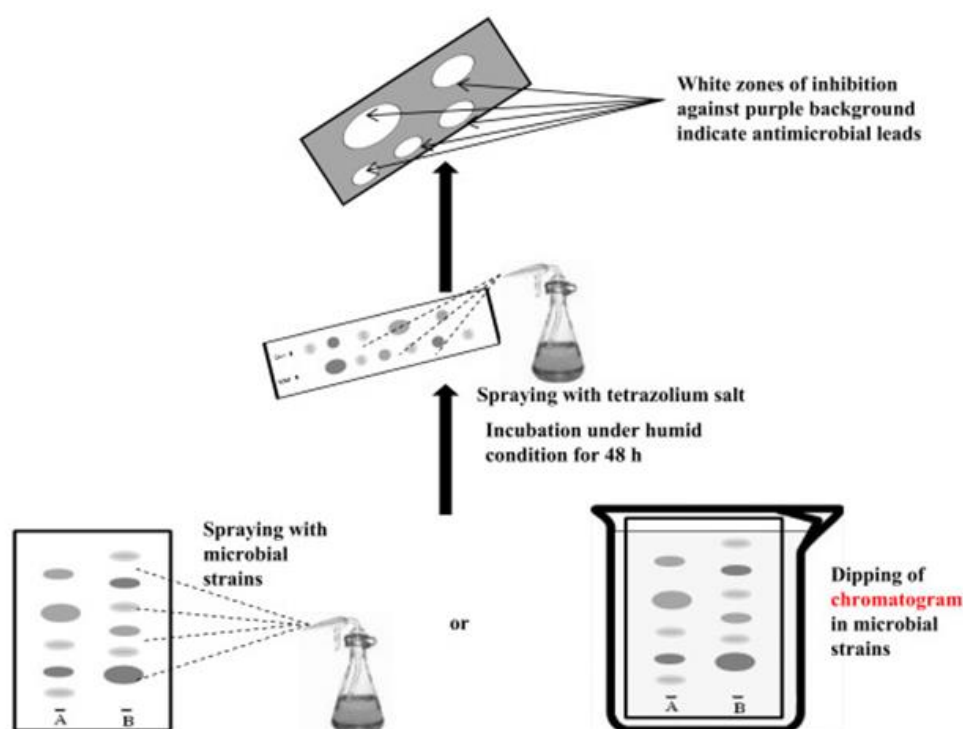
Figure 1.13: Schematic diagram indicates the direct bioautographic process (Dewanjee *et al*., 2015).

Agar overlay is a combination of contact and direct bio-autography. In this method, the chromatogram is covered in a molten, seeded agar medium. After solidification, incubation and staining (usually with tetrazolium dye), the inhibition or growth bands can be seen (Figure 1.14).
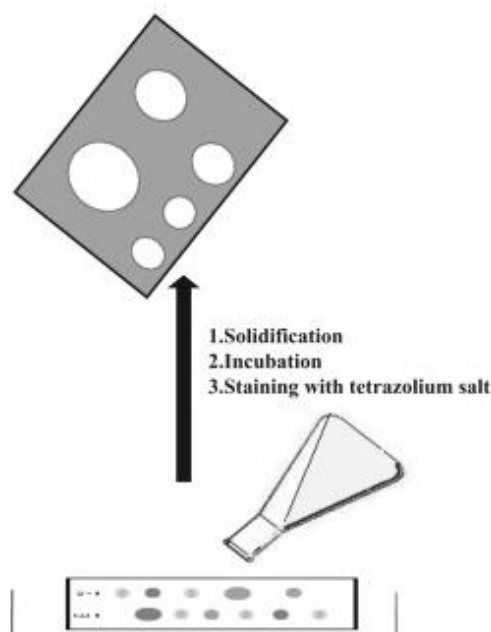
Figure 1.14: Schematic diagram of agar overlay bioautography (Dewanjee *et al*., 2015).

1.5.3 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a widely used technique for the isolation of natural products (Capone *et al*., 2015). In order to fully characterise the active entity, natural products are often isolated following the biological evaluation of a relatively crude extract. The bioactive component is often present only as a minor component in the extract and the resolving power of HPLC is well suited to rapidly process multicomponent samples on both an analytical and preparative scale (Hayes *et al*., 2014).

Gradient elution in which the proportion of organic solvent to water is altered with time may be desirable if more than one sample component is being studied and significantly differ from each other in retention under the conditions employed (Sasidharan *et al*., 2011). Purification of the compound of interest using HPLC is the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions. Identification of compounds by HPLC is a crucial part of any HPLC assay. In order to identify

any compound by HPLC, a detector must first be selected (Hayes *et al*., 2014). Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed. UV detectors are popular among all the detectors because they offer high sensitivity) and also because majority of naturally occurring compounds encountered have some UV absorbance at low wavelengths (Capone *et al*., 2015) (190-210 nm). Figure 1.15 shows a summarised illustration of how HPLC components.
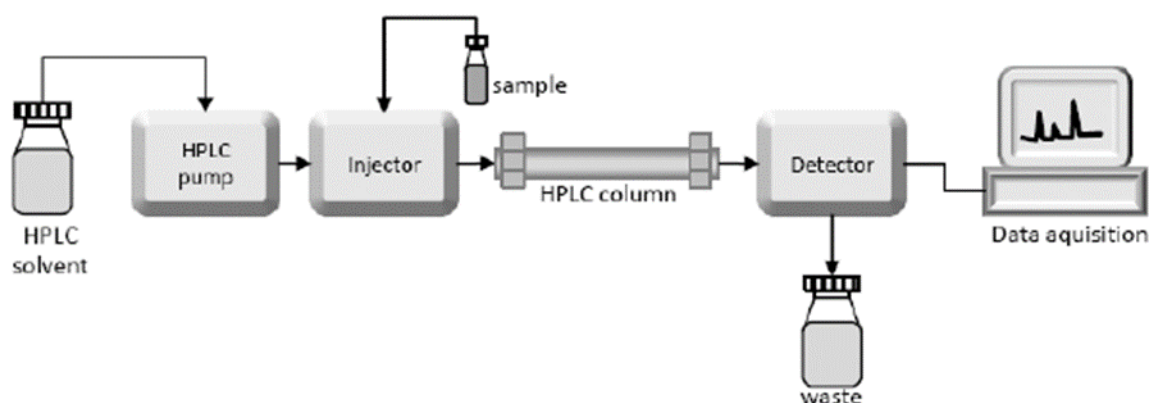
Figure 1. 15: High performance liquid chromatography system (Czaplicki, 2013).

Phenolic acids were extracted from the three date varieties and identified by high-performance liquid chromatography (HPLC) as described by Mansouri *et al*. (2005). One hundred grams of sample was mixed with 300 mL methanol/water (4:1, v/v) for 5 h at room temperature and with continuous agitation. The mixture was then centrifuged for 5 minutes at 6000g and the supernatant was evaporated under vacuum at 40 °C. The residue from the evaporation was treated with 200 mL of water (pH 2 with HCl) and left to rest for 5 min. The resulting mixture was then passed through a glass column (4.6 cm × 15 cm) packed with Amberlite XAD-2 resin (Fluka Chemie; pore size 9 nm, particle size 0.3–1.2 mm). Gallic acid, vanillic acid, coumaric

acid and ferulic acid to name a few and the acids were later tested for antioxidant activity Kchaou *et al*., 2016).

1.6 CHARACTERISATION OF ANTIMICROBIAL COMPOUNDS

Structural determination of compounds uses information from a vast range of spectroscopic techniques (Zhang *et al*., 2018). These include infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy (MS) and UV- visible spectroscopy (UV-Vis) (Altermimi *et al*., 2017). Passing electromagnetic radiation through an organic molecule that absorbs some of the radiation is the basic principle of spectroscopy. Measuring the quantity of absorbed electromagnetic radiation produces a spectrum (Zhang *et al*., 2018). The spectrum is bond specific and depending on the spectra, the structure of the molecule can be elucidated. For structural clarification, scientists use spectra produced from three or four regions, namely visible, ultraviolet, infrared and electrical beam (DeVijlder *et al*., 2018).

1.6.1 Nuclear Magnetic Resonance (NMR) spectroscopy

Magnetic properties of selected atomic nuclei are what NMR is based on, namely, the nuclei of the carbon atom, the hydrogen atom, the proton and an isotope carbon (Vioglio *et al*., 2018). NMR allows researchers to study molecules by noting variances between various magnetic nuclei and giving the positions at which, these differences are found within the nuclei of these molecules (Hansen *et al*., 2016). In addition, it shows the kinds of atoms found in neighbouring groups. In essence, it is able to conclude on the number and type of atoms present in each of these environments.

1.6.2 Fourier-transform infrared spectroscopy (FTIR)

When infrared light passes through an organic compound, some of the frequencies will be absorbed; however, some are transmitted through the sample without absorption happening. IR absorption is related to the vibrational changes that occur inside a molecule when it is exposed to infrared radiation. Essentially infrared spectroscopy can be described as vibrational spectroscopy. Different bonds (C–O, C–C, N–H, C≡C, C=C, C=O and O–H) have different vibrational frequencies (Jeffrey *et al*., 2015; Altermimi *et al*., 2017). The presence of these bonds in a molecule means that its identification is possible by detecting their unique frequency absorption bands in an IR spectrum. Fourier transform infrared spectroscopy (FTIR) is a high-resolution analytical tool to identify the functional groups present in a compound (Karimi and Taherzadeh, 2016). FTIR offers a rapid and non-destructive investigation to fingerprint herbal extracts or powders.

Samples for FTIR can be prepared in a number of ways. For liquid samples, the simplest way is to place one drop of sample between two plates of sodium chloride. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) and then compressed into a thin pellet which can be analysed. Otherwise, solid samples can be dissolved in a solvent such as methylene chloride, and the solution then placed onto a single salt plate. The solvent is then evaporated off, leaving a thin film of the original material on the plate.

1.7 TOXICITY ASSAYS AND *in vivo* EVALUATION

Opoponax essential oil, a chemical component; β-bisabolene and an alcoholic analogue, α-bisabolol, were tested for selectively kill breast cancer cells by Yeo and co-workers in 2016. It was observed that only β-bisabolene, a sesquiterpene constituting 5% of the essential oil, exhibited selective cytotoxic activity for mouse cells (IC50) and human breast cancer cells

(IC50). This loss of viability was because of the induction of apoptosis. β-bisabolene was also effective in reducing the growth of transplanted 4T1 mammary tumours by 37.5% (Yeo *et al*., 2016).

1.8 *In vitro* EVALUATION OF CITRUS FLAVONOIDS

Meiynanto and co-workers (2012) tested citrus flavonoids for the potential to treat cancer. The flavonoids worked either alone or as combination with chemo-preventive agents. The mechanisms of action involved but were not limited to cell cycle modulation, apoptosis induction and antiangiogenic effects. Naturally occurring compounds were also shown to reduce the incidence of resistance and thus increase the efficacy of chemotherapeutics. Oestrogen plays a crucial role in the development and differentiation of breast and endometrial cells, thus may have certain effects in the growth of cancer. *Citrus reticulata* ethanolic extract was potential to be developed as phytoestrogen as its presence in animal trials shows its ability to mimic oestrogens function of strengthening bones and lowering cholesterol levels (Meiynanto and Anindyajati, 2012).

1.9 RATIONALE FOR THE STUDY

The emergence of diseases and drug resistant pathogens coupled with the side effects presented by conventional or synthetic drugs necessitates the discovery of new antibiotics, agrochemicals and chemotherapeutics. *Kigelia africana* fungal endophytes are not well known and thus they offer untapped potential for drug discovery. In 2014, forty endofungal extracts from the Lab 2 culture collection were previously screened for antimicrobial and antioxidant activity with two, namely, ZF 52 and ZF 91, displaying excellent antibacterial activity (minimum inhibitory concentrations of 25 and 0.1953 µg/mL, respectively) against a resistant *P. aeruginosa* strain.

The active anti-*P. aeruginosa* compounds produced by ZF 52 and ZF 91 needed to be purified and more *K. africana* endophytic fungi should be screened for anti-*P. aeruginosa* activity.

1.10 HYPOTHESIS TESTED

It was hypothesized that *K. africana* ZF 52, ZF 91 and other endofungal extracts will display antimicrobial activity against *P. aeruginosa*. It was further hypothesized that the extracts with anti-*P. aeruginosa* activity could be purified and characterised.

1.11 AIMS AND OBJECTIVES

The aim of the study was to perform a phytochemical analysis on the medicinal plant species, and to test *K. africana* ZF 52, ZF 91 and other endofungal extracts for antimicrobial activity against *P. aeruginosa*.

The following objectives were set to test the above hypothesis and achieve the aims of the study:

- To extract biomolecules from endophytic fungal strains, ZF 52, ZF 91 and to screen Lab 2 endophytic fungi culture collections for anti-*P. aeruginosa* activity
- To induce secondary metabolite production in solid substrate fermentation
- To obtain concentrated extracts of secondary metabolites in various solvent systems
- To purify extracts produced by ZF 52, ZF 91 and Lab 2 culture collection using preparative thin layer chromatography, bio-autography and column chromatography
- To characterise the purified anti- *P. aeruginosa* compound

To identify the purified compound by Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) spectroscopy.

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CHAPTER 2: SCREENING EXTRACTS FOR ANTI- *P. aeruginosa* ACTIVITY

2.1 INTRODUCTION

The rise of microbial resistance to antibiotics and the scarcity of new drugs has long been the subject of discussion (Theuretzbacher and Mouton, 2011). The lack of new drug development, misuse and overuse has been attributed to the antibiotic resistance crisis (Ventola, 2015). Alexander Fleming stated in 1945 that the drug penicillin would be in great demand which would lead to an era of antibiotic abuse (Ventola, 2015). The emergence of resistance is driven by three main mechanisms, namely: the rapid increase of antibiotic resistant bacteria due to overexposure to antimicrobial compounds or other contaminants (heavy metals and biocides), horizontal gene transfer of antibiotic resistance genes and recombination and genetic mutation (Berendock *et al*., 2015). Acquiring effective, affordable and novel drugs to treat microbial infections is a global healthcare challenge but mainly in third world countries where more than half of the deaths are due to infectious diseases (Awouafack *et al*, 2012; Srivastava *et al*., 2013; Elisha *et al*., 2017).

It is estimated that 80% of the world's population relies on herb-based medicine. Except for homeopathy, the healing properties of these plants are evaluated by their chemical composition (Ventola, 2015). Many plants are used in medicine with Indian Ayurveda and the Chinese system using roughly 2000 and 5757 plants, respectively (Ventola, 2015). Similar to Asia, Africa is known for its ancient traditional practices and vast number of traditional healers. Due to this, scientists have focused their attention on these plants that have the capacity to heal. Plants have the ability to produce a variety of secondary metabolites, for example, tannins, alkaloids, flavonoids, glycosides, terpenoids, steroids, quinones, coumarins and saponins (Das *et al*., 2010), which have been the source of antimicrobial substances (Fernebro, 2011; Elisha *et al*., 2017).

In the Southern African tropical regions, a commonly encountered medicinal plant is *K. africana.* All parts of this plant have been used in traditional medicine; the fruit is used to treat ulcers, syphilis, abscesses, wounds and rheumatism (Bello *et al*., 2016). The bark and roots are used to treat pneumonia. In West Africa, the fruit is used to treat haemorrhoids, dysentery and gynaecological disorders. The traditional medicinal uses of *K. africana* point to the plants potential as a source of antibacterial compounds. However, there is no scientific research to validate its ethnomedicinal use. There have been studies which examined the plant chemistry, but these were not been linked with bioactivity studies (Das *et al*., 2010; Arkhipov *et al*., 2014; Cook and van Vuuren, 2014). Some studies using the bark extracts showed cytotoxity and good results in treating renal carcinoma and melanoma (Saini *et al*., 2009; Arkhipov *et al*., 2014). Different parts of this plant showed growth inhibition against *Klebsiela pneumonia*, *Proteus vulgaris*, *S. aureus*, *Escherichia coli* and *P. aeruginosa* (Hussain *et al*., 2016).

In recent years, focus has shifted to conservation of medicinal plants which has led to the search for alternative sources of secondary metabolites (Kusari *et al*., 2013). Studies on plant microbiomes indicated that the plant along with its associated microbiome produces bioactive molecules. Investigations of the same plant species located in different geographical regions showed that they produced different bioactive metabolites, which suggested that the endophytic microorganisms produce the bioactive metabolites and not the plants (Idris *et al*., 2013; Kusari *et al*., 2013). Endophytes are bacteria, actinomycetes and fungi that inhabit healthy plants without causing them any harm (Das *et al*., 2017).

Pseudomonas aeruginosa is the leading cause of infection in hospitalised patients. It is resistant to many antimicrobial agents due to its various resistance mechanisms (Joo *et al*., 2011; Nyasulu *et al*., 2012; Holmes *et al*., 2016). The ability of the organism to develop additional resistance during treatment further limits treatment (Hancock and Speert, 2000;

Mudau *et al*., 2013). Risk factors for *P. aeruginosa* infections include presence of previous antibiotic use, admission to an intensive care unit, indwelling devices, prolonged hospital stay, pre-existing diseases and an immuno-compromised status (Harris *et al*., 2002; Joo *et al*., 2011; Mudau *et al*., 2013).

A study conducted by Nyasulu *et al*. (2017) followed the trend of increased *P. aeruginosa* resistance in South African hospitals between 2005 to 2009 and observed a moderate rise in resistance for aminoglycosides (gentamicin 21.7% to 53.7%, tobramycin 38.7% to 60.2% and 25.7% to 39.1% for amikacin), carbapenems (a rise from 45% to 55% for meropenem and imipenem), cephalosporins (cefepime 36.6% to 43.6% and 17.1% to 24.6% for ceftazidime) and ciprofloxacin (25.9% to 53.7%) (Nyasulu *et al*., 2017). The increasing resistance of *P. aeruginosa* to most classes of antibiotics warrants the screening of previously untapped sources for anti-*P. aeruginosa* activity.

2.2 MATERIALS AND METHODS

2.2.1 Growth and maintenance of cultures

Endophytic fungal isolates (previously isolated from leaves of *K. africana*) were obtained from the Lab 2 culture collection and sub cultured onto potato dextrose agar (PDA). The isolates were grown for four days at room temperature (RT), stored at 4°C and used as working stocks (Strobel and Daisy, 2003). *Pseudomonas aeruginosa* ATCC 27853 was sub-cultured from stock culture (Dr Chenia, Microbiology Department in UKZN) and grown on Nutrient agar (NA) at 37°C for 24 h. Long-term endofungal stocks were prepared by inoculating spores from aged plates into 1.5 mL Eppendorf tubes containing 1 mL of 40% glycerol. Bacterial stocks were prepared by growing a loopful of a 24 h plate culture of *P. aeruginosa* into 3 mL nutrient

broth and transferring 0.5 mL of broth culture and 0.5 mL of 40% glycerol into a 1.5 mL Eppendorf tube. Both fungal and bacterial stocks were kept at -80°C.

2.2.2 Production of biomolecules

To induce production of biomolecules, standardised inocula (8 mm blue pipette tip back) from PDA plates were inoculated into rice medium (10 g of rice supplemented with 20 mL peptone water (0.5% peptone and 0.3% sodium chloride)). Solid substrate fermentation (SSF) was performed at room temperature for 30 days (Talonsi *et al*., 2012). In addition, to determine optimum time of bioactive molecule production, aliquots of biomass were extracted weekly, for four weeks.

2.2.3 Extraction of bioactive molecules

Bioactive biomolecules were extracted from mycelial mats using ethyl acetate (EtOAc) (Prabavathy and Nachiyar, 2014). To halt the fermentation process, 10 g of the rice medium containing the biomass was soaked in 50 mL of EtOAc for 24 h under gentle agitation (100 rpm, RT). The biomass and the EtOAc extract were separated by vacuum filtration through Whatman No.1 filter paper. The EtOAc extract containing bioactive compounds was washed with 50 mL demineralized water to remove traces of starch. The organic layers were collected and evaporated using a rotary evaporator. The extract was weighed and resuspended in EtOAc to a final concentration of 10 mg/mL and stored in the refrigerator at 4°C until required.

2.2.4 Assessing anti-*P. aeruginosa* activity

2.2.4.1 Disc diffusion assay

The test strain used was a multidrug resistant *P. aeruginosa* ATCC 27853, which is resistant to β- lactams, aminoglycosides and fluoroquinolones. The test bacteria were inoculated onto nutrient agar (NA) plates and incubated for 24 h at 37°C. For a standardised inoculum for the disc diffusion assay, culture was inoculated in 500 µL of demineralised water and the cell density was standardised to 0.5 McFarland standard using a spectrophotometer (UV 1800). The standardised bacterial cells were spread onto Mueller-Hinton (Merck, Germany) plates using sterile cotton swabs and left to dry. Extracts (100 µL) were saturated on sterile filter paper discs and used as antibiotic discs. The plates were incubated at 37°C for 24 h and the resultant zones of inhibition were measured and scored according to Table 2.1. The experiment was done in triplicate.

Table 2.1: Scoring of activity of extracts (mm) (Chenia, 2013)

0-6	7-9	10-15	16-17	≥18
No activity	No significant activity	Poor activity	Good activity	Significant activity

2.2.4.2 Minimum inhibitory concentration (MIC) determination

The broth dilution microtitre assay was carried out as described by Kwon *et al.* (2007) to determine the minimum inhibitory concentration (MIC). The lowest dilution or concentration which inhibits growth is the MIC (Kwon *et al*., 2007; Zin *et al*., 2007). The standardised test culture inoculum was prepared as described in 2.2.4.1. The bacterial cells were harvested and

standardised to 0.5 McFarland standard in sterile distilled H_2O. A 1:140 dilution of cells was made in Muller Hinton (MH) broth (71 µL of standardised cells and 9.929 mL of MH broth).

Microtitre plates were loaded with the different components as follows: 100 µL of MH broth was added into all the wells, an aliquot of 100 µL of extract was added into well one, mixed and 100 µL was transferred to well two, and so on until well nine. The final 100 µL from well nine was discarded. After addition of extracts, 100 µL of the 1:140 diluted cells were added to all the wells. Plates were incubated at 37°C for 24 h. Thereafter 30 µL resazurin dye was added and further incubated at 37°C for 4 h. Results were read as follows: live cells were pink and dead cells were blue. The highest dilution displaying the blue colour was regarded as the MIC.

2.2.5 Thin layer chromatography

Crude extract (1 µL) was spotted onto thin layer chromatography (TLC) plates (Merck silica gel 60, 20 x 20 cm F254 aluminium sheets: Merck, Germany) and left to develop in various solvent systems (Yu *et al*., 2010). A solvent system of chloroform ($CHCl_3$):methanol (MeOH) and hexane:EtOAc (using 100% $CHCl_3$ or hexane that was increased stepwise by 10% to 100% MeOH or EtOAc, respectively) was screened to assess their separation efficiency (Selvameenal *et al*., 2009). The TLC plates were spotted with the sample using capillary tubes, the plates were placed in the solvent mixture in a developing tank and left to run till the solvent front reached approximately half a centimetre from the top of the plate. TLC plates were viewed under an ultraviolet lamp (254 nm) and developed using 10% H_2SO_4 in MeOH (Selvameenal *et al*., 2009).

2.2.6 Bio-autography

Agar overlay bio-autography was performed by pouring 10 mL of molten agar seeded with 1 mL of *P. aeruginosa* bacterial suspension (0.5 McFarland standard) over the developed

chromatogram and incubating at 37°C for 24 h. After incubation, all plates were sprayed with a 2% (w/v) 2,3,5–triphenyltetrazolium salt stain which stains live cells red (Dewajee *et al*., 2015). Clear zones were taken to represent inhibition of growth by anti-*P. aeruginosa* compounds.

2.2.7 Identification of fungal isolates that produce active compounds of interest

2.2.7.1. Genomic deoxyribonucleic acid isolation

Genomic deoxyribonucleic acid (DNA) isolation was conducted using the ZR Soil Microbe DNA Miniprep™ (Zymo Research, USA) according to the manufacturer's instructions. After extraction, the DNA was analysed by agarose gel electrophoresis. DNA samples were stored at -20°C.

2.2.7.2 Amplification of internal transcribed spacer two region

The universal primers for the fungal internal transcribed spacer two (ITS2) region of the 18S ribonucleic acid (rRNA) gene were used in the PCR reaction (White *et al*., 1990; Ristaino *et al.,* 1998). The ITS primer pair sequences were as follows:

Forward: ITS 5 (5' – GGAAGTAAAAGTCGTAACAAGG – 3')

Reverse: ITS 4 (5' – TCCTCCGCTTATTGATATGC – 3')

The PCR reaction mixture (50 µL) consisted of template DNA, 2.5 µM of each of the forward and reverse primers, 25 mM MgCl$_2$, 10 mM dNTPs, *Taq* DNA polymerase, 10 mM buffer (Thermo Scientific). The reaction mixture was brought to 50 µL using double distilled water. Amplification of the reaction mixture was conducted under the following thermal cycling conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s (Anderson *et al.,* 2003).

2.2.7.3. Sequencing of ITS2 region

The PCR products were visualised using electrophoretic analysis on a 1% agarose gel stained with ethidium bromide. After confirmation that the amplification was successful, amplicons were sequenced using Sanger sequencing at Stellenbosch University, South Africa. The identity of the isolates was determined using the basic local alignment search tool (BLAST) analysis, using the NCBI database (Anderson *et al*., 2003).

2.3 RESULTS

2.3.1 Growth and maintenance of cultures

Potato dextrose agar plates were used to successfully resuscitate thirty-three endophytic fungi as the grown fungi matched the original isolates in terms of varying characteristics and pigmentation. The pigmentation of the mycelia intensified with age from lightly pigmented as the mycelia grew into fresh media (Fig 2.1). This was observed for all fungi growing on PDA.
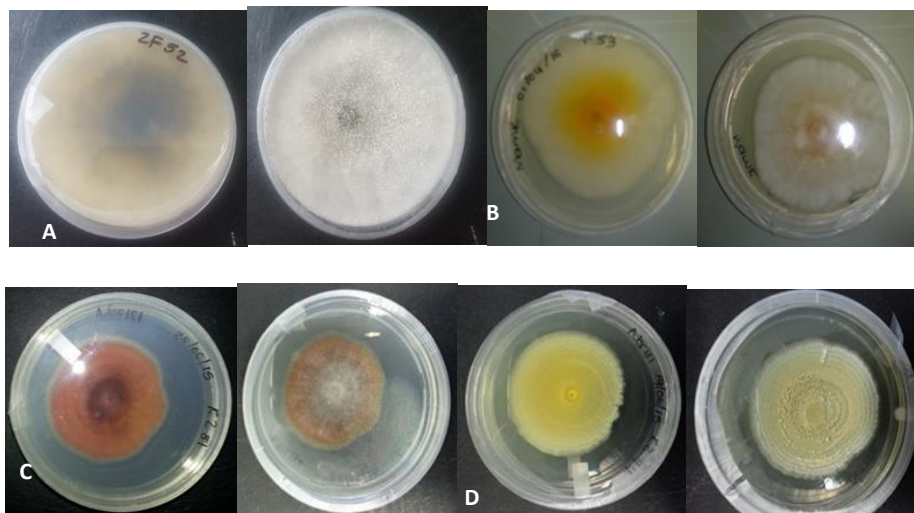
Figure 2.1: Growth of endophytic fungi on potato dextrose agar media for 4 days. A and B: show ZF 52 and ZF 53 fungal isolates (top and bottom); C and D: show KZ 81 and ZF KZ 44.

2.3.2 Production of biomolecules

The solid substrate fermentation reaction contents showed varying pigmentation where initial growth was observed on day four of the 30-day period for fast growing fungi and as delayed as day 15 for slow growing fungi. The presence of white fluffy growths on the surface of the solid substrate was common in all the fermentation vessels. Pigmentation developed with time as observed in Figure 2.2. A characteristic of most of the fermenting biomass was that it matched the pigment observed on PDA plates, with one exception namely ZF 52, which was black and yellow on PDA and black in SSF.
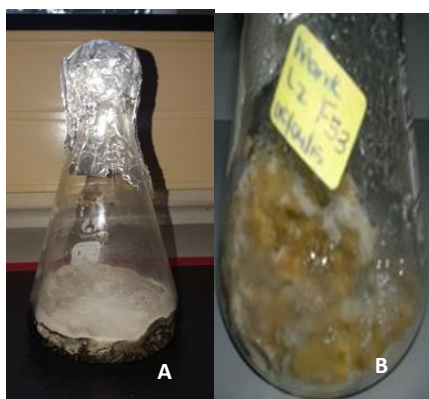
Figure 2.2: Development of mycelial mat growth during the SSF. A: ZF 52, B: ZF 53.

2.3.3 Extraction of bioactive molecules

An assortment of pigments was observed after solvent extraction. The pigmentation of the extracts mostly matched the pigments of the biomass on the PDA plates and SSF reaction vessels, with the exception of ZF 52 whose extract matched only the growth on PDA and not during SSF (Fig 2.3).

Figure 2. 3: Endophytic fungi extracts obtained following solvent extraction.

2.3.4 Assessing anti-*P. aeruginosa* activity

2.3.4.1 Disc diffusion assay

Variable results were obtained using the disc diffusion assay for the 45 extracts. Most extracts did not inhibit *P. aeruginosa*, while a few (three) had significant activity (Figure 2.4 A and B). The size of the zones of inhibition depicted good activity of the extracts. ZF 52 displayed the largest zone of inhibition (40 mm) for T4. Zones of inhibition obtained differed from extract to extract and these ranged from 0-40 mm. ZF 52, ZF 91 and ZF 34 had very good activity, however, due to difficulties encountered in resuscitating long-term cultures from storage, ZF 91 and ZF 34 were not tested further resulting in ZF 52 being the only fungal extract tested further. A time course was conducted in order to observe the difference in activity from week one to week four. The bioactive compounds are produced as early as week as seen in Fig 2.4 C.
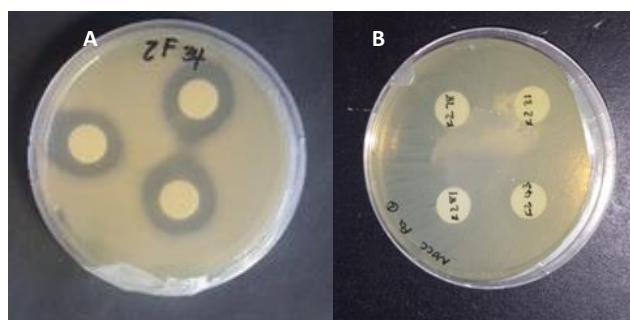
Figure 2.4: Kirby Bauer disc diffusion assay demonstrating anti-*P. aeruginosa* activity of ethyl acetate extracts of endophytic fungi; A: ZF 34 and B: KZ 81, KZ 43, KZ 31 and KZ 78.
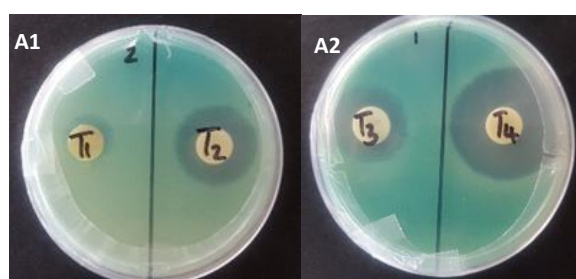

Figure 2.5: Kirby Bauer disc diffusion assay demonstrating anti-*P. aeruginosa* activity of ZF 52 extracts after varying fermentation periods. A1 indicates ZF 52 extract produced after one (T1) and two (T2) weeks and A2 indicates ZF 52 extracts produced after three (T3) and four (T4) weeks.

2.3.4.2 MIC determination

Minimum inhibitory concentrations were determined for those extracts with large inhibition zones, namely, the ZF 52 extracts produced after varying periods of solid substrate fermentation (T1 to T4) (Figure 2.6). All rows A, B, C and D show very strong activity. Extract T4 displayed the strongest activity (lowest MIC) against *P. aeruginosa* while the one with the weakest activity (highest MIC) was T1. The crude extracts developed greater antimicrobial activity over time; the MICs of the extracts were 156.5 µg/mL, 39.06 µg/mL, 78.73 µg/mL and 19.53 µg/mL for T1, T2, T3 and T4, respectively.
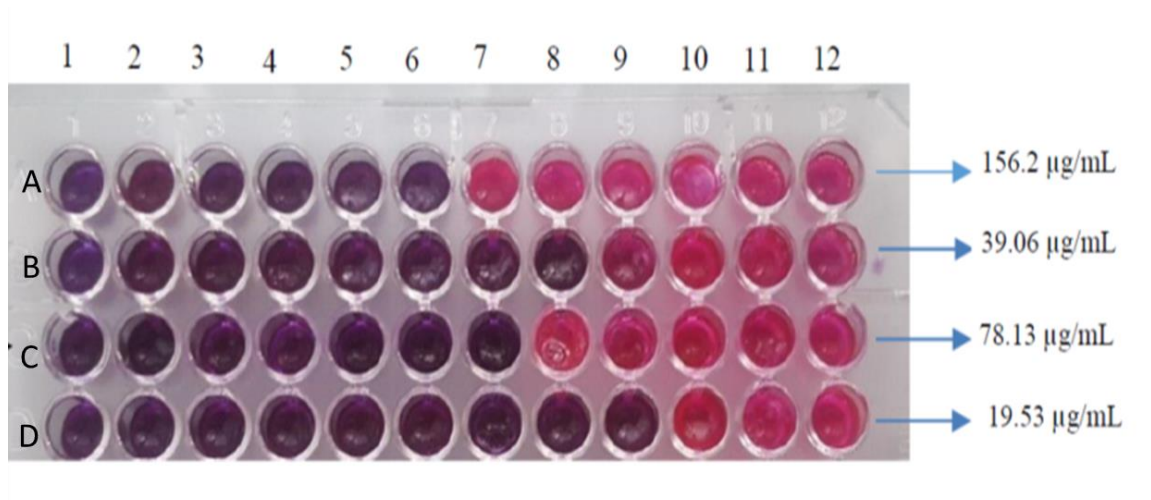
Figure 2.6: Resazurin colour reaction for minimum inhibitory concentration (µg/mL) determination of extracts against *P. aeruginosa.* Row A: 1 week ZF 52 extract 156.3; row B: 2 week ZF 52 extract 39.06; row C: 3 week old ZF 52 extract 78.13 and row D is a 4 week old ZF 52 extract 19.53.

Table 2. 2: Interpretation of the resazurin microtitre assay

Extract	Concentration (µg/mL)											
	1	2	3	4	5	6	7	8	9	10	11	12
	5000	2500	1250	625	312.5	156.3	78.13	39.06	19.53	9.766	4.883	2.441
T1	-	-	-	-	-	-	+	+	+	+	+	+
T2	-	-	-	-	-	-	-	-	+	+	+	+
T3	-	-	-	-	-	-	-	+	+	+	+	+
T4	-	-	-	-	-	-	-	-	-	+	+	+

Keys: +: growth; -: no growth

2.3.5 Thin layer chromatography

All the solvents tested resulted in separation of the different compounds in the crude extract. $CHCl_3$ and EtOAc resulted in little separation of the different compounds in the extract (Figure 2.7 A), with smears being observed. Figure 2.7 B shows better separation resulting from TLC using hexane and EtOAc. Figure 2.8 C showed the separating of the time-course extracts when viewed under UV light. The compound with Rf values around 0.78 was produced from the first week. On the second week the band begins to separate, and the band is seen slightly higher

than the one at T1. Better separation and more compounds are observed from week three while the four-week-old extract shows that the compounds become more concentrated.   The compounds within ZF 52 crude extract are really produced from the first week of SSF, this corroborates Figure 2.5.

Figure 2. 7: TLC analysis of ZF 52 crude ethyl acetate extracts; separated using (A) CDCl$_3$:EtOAc (60:40). A - ZF 52 at four week ethyl acetate extract viewed with staining; (B) - ZF 52 separation using chloroform:ethyl acetate (Rf: 0.22, 0.30, 0.34, 0.43, 0.57 and 0.78) without staining (60:40).
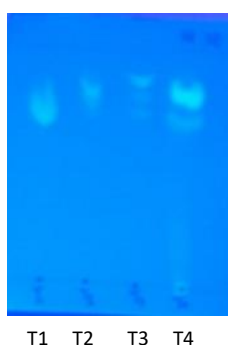
Figure 2.8: TLC analysis ZF 52 crude ethyl acetate extracts of varying ages separated using hexane:EtOAC and viewed under UV. T1- one week old; T2- two week old; T3- three week old and T4 is a four week old ZF 52 ethyl acetate extract.

2.3.6 Bio-autography

Bio-autography revealed that the majority of the compounds resolved on the TLC plates contained bactericidal activity as three (Rf: 0.22, 0.30 and 0.78) bands produced halos after the

dye was sprayed (Figure 2.9). However, the bands close to the origin had no bactericidal activity because these bands appeared pink.

Figure 2. 9: Bio-autogram of *P. aeruginosa* treated ZF 52 extract. Live cells appear pink and dead cells are presented by a halo. A - bio-autogram; B - TLC plate.

2.3.7 Identification

2.3.7.1 Genomic DNA isolation

It was extremely difficult to lyse the mycelia of ZF 52. Poor yields of genomic DNA were obtained after several attempts of using aged mycelia. In the final attempt, a 24 h culture was manually disrupted using forceps. The resultant small mycelia were then subjected to bead bashing as per kit instruction manual but the vortexing time was increased from 30 s to 60 s. A barely visible yield of genomic DNA was obtained visualised on an agarose gel which was stained with ethidium bromide (Figure 2.10).
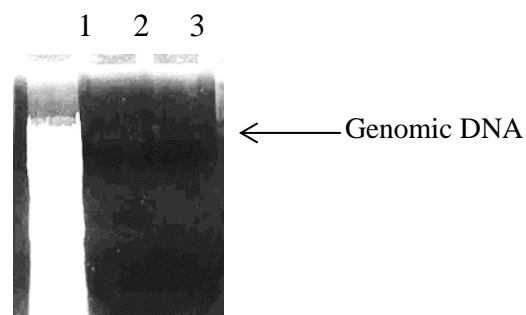
Figure 2. 10: Genomic DNA isolated from ZF 52 on agarose gel. L1: 100 bp plus marker, L2 and L3: ZF52 DNA.

2.3.7.2 PCR amplification

Figure 2.11 shows the gel image of the PCR product obtained after amplification of the ITS2 region of the 18S rRNA gene. The PCR amplification was successful as the expected amplicon size of 600 bp was obtained.
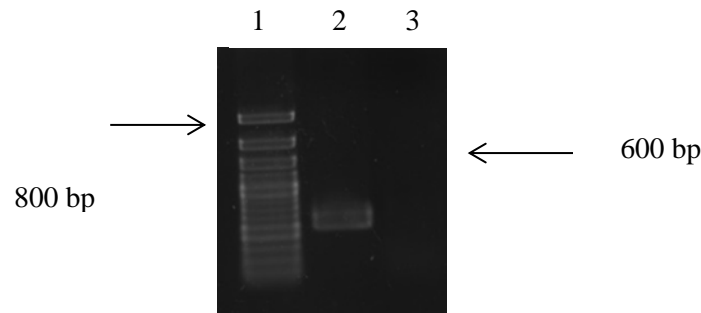

Figure 2.11: Agarose gel image of the ITS2 region of18S rRNA gene after PCR amplification. L1: 100 bp Gene ruler, L2: PCR amplification product, L3: negative control.

2.3.7.3 Identification of ZF52

The 18S PCR amplicon was sequenced at Stellenbosch University, South Africa. Trimmed forward and reverse sequences were aligned and submitted to Genbank for comparison to other sequences. The result returned from Genbank showed ZF 52 has 99% identity to *Neofusicoccum luteum* strain CMW 10309 (Table 2.3).

Table 2.3: BLAST analysis of ZF 52

Description	Max score	Total score	Query cover	E value	Identity	Accession
Neofusicoccum luteum strain CMW 10309 18S ribosomal RNA gene, partial sequence: internal transcribed spacer 1.5 ribosomal	1070	1070	99%	0.0	99%	KF766202. 1

2.4 DISCUSSION

Resuscitation of the fungal endophytes from stock cultures was successful as morphology and pigmentation matched the original plates. Darkly pigmented medicinal plant extracts are reported in literature to possess strong bioactive activity; these include but are not limited to anti-HIV, antimalarial, antimicrobial, antioxidant and anticancer properties. The darkly pigmented endophytic extracts had no activity against *P. aeruginosa* as all three darkly pigmented extracts did not produce zones of inhibition. A study by Sharma *et al*. (2016) described a fungus with olive green pigmentation with extracts containing bioactive metabolites further suggesting that dark pigmented fungi that produce dark extracts are the most likely to contain bioactive metabolites.

In the current study, however, the darkly pigmented endophytic extracts had no activity against *P. aeruginosa* as all three darkly pigmented extracts did not produce zones of inhibition. However, ZF 52, ZF 91 and ZF 34, which were lightly pigmented had good activity against *P. aeruginosa* contrary to Sharma *et al*. (2016). No two extracts had the same colour. It is tempting to assume that they were produced by different fungal species or different strains of the same endophytic fungus as some of the fungi had the same pigmentation and colony morphology. This may be confirmed by identifying all the isolates.

In the antimicrobial activity testing, it was observed that most of the extracts had little to no activity against *P. aeruginosa*. In a previous study, it was observed that *K. africana* endophytic fungal extracts had antimicrobial activity mainly against fungi (Kuali, 2015, Shezi, 2014). This could be because within *K. africana*, beneficial and pathogenic fungi compete for nutrients and shelter thus resulting in fungal endophytes being able to kill most fungi. Out of the 45 extracts screened, only three namely ZF 52, ZF 91 and ZF 34 displayed activity against multi drug

resistant *P. aeruginosa* strain (with 40.33, 22 and 20 mm diameter zones of inhibition, respectively). A study by Ratnaweera and co-workers (2015) also reported variable zones of inhibition (8 to 35 mm) against three Gram-positive bacteria, *Bacillus subtilis* (UBC 344), *Staphylococcus aureus* (ATCC 43300) and MRSA (ATCC33591), two Gram-negative bacteria, *Escherichia coli* (UBC 8161), *P. aeruginosa* (ATCC 27853) and the pathogenic fungus *Candida albicans* (ATCC 90028). They screened eight endofungal extracts from the arid zone invasive plant *Opuntia dillenii* and only two produced zones of inhibition above 30 mm. However, none of these extracts were active against *P. aeruginosa*.

Phenolics, alkaloids and terpenes are the most encountered antibacterial compounds found in medicinal plants and it would be no surprise if the extracts from ZF 52, ZF 91 and ZF 34 also contain these compounds (Chandra *et al*., 2017). Prabukumar *et al*. (2015) tested endophytic fungi CC1, CC2, CC3 and CC4 isolated from *Crescentia cujete* for antibacterial activity. These were evaluated against Gram-negative bacteria *Salmonella typhi* (ATCC 51812), *Shigella flexneri* (MTCC 1457), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae* (ATCC 432), *P. aeurginosa* (ATCC-27853), *E. coli* (ATCC-25922) and Gram-positive bacteria *B. subtilis* (ATCC 441), *S. aureus* (ATCC 25923). These extracts displayed good activity against bacterial human pathogens, in general. Strain CC1 and CC3 crude extracts displayed intermediate inhibition activity against all the human pathogens except *P. aeurginosa* and *S. aureus* while the crude extract of CC4 also inhibited all these human pathogens except for *S. aureus*. The extract of CC2 inhibited the growth of *S. typhi* and *Shigella* sp. The MeOH and EtOAc fungal crude extracts of endophytic fungus *Pestalotiopsis neglecta* BAB-5510 isolated from the leaves of *Cupressus torulosa* inhibited the growth of both Gram-negative and Gram-positive bacterial human pathogens (Sharma *et al*., 2016). These had activity against *S. aureus*, *B. subtilis*, S. *typhimurium* and *E. coli*. However, they displayed no activity against *P. aeruginosa*.

Thin layer chromatography was conducted using several solvent systems before the one giving optimal separation was identified. The most efficient solvent system was hexane:EtOAC (60:40) and it resulted in six distinct spots with Rf values of (0.22, 0.30, 0.34, 0.43, 0.57 and 0.78). In a study conducted by Rashid *et al*. (2017), various solvent systems were utilised with the most effective one being toluene:EtOAC:acetic acid (36:12:5) which resulted in five spots which had Rf values of 0.4, 0.45, 0.46, 0.55, 0.82. Sharma *et al.* (2016) also used TLC to separate the different compounds from the crude extract of *P. niglecta* and the solvent system with optimal resolution was dichloromethane:MeOH (90:10). This yielded two distinct bands with the second band (Rf of 0.79) displaying antibacterial activity.

Antibacterial components were present in the EtOAC extract (Figure 2.9). This was consistent with a study conducted by Jesionek *et al*. (2017) where halos were observed on the bio-autogram against several bacterial test strains. The bio-autogram in the current study showed more than one spot of the developed TLC plate to have anti-*P. aeruginosa* activity. Live cells were seen close to the origin and on the fifth spot indicating that four out of five spots possessed anti-*Pseudomonas* activity. A study by Samrot *et al.* (2016) that utilised TLC and bio-autography to screen extracts from *Mangifera indica* observed a band (Rf of 0.82) that displayed anti-*P. aeruginosa* activity which is not frequently encountered, and which was contrary to the current study. However, in their study, they used a different dye, namely, the MTT dye instead of the 2% (w/v) 2,3,5–triphenyltetrazolium dye. Methanolic extracts of *Ricinus communis* were used to conduct bio-autography with *P. aeruginosa* (PC 002) and *K. pneumoniae* (MTCC 3384) as the test cultures (Sandam and Su, 2015). For *P. pneumoniae* four active bands were observed in total for the fractions with Rf values: 0.200, 0.2750, 0.8875 and 0.9125. One active band with Rf value 0.2750 was observed for *P. aeruginosa*. The methanolic extracts of *R. communis* displayed more activity against *K. pneumoniae* than *P. aeruginosa*.

Sequencing and BLAST analysis of the ITS2 amplicon of ZF 52 had 99% identity to *Neofusicoccum luteum* strain CMW 10309. This fungus was previously identified by Shezi in 2014, this therefore means that culture storage and maintenance, resuscitation and biomolecule production was successful for ZF 52 but was unsuccessful for ZF 91 and ZF 34. Sharma *et al*. (2016) studied the endophytic fungi associated with the *Cupressus torulosa*. Five fungi were isolated and identified as *Alternaria alternata*, *Penicillium oxalicum*, *Daldinia* sp. and *Pestalotiopsis* sp. Idris *et al*. (2013) isolated seven endophytic fungi and identified them as *Aspergillus flavus*, *Aspergillus* sp., *Cladosporium* sp., *Culvularia lunata* and three unknown fungi. A study by Maheswari and Rajagopal (2013) identified 28 endophytic fungi, none of which was *N. luteum*. In contrast Wicaksono *et al*. (2017) isolated *N. luteum* from plants as a pathogen which was inhibited by endophytic *Pseudomonas* sp. The *P. aeruginosa* endophyte was reported to produce diffusible and volatile compounds which inhibited the growth of six botryosphaeriaceous species including *N. luteum*, which is the opposite to the findings of the current study. These finding from these two studies clearly points out the shifting nature of plant-fungal relationships that depend on the balance of antagonism – the interplay of plant, fungal, other organisms and environmental factors (Kusari *et al*., 2012).

2.5 CONCLUSION

Three extracts out of the 45 tested, displayed activity against *P. aeruginosa*. Although the proportion of active to inactive fungal extracts was low, the ones with activity had very significant activity. ZF 91 and ZF 34 were not tested further as resuscitation and storage difficulties were encountered. Separation of ZF 52 revealed the extract to contain more than one compound and the compounds present were shown to have antibiotic activity as 80% of the spots showed anti-*Pseudomonas* activity. The fungus, *N. luteum*, and the subsequent

extract that it produces are not well known, this therefore justifies this study. These findings

promote the purification and further testing the of *N. luteum* extract.

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CHAPTER 3: PURIFICATION AND CHARACTERISATION OF THE *Neofusicoccum luteum* ANTI-*P. aeruginosa* COMPOUND FROM THE ETHYL ACETATE CRUDE EXTRACT

3.1 INTRODUCTION

Endophytic *Botryosphaeriaceae* species have been reported to be in high abundance and encountered very frequently and research has targeted their potential as producers of novel secondary metabolites with anticarcinogenic, antibiotic and fungicidal activity (Saikkonen, 2002; Garcia *et al*., 2012; Dos Santos *et al*., 2016). However, the extent to which some of these species might be considered true endophytes or pathogens undergoing a latent phase still remains in question (Reveglia *et al*., 2018).

Neofusicoccum luteum host colonisation and infection happens mostly through horizontal transmission (individual infections via conidia or ascospores), although vertical transmission (infection through seeds) has been reported (Slippers *et al*., 2013; Lopes *et al*., 2016). Within the plant, species of this genus are able to exist without causing any symptoms and thus remain in the plant as endophytes. The endophytism of *Neofusicoccum* sp. could be the crucial feature aiding their wide distribution since they can be moved effortlessly around the world in cuttings, seeds and also in fruits, subsequently infecting both native and non-native trees in their new-found environments (Lopes *et al*., 2016). *Neofusicoccum luteum* is a plant pathogen and causes dieback and canker in grapevines and tomato cuttings (Altermimi *et al*., 2017; Rezgui *et al*., 2018).

Isolation and purification of bioactive compounds is a technique that has undergone new developments in recent years (Altemimi *et al*., 2015; Atlermimi *et al*., 2017; Kanagavalli and Sadiq, 2018). The technique provides concise separation, isolation and purification and offers an abundance of advanced bioassays (Poongunran *et al*., 2016). When looking for bioactive compounds, the goal is to find an appropriate method to screen the extracts for antimicrobial, antioxidant and or cell toxicity in conjunction with simplicity, speed and specificity (Eruygur *et al*., 2016; Altermimi *et al*., 2017).

Numerous bioactive molecules have been separated and purified by thin-layer and column chromatographic methods (Devika and Koilpillai, 2015). These chromatographic methods are still the mostly used due to their affordability, convenience and availability. Alumina, cellulose, silica and polyamide are the most important materials when separating separating plant-based compounds (Kangavalli and Sadiq, 2018). Using multiple mobile phases to increase polarity is very important for high value separations. Heteronuclear Multiple Bond Correlation (HMBC) experiment correlates chemical shifts of two types of nuclei separated from each other with two or more chemical bonds while the Distortionless enhancement by polarization transfer (DEPT) experiment is able to fully separate the carbon signals. DEPT 45 yields CH, CH2 and CH3 signals positive, DEPT 90 yields only CH signals and DEPT 135 yields CH and CH3 positive while CH2 s negative (Kangavalli and Sadiq, 2018).

Due to the promising results obtained in Chapter 2 regarding the crude extract of *N. luteum*, the fungus *N. luteum* being relatively undocumented and the extract it produces being unknown, the aim herein was to purify and characterise the anti-*P. aeruginosa* compound.

3.2 MATERIALS AND METHODS

3.2.1 Activity-based purification by column chromatography

A glass column was prepared for silica gel gravity chromatography. The column was rinsed with hexane (reagent grade, Merck, India) and left to dry. A cotton plug was placed at the base of the column to prevent the silica from leaking. Activated silica gel (60 mesh, Sigma Aldrich, Switzerland) was then packed into the glass column (length: 53 cm and width: 15 cm) with hexane (Kaur *et al*., 2015). Rotary evaporation was used to concentrate the *N. luteum* crude extract (2 g) which was then mixed with the silica gel in a ratio of 1:1 and loaded into the column.

Optimal separation of compounds was already obtained in 2.3.5. The EtOAc extract (2 g) was subjected to column chromatography with hexane:EtOAc as the mobile phase, starting with 100% hexane. The polarity of the solvent system was gradually increased by 5% for every 100 mL until 100% EtOAc was reached. The polarity of the mobile phase was further increased with 5 and 10% MeOH, sequentially. A total of 27 fractions were collected and combined into four fractions, namely, A (1 – 10, 230 mg), B (11-16, 310 mg), C (17-21, 150 mg) and D (22-27, 310 mg), based on similarities in the TLC profiles of the fractions. The four fractions were further processed by TLC, TLC-bioautography and MIC determinations as described in 2.2.5, 2.2.6 and 2.2.4.2, respectively.

3.2.2 Nuclear magnetic resonance (NMR)

From the MIC determinations conducted on the four fractions, it was observed that only fraction C displayed anti- *P. aeruginosa* activity, thus this fraction was subjected to further purification. Fraction C afforded compound 1 upon crystallization from ethanol. The isolate was dissolved in deuterated chloroform ($CDCl_3$) and subjected to 1 and 2D NMR spectroscopy.

3.3 RESULTS

3.3.1. Purification by activity-based fractionation of the anti-*P. aeruginosa* compound

Column chromatography, bioautographic detection and MIC assays were utilised to purify the anti-*P. aeruginosa* compound of interest. Fractions A-D were subjected to TLC and TLC-bioautography for detection. Smudged spots (no clear bands) were observed from TLC (Figure 3.2) and TLC-bioautography that yield inconclusive results. This was not repeated due to lack of sample; however, MIC determinations were conducted to determine if fractions contained anti-*P. aeruginosa* activity. The MIC results revealed fraction C to be the only fraction with activity against *P. aeruginosa* with an MIC of 2.441 µg/mL (Figure 3.3). Further purification of Fraction C using column chromatography yielded compound 1 with an MIC of 0.6104 µg/mL (Figure 3.4).
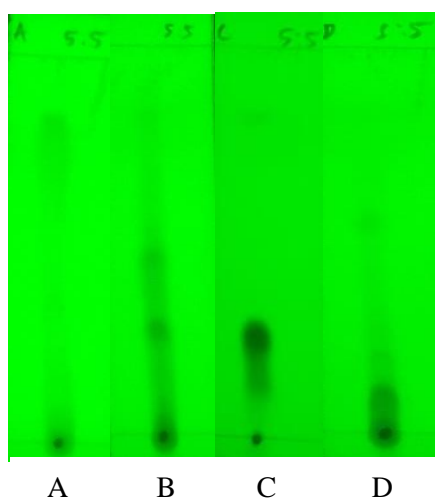
Figure 3. 1: Thin layer chromatography of fractions A, B, C and D.
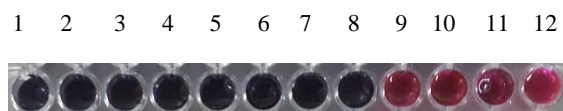
Figure 3. 2: Broth microtitre serial dilution assay (µg/mL) for MIC determination of partially purified pooled fractions. Wells 1-10 contain cells, Muller Hinton broth and sample; Well 11 contains 8% DMSO in water and Well 12 contains more media as a replacement for extract.

Table 3.1: Interpretation of the resazurin microtitre assay for Fraction C

Concentration (µg/mL)											
1	2	3	4	5	6	7	8	9	10	11	12
312.5	156.3	78.13	39.06	19.53	9.766	4.883	2.441	1.221	0.6103	0.3051	0.1521
-	-	-	-	-	-	-	-	+	+	+	+

Keys: +: growth -: no growth
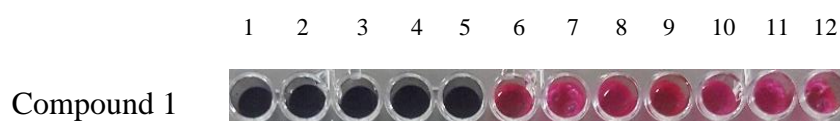
Compound 1

Figure 3. 3: Broth microtitre serial dilution assay (µg/mL) for MIC determination for purified compound 1. Wells 1-10 contain cells, Muller Hinton broth and sample; Well 11 contains 8% DMSO in water and Well 12 contains more media as a replacement for extract.

Table 3.2: Interpretation of the resazurin microtitre assay for compound 1

Concentration (µg/mL)											
1	2	3	4	5	6	7	8	9	10	11	12
9.766	4.883	2.441	1.221	0.6103	0.3051	0.1521	0.0761	0.0380	0.0190	0.010	0.0048
-	-	-	-	-	+	+	+	+	+	+	+

Key: +: growth; -: no growth

3.3.2 Nuclear magnetic resonance and structure elucidation

Compound 1: HR-ESI-MS: m/z 297.1109 [M+Na]$^+$, (calcd for $C_{16}H_{18}O_4Na$, 297.1103). IR (KBr) v_{max} cm^{-1}: 2926, 2879, 1771, 1702, 1612, 1466, 1384, 1200, 1034, 878. ^{1}H NMR (CDCl$_3$, 400 MHz): δ_H: 6.18 (1H, m, H-7), 5.73 (1H, d, J = 1.52 Hz, H-11), 5.00 (1H, brt, J = 4.52 Hz, H-6), 4.96 (1H, dt, J = 13.57 Hz, H-13a), 4.87 (1H, d, J = 13.57 Hz, H-13b), 2.24 (1H, ddd, J = 13.71, 7.66, 5.64 Hz, H-3a), 1.92 (1H, d, J = 4.66 Hz, H-5) (Figure 3.5 A and B), 1.72 – 1.58

(3H, overlapping multiplets, H-1a, 1b, 2b), 1.53 (1H, ddd, $J = 14.28, 7.45, 6.06$ Hz, H-3b), 1.30 (3H, s, H-15), 1.15 (3H, s, H-16) (Figure 3.6).

^{13}C NMR (CDCl$_3$, 400 MHz): δ_C: 180.8 (C-14), 163.6 (C-12), 158.7 (C-9), 132.2 (C-8), 121.8 (C-7), 111.7 (C-11), 71.3 (C-6), 69.5 (C-13), 47.8 (C-5), 42.7 (C-4), 35.0 (C-10), 29.6 (C-1), 27.7 (C-3), 24.7 (C-16), 24.1 (C-15), 17.3 (C-2) (Figure 3.7).

A total of sixteen peaks, comprising two methyl, four methylene, four methine and six quaternary carbons were observed in the ^{13}C NMR spectrum (Figure 3.7) and the Distortionless enhancement by polarization transfer (DEPT) spectra (Figure 3.8) supported these findings. The HMBC spectrum showed ^{3}J correlation between carbonyl at C-11 with methylene protons H-8, methine proton H-10 and methyl protons H-16; and carbonyl at C-2 with H-1b and H-3 (Figure 3.9). ^{1}H NMR was conducted on fraction 17 and compound 1 was isolated as a white solid from the extract of the fungus *N. luteum* with a molecular formula C$_{16}$H$_{18}$O$_4$ (Figure 3.10).
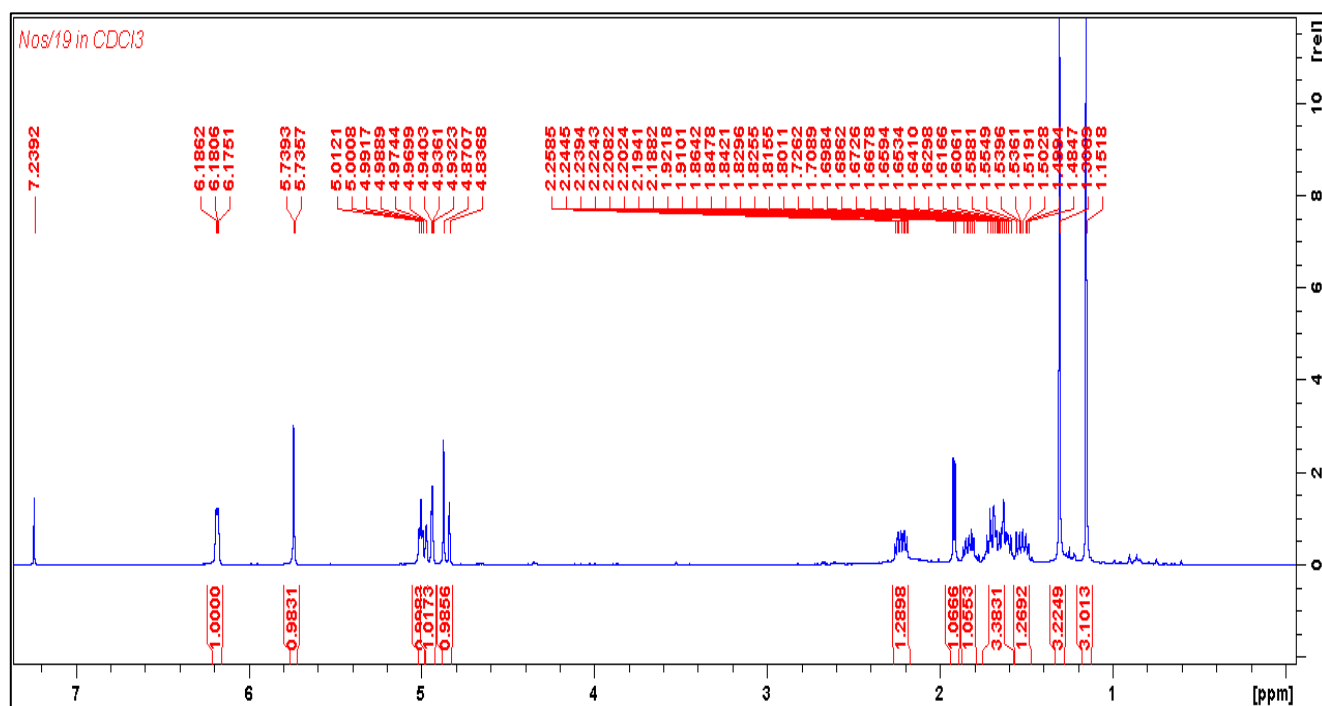
Figure 3.4: H NMR spectrum of compound 1 in CDCl$_3$.
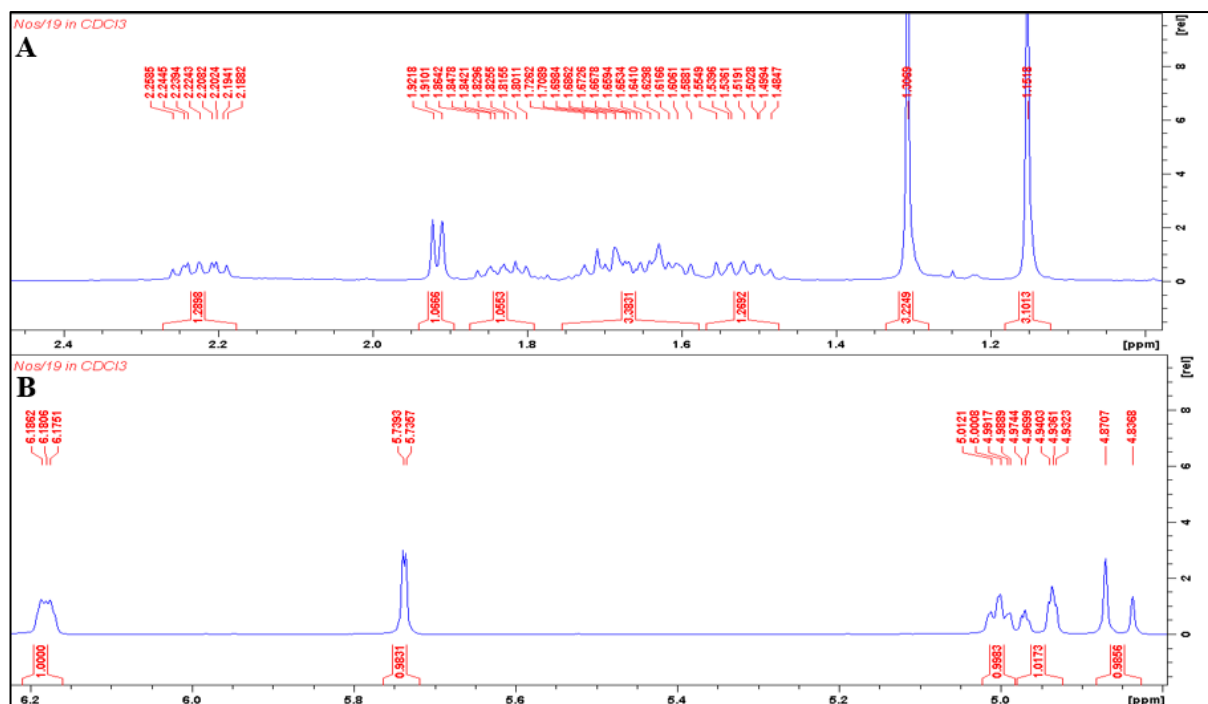
Figure 3.5: Expanded 1H NMR spectrum of compound 1 in CDCl₃ (A and B).
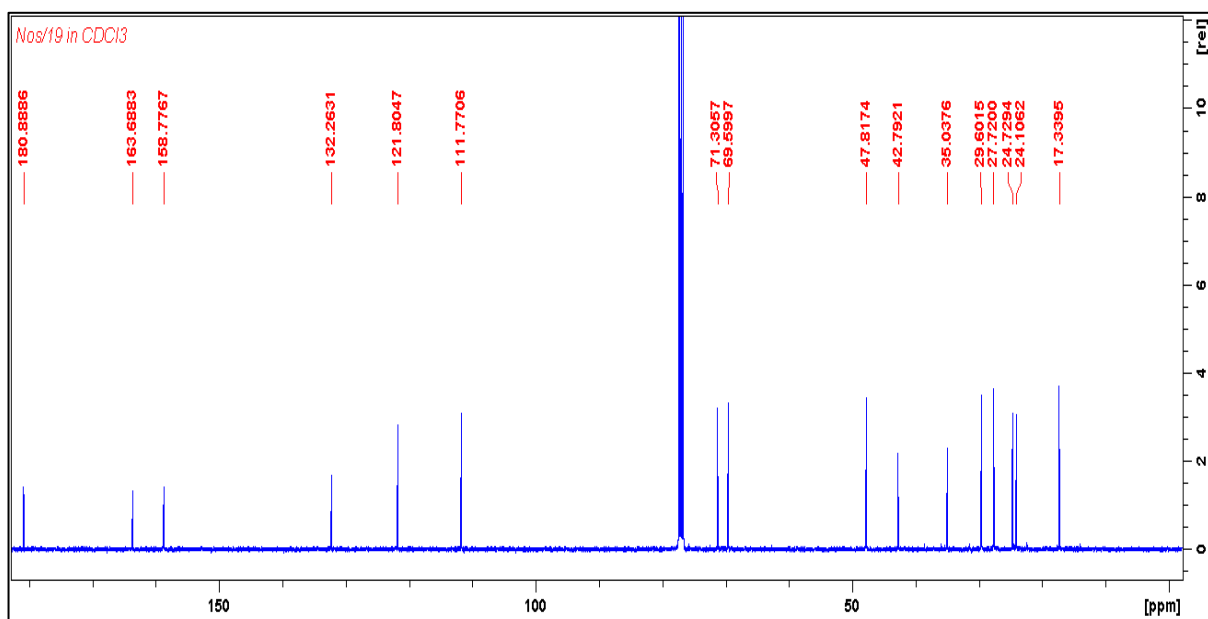
Figure 3.6: 13C NMR spectrum of compound 1 in CDCl₃.
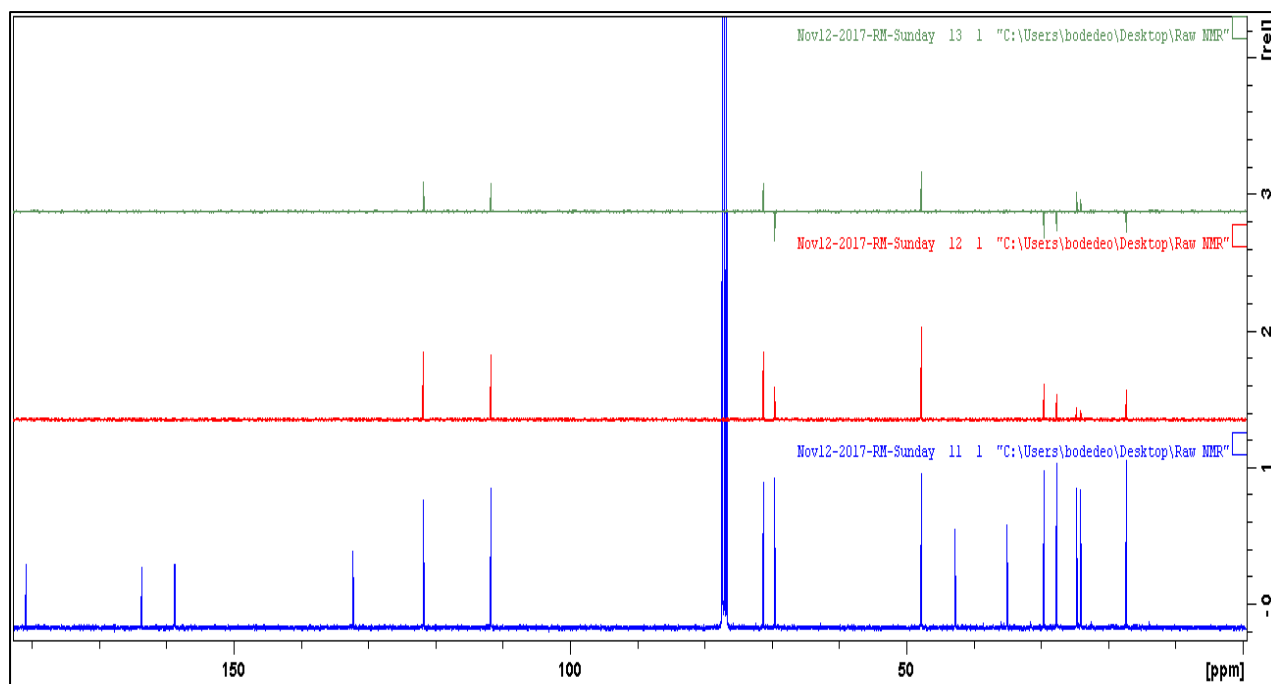
Figure 3.7: DEPT spectra of compound 1.
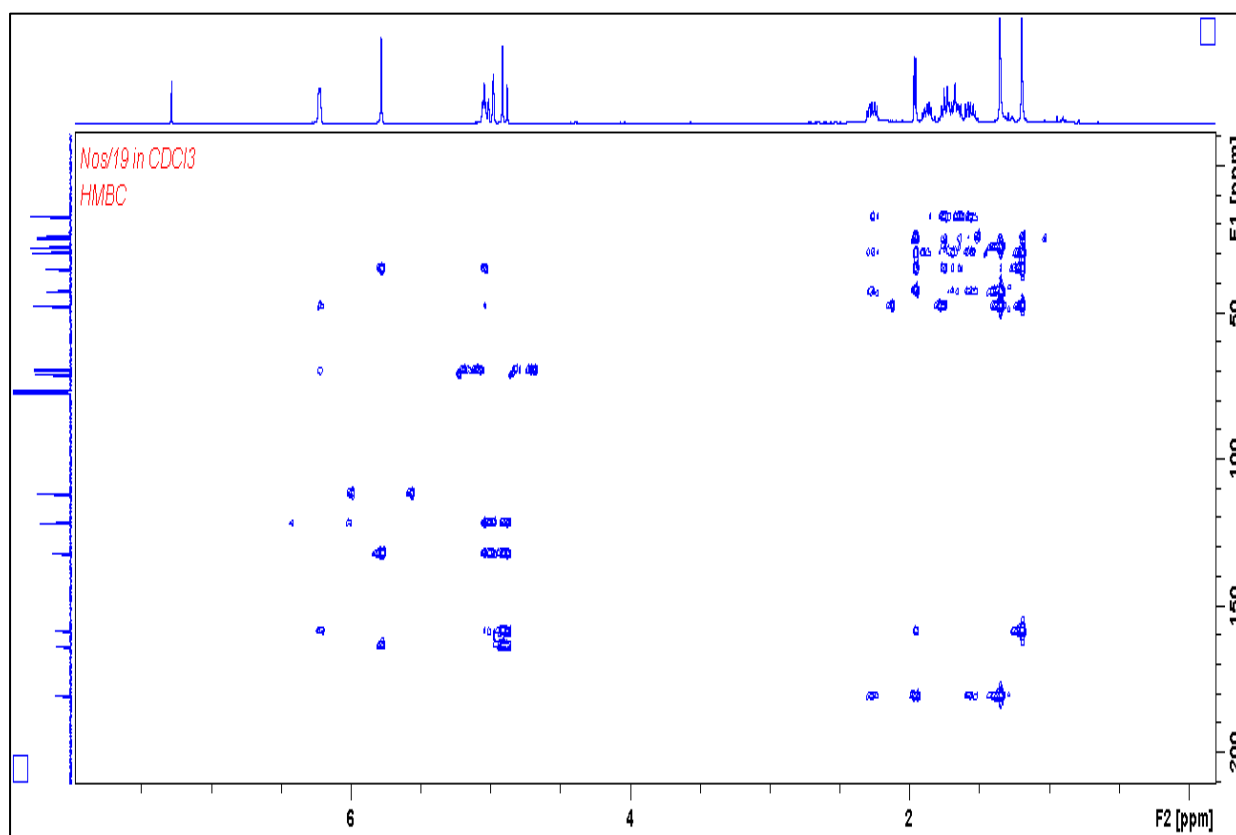
Figure 3.8: HMBC spectrum of compound 1.
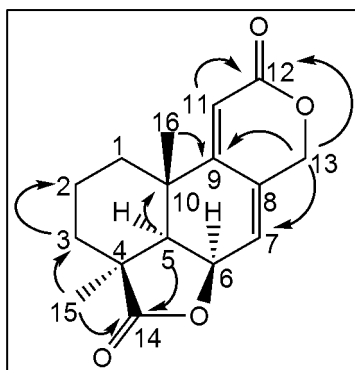
Figure 3.9: Major HMBC correlations observed in compound 1.

3.4 DISCUSSION

Analysis by TLC of these four fractions yielded inconclusive results and forced early MIC determination. The MIC determination showed only fraction C to possess bioactive metabolites which were able to kill *P. aeruginosa* (Figure 3.3). Fraction C was therefore purified and yielded a white crystalline substance, compound 1 (35 mg) which accounted for 1.75% of the crude extract (2 g).

The MIC obtained for compound 1 was exceptional at 0.6103 µg/mL (Figure 3.4) indicating that the pure compound is more active than the partially purified extract (2.441 µg/mL in Figure 3.3) and crude extracts (19.53 µg/mL as seen in Figure 2.6) that contain a range of compounds. A similar study by Fall *et al.* (2017) using an ethanol extract of *Aphania senegalensis* resulted in antibacterial activity against *S. aureus*, *E. coli* and *E. faecalis* with MICs of 0.31 mg/mL, 0.16 mg/mL and 0.08 mg/mL respectively. However, the extract did not show growth inhibition against *P. aeruginosa*. In addition, a study by Malhadas *et al.* (2017) on the antibacterial activity of the EtOAc and MeOH extracts of *Alterneria alternata* fungus showed the extracts to have activity against several bacteria with MICs ranging between 0.095 and 25 mg/mL. The EtOAc extract of the fungus gave an MIC value of 3.125 mg/mL against *P. aeruginosa*, which was higher than the current study. These comparisons indicate that activity of the extracts of *N. luteum* against *P. aeruginosa* is encouraging.

The ^{1}H NMR spectrum (Figure 3.5) showed characteristic alkenyl resonances at δ_H 6.18 (H-7) and 5.73 (H-11) which were confirmed by the ^{13}C NMR chemical shifts at δ_C 121.8 and 111.7, respectively. Oxygenated methine (δ_H 5.00) and methylene (δ_H 4.96 & 4.87) protons were assigned to H-6 and H-13a&b, respectively, while the corresponding oxygenated methine and methylene carbons were observed at δ_C 71.3 (C-6) and 69.5 (C-13), respectively. The 16 peaks observed in Figure 3.7 were also observed by the DEPT spectra (Figure 3.8). Two of the quaternary carbons (δ_C 180.8 and 163.6) were ester carbonyl carbons which confirm the dilactone nature of compound 1 (Figure 3.10).

The locations of the angular methyl protons and the proximity of double bonds to rings B and C were confirmed using HMBC correlations (Figure 3.10) which were deduced from the HMBC spectrum presented in Figure 3.9. There was a correlation between H-11 and the ring C carbonyl. H-13 had correlations with C-12, C-9 and C-7 while the correlation of H-5 to C-14 carbonyl confirms the fusion of ring D with rings A and B. The NMR analysis, supported by the vibrational frequencies (cm^{-1}) of C=O (1771, 1702), C-O (1034), C=C (1612) and C-H (2926) using FTIR justify compound 1 as a dilactone. The dilactone was confirmed with an exact mass m/z 297.1109 for $C_{16}H_{18}O_4Na$. Thus, the molecular formula of compound 1 was deduced to be $C_{16}H_{18}O_4$.

Compound 1 ($C_{16}H_{18}O_4$) was identified as a dilactone (3a,10b-dimethyl-1,2,3,3a,5a,7,10b,10c-octahydro-5,8-dioxa-acephenanthrylene-4,9-dione) and it matched compound 3 in the study of antineoplastic agents conducted by Pettit *et al*. (2003). The same compound was synthesised from a Wieland-Miescher ketone and was similar to oidiolactone C ($C_{16}H_{18}O_5$) (Hanessian *et al*., 2009). This compound occurs both naturally and synthetically. The compound was also tested for antibacterial and antifungal activity against *S. aureus*, *Shigella dysenteriae*, *Penicillium avellaneum*, *C. albicans* and *Saccharomyces cerevisiae*. It displayed anti-*C.*

albicans, anti-*S. cerevisiae* and anti-*P. avellaneum* activity but no antibacterial activity (Lin *et al*., 2009). This suggests that existing compounds (synthetic and natural) need to be tested for new properties as none of the oidiolactones have displayed activity against resistant *P. aeruginosa* in the past.

Oidiolactones were first reported by John *et al*. (1999). The filamentous fungi represented by *Oidiodendron truncatum* and *Oidiodendron griseum* produced the tetranorditerpenoid dilactone, oidiolactones A-D. These were shown to display bioactive properties similar to those produced by cladosporin, a known antifungal, antibacterial and insecticide compound (Hanessian *et al*., 2009).

3.5 CONCLUSION

The purification and characterisation of *N. luteum* ethyl acetate crude extract was successful and proved that the purer a crude extract is; the more antimicrobial activity it possesses. This could be due to antagonistic effects of inactive compounds present in the crude extract. The compound isolated from the ethyl acetate extract of *N. luteum* showed good anti-*P. aeruginosa* activity and this is a step in the right direction in treating infections caused by *P. aeruginosa*.

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CHAPTER 4: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

4.1 SUMMARY OF FINDINGS

Plate stocks and mineral oil slants for endophytic storage are more efficient than glycerol stocks as plate stocks and mineral oil slants stocks resulted in fungal resuscitation with no contamination as opposed to glycerol stocks. Phenotypic traits of the endophytic fungi change slightly in response to being sub-cultured from different stocks over time. Due to fungal extracts being diverse and relatively untested for optimal time for the production of the bioactive compound, it was crucial to set up a time course in order to identify this for the anti-*P. aeruginosa* bioactive compound. Although the compound appears within one week of growth, maximal levels were produced at 4 weeks of growth. Over the various intervals of growth tested, the profiles of compounds varied.

It was important to assess the effect of purification on the anti-*P. aeruginosa* activity of the compound. Crude extracts contain a variety of compounds which either act together to have a combined effect, act against each other or each compound may have entirely different activities. Literature reports that bioactivity increases with purity due to the desired compound being present in higher concentrations and with no impurities. It needs to be noted that some studies have reported purification to have the opposite effect due to the compounds found in the crude extract acting synergistically. Thus, the purer the compound was, the more reduced its ability to inhibit a test organism was.

Isolation of the active metabolite from the crude extract and characterisation showed the compound to be a dilactone (3a,10b-dimethyl-1,2,3,3a,5a,7,10b,10c-octahydro-5,8-dioxa-acephenanthrylene-4,9-dione) which displayed anti-*P. aeruginosa* activity against a resistant strain. This has been a new approach in using this compound which was previously reported

to display antifungal activity and inhibit both lipopolysaccharide-induced interleukin- lj8 and tumour necrosis factor-α production in human whole blood.

4.2 CONCLUSIONS

The aim of this study was to purify, identify and characterise ZF 52 and other *K. africana* endophytic fungi against *P. aeruginosa* which was a relatively new approach. When comparing the MIC of the crude, semi-purified and purified extract, the MIC of the purified extract had the most anti-*P. aeruginosa* activity. Characterisation and identification were successful and achieved by NMR spectroscopy. The isolated bioactive metabolite from the extract of *N. luteum* was identified as a dilactone (3a,10b-dimethyl-1,2,3,3a,5a,7,10b,10c-octahydro-5,8-dioxa-acephenanthrylene-4,9-dione). As purification of the *N. luteum* extract was successful, the hypotheses were accepted. Although the isolated compound is not novel and has previously been shown to have activity against some fungal species, its ability to inhibit the growth of *P. aeruginosa* to this degree is new. This suggests that known compounds need to be screened across a wide range of pathogens and organisms to determine potential activity.

4.3 RECOMMENDATIONS FOR FUTURE WORK

With the vast amount and rapid emergence of bacterial resistance to antibiotics, more testing of the anti-*P. aeruginosa* compound would help reduce the speed at which this pathogen gains resistance. Although the anti-*P. aeruginosa* activity was determined against one resistant strain of *P. aeruginosa*, in order to conclusively realise the activity of this compound it be tested against as many different resistant strains as possible as well as against any clinical strains with emerging resistance.

Column chromatography was used as a purification method, for future studies it may be beneficial to use prep-HPLC for purification as higher yields and greater purity of the purified product are reported to be obtained using this technique. The purified compound 1 isolated in this study is a known diterpene lactone that was first isolated from a marine sponge and demonstrated to have antifungal activity (John *et al*., 1999). This finding highlights the fact that bioactive compounds need to be screened against a wide a panel as possible in order to properly determine their bioactive potential. A compound previously shown to have activity against one pathogen needs to be screened against a wide range of other pathogens, especially newly developing resistant strains, as a potential drug candidate may already be available, but its full spectrum not yet elucidated, and screening would provide a more rapid solution as the compound has already been purified and identified.

The determination of the mode of action (assessing membrane integrity) using a capturing confocal scanning laser microscope is also an important factor and next step in the process in deciding whether or not a compound will be taken to the next level of testing (pre-clinical screening and testing) and if the compound still looks promising, safety and efficacy clinical trials, as well as drug development, formulation and production.

Interdisciplinary research involving Microbiology, Animal biology and Chemistry is key in fast tracking future drug development. This is because different disciplines have access to different materials. It would be interesting to investigate anti-cancer and a- diabetic activities, however the Microbiology department does not have a tissue culture lab for anti-cancer determination, nor does it supply mice for anti-diabetic trails.
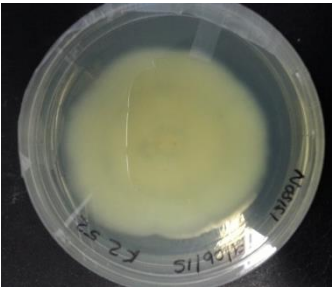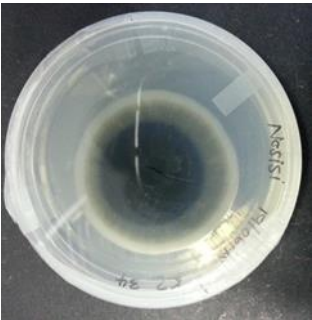
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APPENDIX A

Table A1: Growth of *K. africana* endophytic fungi on PDA.

ISOLATE		Morphology
KZ 52		**Top** Brown centre with outward expansion. Green with a cream edge. **Bottom** Cream with light bottle green
KZ 34		**Top** Green of varying intensity as mycelia expand **Bottom** Navy centre, green expansion and white edge
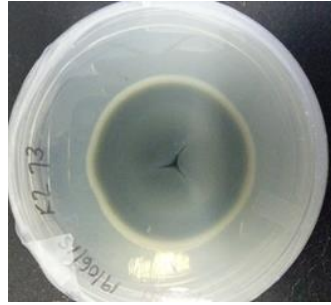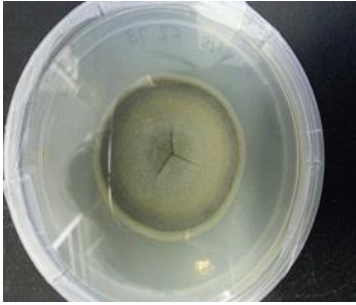
KZ 73			**Top** Different shades of green and is lightest in the centre, with white edge **Bottom** Green with a white edge
KZ 79			**Top** Green, with the lightest shade at the centre **Bottom** Black with light green to white edge
KZ 81			**Top** Red with white woolly matter **Bottom** Brown centre with red carcadia
KZ 72			**Top** Green with white edge **Bottom** Yellow, darkest at the centre, with black extremities
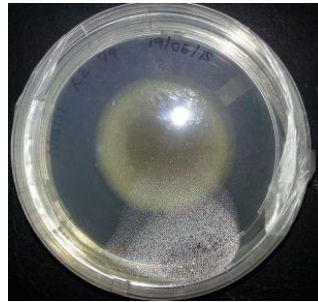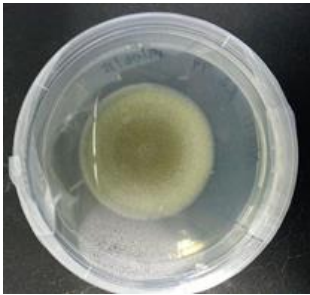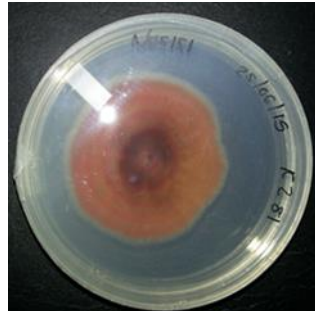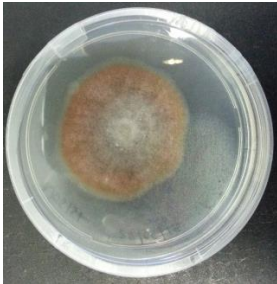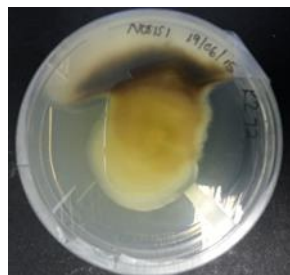
KZ 54
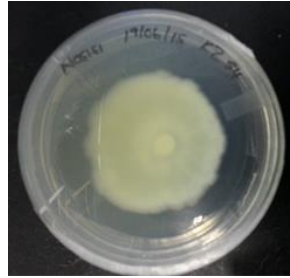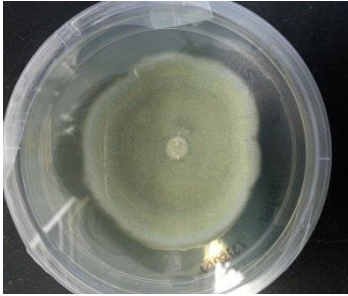
Top
Light green with faint green extremity
Bottom
White and snotty looking

KZ 49

Top
Black, which changes to green as it spreads out
Bottom
Black at the centre and becomes lighter as fungi expands

KZ 44
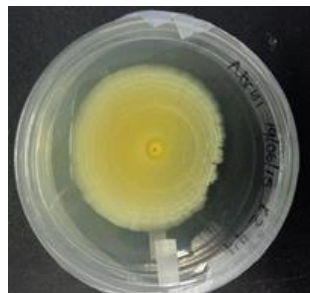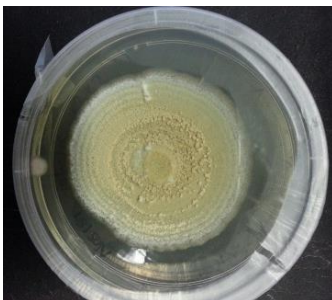
Top
Light green with white fluff
Bottom
Intense yellow and expands to be light yellow

KZ 22
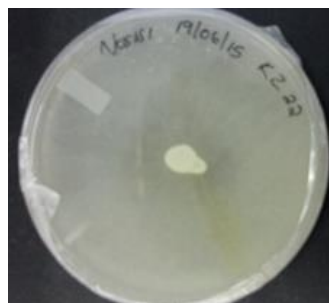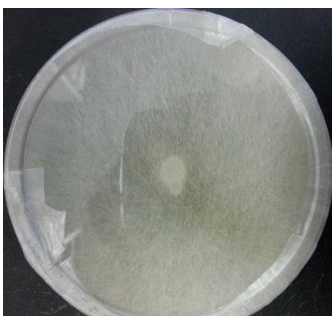
Top
White fluff
Bottom
White centre, growing towards the ends with black dots/spores

| KZ 43 | | | **Top** Light brown with white fluff growing on the surface **Bottom** Yellow with circular yellow rings which become less intense |
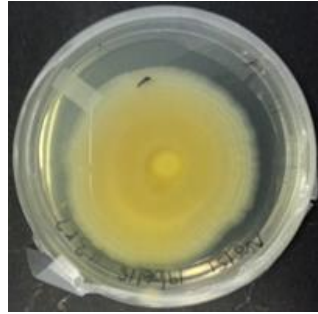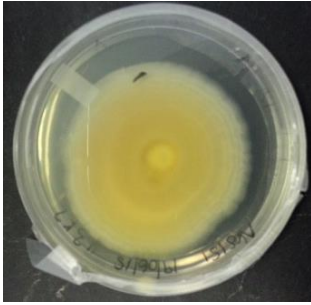
Top
Light brown with white fluff growing on the surface
Bottom
Yellow with circular yellow rings which become less intense

KZ 38
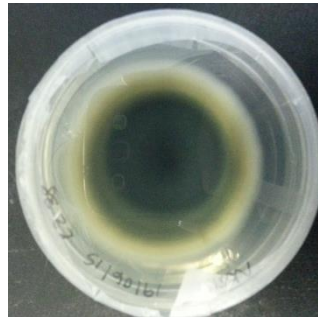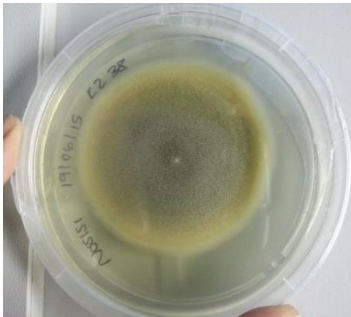
Top
Oldest part of the mycelia is bottle green and becomes faints green as it spreads out
Bottom
Pale green as it spreads to fresh medium, becomes intense green as it ages

KZ 74
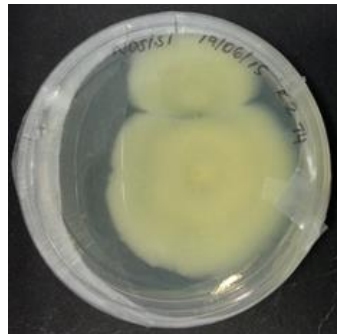
Top
Light green at the centre which becomes darker as it grows out
Bottom
White at the centre which becomes darker as it grows outward

KZ 78
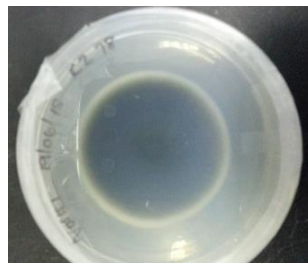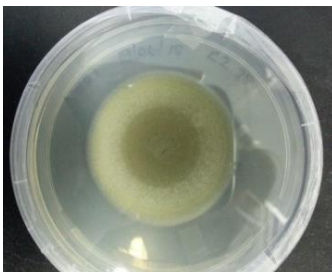
Top
Green centre with white fluff as it grows away from the centre
Bottom
Black with green and white colouration
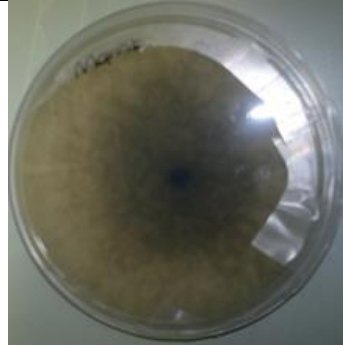
ZF 91			**Top** Black inoculum plug which spreads to be white **Bottom** Becomes white as it spreads to fresh media. The oldest part is black
ZF 65			**Top** Oldest part is black, spreads to be green. Has whooly fluff covering it **Bottom** Bottle green centre, with darker edges
ZF 79			**Top** Black with white fluff covering the surface **Bottom** Black centre, becomes orange as it spreads
ZF 69			**Top** Green centre and grows to have fluff at the extremities **Bottom** Intense orange centre and turns yellow as it grows in fresh media
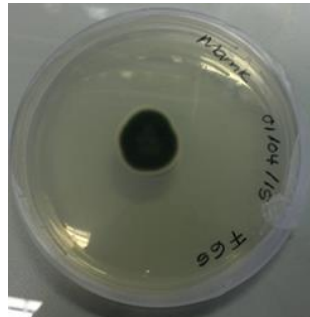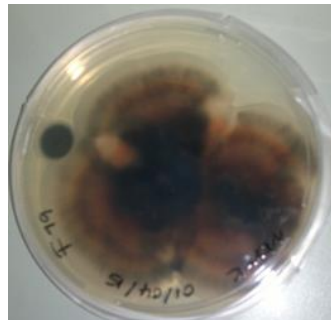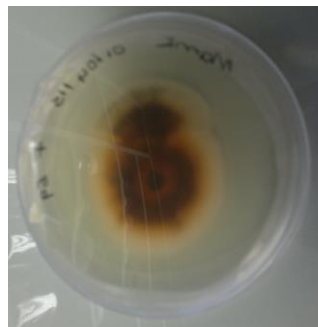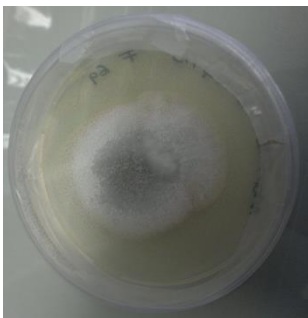
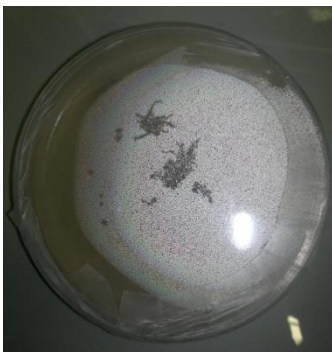| ZF 88		**Top** White centre which spreads to be ivory **Bottom** Pale brown centre which Grows pale yellow
ZF 68		**Top** Black centre which spreads to be yellow **Bottom** Green and spreads
ZF 84		**Top** Yellow at the centre and spreads to have white fluff **Bottom** Black initial growth at the centre and spreads to be pale yellow
ZF 59		**Top** The centre has green growth at the centre **Bottom** Black centre and grows yellow as it spreads to fresh media
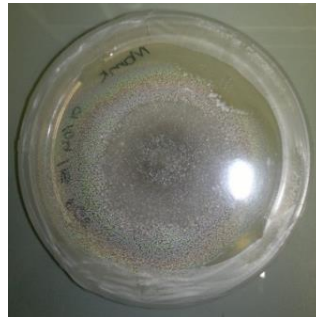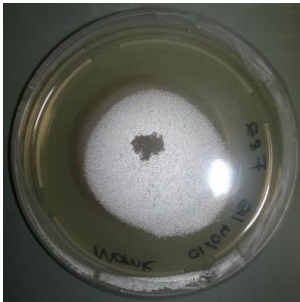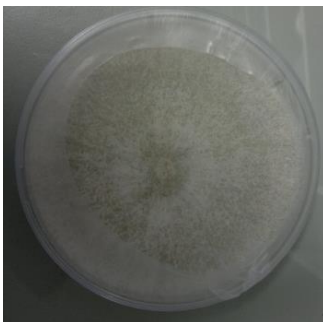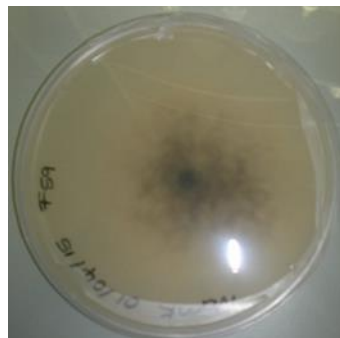
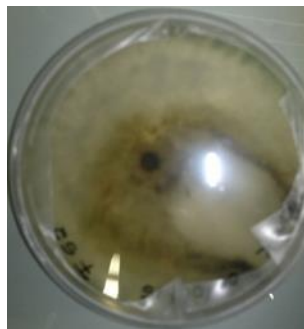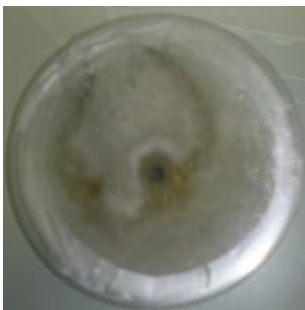|			
ZF 53			**Top** Orange at the centre, becomes white as it spreads to fresh media **Bottom** Intense orange centre and white edges
ZF 96			**Top** Ivory centre with off-white extremities **Bottom** Ivory centre with
ZF 66			**Top** Black centre, spreads white **Bottom** Brown centre, which spreads ivory
ZF 52			**Top** Black centre, spreads yellow and white at the extremities **Bottom** Black with yellow edges
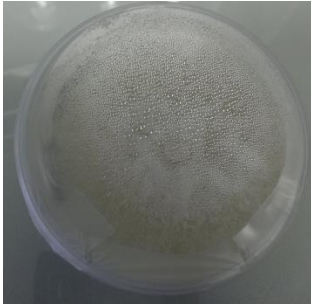
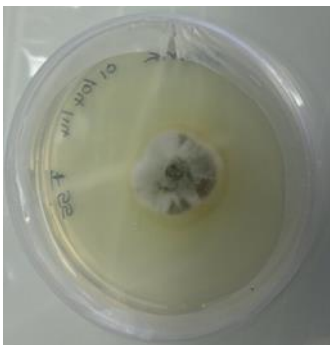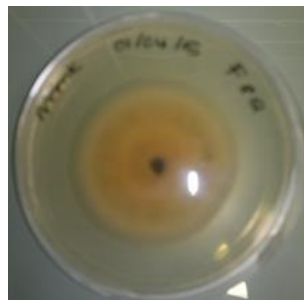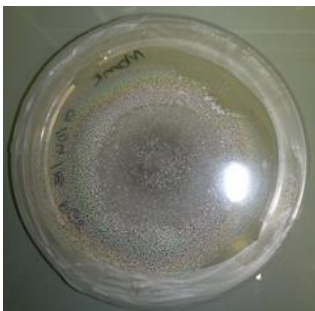|				
ZF 58			**Top** Ivory centre, which spreads white **Bottom** Ivory and becomes white with fresh media
ZF 55			**Top** Black centre, spreads green into fresh media **Bottom** Bottle green centre, becomes less intense green as it spreads to fresh media
ZF 82			**Top** Green centre, becomes yellow as it grows on fresh media **Bottom** Black centre with new mycelia appearing yell-ow
ZF 5			**Top** Mycelia looks green and turns black as it ages **Bottom** Black with ivory edges

APPENDIX B

P. aeruginosa
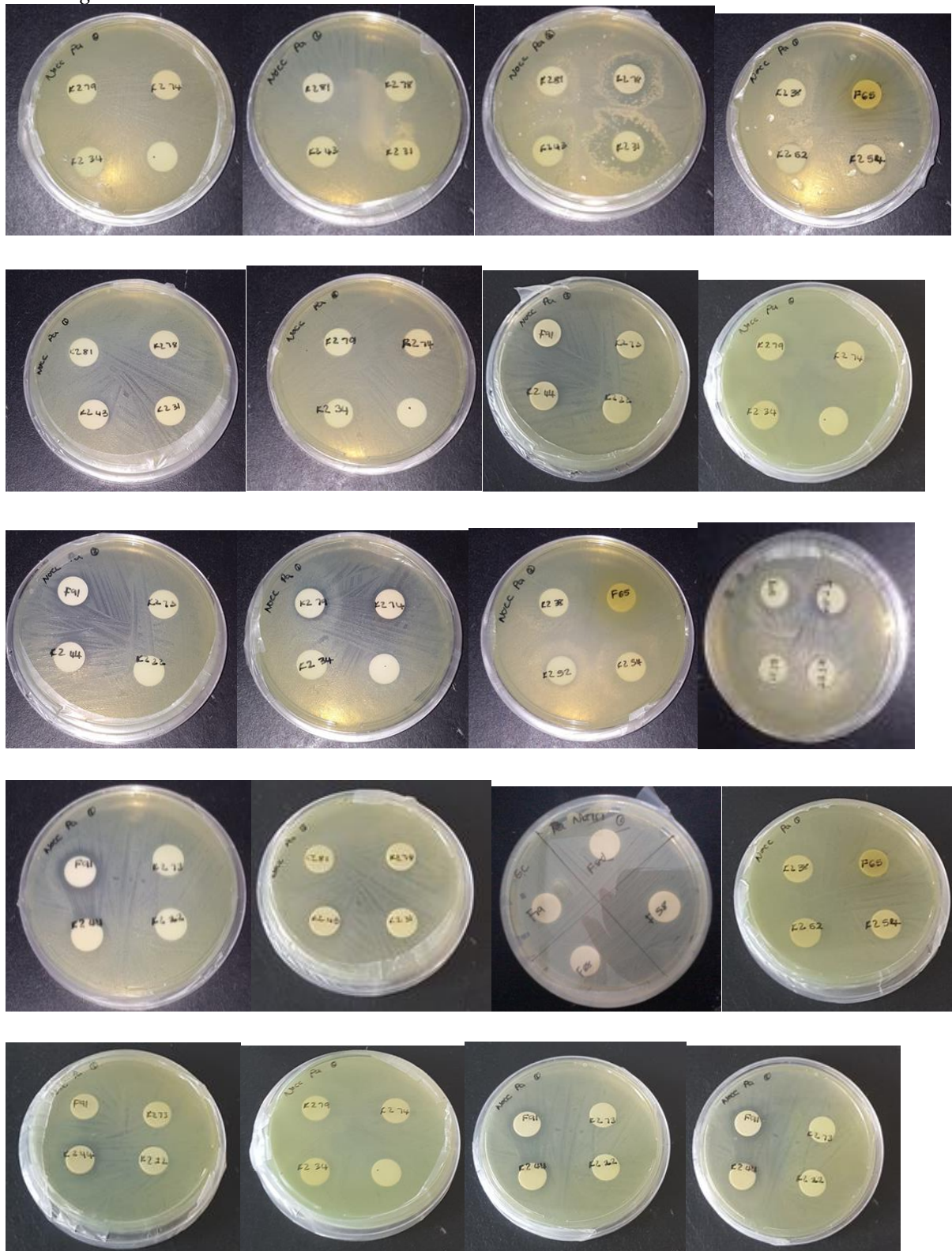
Figure B1.1: Kirby Bauer disc diffusion assay showing zones of inhibition by *K. africana* endofungal

extracts
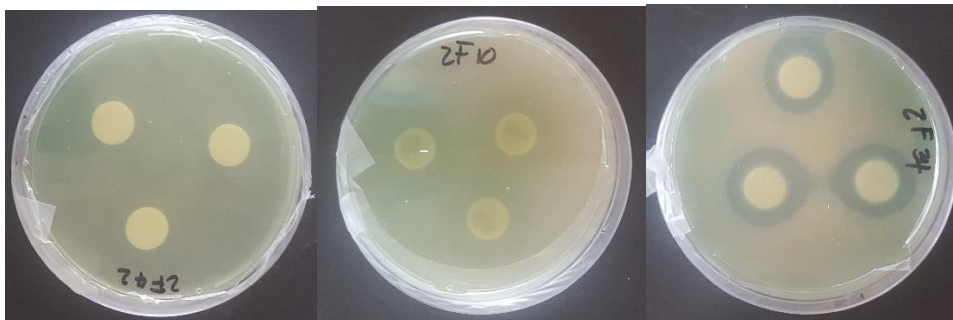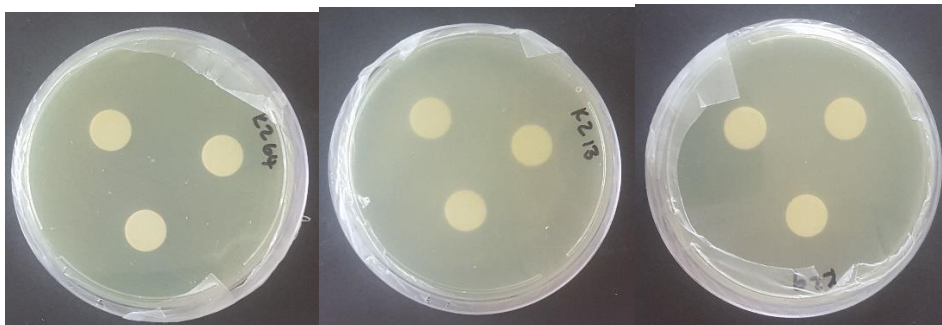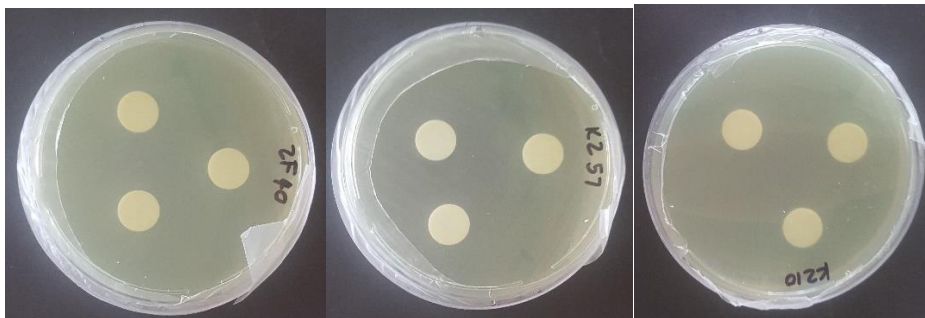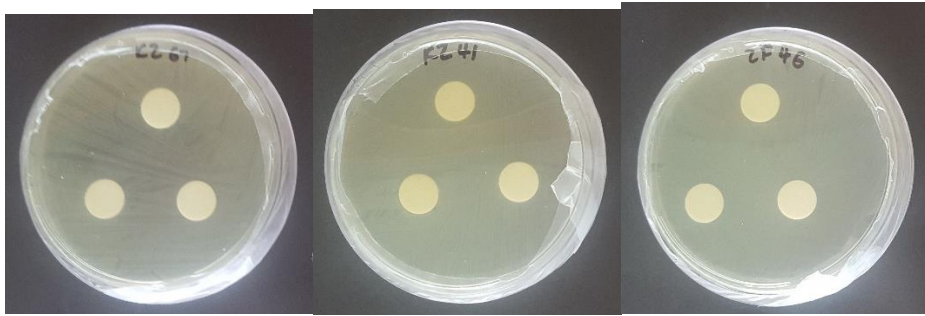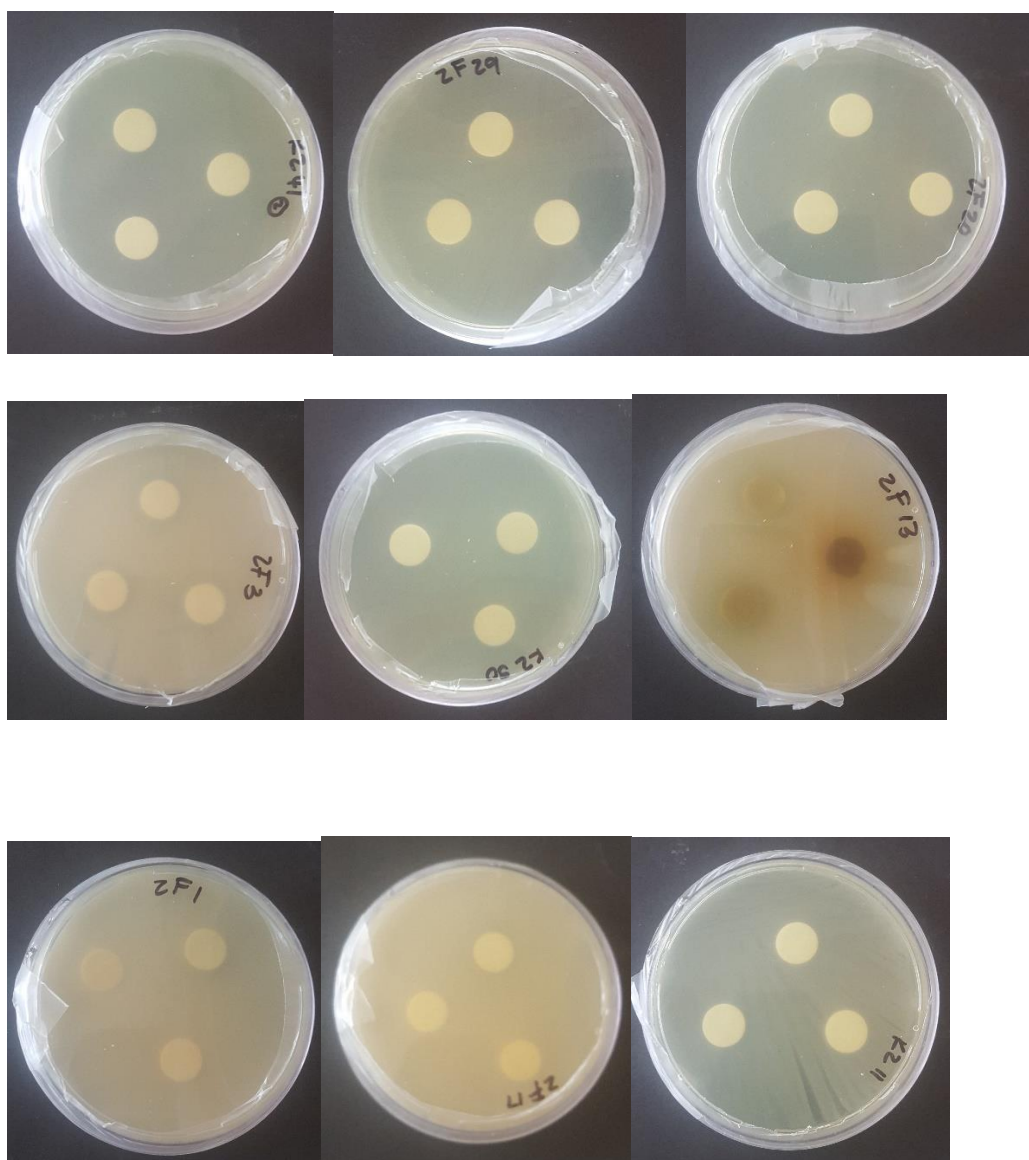
Figure B1.2: Kirby Bauer disc diffusion assay of Lab 2 extract stocks.

Table B1: Raw data from the Kirby Bauer disc diffusion assay for initial screening of extracts

	Zone of inhibition (mm)			Avg	STD
ZF52	42	40	39	40,33333	1,080123
ZF34	22	19	20	20,33333	1,080123
ZF91	20	25	20	22	2.357023
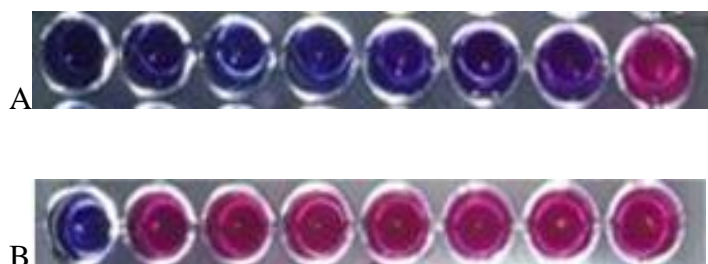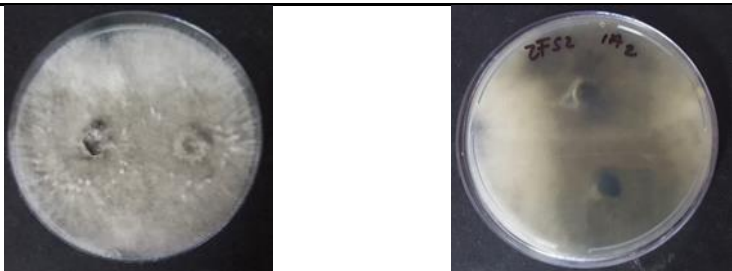
Figure B2: Resazurin colour reaction for MIC determination; A: ZF 52 and B: ZF 91.

Of the screened isolates ZF91, ZF52 and ZF 34 revealed anti-*P. aeruginosa* compounds of interest, however when ZF 91 was sub-cultured from stocks it did not grow and ZF34 was a lab isolate extract and the stock culture wasn't found. Therefore, ZF 52 was the only fungal isolate used for further testing.

APPENDIX C

Table C1: Growth of ZF 52 endophytic fungus on PDA.

| Fungal isolate		Morphology
		Top Older mycelia look black, as it grows on new media it turns cream/light yellow **Bottom** Black centre with new mycelia being yellow
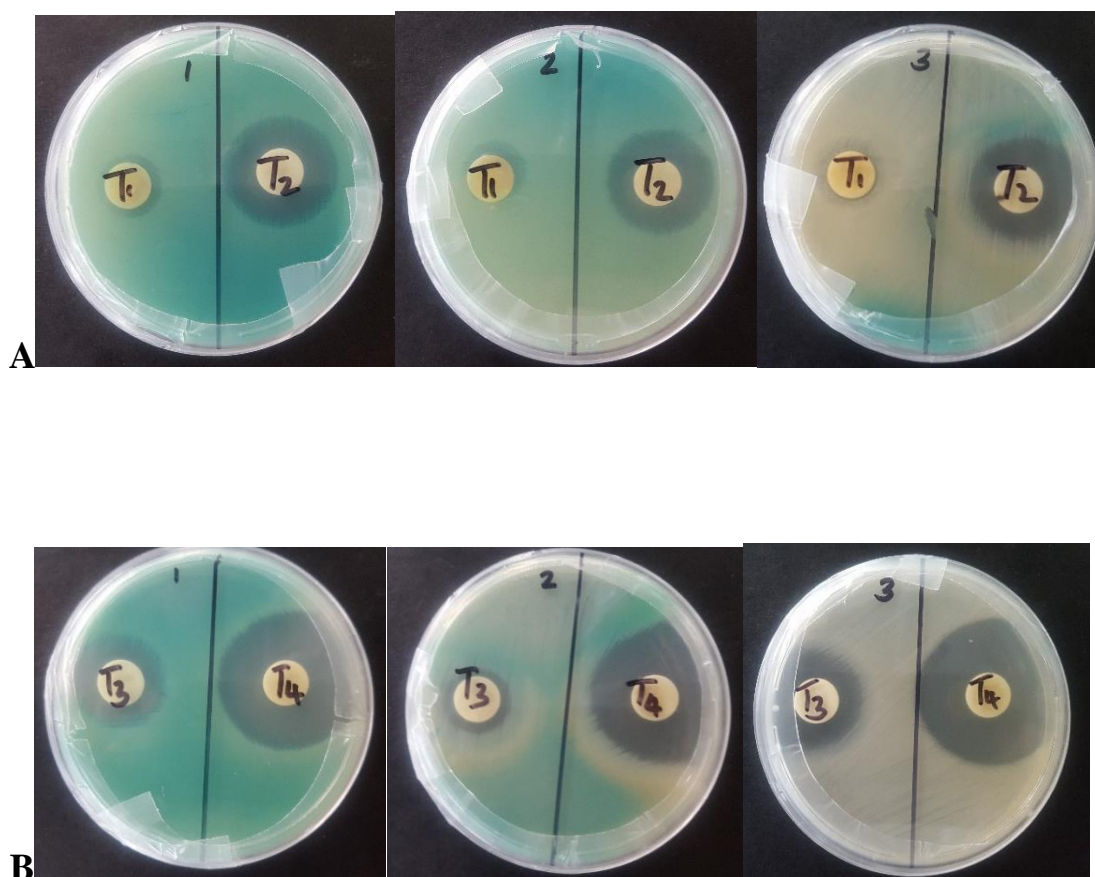
Figure C1: Kirby Bauer disc diffusion assay showing zones of inhibition produced by ZF 52 endofungal extracts against *P. aeruginosa*. A (T1 and T2) and B (T3 and T4).

Table C2: Raw data from the Kirby Bauer disc diffusion assay of the time course extracts.

Extract (days)	Triplicate 1	Triplicate 2	Triplicate 3	Average	Standard deviation
T1	14	17	15	15,33333	1,080123
T2	28	27	27	27,33333	0,408248
T3	25	18	28	23,66667	3,62859
T4	42	40	39	40,33333	1,080123

Figure C2: Resazurin colour reaction for MIC determination of extracts against *P. aeruginosa.*
Row A: 1 week ZF 52 extract (156.3 µg/mL); row B: 2 weeks ZF 52 extract (39.06 µg/mL);
row C: 3 weeks old ZF 52 extract (78.13 µg/mL) and row D is 4 weeks old ZF 52 extract (19.53
µg/mL).

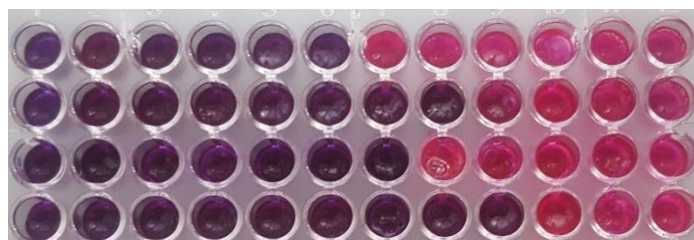Table C3: Interpretation of the resazurin microtitre assay.

Extract age	Concentration (µg/mL)											
	5000	2500	1250	625	312.5	156.3	78.13	39.06	19.53	9.766	4.883	2.441
T1	-	-	-	-	-	-	+	+	+	+	+	+
T2	-	-	-	-	-	-	-	-	+	+	+	+
T3	-	-	-	-	-	-	-	+	+	+	+	+
T4	-	-	-	-	-	-	-	-	-	+	+	+

Identification of ZF 52

Sequencing data:

GGGKACTGCGGAGATCATTACCGAGTTGATTCGAGCTCCGGCTCGACTCTCCCAC

CCTATGTGTACCTACCTCTGTTGCTTTGGCGGGCCGCGGTCCTCCGCACCGGCGC

CCTTCGGGGGGCTGGCCAGCGCCCGCCAGAGGACCATAAAACTCCAGTCAGTGA

ACTTCGCAGTCTGAAAAACAAGTTAATAAACTAAAACTTTCAACAACGGATCTCT

TGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC

AGAATTCAGTGAATCATCGAATC

Table C4: BLAST analysis of ZF 52.

APPENDIX D

Figure D1.1: Thin layer chromatography of crude ethyl acetate extract chloroform:ethyl acetate.

Figure D1.2: Thin layer chromatography of crude ethyl acetate extract chloroform:ethyl acetate stained with 5% methanol in sulphuric acid.

Figure D2: Thin layer chromatography of ethanol and methanol extracts using chloroform:Methanol.
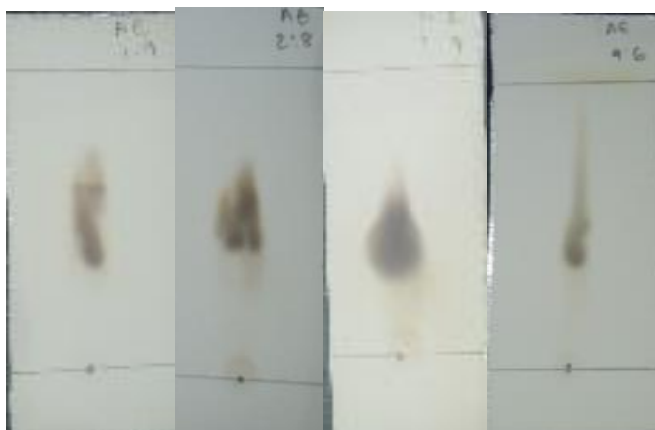
Figure D3: Thin layer chromatography of ZF 52 ethyl acetate extract using acetonitryl:ethyl acetate
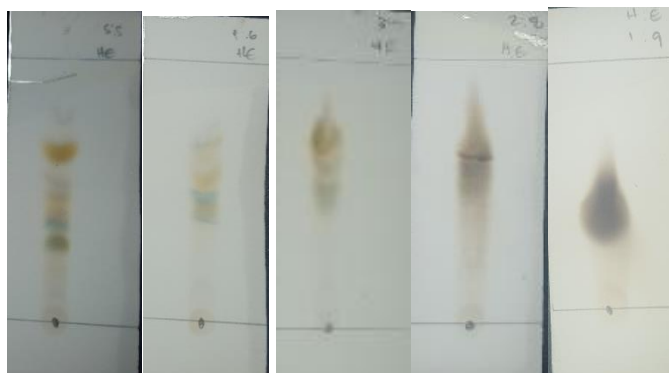
Figure D4: Thin layer chromatography of ZF 52 ethyl acetate extract using hexane:ethyl acetate
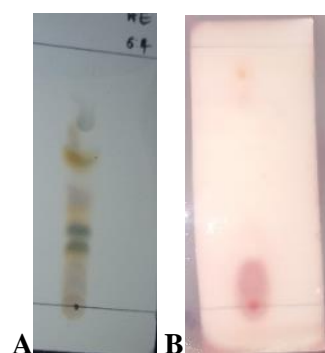
Figure D5: (A)Thin layer chromatography and (B) bioautography of the ZF 52 crude extract.

APPENDIX E
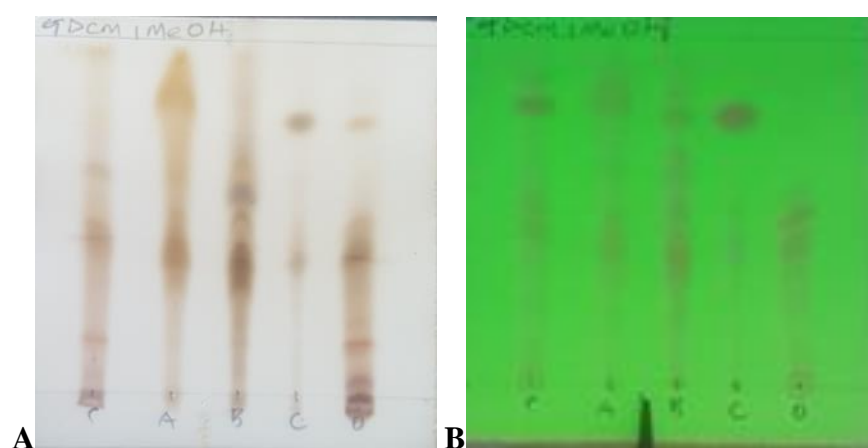
Figure E1: Thin layer chromatography of partially purified extract, with sub-fractions A, B, C and D.

Figure E2: Broth microtitre serial dilution assay for MIC determination of partially purified purified pooled fractions C

Figure E3: Broth microtitre serial dilution assay for MIC determination for purified compound 1.

APPENDIX F
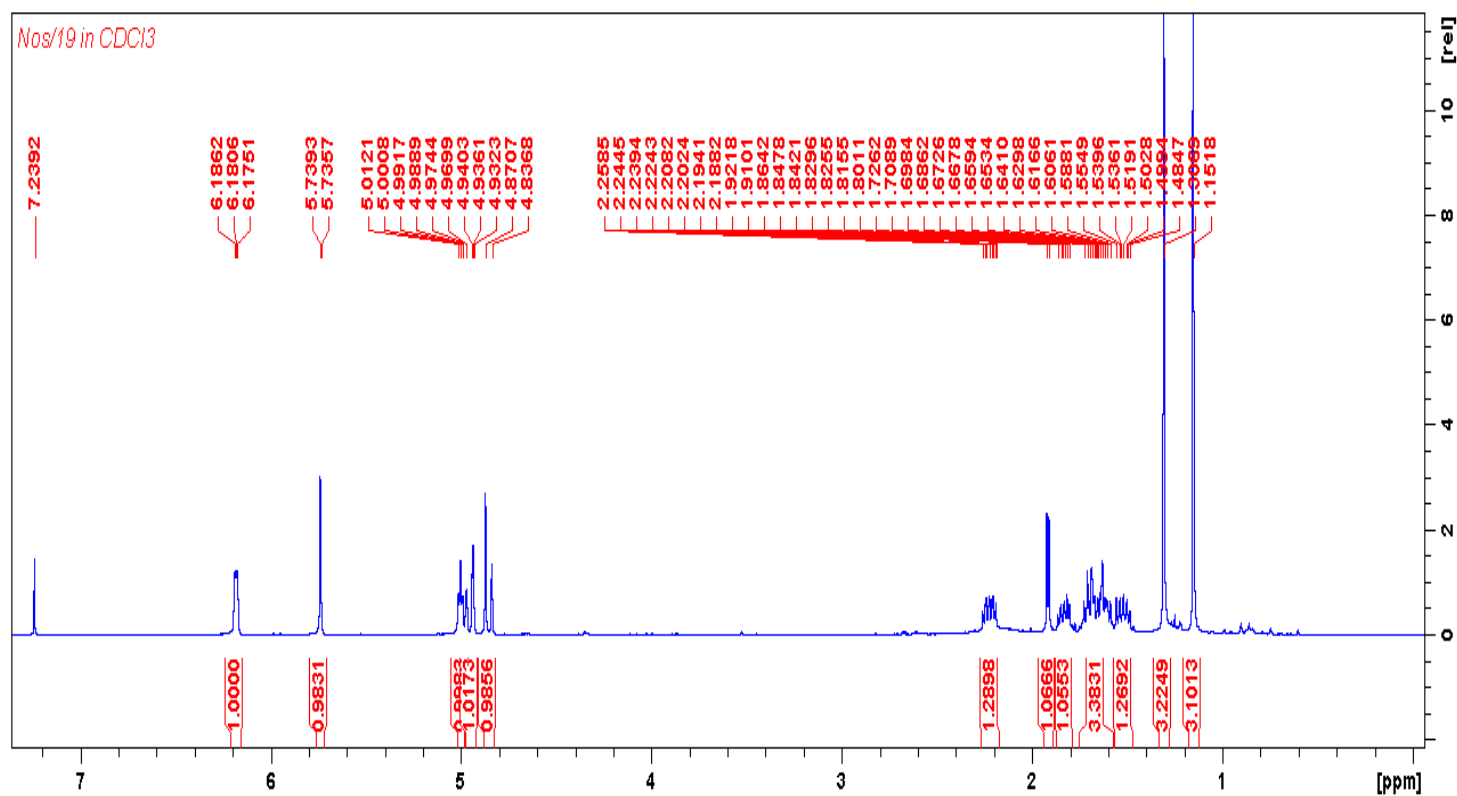
Figure F1: 1H-NMR (chloroform d$_1$, MHz) of compound 1.
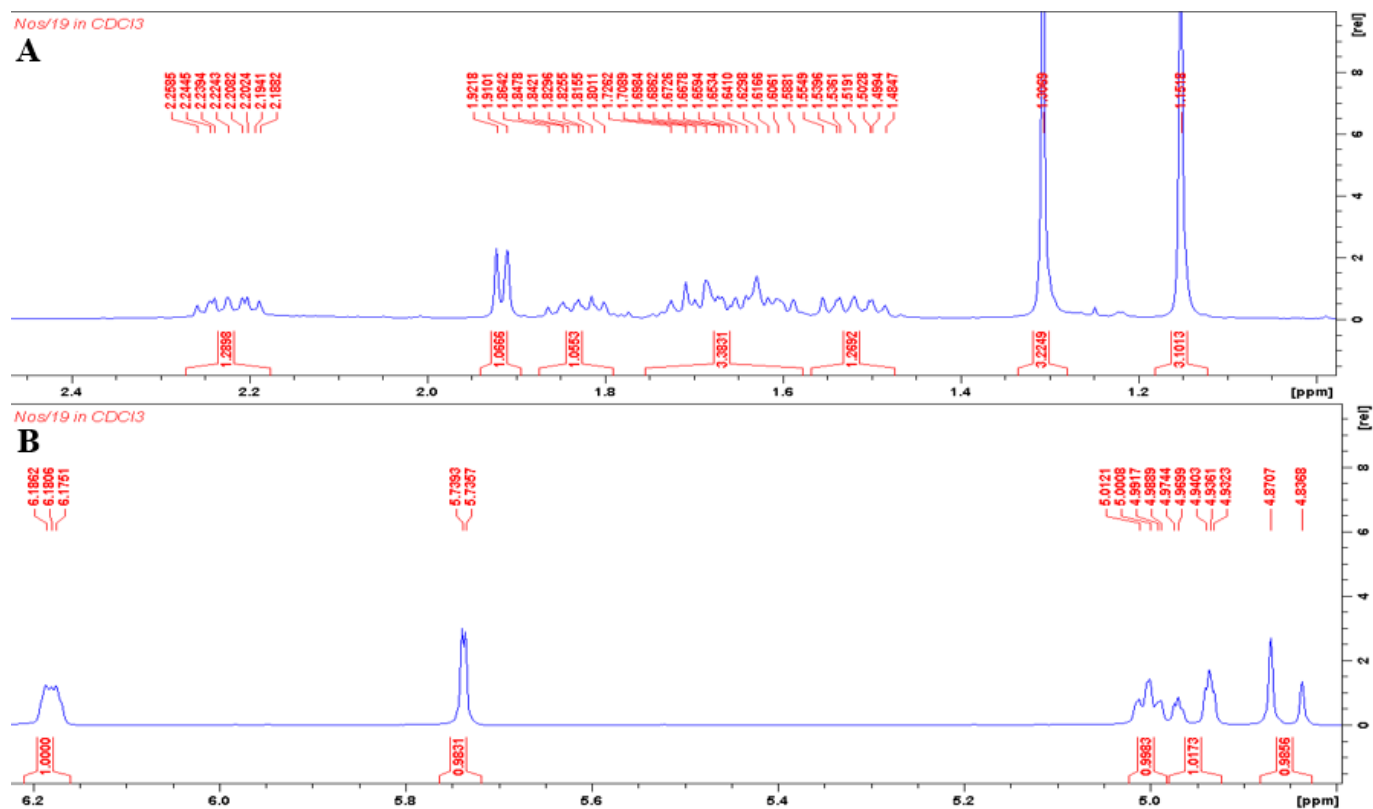
Figure F2: 1H-NMR (chloroform d$_1$, MHz) of compound 1 (A and B).
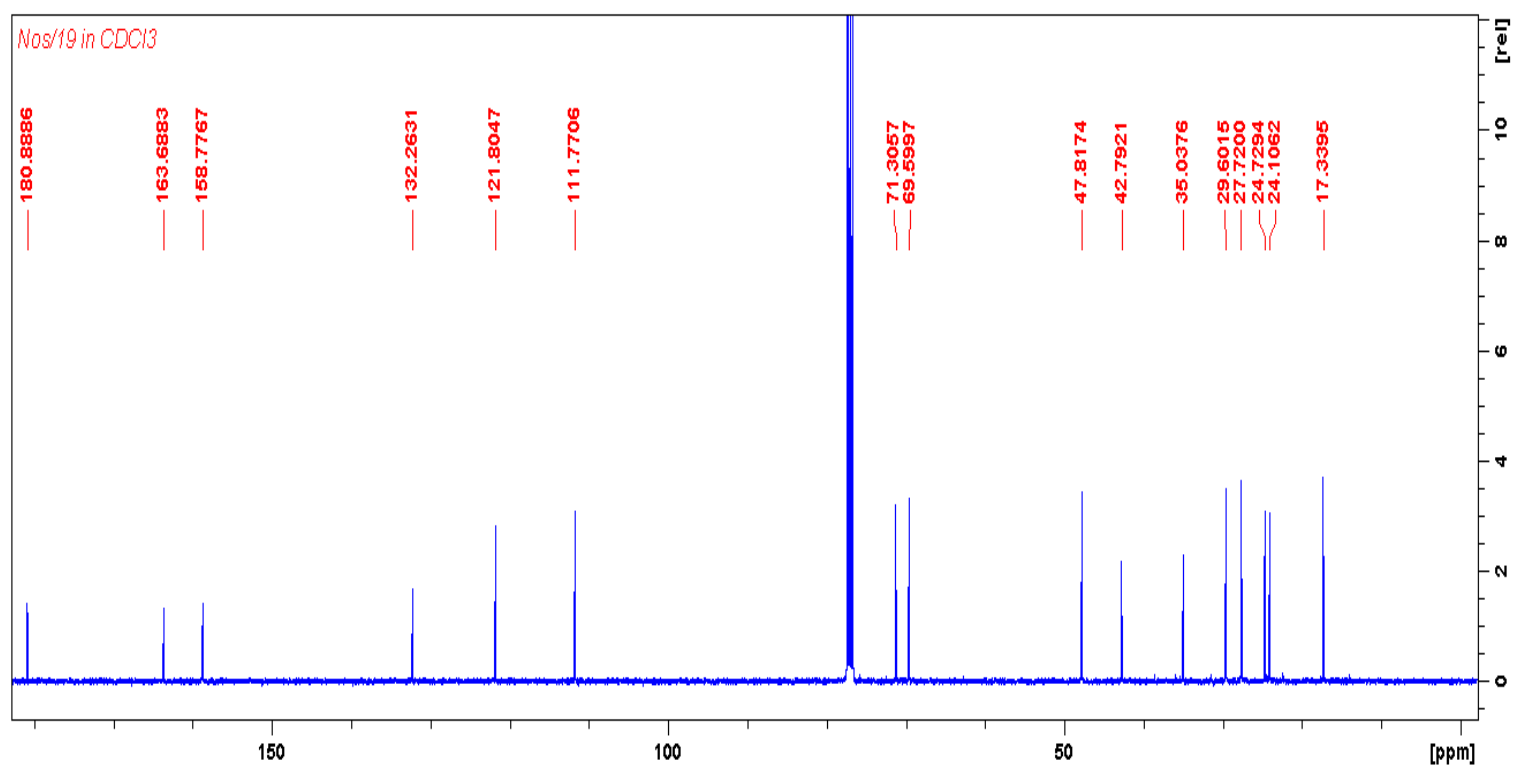
Figure F3: 13C-NMR of bioactive fraction compound 1.
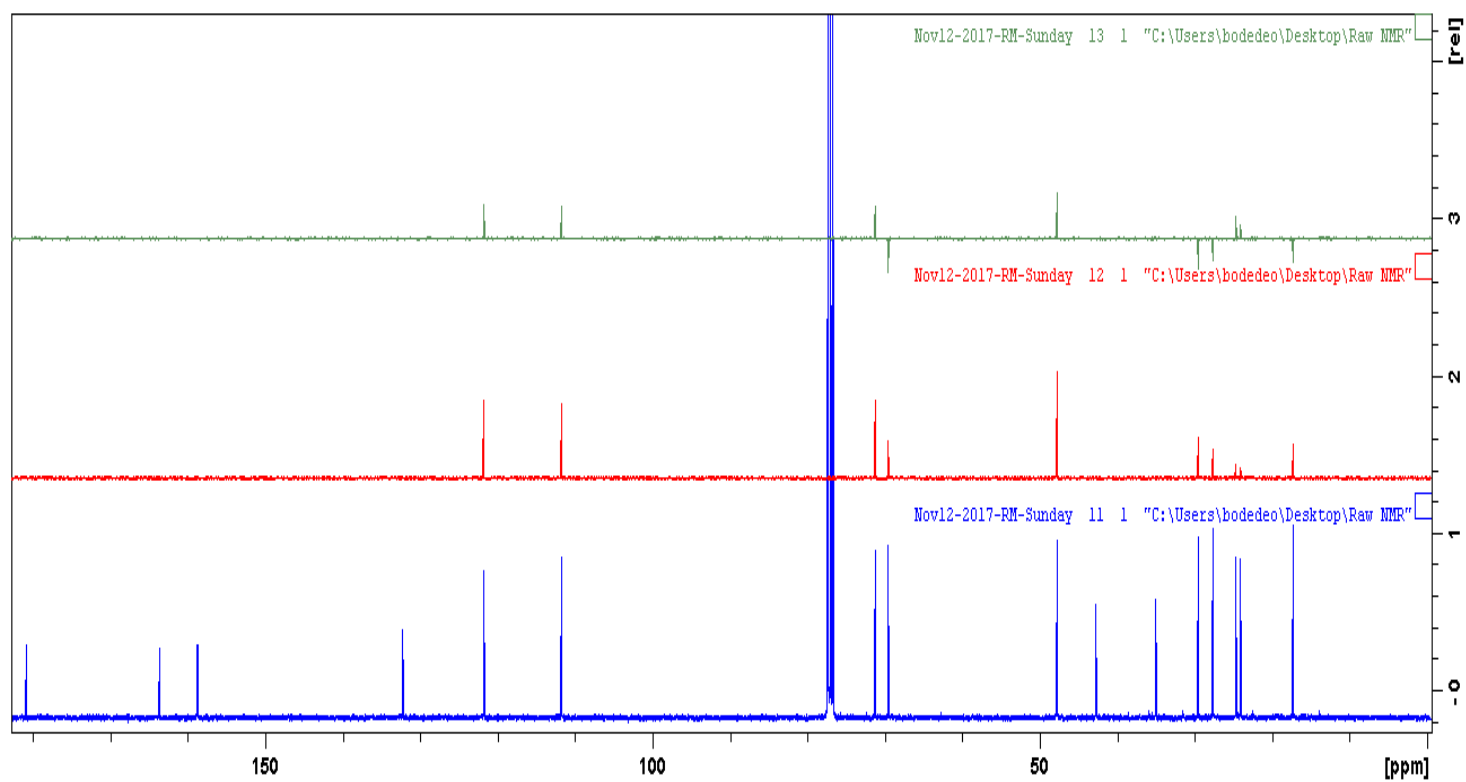
Figure F4: ^{13}C Dept 90 and Dept 135 of bioactive fraction superimposed.
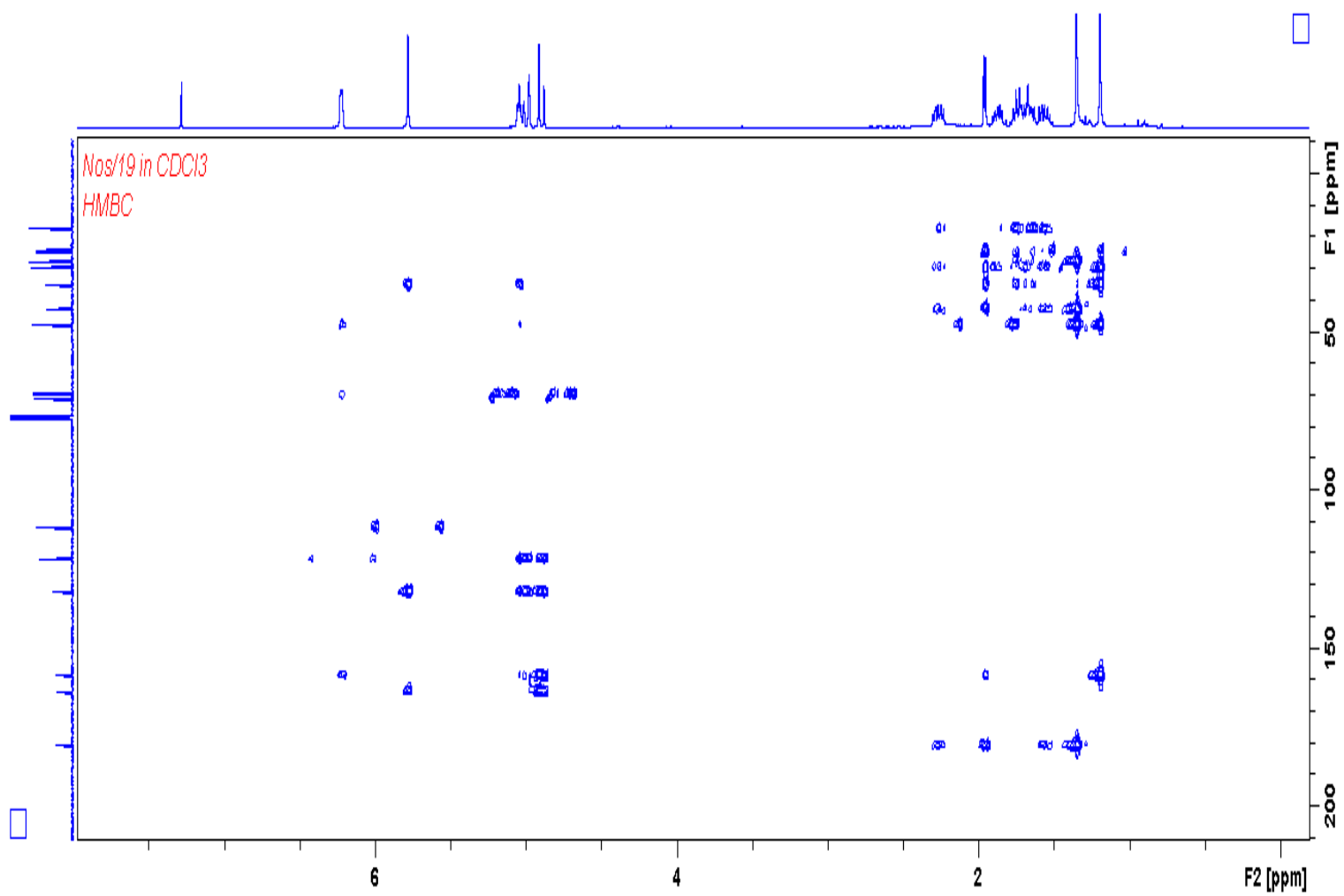
Figure F5: ^{1}H- ^{13}C HMBC NMR spectrum of compound 1.
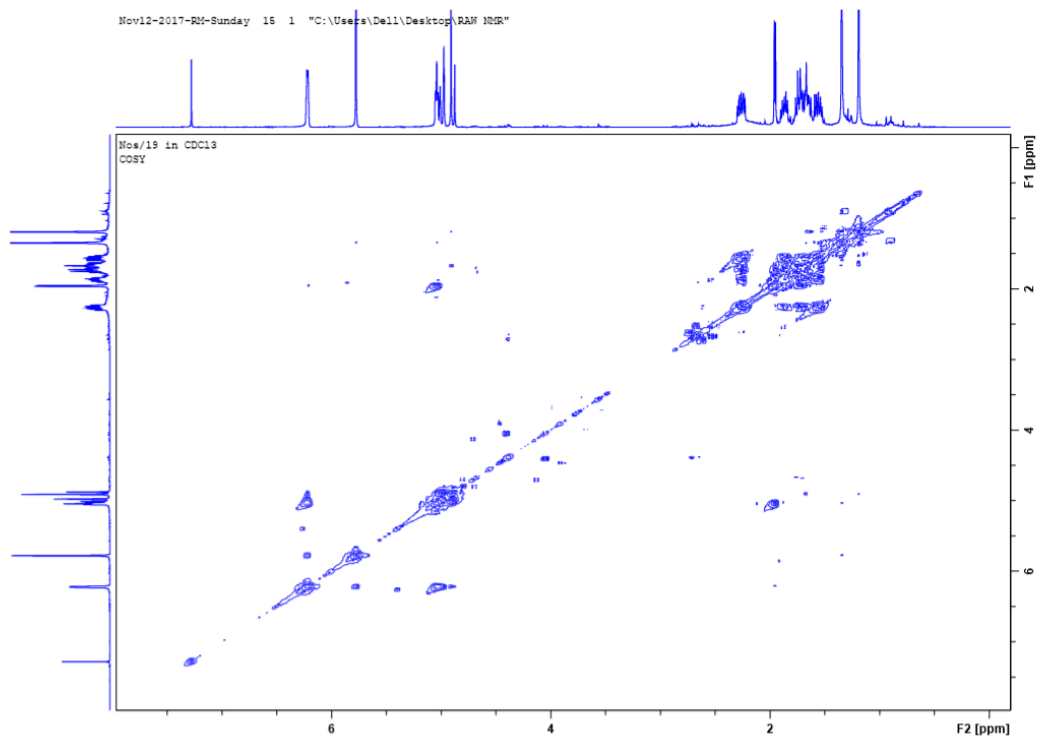
Figure F6: COSY ¹H-NMR spectrum of fraction compound 1.
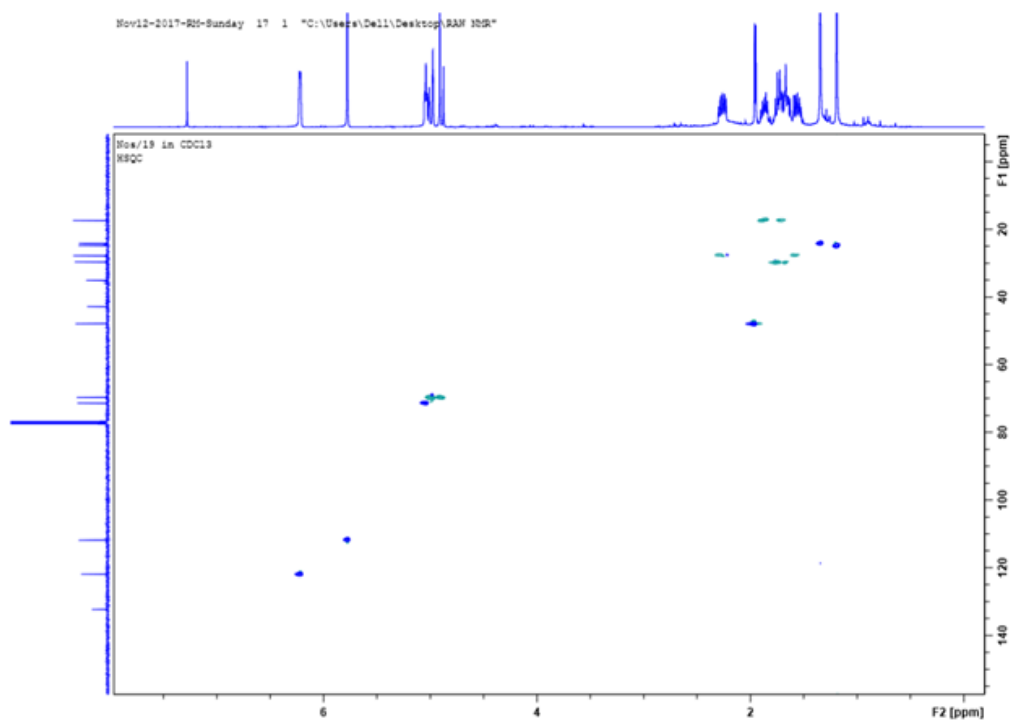
Figure F7: HSQC NMR spectrum of compound 1.
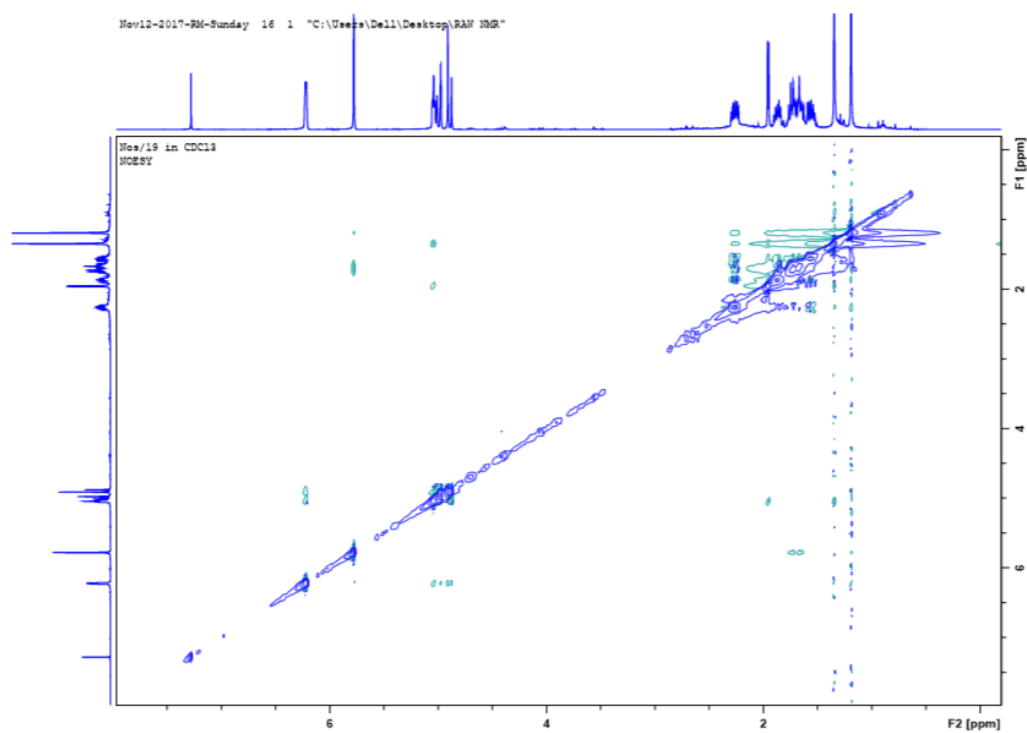
Figure F8: 2-D NOESY spectrum of compound 1.
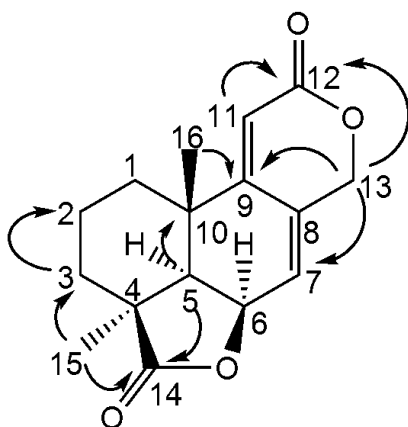
Figure F9: Major HMBC correlations observed in compound 1.
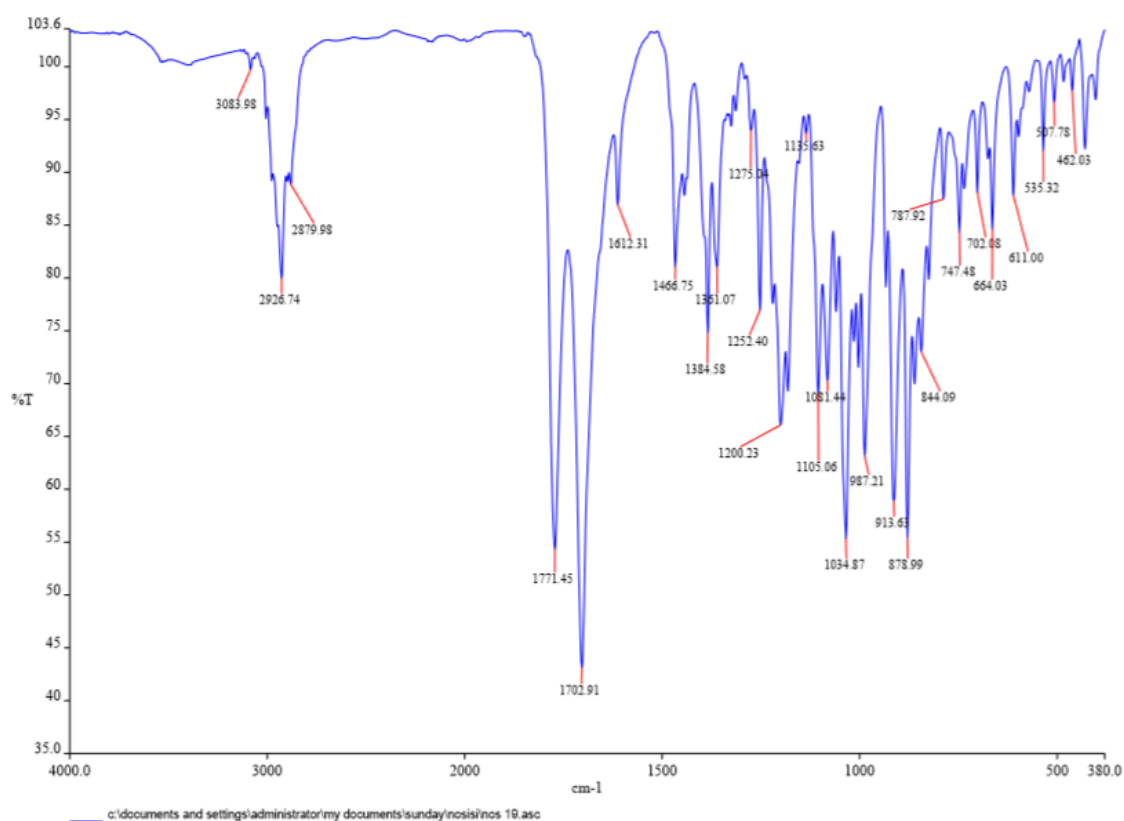
Figure F10: FTIR spectrum of compound 1.

APPENDIX G

Table G1: Crystal data and structure refinement for 18zs_bm_s1_0ma.

Table G1: Crystal data and structure refinement for 18zs_bm_s1_0ma.	
Identification code	18zs_bm_s1_0ma
Empirical formula	$C_{16}H_{18}O_4$
Formula weight	274.30
Temperature/K	100
Crystal system	monoclinic
Space group	C2
a/Å	18.120(3)
b/Å	7.6922(13)
c/Å	12.393(4)
α/°	90
β/°	129.372(6)
γ/°	90
Volume/Å³	1335.3(5)

Z	4
ρ_{calc}g/cm^3	1.364
μ/mm^{-1}	0.097
F(000)	584.0
Crystal size/mm^3	0.29 × 0.18 × 0.11
Radiation	MoKα ($\lambda = 0.71073$)
2Θ range for data collection/°	4.252 to 56.676
Index ranges	-24 ≤ h ≤ 24, -10 ≤ k ≤ 10, -16 ≤ l ≤ 16
Reflections collected	16326
Independent reflections	3281 [$R_{int} = 0.0458$, $R_{sigma} = 0.0296$]
Data/restraints/parameters	3281/1/183
Goodness-of-fit on F^2	1.061
Final R indexes [I>=2σ (I)]	$R_1 = 0.0387$, $wR_2 = 0.1015$
Final R indexes [all data]	$R_1 = 0.0407$, $wR_2 = 0.1032$
Largest diff. peak/hole / e Å^{-3}	0.40/-0.23
Flack parameter	0.2(3)

Table G2: Fractional Atomic Coordinates (×10^4) and Equivalent Isotropic Displacement Parameters.

Fractional Atomic Coordinates (×10^4) and Equivalent Isotropic Displacement Parameters (Å^2×10^3) for 18zs_bm_s1_0ma. U_{eq} is defined as 1/3 of of the trace of the orthogonalised U_{IJ} tensor.

Atom	x	y	z	U(eq)
O4	6223.6(11)	-2781(2)	6527.3(17)	27.5(4)
O3	7745.1(10)	-2460.1(18)	8450.4(15)	21.4(3)
O2	10496.6(10)	2835.2(19)	12525.0(15)	21.4(3)
O1	10445.4(11)	5510(2)	11854.1(16)	26.3(3)
C11	6970.5(14)	-2024(3)	7120(2)	20.0(4)
C9	7233.6(13)	-609(3)	6558(2)	18.6(4)
C10	8087.3(13)	215(2)	7927.9(19)	15.8(4)
C5	7797.6(13)	1635(3)	8484.6(19)	17.1(4)
C4	8730.7(13)	2177(3)	9861(2)	17.3(4)
C3	9070.0(14)	3800(2)	10260(2)	19.0(4)
C2	10033.9(14)	4140(3)	11588(2)	20.1(4)
C12	8553.2(14)	-1345(2)	8899(2)	18.5(4)
C8	6433.4(14)	659(3)	5494(2)	22.2(4)
C7	6323.6(14)	2332(3)	6060(2)	24.1(4)
C6	7271.0(14)	3051(3)	7367(2)	22.1(4)
C15	7155.5(14)	993(3)	8826(2)	20.9(4)
C14	9293.6(13)	745(2)	10840.0(19)	17.5(4)
C13	9174.9(13)	-904(3)	10421(2)	18.3(4)
C1	9957.9(14)	1298(3)	12341(2)	20.5(4)
C16	7562.4(15)	-1609(3)	5847(2)	24.7(4)

Table G3: Anisotropic Displacement Parameters ($\text{Å}^2 \times 10^3$) for 18zs_bm_s1_0ma. The Anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2a^{*2}U_{11}+2hka^*b^*U_{12}+\ldots]$.

Anisotropic Displacement Parameters ($\text{Å}^2 \times 10^3$) for 18zs_bm_s1_0ma. The Anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2a^{*2}U_{11}+2hka^*b^*U_{12}+\ldots]$.					
Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O4	22.4(7)	29.3(8)	33.1(8)	-2.6(6)	18.8(7)	-7.2(6)
O3	20.6(7)	20.2(7)	25.9(7)	0.5(6)	15.9(6)	-2.2(5)
O2	18.0(7)	21.7(7)	22.6(6)	-1.4(5)	12.0(6)	-1.6(5)
O1	23.5(7)	22.6(7)	30.5(7)	-2.6(6)	16.1(6)	-3.7(6)
C11	21.2(9)	18.8(8)	26.5(9)	-2.0(7)	18.1(8)	-1.0(7)
C9	16.7(9)	22.7(10)	20.9(9)	-1.4(7)	14.1(8)	-1.6(7)
C10	14.5(8)	18.6(8)	19.5(8)	-0.3(7)	13.2(7)	-0.6(7)
C5	15.0(8)	17.9(8)	21.4(9)	1.2(7)	12.9(8)	0.9(7)
C4	16.4(8)	20.8(9)	21.1(8)	0.3(7)	15.0(7)	1.4(7)
C3	18.0(9)	19.1(9)	23.6(9)	0.4(7)	14.9(8)	1.7(7)
C2	20.6(9)	21.7(9)	24.3(10)	-2.5(8)	17.2(8)	0.0(8)
C12	17.1(8)	18.5(8)	24.2(9)	0.0(7)	15.1(8)	-0.1(7)
C8	19.5(9)	26(1)	21.5(9)	2.7(8)	13.2(8)	1.6(8)
C7	18.0(9)	24.5(9)	24.6(9)	2.8(8)	11.1(8)	2.3(8)
C6	20.4(9)	19.4(9)	25.0(9)	3.3(8)	13.6(8)	2.5(7)
C15	17.2(9)	26(1)	25.2(9)	-1.1(8)	16.0(8)	-0.3(7)
C14	14.8(8)	21.6(9)	21.0(8)	1.3(7)	13.7(7)	0.3(7)
C13	16.3(8)	20.3(9)	22.6(9)	3.4(7)	14.4(8)	1.8(7)
C1	19.6(9)	22.9(9)	21.3(8)	0.5(8)	14.1(8)	-1.1(7)
C16	22.5(10)	32.5(11)	25.1(9)	-5.8(8)	18.0(8)	-1.0(8)

Table G4: Bond Lengths for 18zs_bm_s1_0ma.

Bond Lengths for 18zs_bm_s1_0ma.					
Atom	Atom	Length/Å	Atom	Atom	Length/Å
O4	C11	1.204(3)	C5	C4	1.511(3)
O3	C11	1.366(2)	C5	C6	1.529(3)
O3	C12	1.469(2)	C5	C15	1.550(3)
O2	C2	1.350(2)	C4	C3	1.340(3)
O2	C1	1.456(2)	C4	C14	1.468(3)
O1	C2	1.210(3)	C3	C2	1.477(3)
C11	C9	1.523(3)	C12	C13	1.499(3)
C9	C10	1.531(3)	C8	C7	1.538(3)
C9	C8	1.539(3)	C7	C6	1.533(3)
C9	C16	1.546(3)	C14	C13	1.336(3)
C10	C5	1.550(3)	C14	C1	1.500(3)
C10	C12	1.520(3)			

Table G5: Bond Angles for 18zs_bm_s1_0ma.

Atom	Atom	Atom	Angle/°	Atom	Atom	Atom	Angle/°
C11	O3	C12	109.14(15)	C15	C5	C10	114.98(16)
C2	O2	C1	118.69(15)	C3	C4	C5	126.65(18)
O4	C11	O3	120.98(19)	C3	C4	C14	118.57(17)
O4	C11	C9	128.6(2)	C14	C4	C5	114.77(17)
O3	C11	C9	110.33(16)	C4	C3	C2	121.19(18)
C11	C9	C10	100.26(15)	O2	C2	C3	118.48(18)
C11	C9	C8	116.55(16)	O1	C2	O2	118.31(17)
C11	C9	C16	104.52(17)	O1	C2	C3	123.13(19)
C10	C9	C8	115.32(16)	O3	C12	C10	103.99(15)
C10	C9	C16	110.76(15)	O3	C12	C13	112.41(16)
C8	C9	C16	108.75(16)	C13	C12	C10	114.46(16)
C9	C10	C5	113.32(15)	C7	C8	C9	117.64(16)
C12	C10	C9	102.35(15)	C6	C7	C8	113.69(17)
C12	C10	C5	112.25(15)	C5	C6	C7	109.69(16)
C4	C5	C10	104.15(14)	C4	C14	C1	113.75(17)
C4	C5	C6	115.72(17)	C13	C14	C4	122.34(18)
C4	C5	C15	106.03(15)	C13	C14	C1	123.80(18)
C6	C5	C10	106.96(15)	C14	C13	C12	120.76(18)
C6	C5	C15	109.20(16)	O2	C1	C14	110.82(16)

Table G6: Torsion Angles for 18zs_bm_s1_0ma.

A	B	C	D	Angle/°	A	B	C	D	Angle/°
O4	C11	C9	C10	159.3(2)	C4	C3	C2	O2	-12.9(3)
O4	C11	C9	C8	34.1(3)	C4	C3	C2	O1	163.9(2)
O4	C11	C9	C16	-85.9(3)	C4	C14	C13	C12	8.7(3)
O3	C11	C9	C10	-24.4(2)	C4	C14	C1	O2	-47.5(2)
O3	C11	C9	C8	-149.55(17)	C3	C4	C14	C13	-159.64(19)
O3	C11	C9	C16	90.41(18)	C3	C4	C14	C1	24.1(2)
O3	C12	C13	C14	-118.8(2)	C2	O2	C1	C14	43.1(2)
C11	O3	C12	C10	20.64(19)	C12	O3	C11	O4	179.32(18)
C11	O3	C12	C13	145.00(17)	C12	O3	C11	C9	2.7(2)
C11	C9	C10	C5	-86.00(18)	C12	C10	C5	C4	61.39(19)
C11	C9	C10	C12	35.10(17)	C12	C10	C5	C6	-175.61(15)
C11	C9	C8	C7	91.0(2)	C12	C10	C5	C15	-54.2(2)
C9	C10	C5	C4	176.73(15)	C8	C9	C10	C5	40.0(2)
C9	C10	C5	C6	-60.3(2)	C8	C9	C10	C12	161.13(16)
C9	C10	C5	C15	61.2(2)	C8	C7	C6	C5	-54.1(2)
C9	C10	C12	O3	-35.00(17)	C6	C5	C4	C3	10.0(3)
C9	C10	C12	C13	-158.03(16)	C6	C5	C4	C14	-171.15(16)
C9	C8	C7	C6	33.3(2)	C15	C5	C4	C3	-111.2(2)

C10	C9	C8	C7	-26.2(2)	C15	C5	C4	C14	67.6(2)
C10	C5	C4	C3	127.1(2)	C15	C5	C6	C7	-58.2(2)
C10	C5	C4	C14	-54.1(2)	C14	C4	C3	C2	6.6(3)
C10	C5	C6	C7	66.8(2)	C13	C14	C1	O2	136.24(19)
C10	C12	C13	C14	-0.4(3)	C1	O2	C2	O1	169.16(18)
C5	C10	C12	O3	86.82(17)	C1	O2	C2	C3	-13.9(2)
C5	C10	C12	C13	-36.2(2)	C1	C14	C13	C12	-175.40(17)
C5	C4	C3	C2	-174.61(17)	C16	C9	C10	C5	164.06(16)
C5	C4	C14	C13	21.4(3)	C16	C9	C10	C12	-74.84(19)
C5	C4	C14	C1	-154.89(16)	C16	C9	C8	C7	-151.27(18)
C4	C5	C6	C7	-177.67(17)					

Table G7: Hydrogen Atom Coordinates ($\mathring{A}\times10^4$) and Isotropic Displacement Parameters ($\mathring{A}^2\times10^3$) for 18zs_bm_s1_0ma.

| **Hydrogen Atom Coordinates ($\mathring{A}\times10^4$) and Isotropic Displacement Parameters ($\mathring{A}^2\times10^3$) for 18zs_bm_s1_0ma.**				
Atom	*x*	*y*	*z*	U(eq)
H10	8534.62	721.69	7798.21	19
H3	8683.59	4749.02	9677.96	23
H12	8948.36	-1970.02	8716.33	22
H8A	6537.25	998.96	4828.79	27
H8B	5820.34	21.03	4956.59	27
H7A	6014.79	3230.62	5320.78	29
H7B	5895.88	2093.87	6282.03	29
H6A	7670.96	3450.62	7127.97	27
H6B	7147.19	4058.29	7729.51	27
H15A	6580.78	445.15	7997.27	31
H15B	6972.35	1983.45	9108.79	31
H15C	7507.41	145.45	9588.83	31
H13	9488.3	-1808.37	11095.01	22
H1A	9585.47	1553.5	12655.52	25
H1B	10405.25	337.9	12921.88	25
H16A	8042.21	-2476.07	6495.48	37
H16B	7840.3	-791.1	5585.95	37
H16C	7013.12	-2193.22	5008.32	37

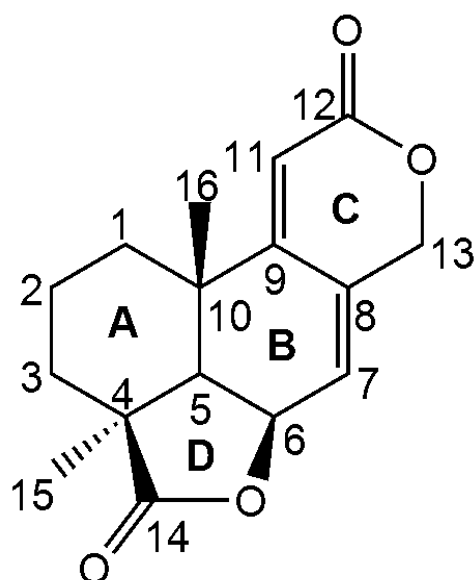**Experimental**
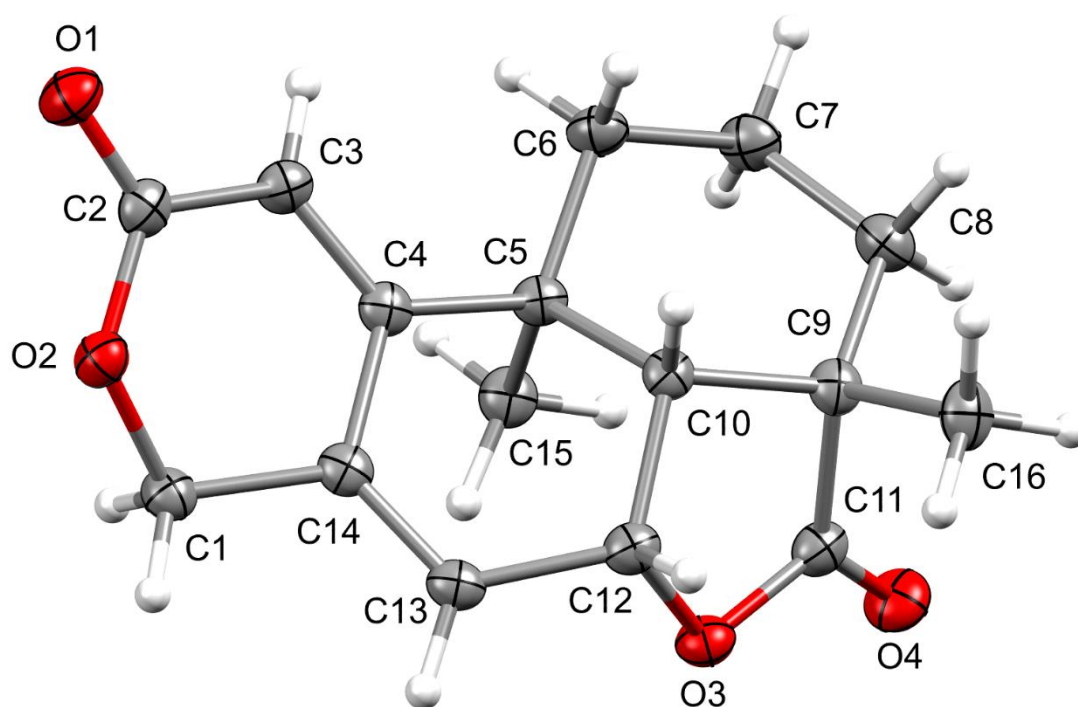
Figure G1: Single-crystal X-ray structure and numbering scheme of compound 1.

Figure G2: Structure of CJ-14445.