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Evaluating the Activity of Novel Natural and Synthetic Antifungal Agents Against Select Pathogenic Yeast

by

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Dissertation

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Co-supervisor: Prof Marianne Cronjé

May 2023

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



_____ (Signature of candidate)

23rd day of May 2023 at the University of the Witwatersrand, Johannesburg.

ABSTRACT

Fungal infections are believed to affect over a billion people worldwide, with more than 150 million people estimated to be suffering with serious or fatal fungal diseases. Many of these infections are caused by opportunistic fungal pathogens within the *Candida* and *Cryptococcus* genera. Current treatment options are limited, costly and can come with significant toxic side effects. This has contributed towards the emergence of novel, multidrug resistant pathogens. With the rise of microbial resistance, it is imperative that efforts are made to provide alternative antifungal treatments that are not only effective, but also non-toxic. To this end, plant based extracts and silver compounds serve as promising treatment opportunities. This study screened a variety of novel silver (I)-phosphine compounds and native plant extracts for their antifungal and antibiofilm activity against pathogens in the *Candida* and *Cryptococcus* genera. Furthermore, the toxicity of select compounds was assessed using *Caenorhabditis elegans* as a model organism. Lastly, the efficacy of select compounds was assessed *in vivo* through the use of the *C. elegans* infection model.

In **Chapter 1,** important fungal pathogens within the *Candida* and *Cryptococcus* genera are examined. The difficulties associated with the treatment of systemic infections including resistance mechanisms, such as biofilm formation, employed by fungal pathogens is reviewed. The importance of the need for new antifungal treatment opportunities is stressed with silver-based molecules and plant extracts serving as promising antifungal treatment opportunities. Lastly, the use of *C. elegans* as a model organism to assess toxicity and efficacy of novel antifungal drugs is assessed.

In **Chapter 2**, seven novel silver (I)-phosphine complexes (SPC) as well as eleven native plant extracts are screened for their antifungal activity against a variety of clinically important pathogens within the *Candida* and *Cryptococcus* genera. This screening showed SPC 7 as wells as the ethanolic extracts of *Hypoxis hemerocallidea*, *Kigelia africana* and *Pelargonium zonale* possess promising antifungal activity. As such the minimum inhibitory and fungicidal concentrations for those compounds were determined.

Chapter 3 examines the antibiofilm properties of the *K. africana* extract and SPC 7 against pathogens within the *Candida* and *Cryptococcus* genera. The results demonstrated that neither of the compounds were able to prevent or disrupt biofilm formation, however when the biofilms were exposed to SPC 7, reductions in the metabolic activity of the biofilms was observed.

In **Chapter 4**, the toxicity of SPC 7 and the ethanolic extracts of *H. hemerocallidea, K. africana* and *P. zonale* is assessed using *C. elegans* as a model organism. The *K. africana* extract was found to be the least toxic of the plant extracts with SPC 7 also showing low levels of toxicity against the nematode. As such, the efficacy of those compounds was assessed *in vivo* against *Candida auris* and *Cryptococcus neoformans* infected nematodes. The infected nematodes showed significant reductions in mortality when treated with SPC 7.

As such, SPC 7 was identified as a promising antifungal treatment showing potent fungistatic, fungicidal, and antibiofilm properties against *Candida* and *Cryptococcus* pathogens. Furthermore, SPC 7 shows to exhibit low toxicity towards nematodes while also improving the lifespan of *C. auris* and *C. neoformans* infected nematodes. Thus, future work should be conducted to elucidate the mechanisms of action to further assess its suitability as a novel antifungal treatment.

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LIST OF SYMBOLS AND ABBREVIATIONS

ABC-T: (ATP)-binding cassette transporters

AIDS: acquired immunodeficiency virus

AmB: Amphotericin B

AP: *Hypoxis hemerocallidea*

ATP: Adenosine Triphosphate

BHI: brain heart infusion

CSLI: Clinical and Laboratory Standards Institute

CT: computed tomography

CV: crystal violet

DMSO: Dimethyl sulphoxide

eABCC: Angela Botes culture collection

ECM: extracellular matrix

EPS: extracellular polymetric substances

GXM: glucuronoxylomannan

HIV: human immunodeficiency virus

INT: iodonitrotetrazolium

K: *Kigelia africana*

LC50: Lethal concentration that kills 50% of the population

M: molar

MFC: minimum fungicidal concentrations

MFS-T: major facilitator transporters

MHA: Mueller Hinton agar

MIC: minimum inhibitory concentration

MFC: minimum fungicidal concentration

MM: minimal media

MRI: magnetic resonance imaging

neg: negative

NET: neutrophil extracellular trap

NICD: National Institute for Communicable Diseases

P: *Pelargonium zonale*

pos: positive

ROS: reactive oxygen species

rpm: revolutions per minute

SA: South Africa

SDA: Sabouraud dextrose agar

SEM: standard error of the mean

SPC: silver (I)-phosphine complex

TB: Tuberculosis

v/v: volume/volume percent

w/v: weight/volume percent

XTT: sodium 3´-[1- (phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate]

YPD: Yeast Peptone Dextrose

ZOI: Zone of inhibition

CHAPTER 1
Literature Review

1.1 Introduction

Fungal related diseases and infections are thought to affect over a billion people worldwide (Bongomin *et al.*, 2017). Recent estimates have determined that more than 150 million people are suffering with serious or fatal fungal diseases globally (Bongomin *et al.*, 2017). In South Africa (SA) there is a large proportion of individuals who are immunocompromised. These people include more than 7.5 million people who are living with human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS), half a million people suffering with tuberculosis (TB) and those with various cancers and pulmonary diseases (Naidoo *et al.*, 2017; Lehohla, 2015). Fungi have been found to play a significant role in nosocomial infections often acting as opportunistic pathogens (Badiee and Hashemizadeh, 2014). Consequently, many immunocompromised individuals have their conditions exacerbated by fungal infections (Schwartz *et al.*, 2019). As such there exists a high economic and healthcare-associated burden related to the treatment of fungal diseases with over 3.2 million people affected in SA each year (Schwartz *et al.*, 2019).

1.2 Fungal Pathogens of Clinical Prevalence

Fungal infections can range from superficial and mucosal to more severe allergic and systemic infections with most arising from either skin contact with the fungus or inhalation of infectious spores (Bongomin *et al.*, 2017; de Pauw, 2011). Globally, the most commonly occurring fungal diseases include those caused by the opportunistic pathogens within the *Candida* and *Cryptococcus* genera (de Pauw, 2011).

1.2.1 *Candida* Infections

Candida is genus of yeast that encompasses about 150 different species (Brandt and Lockhart, 2012). Most species of *Candida* are known to be harmless to humans, often located on mucosal surfaces such as epithelial surfaces and within the gastrointestinal tract (Kühbacher *et al.*, 2017; Pérez, 2021). As such, they make up an important part of the gut and skin microbiota acting as commensals or endosymbionts (Kühbacher *et al.*, 2017; Pérez, 2021). However, when disruptions to the microbiome occur or the immune system is compromised, some species of *Candida* have the ability to cause disease and are known as opportunistic pathogens (Turner and Butler, 2014). As such, higher isolation rates of these opportunistic *Candida* pathogens are

often found amongst immunocompromised patients including the elderly and those receiving medical care (Richardson and Rautemaa, 2009).

Candida related diseases are often superficial, primarily affecting the skin and mucosal membranes resulting in various candidiasis including oesophageal, oral and vulvovaginal (Kullberg and Arendrup, 2015). Once an infection has spread from the mucosal membranes to the bloodstream it is known as candidemia. These systemic infections can spread through the bloodstream to other parts of the body including the kidneys, liver and brain (Kullberg and Arendrup, 2015). This invasive candidemia is oftentimes fatal, particularly in individuals who are immunocompromised. Studies show the crude mortality rate attributed to various candidiasis to be between 19 – 60% depending on the species (Morgan *et al.*, 2005; Hirano *et al.*, 2015; Flevari *et al.*, 2013). In SA, candidemia has been found to have a mortality rate of 43 – 55% (Hussain *et al.*, 2022; Van Schalkwyk *et al.*, 2019). Furthermore, *Candida* species account for the large majority of nosocomial fungal infections, and it is approximated that 8 –13% percent of all hospital acquired blood stream infections arise as a result of them (Magill *et al.*, 2018; Wisplinghoff *et al.*, 2004).

The mechanisms by which *Candida* causes infection varies widely between different species as well as the location of infection (Mendonca *et al.*, 2022). In general, *Candida* first attach to the host cell using a variety of molecules including adhesins, ligands and integrins (Naglik *et al.*, 2011; Ciurea *et al.*, 2020). Following this, the fungal cells must invade the host cells which occurs through endocytosis or by active penetration, mainly through hyphae formation, depending on the species of *Candida* (Naglik *et al.*, 2011). The pathogens utilise a wide arsenal of virulence factors to aid in pathogenesis. These include biofilm formation, morphological changes, production of hydrolytic enzymes, production of stress tolerance proteins, as well as inducing the expression of a wide variety of molecules that aid in host defence suppression and evasion (Staniszewska, 2020; Talapko *et al.*, 2021; Rossato and Colombo, 2018; Richardson and Rautemaa, 2009). Systemic infections arise from the unchecked growth of these pathogens which are able to make their way into the bloodstream and are further spread to other organs around the body (Kullberg and Arendrup, 2015).

The treatment of *Candida* infections also varies widely depending on the species and location of infection (Mendonca *et al.*, 2022). Many infections manifest superficially on the skin and can be easily diagnosed and treated with a variety of topical medications (Taudorf *et al.*, 2019).

However, diagnosis and treatment of systemic infections can be much more complicated. Usually bloodstream infections can be confirmed with a blood culture analysis, however these methods have long growth and turnaround times and the tests utilised have low sensitivity of around 50% (Mendonca *et al.*, 2022). Additionally, these methods are often unable to differentiate between various species of *Candida* which may lead to inappropriate treatments being administered (Mendonca *et al.*, 2022). A few treatments are available for the treatment of candidiasis including various formulations of amphotericin B as well as azole and echinocandin based treatments however, these are often expensive and they are not always effective against all strains (Spellberg *et al.*, 2006). These factors complicate and lengthen the diagnosis process which delays patient treatment and ultimately leads to poorer patient outcomes as well as higher associated costs (Barantsevich and Barantsevich, 2022).

The main culprit responsible for many of these infections is *Candida albicans* however non-albicans species such as *C. glabrata, C. krusei, C. parapsilosis* and *C. tropicalis* have also been implicated in an increasing number of bloodstream infections (Hajjeh *et al.*, 2004). Collectively these species are responsible for over 95% of all invasive *Candida* infections (Hajjeh *et al.*, 2004). However, recently there has been an emergence of a number of invasive infections caused by a new isolate named *Candida auris* (Ademe and Girma, 2020).

The first isolate of *C. auris* was obtained in 2009 in Japan and was notable for its ability to grow in the presence of the potent fungicide, micafungin, which is used as a first line therapy to treat various invasive candidiasis (Satoh *et al.*, 2009). *Candida auris* is known to cause severe invasive infections however it is particularly worrisome for its role as an emerging novel multidrug resistant pathogen (Ademe and Girma, 2020). In the short time since its discovery, *C. auris* infections have surged worldwide and now accounts for the highest proportion of non-albicans *Candida* infections, surpassing once predominant species such as *C. glabrata* and *C. tropicalis* (Ahmad and Alfouzan, 2021). The significance of this pathogen lies in its high incidence of multidrug resistance. While resistance profiles vary between *C. auris* isolates, reduced susceptibility to the main classes of antifungal drugs including azoles, echinocandins and polyenes is observed (Navalkele *et al.*, 2017). This makes *C. auris* infections difficult to treat, thus resulting in high mortality rates (Welsh *et al.*, 2017). In fact, studies have estimated the global crude mortality rates to lie in the range of 30 - 60% (Osei Sekyere, 2018; Lockhart *et al.*, 2017; Al Maani *et al.*, 2019) and have calculated that the overall infection mortality rate lies at around 52% (Al-Rashdi *et al.*, 2021; Al Maani *et al.*, 2019). Furthermore, *C. auris* has

shown a propensity for plastics (including equipment commonly used in hospital settings such as catheters, scopes and prosthetics) which makes it likely to be involved in nosocomial infections (Welsh *et al.*, 2017; Pfaller and Diekema, 2004). Coupled with the fact that *C. auris* is known to also persist after exposure to standard disinfectants such as some ammonia-based compounds there is cause for concern over the threat of its spread (Ku *et al.*, 2018). In fact, *C. auris* was recently implicated in a number of healthcare facility outbreaks across numerous countries including SA (Govender *et al.*, 2018). Furthermore, national surveillance of *C. auris* infections over a four-year period in SA show a rise in the number of infections observed (Govender *et al.*, 2018) and estimates 10% of all candidemia cases to be attributed to *C. auris* (Govender *et al.*, 2018). These numbers are only expected to rise in lieu of the COVID-19 pandemic which resulted in a large majority of immunocompromised patients requiring hospital care. Thus, this pathogen has shown cause for concern as a serious epidemiological threat if not adequately controlled.

1.2.2 *Cryptococcus* Infections

Cryptococcus is a genus of yeasts which was first described in the early 1900's and has now come to encompass around 30 different species (Barnett, 2010). It includes several medically important species including *C. neoformans* and *C. gattii* which are estimated to affect over one million people globally every year (Park *et al.*, 2009; Pappas, 2013). Cryptococcosis is termed as an infection caused by invasive pathogens within the *Cryptococcus* genera that has been estimated to have a case-fatality ratio of around 12% in the North America (Mirza *et al.*, 2003). The incidence is increased in sub-Saharan Africa where it is estimated that over 200 000 people are infected with *Cryptococcus* annually which results in around 135 000 deaths each year (Rajasingham *et al.*, 2017). Immunocompetent patients are often infected by *C. gattii* and *C. tetragattii* whereas immunocompromised patients are affected mainly by *C. deneoformans* and *C. neoformans* (Pappas, 2013).

In immunocompromised patients, infection often manifests as severe diseases such as cryptococcal meningitis or pulmonary cryptococcosis. Studies have shown that cryptococcosis is the most common form of meningitis in people living with HIV/AIDS in sub-Saharan Africa and accounts for 15% of AIDS-related deaths globally (Rajasingham *et al.*, 2017). In fact, it is one of the leading causes of death amongst HIV-infected individuals, second only to tuberculosis (Rajasingham *et al.*, 2022; Mada *et al.*, 2020). Although cryptococcal infections

are predominantly associated with HIV/AIDS, members of other immunocompromised groups such as those with cancers, liver cirrhosis, diabetes, organ transplant recipients and those on long-term corticosteroid treatments are also at high risk of developing cryptococcosis (Mada *et al.*, 2020; Lin *et al.*, 2015).

The most common culprits for most cases of cryptococcosis are *C. neoformans* and *C. gattii* (Maziarz and Perfect, 2016). These pathogens occur worldwide and are commonly found in decaying wood and soil contaminated by bird droppings (Levitz, 1991). A person can become infected with *Cryptococcus* after exposure and inhalation of the desiccated fungal cells (Kwon-Chung *et al.*, 2014). It is believed that people may also breath in cryptococcal basidiospores during their childhood which then colonise the airways (Kwon-Chung *et al.*, 2014). Although most immunocompetent people remain asymptomatic and can clear the infection without the onset of disease, it has been seen that latent infections may be established and can be triggered if the person becomes immunocompromised (Hommel *et al.*, 2019). Following the inhalation of desiccated cryptococcal yeast cells or basidiospores, the cells travel through the airways and into the lungs. It is here they encounter the alveolar macrophages which go on to recruit other immune cells with the aid of cytokines to stimulate an immune response (Kwon-Chung *et al.*, 2014). In an immunocompetent host, the infection is usually eliminated following the appropriate immune response such as phagocytosis and lysosome activity, however in immunocompetent hosts the cryptococcal cells are allowed to proliferate (Elsegeiny *et al.*, 2018; Sabiiti *et al.*, 2014). When this proliferation goes unchecked, fungal masses develop which may start to impair the function of the affected organ, usually the lung (Tan *et al.*, 2021). Some of the fungal cells may separate from the mass and can be carried by the bloodstream to other parts of the body, such as the brain and central nervous system, where they attach and continue to proliferate (Tan *et al.*, 2021). In immunocompromised individuals when the infection cannot be cleared, it can remain in the lungs and cause pulmonary pneumonia and if it disseminates to the brain or nervous system it can result in cryptococcal meningitis (Abassi *et al.*, 2015). In rare instances, blood stream infections may be established too (Perfect and Bicanic, 2015). Cryptococcal infections are not thought to be contagious and person-to-person, and thus nosocomial infections, are also uncommon (Miozzo *et al.*, 2010; Perfect and Bicanic, 2015).

The diagnosis of cryptococcosis usually occurs after the onset of symptoms (Perfect and Bicanic, 2015). Due to the prevalence of cryptococcosis in patients with HIV/AIDS and

transplant patients, a diagnosis can often be made fairly quickly and those patients can start receiving treatment within two to three weeks (Perfect and Bicanic, 2015). Matters are complicated for the diagnosis of those without these major risk factors. In these cases, it can sometimes take twice as long to receive a correct diagnosis and treatment, which significantly decreases their prognosis (Perfect and Bicanic, 2015; Bratton *et al.*, 2012). In fact, it is estimated that the mortality rate is as high at 30% for those who are diagnosed late in the infectious process (Perfect *et al.*, 2010). Computed tomography (CT) scans and magnetic resonance imaging (MRIs) are often employed to show pulmonary nodules or meningitis however these diseases present similarly to symptoms caused by tuberculosis, cancers, or bacterial and viral infections. Thus, tissue biopsy samples and serological testing of spinal fluid are usually performed to confirm cryptococcosis (Maziarz and Perfect, 2016).

Once a positive diagnosis is confirmed, a few treatment opportunities exist. Most notably, amphotericin B, fluconazole and flucytosine are routinely administered (Perfect and Bicanic, 2015). However medications used for treatment may vary depending on the condition of the patient, as well as the secondary diseases and symptoms they may be experiencing. While immunocompetent people require treatment for a few weeks, immunocompromised patients often require treatment for years or even for their lifetime to prevent reactivation of the infection (Perfect and Bicanic, 2015). Although these treatments may be effective in clearing the fungal pathogen, they also come with several disadvantages. The main ones being that they are not widely available, their use is also hampered by cost, and amphotericin B in particular has shown to have severe adverse effects such as nephrotoxicity (Loyse *et al.*, 2013). In conjunction with issues of poor long-term treatment adherence, these cryptococcal infections can become difficult to adequately treat.

1.3 Resistance Mechanisms

It is important to understand the strategies employed by fungal pathogens that allow them to become resistant. Fungi may have intrinsic resistance whereby a species may be able to resist the activity of an antifungal through its inherent structural and functional characteristics (Cowen *et al.,* 2015). For example, the antifungal having a lack of affinity for the fungal target or through the use efflux pumps in the pathogen (Cowen *et al.,* 2015). Acquired resistance occurs when the fungal pathogen gains the ability to resist a particular antifungal to which it

was previously susceptible (Cowen *et al.*, 2015). In general, antifungal resistance is acquired as a result of changes that modify the interaction between the drug and target (Fisher *et al.*, 2022). Some examples of this include: modifying the binding site, overexpressing the drug target, altering the permeability of the fungal cell membrane, or increased drug export by means of efflux pumps (Bhattacharya *et al.*, 2020). The mode of resistance depends on the mechanism of action the antifungal drug compounds themselves exert.

Azoles such as fluconazole are commonly employed in the treatment of candidiasis and thus *Candida* species have developed a multitude of resistance strategies which can be observed in drug-resistant clinical isolates (Sanguinetti *et al.*, 2005). One of the main strategies includes over expressing membrane transporter proteins in a process known as drug efflux (Bhattacharya *et al.*, 2020). The increased expression of Adenosine Triphosphate (ATP)-binding cassette transporters (ABC-T) and major facilitator transporters (MFS-T) have been implicated in azole resistance (Sanguinetti *et al.*, 2005). The overexpression of genes encoding for these proteins has been observed in azole resistant *Candida* clinical isolates (Bhattacharya *et al.*, 2020; Calabrese *et al.*, 2000; White *et al.*, 2002). Additionally, studies show that azole resistant strains of *C. albicans* can become susceptible to azole derivatives when certain genes that encode for transporter proteins are disrupted (Tsao *et al.*, 2009). These trends have also been exhibited in other species such as *C. glabrata* (Sanguinetti *et al.*, 2005; Culakova *et al.*, 2015), *C. neoformans* (Lamping *et al.*, 2010) and notably in *C. auris* (Bhattacharya *et al.*, 2019; Rybak *et al.*, 2020). Additionally, azole drug resistance is also seen to be mediated by alterations to the ergosterol biosynthesis pathways. Azoles target multiple steps within the ergosterol pathway, thus fungal cells combat this by overexpressing some genes that encode for affected vital biosynthetic enzymes (Arthington-Skaggs *et al.*, 1999; Sanglard *et al.*, 2003; Xu *et al.*, 2007).

Work has also been done to characterise the mechanisms employed by fungi to combat polyene and echinocandins, however they are not as well characterised as azole resistance. Resistance against polyenes, like amphotericin B, has also been linked to changes within the ergosterol pathway in *C. albicans* and *C. neoformans* (Sionov *et al.*, 2012; Vandeputte *et al.*, 2012).

A variety of other resistance strategies are employed by fungi including the induction of chromosomal abnormalities, implementation of cellular stress enzymes, and importantly biofilms (Cowen *et al.*, 2015). Understanding the resistance strategies that fungi employ allows

for the development of drugs with new targets in mind. These strategies developed due to the repeated use of the limited antifungal drugs available, thus emphasising the need for new antifungal treatments. Understanding the mechanisms also opens the door for therapies that supress fungal resistance and allow for standard therapies to work efficiently.

1.4 Biofilms

Biofilms are described as structured communities of microorganisms that have the ability to stick not only to surfaces, but also to each other (Ramage *et al.*, 2012). A defining feature of biofilms is their ability to produce and enclose themselves within a protective extracellular matrix (ECM) composed of extracellular polymetric substances (EPS) (López *et al.*, 2010). This complex matrix allows for several advantages for the microbial community, the main one being protection from the environment and physical and chemical stressors (Ramage *et al.*, 2012). A variety of clinically important fungi have demonstrated the ability to form biofilms including species within the *Candida* and *Cryptococcus* genera (Kernien *et al.*, 2018). Biofilms are also known to play a key role in fungal resistance which complicates therapeutic strategies (Chandra and Mukherjee, 2015). Thus, there has been growing interest in the role that fungal biofilms play in fungal infections.

1.4.1 Biofilm Formation

To understand the clinical role that biofilms play in resistance and infection it is important to understand how and why they form. Biofilm formation consists of a few main phases, namely: adherence to a surface, proliferation to form a fungal community, the production of the ECM, and dissemination (Cavalheiro and Teixeira, 2018). The formation begins with the adhesion of planktonic or free-floating microorganisms to a surface (Ramage *et al.*, 2012). The surface may be biotic such as mucosal surfaces or abiotic such as medical devices (Harriott *et al.*, 2010; Kojic and Darouiche, 2004). Following adhesion, the yeast cells will usually form a distinct colony (Cavalheiro and Teixeira, 2018). During this phase, the colonies will start organising themselves and will begin producing and secreting EPS (Cavalheiro and Teixeira, 2018). The EPS include molecules such as proteins, lipids, carbohydrates and DNA (Baillie and Douglas, 2000; Martins *et al.*, 2010). Studies have also shown the ECM to contain materials found in the surrounding environment with one study demonstrating that a significant portion of the

proteins found in the ECM were obtained from the host (Nett *et al.*, 2015; Kernien *et al.*, 2018). These components account for the distinctive three-dimensional structure of the biofilm (Cavalheiro and Teixeira, 2018). During the maturation phase a distinctive, thick layer of EPS is formed (Cavalheiro and Teixeira, 2018). The composition and structure of the mature ECM differs between the different fungal genera and species. Lastly, the mature biofilm releases budding daughter cells which become detached and migrate to form more biofilm (Chandra and Mukherjee, 2015). This allows for the colonisation of the biofilm over a surface but can also allow for the propagation of the biofilm in new areas, such as different areas of the body during infection that were not previously colonised (Cavalheiro and Teixeira, 2018; Chandra and Mukherjee, 2015).

1.4.2 Clinical Relevance of Biofilms

Fungal infections are well known to mainly affect those who are immunocompromised (Schwartz *et al.*, 2019). This makes health-care settings a prime location for the spread of these infections where patients are likely to already be in a vulnerable state. Studies show that biofilm formation is a major cause of persistent fungal infections and plays a critical role in invasive fungi/candidiasis (Andes *et al.*, 2012; Ala-Houhala and Anttila, 2020) and estimates attribute more than 500 000 deaths to biofilm-associated infections per year (Müller *et al.*, 2011). Thus, biofilms pose a huge threat to human health.

Biofilms can progress during the course of infection to affect many different parts of the body, but they are most often seen to adhere to mucosal tissues (Ganguly and Mitchell, 2011). They are often found to colonise the oral cavity along with the gastrointestinal and genitourinary tracts (Ganguly and Mitchell, 2011). Infections may initially arise as a result of an altered or dysregulated microbiome whereby pathogens which were usually controlled begin to flourish. This can happen to people on certain medications and is especially common in those treated with antibiotics (Krčméry *et al.*, 1999). However, they may also be introduced to these pathogens due to breaks in sterility and improper hygiene. Biofilms also have a propensity to colonise abiotic surfaces. This includes medical equipment and devices routinely used in hospital settings (Harriott *et al.*, 2010). This is of major importance for indwelling medical devices such as various catheter types, vascular access devices, prosthetics and other synthetic implants (Donlan, 2001; Kojic and Darouiche, 2004). In most cases once the biofilm has begun development, it becomes immensely more difficult to treat and eradicate, with removal of the

medical device often being the only option (Pfaller and Diekema, 2007; Andes *et al.*, 2012). The formation of biofilms on these devices is highly correlated with dissemination of the pathogen leading to bloodstream infections with high associated mortality rates (Donlan, 2001; Tumbarello *et al.*, 2007). Estimates show almost 80% of patients with invasive candidiasis have had indwelling medical devices, thus a high correlation exists between biofilm formation and increased infection severity (Andes *et al.*, 2012). As such it is important to understand what makes these biofilms so robust and difficult to treat to overcome these issues.

1.4.3 Factors Contributing to Biofilm Resistance

Biofilms pose such a challenge as they demonstrate increased antimicrobial tolerance and are able to evade host immune responses (Roilides *et al.*, 2015). Studies show that biofilms exhibit much higher rates of resistance when compared to their planktonic counterparts (Sherry *et al.*, 2017; Hawser and Douglas, 1995). This robustness of biofilms is due in large part to their structural complexity and the formation of the ECM (Roilides *et al.*, 2015). Biofilms also undergo many of the same changes that would be employed in their planktonic state, however the effect of these resistance strategies is amplified due to the increased cell density of the biofilm structure (Ramage *et al.*, 2012; Perumal *et al.*, 2007). Some of these resistance strategies include: altering drug-target affinity, overexpression of membrane transport proteins for drug efflux, increased stress responses, and inducing fungal cell wall changes to reduce absorption of drugs (Íñigo and Del Pozo, 2018). The ECM also offers a new strategy by acting as a physical barrier between antifungal drugs and the fungal cells (Cavalheiro and Teixeira, 2018). Some studies show that EPS components such as β-glucan are able to bind antifungals such as amphotericin B in the ECM, thus reducing their bioavailability and efficacy (Nett *et al.*, 2007). Another unique trait exhibited by biofilms is the production of persister cells. These are dormant and non-dividing cells which are highly tolerant to antimicrobial drugs (LaFleur *et al.*, 2006). Antimicrobial drugs can bind to the specific targets, however they are not able to inhibit the function of those cells as a result of their dormancy (LaFleur *et al.*, 2006).

In addition to implementing resistance strategies, biofilms are also able to effectively evade host immune responses. For various *Candida* species, neutrophils are usually the immune responders that are employed to gain control of the infection (Kolaczkowska and Kubes, 2013). They utilise a wide arsenal of responses including the induction of phagocytosis, production of

reactive oxygen species (ROS) and inducing neutrophil extracellular trap (NET) release (Kolaczkowska and Kubes, 2013). While the degree of immune evasion varies between species, it is seen that neutrophils fail to release NETs when in the presence of *Candida* biofilms (Johnson *et al.*, 2016). This is believed to be as a result of the concealment of cell surface components, such as mannan and β-glucan, by the ECM which 'hides' the biofilm from immune recognition thus preventing the release of NETs (Johnson *et al.*, 2016; Mitchell *et al.*, 2015). *Candida* biofilms are also capable of resisting attacks from monocytes and macrophages (Alonso *et al.*, 2017). They employ a variety of inhibitory strategies including downregulating cytokines which aid in phagocyte activation (Kernien *et al.*, 2018) and impairing the migration capacity of macrophages (Alonso *et al.*, 2017).

Many studies focus on *Candida* based biofilms due to their frequency in clinical settings however some work has also been done to investigate the host evading properties of *Cryptococcus* biofilms too. Biofilm producing species of *C. neoformans* produce a protective capsule that is comprised of various polysaccharides such as mannoprotein, galactoxylomannan and glucuronoxylomannan (GXM) (Martinez and Casadevall, 2015). These components are shed into the ECM during biofilm formation and allow for surface and cell adhesion (Martinez and Casadevall, 2015). In particular, GXM seems to play a key role in evading host reactions by inhibiting several key immune responses such as neutrophil function, phagocytosis, NET production and defensins (Martinez and Casadevall, 2015; Vecchiarelli, 2000).

1.4.4 Biofilm Treatments

Limited treatments are available to combat or disrupt biofilm formation once a mature biofilm has formed. Thus, prevention of biofilm formation is preferred by implementing adequate therapeutic measures early in the course of infection. However, factors such as poor screening and misdiagnoses can complicate these matters.

In terms of biofilms on indwelling devices, treatment opportunities are almost non-existent with the only course of action being removal of the affected device (Donlan, 2001). To combat this, there have been strides to develop the surface coatings and structures of medical devices to inhibit biofilm formation (Donlan, 2001).

As discussed, biofilms generally exhibit increased tolerance or resistance to antifungal treatments when compared to their planktonic counterparts. Thus much higher concentrations of the antifungal agents are required to display effective antibiofilm activity (Ramage *et al.*, 2001). However, treatment at these concentrations can be considered toxic and unsafe as is the case for polyenes such as amphotericin B (Ramage *et al.*, 2002; Pierce *et al.*, 2013). Liposomal formulations of amphotericin are more tolerated however they are not as widely available and can come with increased costs (Kuhn *et al.*, 2002). Additionally, biofilms are seen to show high levels of intrinsic resistance against some antifungals such as azoles (Pierce *et al.*, 2013). Only echinocandins have been seen to display antibiofilm activity at accepted therapeutic conditions against *Candida* biofilms (Uppuluri *et al.*, 2011; Tits *et al*., 2020).

There has also been some research into combination therapies with various antifungals and even with antibiotics to combat biofilms, however these are still in the early stages before they can be implemented in clinical settings (Del Pozo *et al.*, 2011; Iñigo *et al.*, 2012). And despite the high association of biofilms with severity of disease much less research is done to establish how developing treatments would fare against biofilms as opposed to planktonic cells. The failure of antifungal drugs and the immune response due to the increased resistance of biofilms makes them incredibly difficult to treat. With the increasing number of immunocompromised individuals who are required to be in a hospital setting or those who will be requiring the aid of medical devices, it is important to reduce the risk of adverse outcomes by having effective therapeutic treatments readily available to treat biofilms. With the current opportunities to treat biofilms severely lacking, it is imperative to search for novel treatments that can either disrupt biofilm formation or prevent it altogether.

1.5 Difficulties and Current Treatment of Systemic Fungal Infections

These life-threatening fungal pathogens have demonstrated increasing prevalence and pathogenicity worldwide (Bongomin *et al.*, 2017). This is largely in part due to mechanisms of resistance developed by the microbes against the antifungal treatments that are currently utilised (Clark and Drummond, 2019; Fisher *et al.*, 2018). When paired with the fact that limited treatments exist for many fungal infections this complicates implementing adequate

therapeutic approaches. Additionally, the widespread use of antifungals in the agricultural and industrial sectors has also contributed to the rise of drug-resistant and novel fungal pathogens (Fisher *et al.*, 2018; Pfaller and Diekema, 2004).

The scope of antifungal treatments is quite limited with only a few classes of molecules being utilised in clinical settings (Roemer and Krysan, 2014). Aside from inaccurate notions that fungi are a lesser burden on society and thus require fewer research inputs into drug development, they also come with intrinsic challenges in that they are eukaryotic and thus more closely related to their human hosts than other microbial pathogens (Kathiravan *et al.*, 2012). This greatly narrows the possible range of drug targets to those that are distinctly fungal as not to interrupt any biochemical processes that are shared amongst host and pathogen. These main drug targets include those that account for the interruption of the synthesis of the cell wall, sterols, nucleic acids and proteins (Roemer and Krysan, 2014; Scorzoni *et al.*, 2017).

Drugs that fall in the categories of azoles, echinocandins and polyenes are commonly used to treat systemic fungal infections (Kischkel *et al.*, 2020). The type of drug administered depends on the causative fungal pathogen, the severity of infection as well as the clinical condition of the affected patient. Polyenes, such as amphotericin B, works form complexes with ergosterol that disrupts the membrane and causes increased membrane permeability and cytoplasmic leakage (Kathiravan *et al.*, 2012). Azoles are a group of drugs that prevent the formation of ergosterol at various points in the biosynthetic pathway and include drugs such as fluconazole and itraconazole (Kathiravan *et al.*, 2012; Kischkel *et al.*, 2020). Glucan synthase inhibitors, such as the echinocandins micafungin and caspofungin, result in lysis of the cell by hindering the addition of glucose to the glucan polymer that makes up a component of the cell wall (Kischkel *et al.*, 2020). Of these limited treatment options many of the available antifungals are expensive and may not be available in certain areas (Kathiravan *et al.*, 2012). This results in the use of antifungal drugs that are not effective for the fungal disease they are meant to treat; further contributing to the development of resistance strategies.

Furthermore, some antifungal drugs have also been shown to have adverse side effects against the patient. For example amphotericin B has been seen to cause cellular toxicity in the host (as it interacts with mammalian sterols) as opposed to that of the pathogen (Kischkel *et al.*, 2020). Other drugs such as fluconazole and itraconazole are generally seen to be more tolerated in

patients but come with the caveat that they are generally not as effective in the treatment of systemic fungal infections (Yonga, 1995).

It is clear that there are many challenges in the way of effectively treating systemic fungal infections. From the limited range of drugs to the serious side effects that have stunted drug selection. With the mounting threat of antifungal resistance, it is imperative to find drug candidates that can combat these problems.

1.6 Plant Extracts in Drug Discovery

For centuries plant material has acted as the primary source of medicine (Salim *et al.*, 2008; Dekebo, 2019). In modern times, traditional plant-based medicines still prove to be an important resource in developing and rural areas (Salim *et al.*, 2008). While traditional medicine practices are not widely utilised anymore, plants have continued to provide society with a variety of modern medicines as their use shifted from herbal remedies to the isolation of specific active compounds (Salim *et al.*, 2008; Balunas and Kinghorn, 2005). In the early 19th century, the first semi-synthetic pure drug was introduced to the market (Diasd *et al.*, 2012). This drug was known as aspirin which was based on a natural compound known as salicin derived from the *Salix alba* plant (Dias *et al.*, 2012). This led to a rapid surge to identify bioactive plant compounds for pharmaceutical use which resulted in the development of drugs such as codeine and quinine which are still used today (Veeresham, 2012). It is estimated that around 40-50% of all drugs currently in circulation have been derived from plants (Veeresham, 2012). Not only can their natural bioactive products be used directly for medicinal applications, but they also play a role in the production of synthetic molecules as many plant-based bioactive compounds serve as the template for the chemical models and design of synthetic drugs (Veeresham, 2012; Katiyar *et al.*, 2012). This has led to the development of drugs that are used to treat a variety of diseases from cancers to neurological disorders and microbial infections (Heinrich and Teoh, 2004). Plants therefore play an indispensable role in the drug discovery process.

1.6.1 Plants as Antimicrobial Agents

Despite the array of antimicrobial treatments developed over the past few decades the incidence of antimicrobial resistance is still on the rise (Brusselaers *et al.*, 2011; Lipsitch, 2001). Thus, the search for new compounds that possess antimicrobial activity is more important than ever to combat this alarming trend. To aid in this endeavour, plant products offer an interesting solution. Plants serve as the host to a plethora of natural products and metabolites that may be utilised in the drug discovery process (Ginovyan *et al.*, 2017). These plant-derived compounds may target different sites compared to traditional antimicrobials and consequently may possess unique mechanisms of action against the microorganisms (Ahmad and Beg, 2001; Petrosyan *et al.*, 2015). Natural products are m It is estimated that less than 1% of the world's plant species have been screened for their phytochemical properties, thus there is potential for the discovery of novel antimicrobial compounds (Dias *et al.*, 2012; Cragg and Newman, 2005).

Plants contain an abundance of secondary metabolites and other phytochemicals (Hussein and El-Anssary, 2019). Secondary metabolites are termed as compounds which are not directly responsible for the normal development and reproduction of the plant, but rather they give the plant a competitive advantage in the environment to improve survival (Teoh, 2016). They largely play a role in plant defence by offering protection form herbivory, other plants, and pathogens (Teoh, 2016). These secondary metabolites are often specific to species and have been used by humans as a source of medicines and recreational compounds (Hussein and El-Anssary, 2019; Teoh, 2016). These metabolites are grouped according to their chemical structures, the main classes being alkaloids, phenolics and terpenes (Hussein and El-Anssary, 2019). Plants are also subject to fungal pathogens and infection and as such need adequate defence mechanisms to ensure their survival (Pusztahelyi *et al.*, 2015). The secondary metabolites that usually prevent fungal infection are found within the following classes: non-ribosomal peptides, polyketides, and terpenes (Pusztahelyi *et al.*, 2015). Thus, it is likely that these compounds will also be effective against some human pathogens.

1.6.2 Preparation of Plant Extracts

Plant extract refers to the compounds, metabolites or other substances that are removed from a plant by physical or chemical methods for further use (Abubakar and Haque, 2020). For

antifungal drug discovery, the extraction processes aim to isolate the abundance of secondary metabolites and phytochemicals to further assess their properties (Dekebo, 2019). A wide variety of methods exist to aid in this process including infusion, maceration, microwave and ultrasound assisted extraction, and solvent based extraction (Abubakar and Haque, 2020; Dekebo, 2019). The method utilised depends on several factors including the types of metabolites that need to be extracted, the intended use of the extracts, as well as equipment and cost restrictions (Abubakar and Haque, 2020). With these considerations in mind, a solvent-based extraction method is commonly utilised for antimicrobial testing (Sultana *et al.*, 2009; Alara *et al.*, 2020). There are a variety of solvents to choose from depending on the properties of the metabolites that one wishes to extract. Solvents such as water and alcohols are polar and are able to extract substances that are polar, while chloroform and ethers are useful for the extraction of non-polar substances (Abubakar and Haque, 2020). For this reason, many studies use aqueous and ethanolic solvents as they also come with the benefit of being non-toxic, widely available, and cost-effective (Sultana *et al.*, 2009).

1.6.3 Plant Extracts as Antifungals

With the huge potential plant extracts pose for the discovery of novel antifungal drugs there has been significant interest in studying their antimicrobial properties worldwide (Andriani *et al.*, 2021; Silva *et al.*, 2020). These studies focus their efforts on evaluating native plant extracts against a few clinically important fungal pathogens. To name a few, plant extracts with potent fungistatic and fungicidal properties have been established worldwide with native plants from Brazil (Oliveira *et al.*, 2014; Bonifacio *et al.*, 2019; Pedroso *et al.*, 2019), Ghana (Suurbaar *et al.*, 2017) and SA (Mahlo *et al.*, 2016; Shai *et al.*, 2008). It should be noted that these studies mainly evaluate the efficacy of the plant extracts against *C. albicans* and *C. neoformans*, with very few studying other species within the *Candida* and *Cryptococcus* genera. Thus, there is a need to not only investigate the antifungal properties of a variety of plants, but also test them against a wider variety of fungal species.

Using plant extracts as potential antifungal treatments also comes with the advantages of being cheaper to purchase and manufacture than traditional synthetic drugs (Vaou *et al.*, 2021). Additionally, depending on the preparation and application, the use of native plants can be less toxic and more readily available to communities (Vaou *et al.*, 2021) Considering the

widespread biodiversity of SA, containing over 24 000 plant species with more than 50% being endemic, there is huge potential for the discovery of novel antifungals (Mamathaba *et al.*, 2022).

1.7 Silver as an Antifungal

Before the introduction of antibiotics, silver and silver based molecules were widely used as antimicrobial and antiseptic agents for the treatment of topical infections, burns and other wounds (Wlodkowski and Rosenkranz, 1973; Clement and Jarrett, 1994). Today silver based antimicrobial treatments including ointments and colloidal solutions are confined mainly to those that address burns using silver sulfadiazine (Wlodkowski and Rosenkranz, 1973). Silver has also been utilised in water sterilisation processes and shows promise for therapeutic applications including anti-cancer and anti-inflammatory therapies (Kyros *et al.*, 2010; Engelbrecht *et al.*, 2018). There is also much research supporting silver nanoparticles as viable therapeutics (Panáček *et al.*, 2009; Kim *et al.*, 2008). It has also been included in coatings used for medical equipment and devices such as catheters to reduce microbial growth and their associated health effects (Rupp *et al.*, 2004). Importantly, silver is not known to interfere with mammalian biological processes and has demonstrated low toxicity in mammalian cells which makes it a good treatment option (Lansdown, 2006). Much of the silver compounds are excreted or accumulate at low levels in the body with no adverse effects. Topical treatments such as silver sulfadiazine have been shown to be remarkably safe with rare occurrences of serious side effects since being utilised from the 1960s (Fuller, 2009).

It has also been proven that silver itself exerts antimicrobial effects (Berger *et al.*, 1976). While the mechanisms surrounding silver's antimicrobial properties have not been fully elucidated, several theories exist to explain its mode of action (Berger *et al.*, 1976). Silver is known to bind to many cellular components including lipids, nucleic acids and other proteins, but it is generally seen to affect the cell wall and cell membranes of microbes (Clement and Jarrett, 1994; Coward *et al.*, 1973). Silver is thought to disturb the integrity of the cell wall by interacting with electrons and thus affecting permeability as well as impairing the function of critical surface enzymes. It is also thought to intracellularly affect cell metabolism, play a role in nucleic acid damage by the formation of ROS which leads to mitochondrial dysfunction (Thurman *et al.*, 1989; Berners-Price *et al.*, 1994). Much of our limited understanding of

silver's mechanism of action as an antimicrobial stems from experiments performed on bacteria and far less is known about how it acts against fungal species. Nevertheless, silver-based molecules have been shown to possess promising antifungal activity in several studies (Gandra *et al.*, 2017; Panáček *et al.*, 2009; Berners-Price *et al.*, 1988).

Despite evidence showing the antifungal activity of silver-based molecules there are still no treatments available with these compounds to treat systemic fungal infections. A common problem with many silver based drugs is that in physiological conditions silver ions bind to and precipitate with free chlorine atoms or to bind cellular enzymes, thus reducing their bioavailability (Berners-Price *et al.*, 1988). Since the activity of these complexes is directly associated with their stability, solubility and lipophilicity it becomes beneficial to enlist the help of suitable ligands that can help achieve and overcome these issues while also increasing target selectivity. One such category of ligands of interest are the phosphine ligands.

These phosphine ligands are lipophilic and are seen to be soluble in organic solvents (Katti, 1996; Kim *et al.*, 2005). A variety of phosphine ligands have been used to increase the solubility of a range of molecules including proteins, metals and some nanoparticles (Bergin *et al.*, 2016; Herbert and Molloy, 1998). Previous studies have shown metal phosphine complexes, such as gold (I) complexes, to have improved solubility and distribution when bound to various phosphine ligands for the treatment of diseases such as arthritis and cancer (Berners-Price and Sadler, 1988). When bound to silver (I) with specific configurations the resulting silver phosphine molecules have been found to possess promising antimycotic properties (Gandra *et al.*, 2017). Various silver phosphine complexes have been shown to reduce or even inhibit cellular respiration, ergosterol levels, cytochrome biosynthesis and mitochondrial function in certain *Candida* species (Berners-Price *et al.*, 1994; Berners-Price *et al.*, 1988; Coyle *et al.*, 2003). Thus, silver phosphine complexes appear to be a promising antifungal treatment that should be studied further to elucidate their antifungal properties in a wider variety of fungal species.

1.8 *Caenorhabditis* elegans

Caenorhabditis elegans is a small invertebrate nematode which is gaining popularity as a means to study the pathogenesis of microbes as well as its ability to act as a model system for drug discovery (Desalermos *et al.*, 2011; Madende *et al.*, 2020). These worms are free-living and are found naturally in humid, temperate areas prominently isolated where there is an abundance of microbes such as in soil and decaying vegetation (Schulenburg and Félix, 2017). In their natural environment, *C. elegans* has been found to eat bacteria from a variety of genera including *Acetobacteriaceae, Bacillus, Enterobacteriaceae, Comomonas* and *Pseudomonas* (Zečić *et al.*, 2019). Although it is not their preferred food source, studies studying the gut of *C. elegans* have found that they consume yeast cells and degraded plant tissue as a way to meet their nutritional needs (Zečić *et al.*, 2019; Félix and Duveau, 2012).

The *C. elegans* nematode was first isolated from soil in the early 1900's with early work focusing on establishing a uniform laboratory strain (Nigon and Dougherty, 1950). Nematodes within the *Caenorhabditis* genera such as *C. briggsae* and *C. elegans* gained popularity in the 1960s after Sydney Brenner began studying their ability to function as a simple, eukaryotic genetic model to elucidate the genes and pathways involved in processes within the field of development and neurobiology (Crick *et al.*, 1961). Since then numerous studies have been performed using *C. elegans* and its related mutants to research topics including cell lineage, programmed cell death, ageing, neural networks, behaviour, evolutionary biology and pathogenesis to name a few (Meneely *et al.*, 2019). Thus *C. elegans* has laid the foundation for a wide array of notable discoveries and its importance as a model organism has been cemented.

The anatomy of *C. elegans* presents with a cylindrical body shape that is tapered at both ends and is unsegmented (Altun *et al.*,2006). They consist of an outer tube which consists of the cuticle, epithelial, nervous, muscular and excretory systems and an inner tube consisting of the reproductive and alimentary systems (Altun *et al.*, 2006). These tubes are separated by a pseudocoelomic cavity. Naturally, *C. elegans* can be found as hermaphrodites (XX) or males (XO). Males are found in populations at a much lower frequency (1 in 500 worms in a population) whereas hermaphroditic worms are found in abundance (Riddle *et al.*, 1997). Hermaphroditic worms possess the ability to produce a limited number of sperm which allows them to self-fertilise in the absence of a male nematode (Riddle *et al.*, 1997).

The life cycle of *C. elegans* consists of a few distinct stages including the embryonic, larval and adulthood phase (Altun *et al.*, 2006). The embryonic stage further consists of two main steps: proliferation and organogenesis (Altun *et al.*, 2006; Sulston *et al.*, 1983). The proliferation process includes the division of a single cell into multiple undifferentiated cells. The initial stages of proliferation takes place within the uterus of the nematode with the embryo being laid within an egg when it reaches around 30 cells (Sulston *et al.*, 1983). The egg is composed of layers of vitelline, chitin and proteoglycans which act as a protective shield for the embryo against environmental stresses whilst also being selectively permeable (Mkandawire *et al.*, 2021; Stein and Golden, 2018). This allows for the egg to assess the external conditions and remain in the environment for extended periods of time, allowing for hatching when conditions are favourable (Mkandawire *et al.*, 2021). Following the egg being laid, the cells continue to proliferate until there are 550 undifferentiated cells. Following this, the process of organogenesis is initiated. Organogenesis includes the process by which the undifferentiated cells undergo terminal differentiation. This process results in fully differentiated organs and tissues that make up the main body of the nematode (Sulston *et al.*, 1983). During this stage the nematode also undergoes elongation to transform the body into its distinct longer and slimmer profile (Hall *et al.*, 2017). It is at this point that the nematode gains the ability to move within the egg which signals that the hatching stage is near and thus the end of the embryonic stage (Hall *et al.*, 2017).

During hatching, the eggshell is weakened near the head of the nematode which allows it to break through the egg and into the environment. This signals the onset of post embryonic development which occurs after hatching once the larvae has begun feeding (Altun *et al.*, 2006). The larvae pass through four distinct stages (L1 - L4) before reaching adulthood (Figure 1.1). The end of each of these stages is marked by the moulting of the existing cuticle which is replaced by a newly synthesised, stage specific cuticle (Altun *et al.*, 2006).

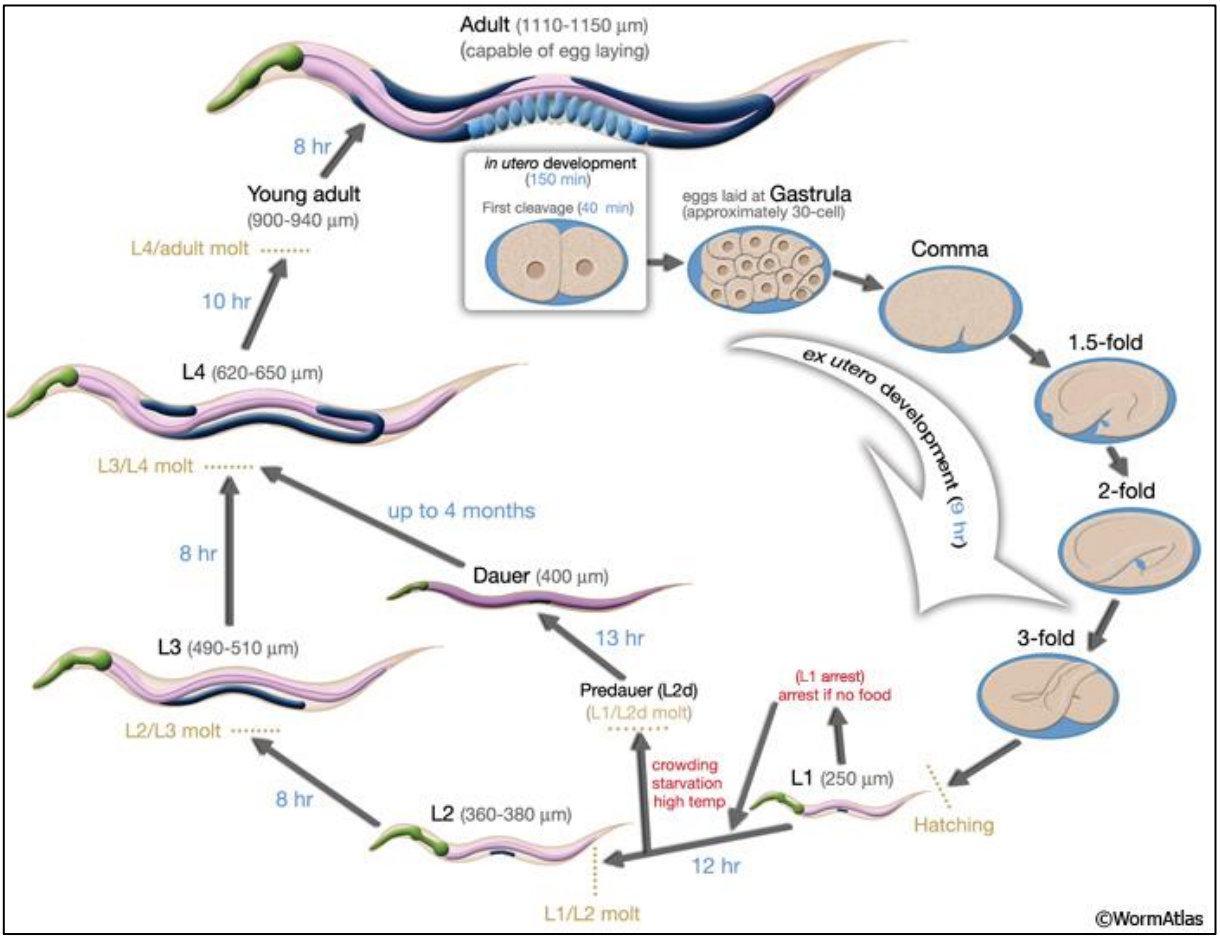

Figure 1.1: The typical life cycle of *Caenorhabditis elegans* at 22°C (Altun and Hall, 2009).

The time it takes for a fertilised egg to develop into an egg laying adult varies due to a variety of factors including the availability of a suitable food source and, importantly, the temperature (Altun *et al.*, 2006; Stiernagle, 1999). Most species of *C. elegans* typically grow at temperatures between 15 to 25°C and once developed they can live for around two to three weeks under ideal conditions. Nematodes tend to grow at a quicker rate at the upper limit of their temperature requirements. As a result they can grow up to two times quicker at 25°C than at 16°C (Stiernagle, 1999). Under ideal conditions it can take *C. elegans* around 39 hours to reach adulthood from fertilisation at 25°C and around 75 hours at 16°C. Their quick generation time and life cycle along with a myriad of other factors enable *C. elegans* to be used as a suitable organism for molecular studies.

1.8.1 The role of *C. elegans* as a Model Organism

There are a host of advantages that make *C. elegans* an ideal candidate for research as a model organism. These organisms are favourable as a result of their small size with adult worms typically growing to around 1 mm in length (Stiernagle, 1999). This makes it possible to keep a large number of worms within minimal space constraints and minimal resources. Additionally, *C. elegans* has simple growth requirements and low maintenance compared to more advanced mammalian model organisms. These nematodes grow at temperatures between 15 - 25°C and can be cultured on Petri dishes that are seeded with a strain of *Escherichia coli* (OP50) (Stiernagle, 1999). Due to their hermaphroditic nature, *C. elegans* are able to self-fertilise (Altun *et al.*, 2006). These nematodes can produce around 300 - 350 offspring through self-fertilisation, and up to 1500 if they mate with males, which allows for the easy cultivation and maintenance of nematode collections in a laboratory setting (Altun *et al.*, 2006). This is further aided by their quick generation time whereby it can take only three to six days for an egg to grow into a mature adult which has a life span of two to three weeks (Altun *et al.*, 2006). Despite the array of advantages presented for *C. elegans* as a practical model organism it seems far-fetched to imagine their usefulness for studying human diseases. This begs the question of how these non-mammalian worms are able to give insight to human diseases.

Although small, these worms consist of several organ systems that can be found in more complex organisms including digestive, nervous and reproductive systems (Apfeld and Alper, 2018). Various studies have found that *C. elegans* has approximately 30 - 60% of genes with orthologs and homologs in mammals (Lai *et al.*, 2000). Using comparative genomics, one study found at least 83% of the nematode proteome to have homologous human genes with a large number of the nematode proteins matching gene transcripts that have been identified in humans (Lai *et al.*, 2000). Additionally these researchers found only around 11% of the proteome to be specific to nematodes, indicating high homology between *C. elegans* and humans (Lai *et al.*, 2000). Thus, these nematodes are suitable candidates to study a variety of human diseases. The mechanisms and biochemical pathways behind several human diseases have already been studied utilising *C. elegans* as a model organism. This includes neurodegenerative diseases like Parkinson's and Alzheimer's (Markaki and Tavernarakis, 2020). Additionally, *C. elegans* has played an important role in understanding microbial pathogenesis and the discovery of novel candidate drugs (Ahamefule *et al.*, 2021).

As discussed earlier, the search to find suitable candidate drugs to treat the rise of resistant fungal pathogens is thwarted by hurdles including issues of drug solubility and notably, drug toxicity. While *in vitro* studies can elucidate a variety of properties about potential drug candidates these methods cannot infer the effects the drug will have when introduced to a complex living organism. Thus, it is imperative to have a means by which to test the effects a candidate drug molecule may have on an organism *in vivo* to assess its suitability as a potential treatment. To aid in this, *C. elegans* models have been used to assess the cytotoxicity of several bioactive compounds (Ahamefule *et al.*, 2021). Compounds sourced from the ocean, plants as well as some other repurposed compounds have been assessed for their toxicity (Ahamefule *et al.*, 2021; Shu *et al.*, 2016; Pedroso *et al.*, 2019) using *C. elegans*. Remarkably, studies have found there is a good correlation between the relative toxicity of compounds observed in nematode models to that of rodent models (Williams and Dusenbery, 1988). Compounds such as nano silver (Hunt, 2017), metal salts (Hunt, 2017), and organophosphates (Cole *et al.*, 2004) have demonstrated a correlation between the lethal concentrations that kill 50% of the population (LC50) of nematodes to that of the LC50 in rodent models (Hunt, 2017). Additionally, *C. elegans* toxicity models have also been shown to be powerful predictors of compounds that are not only lethal, but those that can also cause reproductive and developmental anomalies in mammals. One study showed that within a cohort of test compounds known to cause adverse developmental effects in mammals, *C. elegans* models also showed compromised egg viability for around 89% of those compounds (Harlow *et al.*, 2016). Although mammalian models remain the golden standard for toxicity testing, *C. elegans* serves as a suitable intermediary between *in vivo* and mammalian models to screen potential compounds.

The next hurdle in the drug discovery process is ensuring the compounds are effective *in vivo* with all the difficulties presented within a complex living organism. This includes issues of compound solubility, absorption and pathogen-host interactions including immunity to name a few (O'Reilly *et al.*, 2014). It has been found that *C. elegans* are highly susceptible to a variety of human pathogens across a range of genera including *Candida* and *C*ryptococcus (Tan *et al.*, 1999). A variety of similar innate immune system signal cascades are found to be present between *C. elegans* and humans (Kim *et al.*, 2002). Thus, the nematode is an ideal candidate to not only study the pathogenesis of microbes but can also act as an initial screening tool to assess the efficacy of novel bioactive compounds.

Mutant *C. elegans* strains are typically used in these studies to help overcome some of the challenges associated with this infection model type. Examples of these mutations include the *glp-4* and *sek-1* mutations (Rastogi *et al.*, 2015; Hoyt *et al.*, 2017). The *glp-4(bn2)* mutation includes a germ-line proliferation defect that occurs during larval development (Rastogi *et al.*, 2015). This makes the nematodes temperature sensitive and as such they lack the ability to produce gonads and progeny at temperatures higher than 25°C (Miyata *et al.*, 2008). This becomes useful for tracking population numbers over an extended period of time. Additionally, the *sek-1(km4)* mutation inhibits the correct functioning of protein kinase activity which are vital for regulating the signal transduction pathways that govern the innate immune system and the expression of immunity genes (Hoyt *et al.*, 2017). This mutation thus allows the nematodes to be immunocompromised such that they can be more easily infected with the pathogenic fungi (Kim *et al.*, 2002). Following successful establishment of infection in the organism the efficacy of the compounds can be tested. This is evaluated by testing if the compounds can prevent, rescue or improve the life span of the infected *C. elegans* worms (Eng and Nathan, 2015). Several studies have shown that infected nematodes show improved life expectancy when treated with the various compounds compared to the untreated controls (Sifri *et al.*, 2003; Durai and Balamurugan, 2012; Eng and Nathan, 2015). Thus, *C. elegans* serve as useful model organisms to study the pathogenicity of microbes as well as the efficacy of compounds *in vivo*.

1.9 Importance of Research

With the threat of emerging microbial resistance, it is imperative that substantial efforts are made to address this issue, especially in the case of antifungal treatments. The misadministration of the limited arsenal of the available antifungal drugs has led to the emergence of novel multidrug resistant pathogens such as *C. auris*. Furthermore, notions that fungi are a lesser burden on society than other microbes has led to fewer research inputs in the search for novel treatments. Clinically important pathogens within the *Candida* and *Cryptococcus* genera place a huge burden on the healthcare system, especially in developing countries where there are huge populations of immunocompromised people living with HIV/AIDS and TB. The current treatment options are often costly, can come with significant toxic side effects, and are often not available in certain areas. Thus, it is imperative that efforts are made to aid in this endeavour to provide alternative treatment opportunities. Promising

treatment opportunities lie in that of silver-based compounds as well as native plant extracts. Both silver and plant extracts have gained renewed interest as potential antimicrobial treatments, however the research is often focussed on bacterial strains and only a few fungal strains such as *C. albicans* and *C. neoformans*. As such it is important to test not only a variety of potential compounds for their efficacy but also a wider range of pathogens within the *Candida* and *Cryptococcus* genera. In addition, despite the crucial role biofilms play in establishing persistent and severe infections, much research is only directed towards establishing the efficacy of new compounds towards planktonic cells and does not further consider their effect on biofilms. Identifying compounds that have antibiofilm properties could play a role in limiting the severity of disease in patients. Lastly, a common limitation of the available antifungal drugs is that they possess a degree of toxicity towards the host. It is therefore important to not only find compounds that are effective antifungals, but also possess low host toxicity. The model organism *C. elegans* can assist in this regard to act as an initial screening tool to assess the toxicity of the test compounds. Additionally, compounds that are effective *in vitro* may not always function the same when introduced to a complex, living system. Thus, the efficacy of the compounds may be assessed *in vivo* through the use of the *C. elegans* infection model. This study aims to address several research gaps by through a variety of methods to ultimately aid in the search for novel antifungal compounds.

1.10 Aims and Objectives

Aim 1: To investigate the antifungal activity of the silver (I)-phosphine complexes and native plant extracts against fungal pathogens within the *Candida* and *Cryptococcus* genera.

1. Obtain crude plant extracts from a variety of native plant species using a variety of extraction solvents.
2. Screen the test organisms for susceptibility to the compounds using disc diffusion assays.
3. Determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC) of the various compounds.

Aim 2: To determine the anti-biofilm activity of the novel silver (I)-phosphine complex 7 and *Kigelia africana* extract against fungal pathogens within the *Candida* and *Cryptococcus* genera.

1. Determine if the compounds inhibit or degrade biofilms by assessing the density of the treated fungal cells using the crystal violet.
2. Determine if the compounds inhibit or degrade biofilms by assessing the metabolic activity of the treated fungal cells using the XTT assay.

Aim 3: Assess the toxicity and efficacy of the novel silver (I)-phosphine complex 7 and native plant extracts using *Caenorhabditis elegans* as a model organism.

1. *Caenorhabditis elegans* will be used to assess the toxicity of the compounds at therapeutic dosages.
2. *Caenorhabditis elegans* will be used as a model of pathogenesis using pre-infection and co-inoculation protocols.

1.11 References

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CHAPTER 2
Evaluating the antifungal activity of novel natural and synthetic compounds against select pathogenic yeast

2.1 Abstract

Serious and fatal fungal related diseases affect over 150 million people each year, the causative agents usually being pathogens within the *Candida* and *Cryptococcus* genera. The limited treatment opportunities available for the combatting these infections has contributed towards the emergence of novel multidrug resistant pathogens such as *Candida auris*. Therefore, it is imperative to find novel treatments to address this issue of antimicrobial resistance. As such this study screened and evaluated the antifungal activity of a variety of novel silver (I)-phosphine complexes (SPC) and native plant extracts against select pathogenic fungi. Through the use of disc diffusion assays, it was found that the SPC 7 as well as ethanolic extracts of *Hypoxis hemerocallidea, Kigelia africana* and *Pelargonium zonale* had promising antifungal activity. The determinations of minimum inhibitory concentrations (MICs) revealed SPC 7 to cause inhibitory effects against all the test organisms (< 0.25 mg/mL). The *P. zonale* extract has the best fungistatic activity, followed by *K. africana* and then *H. hemerocallide.* Minimum fungicidal concentration determinations found that of the extracts, only *K. africana* possessed fungicidal properties however it was only effective against the *Cryptococcus* strains (40 – 80 mg/mL). Additionally, SPC 7 showed fungicidal effects against all the test organisms (2 – 8 mg/mL). As such, SPC 7 and *K. africana* show promise as antifungal treatments and should be studied further.

2.2 Introduction

Fungi describes a diverse group of eukaryotic microorganisms for which there are over 1.5 million different fungal species (Hawksworth, 2001). Of this, it is estimated that around 300 species are pathogenic to humans (Hawksworth, 2001) and these pathogenic fungi are estimated to affect over a billion people worldwide (Bongomin *et al.*, 2017). Although fungal infections are often overlooked as a serious threat to human health, it is estimated that more than 150 million people are suffering with serious or fatal fungal diseases globally, which results in the death of over 1.6 million people annually (Bongomin *et al.*, 2017). Fungal pathogens are often opportunistic and often affect those who are immunocompromised (Riley, 2021). In South Africa (SA), a large proportion of individuals are immunocompromised as a result of the prevalence of human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS), tuberculosis as well as various cancers and pulmonary

diseases (Naidoo *et al.*, 2017). As such there exists a high economic and healthcare-associated burden related to the treatment of fungal diseases with over 3.2 million people affected in SA each year (Schwartz *et al.*, 2019).

A large majority of serious and systemic fungal infections are caused by pathogens within the *Candida* and *Cryptococcus* genera (de Pauw, 2011). *Candida* infections usually cause infections at various mucosal membranes of the body including oesophageal, oral and vulvovaginal (Kullberg and Arendrup, 2015). Inadequate and inefficient treatment can lead to the spread of the infection through the bloodstream to other parts of the body, known as invasive candidemia (Kullberg and Arendrup, 2015). This is oftentimes fatal and estimates show the crude mortality rate attributed to various candidemias to be anywhere from 19 - 60% (Hirano *et al.*, 2015; Flevari *et al.*, 2013). Additionally, *Candida* species account for a large majority of nosocomial infections, accounting for approximately 8 - 13% percent of all hospital acquired blood stream infections (Magill *et al.*, 2018; Wisplinghoff *et al.*, 2004).

Various cryptococcal infections are seen to affect over one million people globally every year (Park *et al.*, 2009; Pappas, 2013). Estimates show that various cryptococcal related diseases are globally estimated to kill over 180 000 people annually, with a mortality rate of up to 70% in some sub-Saharan African countries (Rajasingham *et al.*, 2017). Immunocompetent patients are often infected by *C. gattii* and *C. tetragattii* whereas immunocompromised are affected mainly by *C. deneoformans* and *C. neoformans* (Pappas, 2013). Infection often manifests as severe diseases such as cryptococcal meningitis or pulmonary cryptococcosis which can be fatal. Studies have shown that cryptococcosis is the most common cause for meningitis in people living with HIV/AIDS in sub-Saharan Africa and accounts for 15% of all AIDS-related deaths globally (Rajasingham *et al.*, 2017). In fact, it is one of the leading causes of death amongst HIV-infected individuals, second only to tuberculosis (Rajasingham *et al.*, 2022; Mada *et al*., 2020). Although cryptococcal infections are associated with HIV/AIDS, members of other immunocompromised groups such as those with cancers, liver cirrhosis, diabetes, organ transplant recipients and those on long-term corticosteroid treatments are also at high risk of developing cryptococcosis (Mada *et al*., 2020; Lin *et al.*, 2015).

Despite the increasing prevalence exhibited by these fungal pathogens worldwide, there are

still limited treatments opportunities available for combatting these diseases. The scope of antifungal treatments is quite limited with only a few classes of molecules being utilised in clinical settings (Roemer and Krysan, 2014). Aside from inaccurate notions that fungi are a lesser burden on society and thus require fewer research inputs into drug development, they also come with intrinsic challenges in that they are eukaryotic and thus more closely related to their human hosts than other microbial pathogens (Kathiravan *et al.*, 2012). This greatly narrows the possible range of drug targets to those that are distinctly fungal as not to interrupt any biochemical processes that are shared amongst host and pathogen. Of these limited treatment options many of the available antifungals are expensive and may not be available in certain areas (Kathiravan *et al.*, 2012). This results in the use of antifungal drugs that are not effective for the fungal disease they are meant to treat which contributes to the development of fungal resistance strategies. Furthermore, some antifungal drugs have also been shown to have adverse side effects. For example, amphotericin B has been seen to cause cellular toxicity in the host (as it interacts with mammalian sterols) as opposed to that of the pathogen (Kischkel *et al.*, 2020). Other drugs such as fluconazole and itraconazole are generally seen to be more tolerated in patients but come with the caveat that they are generally not as effective in the treatment of systemic fungal infections (Yonga, 1995). As such, the misuse of the limited arsenal of antifungal drugs has resulted in the rise of resistance mechanisms being implemented by fungal pathogens, which further complicates treatment.

Of note, is the pathogen *C. auris* which is characterised for its role as an emerging novel multidrug resistant pathogen (Ademe and Girma, 2020). This pathogen has been found to have reduced susceptibility to many of the current antifungal classes of drugs including azoles, echinocandins and polyenes (Navalkele *et al*., 2017). As such, *C. auris* infections are difficult to treat and are associated with high mortality rates in the range of 30 - 60% (Osei, 2018; Lockhart *et al.*, 2017; Al Maani *et al.*, 2019). This pathogen has already been implicated in a number of healthcare facility outbreaks across numerous countries including SA (Govender *et al.*, 2018), and surveillance studies show an overall increase in the number of cases observed each year (Govender *et al.*, 2018). Therefore, it is imperative to find alternative treatment options to aid in the control of these fungal infections.

Currently, there are several promising treatment opportunities to aid in this endeavour. Silver was previously widely utilised as an antimicrobial treatment before the development of

antibiotics (Wlodkowski and Rosenkranz, 1973; Clement and Jarrett, 1994). Although not widely used now, silver is still used in some topical antimicrobial treatments (Wlodkowski and Rosenkranz, 1973). The need for new antimicrobials to address the issue of microbial resistance has led to a resurgence in the popularity of silver-based molecules. One main advantage of using silver-based molecules includes its low toxicity towards mammalian cells and organisms which makes it a good treatment option (Lansdown, 2006). However, a common problem with many silver based drugs is that in physiological conditions silver ions bind to and precipitate with free chlorine atoms or to bind cellular enzymes, thus reducing its bioavailability (Berners-Price *et al.*, 1988). Since the activity of these complexes is directly associated with their stability, solubility and lipophilicity, it becomes beneficial to enlist the help of suitable ligands that can help achieve and overcome these issues. Phosphine ligands have been used to increase the solubility of a range of molecules including proteins, metals and some nanoparticles (Bergin *et al.*, 2016; Herbert and Molloy). Previous studies have also shown that when bound to silver (I) with specific configurations the resulting silver phosphine molecules possess promising antimycotic properties (Gandra *et al.*, 2017; Berners-Price *et al.*, 1994; Berners-Price *et al.*, 1988; Coyle *et al.*, 2003).

In addition to synthetic molecules such as the novel silver (I)-phosphine molecules, another promising treatment opportunity lies in that of natural plant extracts. Plant-based medicines were once utilised as the primary source of medicine for centuries before the development of modern medicines and are still utilised today by some traditional healers in developing and rural areas (Salim, Chin and Kinghorn, 2008; Dekebo, 2019). Although plants are not directly utilized in modern medicines, it is estimated that around 40 - 50% of all drugs currently in circulation have been derived from natural plant bioactives (Veeresham, 2012), or have acted as the template for the chemical models and design of synthetic drugs (Veeresham, 2012; Katiyar *et al.*, 2012). As such, plants play a vital role in the drug discovery process. Plants contain an abundance of secondary metabolites and phytochemicals which play a role in plant protection, including defence against plant pathogens (Teoh, 2016). With less than 1% of the world's plants being screened for their phytochemical properties, there is huge potential for the discovery of novel antimicrobial compounds (Dias, Urban and Roessner, 2012; Cragg and Newman, 2005).

Thus, it is imperative to find novel treatments to address the issue of antimicrobial resistance.

This study will therefore screen and evaluate the antifungal activity of a variety of novel synthetic and natural compounds against select pathogenic fungi in the *Candida* and *Cryptococcus* genera.

2.3 Aims and Objectives

This study aimed to investigate the antifungal activity of the novel silver (I)-phosphine complexes and native plant extracts against fungal pathogens within the *Candida* and *Cryptococcus* genera.

This was achieved by:

1. Obtaining crude plant extracts from a variety of native plant species using ethanol and methanol as extraction solvents.
2. Screening the test organisms for susceptibility to the compounds using disc diffusion assays.
3. Determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC) of the various compounds.

2.4 Materials and Methods

2.4.1 Maintenance of Yeast Isolates

Seven test organisms from the fungal genera *Candida* and *Cryptococcus* (Table 2.1) were utilised during this study to determine the antifungal effects of the plant extracts. The *Candida auris* strains were kindly provided by Dr Aijaz Ahmad (Department of Clinical Microbiology and Infectious Diseases; The University of the Witwatersrand) (National Institute for Communicable Diseases (NICD) ethics approval number M140159) and the remaining isolates were retrieved from the culture collection of Dr Angela Botes (ABCC; School of Molecular and Cell Biology; The University of the Witwatersrand). All test organisms were sub-cultured on Sabouraud dextrose agar (SDA) and incubated at 37°C before use. *Candida* strains were grown for 48 hours while *Cryptococcus* strains were grown for 72 hours.

Table 2.1: Pathogenic Fungal Strains. A range of pathogenic fungal isolates chosen for their clinical relevance from the *Candida* and *Cryptococcus* genera.

Culture collection number	Fungal Strain Name	Source
eABCC239	*Candida auris* (intermediate resistance to AmB – 1 µg/mL)	Dr. Aijaz Ahmad
eABCC240	*Candida auris* (low resistance to AmB – 0.25 µg/mL)	Dr. Aijaz Ahmad
eABCC241	*Candida auris* (high resistance to AmB – 4 µg/mL)	Dr. Aijaz Ahmad
eABCC15	*Cryptococcus deneoformans*	Dr. Angela Botes
eABCC50	*Cryptococcus gattii*	Dr. Angela Botes
eABCC16	*Cryptococcus neoformans*	Dr. Angela Botes
eABCC51	*Cryptococcus tetragattii*	Dr. Angela Botes

AmB – Amphotericin B. AmB resistance values calculated by the NICD. NICD ethics approval number for *Candida auris* strains: M140159.

2.4.2 Preparation of Silver (I)-phosphine complexes

Each novel compound consists of a silver salt and ligand in varying ratios. The compounds were dissolved in either sterile distilled water or 100% (v/v) dimethyl sulfoxide (DMSO) and were stored at 4°C in the dark. All compounds were heated at 70°C for 30 minutes before use. These compounds were synthesised at The University of Johannesburg and were kindly provided by Dr. Zelinda Engelbrecht and Prof. Marianne Cronjé (School of Molecular and Cell Biology; The University of the Witwatersrand).

2.4.3 Preparation of Plant Extracts

Dried plant material was obtained from Medico Herbs (https://medicoherbs.co.za/) and are listed in Table 2.2. The plant material was ground into a fine powder using a standard mortar and pestle. Extracts were prepared by combining the ground plant material with the appropriate solvent; either 95% ethanol (v/v) or methanol; in a 1:10 ratio whereby 10 mL of solvent was mixed with 1 g of plant material (Ahmad *et al.*, 2020). The mixtures were vortexed vigorously for five minutes and left to stand undisturbed for one hour at room temperature to allow for extraction. The mixtures were centrifuged for 10 minutes at 1400 × g at room temperature. The supernatants of each mixture were filtered through Whatman No. 1 filter paper into pre-weighed 50 mL Falcon tubes. The filtrate was left to air-dry at room temperature with the aid of a fan to expedite the evaporation process. Once the extracts were dry, the Falcon tubes were weighed, and the extract was resuspended in sterile water to a final concentration between 150 – 200 mg/mL. The extracts were stored at 4°C in the dark until needed.

Table 2.2: Dried plant material utilised in this study. Active compounds were extracted from dried, ground plant material using either ethanol or methanol as the extracting solvent.

Scientific Name	Common Name	Solvent Used
Agathosma betulina	Buchu	Methanol
Curcuma longa	Turmeric	Ethanol
Harpagophytum procumbens	Devil's Claw	Ethanol
Helichrysum petiolare	Imphepho	Ethanol
Hoodia gordonii	Hoodia	Ethanol
Hypoxis hemerocallidea	African Potato	Ethanol
Kigelia africana	Kigelia	Ethanol
Leonotis leonurus	Wild Dagga	Ethanol
Pelargonium zonale	Pelargonium	Ethanol
Rhododendron indicum	Wild Rosemary	Ethanol
Zingiber officinale	Ginger	Ethanol

2.4.4 Diffusion Assays

The diffusion assay serves as a simple and efficient way to determine which antifungal agents display antifungal activity against the test organisms to decide which are appropriate for further consideration in this study. This experiment was carried out in accordance with the Clinical and Laboratory Standards Institute (CSLI) broth microdilution guidelines with some modifications (Vandenbossche *et al.*, 2002; Ferraro, 2000).

All strains were cultured as previously described. Five distinct colonies were picked and suspended in sterile physiological saline solution (0.85% (w/v) sodium chloride) and vortexed until fully resuspended. A sterile cotton swab was dipped into the solution, the excess fluid was removed, and the swab was used to evenly streak the surface of a Mueller Hinton agar (MHA) plate from top to bottom. This procedure was repeated at least six times with a 90° rotation of the plate each time (Jorgensen and Ferraro, 1998).

The test assay was performed by adding aliquots of the various compounds to 6 mm Whatman paper antibiotic discs. All the plant extracts were adjusted to a concentration of 50 mg/mL in sterile water before a 40 µL aliquot of each extract was added to 6 mm Whatman paper antibiotic discs, making the effective concentration 2 mg/mL. The silver compounds were adjusted to a concentration of 10 mg/mL in sterile water (or 100% (v/v) DMSO only for SPC 7) and a 20 µL aliquot (0.2 mg/mL effective concentration) of each was added to the antibiotic discs. These discs were left to dry and were then sterilely placed onto the surface of the pre-inoculated agar. Additionally, a 5 µL aliquot of the silver complexes (10 mg/mL) was added directly onto the surface of the inoculated agar itself (effective concentration of 0.05 mg/mL). Positive controls were prepared by adding various concentrations of the antifungal amphotericin B (1, 5 and 10 µg) to the antibiotic discs. The negative controls were prepared in the same fashion using 20 µL of physiological saline solution or 100% DMSO.

All plates were inverted and incubated at 37°C. The *Candida* strains were incubated for 48 hours while *Cryptococcus* strains were incubated for 72 hours. Following the appropriate incubation time, the zones of inhibition were measured to the nearest millimetre. All control and experimental diffusion assays were performed in duplicate.

2.4.5 Minimum Inhibitory Concentration (MIC)

A minimal media solution (MM) (30 mM glucose, 20 mM magnesium sulphate, 58.8 mM potassium dihydrogen phosphate, 26 mM glycine, 330 mM 3-(N-morpholino)propanesulfonic acid and 6 µM thiamine hydrochloride) was prepared and adjusted to pH 7.0 after which it was filter sterilised and stored at room temperature. Fresh sub-cultures were prepared for each fungal isolate as is described in section 2.4.1. A colony of each isolate was picked and added to a 5 mL aliquot of MM, briefly vortexed and incubated at 37°C on a rotary wheel for 48 hours. Following incubation, the cells were harvested by centrifugation (5000 × g, 5 mins), the supernatant was discarded, and the cell pellet was resuspended in 1 mL of fresh MM to make stock solutions. The cell concentrations of the stock solutions were determined by means of a haemocytometer by using the following formula: cell concentration = (average cell count/16) × dilution factor × 4×10^6. Working cell solutions were made up to a concentration of 2×10^6 cells/mL using MM.

In this experiment only the best performing compounds identified from the disc diffusion assays were utilised further. For the plant extracts this included *K. africana*, *H. hemerocallidea* and *P. zonale*. For the silver compounds, only SPC 7 resuspended in sterile distilled water was utilised. Thus, serial dilutions of the test compounds were prepared using fresh MM. The plant extracts were tested in the range of 1 – 80 mg/mL while the silver compound was tested in the concentration range of 0.25 – 8 mg/ml. Amphotericin B was used as a positive test control, being tested in the range of 0.25 – 8 µg/mL. The negative control included an aliquot of MM while the growth control included the addition of cells to the MM but no test compounds.

The MIC was performed using flat-bottomed 48-well plates. A 100 µL aliquot of the serially diluted test compounds was added to each well, followed by a 100 µL aliquot of the working cell solution for each isolate. Thus, all wells were made up to a final volume of 200 µL with a cell concentration of 1 x 10^6 cells/mL. Each test condition/concentration was tested in triplicate. The plates were sealed with parafilm, covered with foil and incubated at 35°C with shaking at 100 rpm. The *Candida* plates were incubated for 48 hours and the *Cryptococcus* plates for 72 hours.

The MIC values for each compound were determined using two different methods. For the plant extracts the MIC was defined as the lowest concentration for each compound at which the wells appear optically clear and lack any visible cell growth. For the silver compound and the amphotericin controls, the MIC was determined using the iodonitrotetrazolium (INT) colourimetric assay. Two different approaches were utilised due to the different nature of the synthetic and natural compounds.

2.4.5.1 INT Assay

Following the appropriate incubation of the MIC samples, the INT solution was freshly prepared to a concentration of 0.2 mg/mL with sterile water. A 40 µL aliquot of the INT solution was added to each well, the plates were resealed and were left to incubate for 24 hours at 37°C with shaking at 100 rpm. Those wells containing viable cells displayed the reduction of the light-yellow INT dye to a pink colour. The MIC was defined as the lowest concentration for each compound that prevented the colour change of the dye and therefore prevented microbial growth.

2.4.6 Minimum Fungicidal Concentration (MFC)

The MFC was determined by using the same plates prepared for the MIC assay. An aliquot of 10 µL was taken from each well and was spread onto an SDA plate using the hockey stick method (Espinel-Ingroff *et al.*, 2002). The plates were inverted and incubated at 37°C. The *Candida* plates were incubated for 48 hours, and *Cryptococcus* plates for 72 hours. Following incubation, the number of colonies on each plate was counted and the MFC for each compound was determined at the lowest concentration at which three or fewer than three colonies grew.

2.5 Results

The identification and development of novel antifungal treatments is vital to help combat the rising issue of antimicrobial resistance (Prestinaci *et al.*, 2015). Antifungal activity refers to those compounds that have the ability to inhibit the growth of fungi by means of fungistatic or fungicidal mechanisms (Ferarro, 2000). Inhibiting the growth of fungi is an essential property for an effective antifungal therapy to allow for a host to successfully eliminate the pathogen.

As such the antifungal activity of the plant extracts and synthetic silver (I)-phosphine complexes (SPCs) was investigated by means of diffusion assays and determining the minimum inhibitory and fungicidal concentrations.

2.5.1 Diffusion assays

Screening of the plant extracts and SPCs was performed first to identify those compounds that showed the best antifungal activity, which would be further utilised in subsequent experiments. The compounds were added to paper discs at set concentrations, and the impregnated discs were added to inoculated plates (Ferraro, 2000; Vandenbossche *et al.*, 2002). Following an incubation period, the plates were examined for the formation of zones of clearance or zones of inhibition (ZOI). This clear zone indicates that the fungi failed to grow in the presence of the compound and suggests that the fungi is susceptible to the compound (Ferraro, 2000; Vandenbossche *et al.*, 2002).

2.5.1.1 Plant Extracts

Of the eleven plant extracts tested *C. longa, H. hemerocallidea, H. procumbens, K. africana, P. zonale* showed varying degrees of antifungal activity based on the ZOI (Table 2.3). The *A. betulina* and *Z. officinale* extracts were only shown be to be effective against one or two species within the *Cryptococcus* genera. The *H. gordonii, H. petiolare, L. leonurus and R. indicum* extracts showed no antifungal activity against any of the fungi tested. The *H. hemerocallidea* and *P. zonale* extracts showed antifungal activity against fungi within the *Candida* and *Cryptococcus* genera (Figure 2.1) while *C. longa*, *H. procumbens* and *K. africana* activity was confined to only that of *Cryptococcus* species.

Although *C. longa* showed good antifungal activity against the fungi it was decided not to further utilise it as its activity has previously been well characterised. The *H. procumbens* extract showed better activity against *C. neoformans* with a 20.5 mm ZOI compared to the 10.5 mm ZOI of the *K. africana* extract, however overall *K. africana* showed better activity against the other species such as *C. gattii* and *C. tetragattii* with larger ZOIs. As such, subsequent experiments further examined the antifungal activity of *K. africana* as well as *H. hemerocallidea* and *P. zonale* due to their broad activity (Figure 2.1).

Table 2.3: The mean zones of inhibition (mm) of a variety of fungal species when treated with crude plant extracts using the disc diffusion method. The various plant extracts were added to the paper discs at an effective concentration of 2 mg/mL. Amphotericin B (5 µg) was used as positive control and distilled water as a negative control. The ± values indicate the standard deviation (n = 2).

	Agathosma betulina	*Leonotis leonurus*	*Hypoxis hemerocallidea*	*Curcuma longa*	*Pelargonium zonale*	*Hoodia gordonii*	*Kigelia africana*	*Zingiber officinale*	*Harpagophytum procumbens*	*Rhododendron indicum*	*Helichrysum petiolare*	*Amphotericin B*	*Distilled water*
Cryptococcus deoneoformans (eABCC15)	9.5 (± 0.7)	0	26 (± 1.4)	6.5 (± 2.1)	14 (± 1.4)	0	0	0	0	0	0	16 (± 0)	0
Cryptococcus neoformans (eABCC16)	0	0	28 (± 2.8)	0	9.5 (± 0.7)	0	10.5 (± 2.1)	0	20.5 (± 0.7)	0	0	22.5 (± 0.7)	0
Cryptococcus gattii (eABCC50)	0	0	21.5 (± 0.7)	9.5 (± 0.7)	8.5 (±- 0.7)	0	11.5 (± 0.7)	5 (± 0)	2	0	0	22 (± 0)	0
Cryptococcus tetragattiii (eABCC51)	0	0	18 (± 1.4)	4.5 (± 3.5)	7.5 (± 3.5)	0	2 (± 0)	6.8 (± 5.3)	0.5 (± 0)	0	0	21.5 (± 0.7)	0
Candida auris (eABCC239)	0	0	7.3 (± 1.1)	0	7.5 (± 0.7)	0	0	0	0	0	0	10.5 (± 0.7)	0
Candida auris (eABCC240)	0	0	0	0	7.5 (± 0.7)	0	0	0	0	0	0	14 (± 1.4)	0
Candida auris (eABCC241)	0	0	0	0	7.8 (± 0.4)	0	0	0	0	0	0	8 (± 0)	0

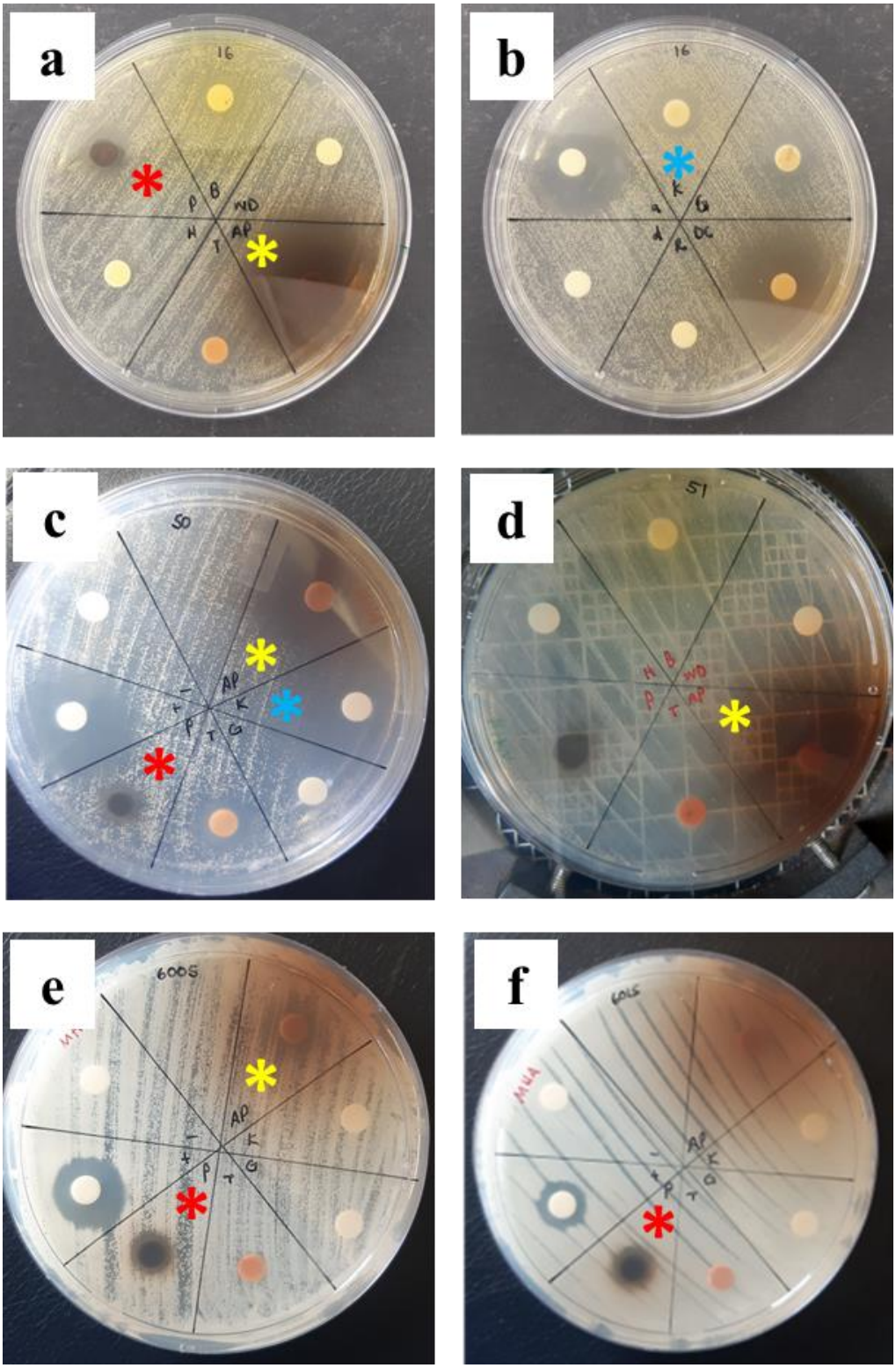

Figure 2.1: Diffusion assays using plant extracts. The various plant extracts were added to the paper discs at an effective concentration of 2 mg/mL. Amphotericin B (5 µg) was used as positive control and distilled water as a negative control. All assays were performed in duplicate. The stars denote zones of inhibition caused by the best performing plant extracts: yellow - *Hypoxis hemerocallidea*; red - *Pelargonium zonale*; blue - *Kigelia africana*. (a, b) *Cryptococcus neoformans* (eABCC16); (c) *Cryptococcus gattii* (eABCC50); (d) *Cryptococcus tetragattii* (eABCC51); (e) *Candida auris* (eABCC239); (f) *Candida auris* (eABCC240).

2.5.1.2 SPCs

When the SPCs were added to the paper discs no ZOIs could be seen for SPCs 1 - 6 thus seemingly indicating they possessed no antifungal activity (Table 2.4 and Figure 2.2). To confirm their apparent lack of activity, the SPCs were spotted directly onto the inoculated agar and left to incubate. This revealed that SPCs 1 - 6 do have some degree of inhibition as can be seen by the localised ZOIs in Figure 2.2, however their poor solubility prevents them from diffusing from the paper disc. SPC 7 is a reformulated version of SPC 6, with the goal being to increase the solubility of the compound. This increase in solubility is confirmed whereby a small ZOI can be seen around the disc when it is resuspended in distilled water (Figure 2.2). A larger ZOI is seen when the compound is dissolved in DMSO, indicating improved solubility in the DMSO. Ultimately SPC 7 resuspended in distilled water was carried forward in subsequent experiments to assess the true antifungal activity of the compound without interference of the DMSO.

Thus, the best performing test compounds were identified (that being *K. africana, H. hemerocallidea*, *P. zonale* and SPC7) and these compounds were carried forward for the subsequent experiments.

Table 2.4: The mean zones of inhibition (mm) of a variety of fungal species when treated with novel silver (I)-phosphine complexes using the disc diffusion method. The ± values indicate the standard deviation of the values (n = 2).

	SPC 1	SPC 2	SPC 3	SPC 4	SPC 5	SPC 6	SPC 7	SPC 7 DMSO	Distilled water	DMSO
Cryptococcus deoneoformans (eABCC15)	0	0	0	0	0	0	0.8 (± 0.4)	2.3 (± 0.4)	0	0
Cryptococcus neoformans (eABCC16)	0	0	0	0	0	0	1.3 (± 0.4)	3 (± 0)	0	0
Cryptococcus gatti (eABCC50)	0	0	0	0	0	0	0	2.5 (± 0.7)	0	0
Cryptococcus tetragattii (eABCC51)	0	0	0	0	0	0	0.8 (± 0.4)	2.5 (± 0)	0	0
Candida auris (eABCC239)	0	0	0	0	0	0	2 (± 0)	4.5 (± 0.7)	0	0
Candida auris (eABCC240)	0	0	0	0	0	0	0.8 (± 0.4)	3 (± 0)	0	0
Candida auris (eABCC241)	0	0	0	0	0	0	1.8 (± 0.4)	4.8 (± 0.4)	0	0

SPC - silver (I)-phosphine complexes; DMSO – Dimethyl sulphoxide.

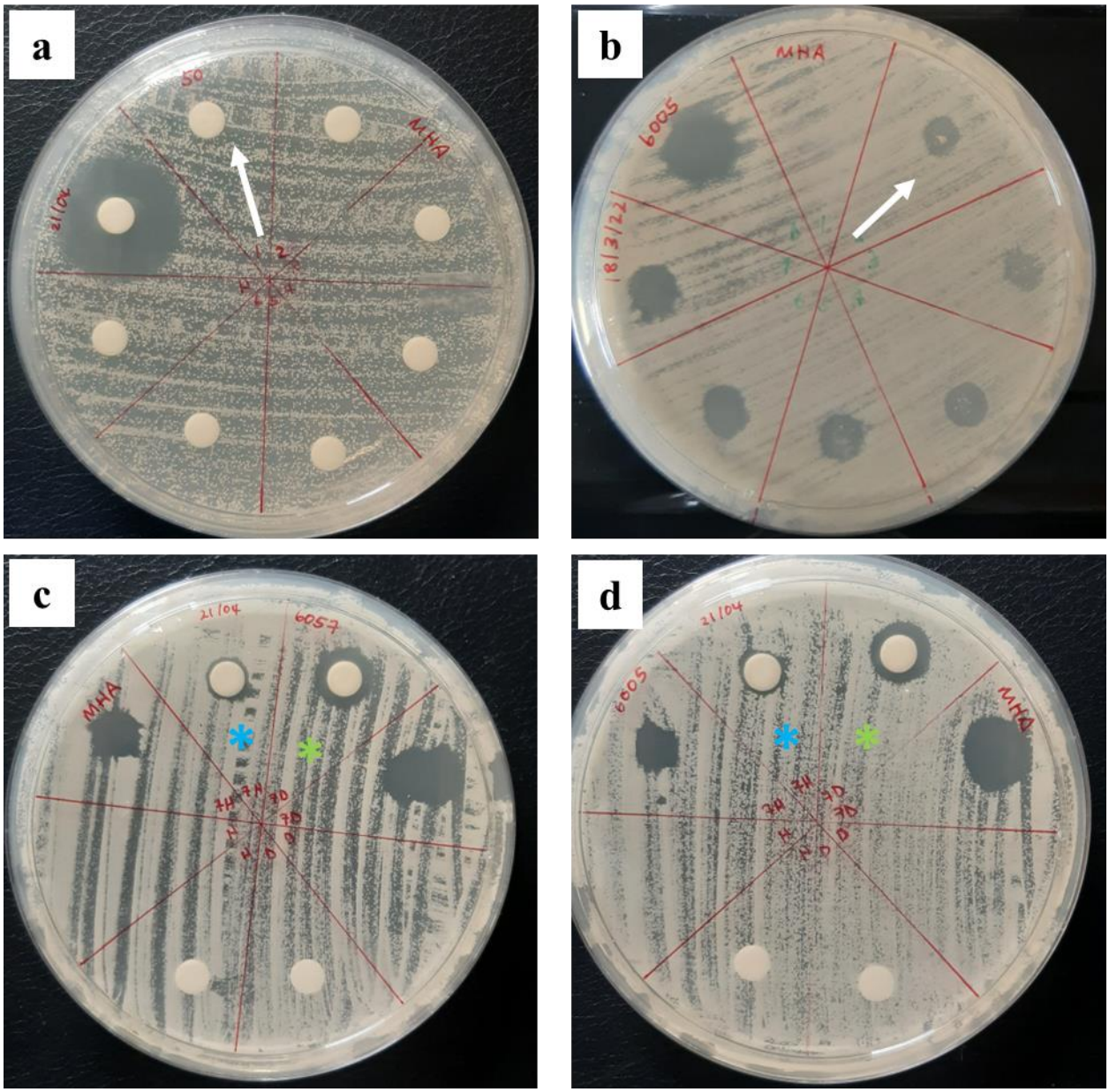

Figure 2.2: Diffusion assay using silver (I)-phosphine complexes. A 10 µL aliquot of 10 mg/mL solution of each compound was added to each disc (a) or spotted directly onto the agar (b). Amphotericin B (5 µg) was used as positive control and distilled water as a negative control. (a*) Cryptococcus gattii* (eABCC50); clockwise from the arrow – SPC1-SPC6, distilled water, amphotericin B. (b) *Candida auris* (eABCC239); clockwise from the arrow – SPC1-SPC6, amphotericin B, distilled water. (c and d) blue star – SPC 7 in distilled water; green star – SPC 7 in DMSO; (c) *Candida auris* (eABCC241); (d) *Candida auris* (eABCC239).

2.5.2 Minimum Inhibitory Concentrations

The MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism (Espinel-Ingroff *et al.*, 2002). For the plant extracts the MIC was defined as the lowest concentration for each compound at which the wells appear optically clear and lack any visible cell growth. In general, a much higher concentration (80 or more than 80 mg/mL) of the *H. hemerocallidea* extract was required to inhibit the growth of the fungal isolates, except for *C. deneoformans* which was inhibited at 20 mg/mL (Table 2.5). The *K. africana* extract also appeared to inhibit *C. deneoformans* the best at 20 mg/mL. Additionally, *K. africana* showed to be better at inhibiting the *Cryptococcus* species than the *Candida* species, with inhibition in the range of 20 – 40 mg/mL for *Cryptococcus* and 40 – 80 mg/ml for *Candida*. Lastly, the *P. zonale* extract appeared to possess the best inhibitory properties of the plant extracts. It showed inhibitory effects for all *Candida* and *Cryptococcus* extracts in the range of 20 – 40 mg/mL.

For the SPCs and controls a colourimetric dye was used to ascertain the viability of the isolates after treatment with the compound as various concentrations. The dye is reduced from a light-yellow colour to pink in the presence of viable cells, thus the darker the pink colour of the well, the poorer the compound was at inhibiting the growth of the isolates (Berridge and Patries, 2005). Conversely, light-yellow wells indicated that the compounds inhibited the isolate's growth at a that concentration. Interestingly, the SPC 7 showed fungistatic properties with less than 0.25 mg/mL required to inhibit the growth of all fungal isolates (Table 2.5 and Figure 2.3).

Table 2.5: Minimum inhibitory concentrations (mg/mL) of crude plant extracts and a silver (I)-phosphine complex against various fungal species within the *Candida* and *Cryptococcus* genera.

	Compound	K	AP	P	SPC 7	AmB
		Minimum Inhibitory Concentration (mg/mL)				(µg/mL)
Fungal species	*Cryptococcus deneoformans* (eABCC15)	20	20	20	<0.25	4
	Cryptococcus neoformans (eABCC16)	40	80	20	<0.25	2
	Cryptococcus gattii (eABCC50)	40	>80	40	<0.25	4
	Cryptococcus tetragattii (eABCC51)	40	80	20	<0.25	4
	Candida auris (eABCC239)	40	80	40	<0.25	2
	Candida auris (eABCC240)	80	>80	20	<0.25	0.5
	Candida auris (eABCC241)	80	>80	40	<0.25	>8

K – *Kigelia africana*; AP – *Hypoxis hemerocallidea*; P - *Pelargonium zonale;* SPC7 – Silver (I)-phosphine complex 7; AmB – Amphotericin B. Each condition was tested in triplicate.

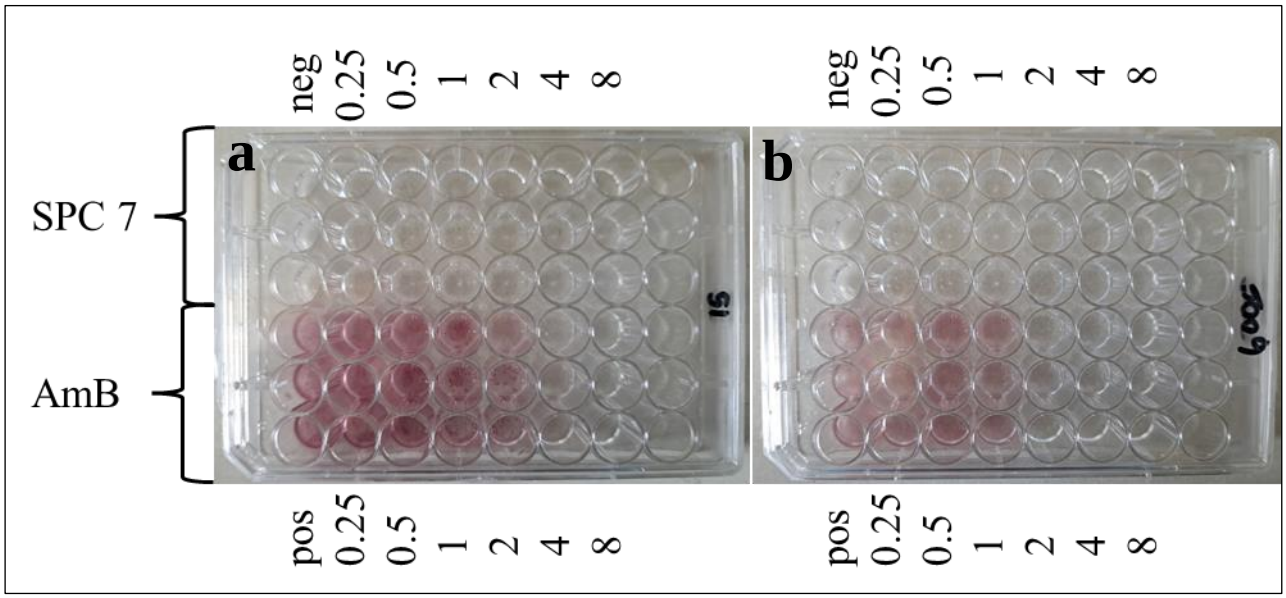

Figure 2.3: Minimum inhibitory concentrations (MIC) using iodonitrotetrazolium (INT) colourimetric assay. SPC 7 – silver (I)-phosphine complex 7; AmB – Amphotericin B; neg – negative control for SPC 7 (minimal media only); pos – positive control (fungal cells in minimal media only). a – *Cryptococcus deneoformans* (eABCC15); b – *Candida auris* (eABCC239). Fungal cells (1 x 10^6 cells/mL) were treated with SPC 7 compound (top row) with a concentration range of 0.25-8 mg/mL as well as AmB (bottom row) at a concentration range of 0.25-8 µg/mL. Following incubation of the plates (24 hours for *Candida* and 48 hours for *Cryptococcus*) a 40 µL aliquot (0.2 mg/mL) of the INT dye was added to each well and incubated for 24 hours at 37°C. The MIC was determined at the lowest concentration where the wells appeared clear. The assay was completed in triplicate.

2.5.4 Minimum Fungicidal Concentrations

The MFC is defined as the lowest compound concentration at which three or fewer than three colonies grow (Espinel-Ingroff *et al.*, 2002). Despite showing promising fungistatic activity, the *P. zonale* extract showed no fungicidal activity against any of the test strains even at the highest concentration tested of 80 mg/mL (Table 2.6). The *H. hemerocallidea* extract was seen to only display fungicidal activity against *C. deneoformans.* The *K. africana* extract showed fungicidal activity against the *Cryptococcus* strains however not against the *Candida* strains. The SPC 7 displayed fungicidal activity against all the test strains in both the *Candida* and *Cryptococcus* genera. As expected, the MFCs were higher than that of the MICs (Table 2.5).

Table 2.6: Minimum fungicidal concentrations (mg/mL) of crude plant extracts and a silver (I)-phosphine complex against various fungal species within the *Candida* and *Cryptococcus* genera.

	Compound	K	AP	P	SPC 7	AmB
		Minimum Fungicidal Concentration (mg/mL)				(µg/mL)
Fungal species	*Cryptococcus deneoformans* (eABCC15)	40	40	>80	1	4
	Cryptococcus neoformans (eABCC16)	40	>80	>80	1	2
	Cryptococcus gattii (eABCC50)	80	>80	>80	2	4
	Cryptococcus tetragattii (eABCC51)	80	>80	>80	8	4
	Candida auris (eABCC239)	>80	>80	>80	2	8
	Candida auris (eABCC240)	>80	>80	>80	2	>8
	Candida auris (eABCC241)	>80	>80	>80	1	>8

K – *Kigelia africana*; AP – *Hypoxis hemerocallidea*; P - *Pelargonium zonale;* SPC7 – Silver (I)-phosphine complex 7; AmB – Amphotericin B.

2.6 Discussion

The rise of antimicrobial resistance has resulted in the urgent need for the discovery and development of new therapeutic treatment options (Fisher *et al.*, 2018). As such this research was performed to aid in the quest for novel treatments (Fisher *et al.*, 2022). An array of experiments were performed to establish the antifungal activity of various natural and synthetic compounds.

2.6.1 Screening of Compounds

The first step in the discovery process involved screening the various compounds for antifungal activity against the select fungal pathogens (Katiyar *et al.*, 2012). This was done in an effort to ascertain the best performing compounds which would then be utilised for further studies. Successful screening negates the need for using time and resources that would be spent trying to define parameters of ineffective antimicrobials (Zgoda and Porter, 2001). Eleven plant extracts were assessed as well as seven novel SPCs.

Initial screening employed the technique of the diffusion assay. This is a standardised method to screen for the ability of a compound to inhibit the growth of a microorganism (Hudzicki, 2009). The general principle behind the technique is that when the paper discs are impregnated with a compound and subsequently placed onto an agar plate, the solvent from the disc is absorbed into the agar, and with it the compound also diffuses into the agar (Hudzicki, 2009; Jorgensen and Turnidge, 2015). The concentration of the compound is highest closest to the disc and the degree of diffusion is dependent on the solubility properties of the compounds themselves (Hudzicki, 2009; Bauer, 1966). When the agar plate is inoculated with the microorganism, the addition of the impregnated disc to the agar allows for the concurrent growth of the microorganism and the diffusion of the compound. If a microorganism is susceptible to the compound it will not grow in its presence resulting in a clear ZOI around the discs. The critical mass is the point at which the concentration of the compound is no longer effective against the microorganism thereby allowing for its growth as is demonstrated in Figures 2.1 and 2.2.

2.6.1.1 Plant Extracts

Of the eleven extracts tested, *H. gordonii, H. petiolare, L. leonurus and R. indicum* showed no apparent activity against the test organisms as is seen by the lack of clear zones around the discs (Figure 2.1 and Table 2.3). According to the literature, *H. gordonni* has not previously been assessed in depth for its antifungal activity while *H. petiolare* only showed antifungal activity when acetone was used as a solvent (Akinyede *et al.*, 2021). Additionally, *R. indicum* has previously shown activity against *Saccromyces cervesiae* however, it has not shown to have antifungal activity against test organisms in the *Candida* and *Cryptococcus* genera (Judzentiene *et al.*, 2020). While *L. leonurus* has shown a degree of activity against

C. neoformans (Wanas *et al.*, 2016) the extraction solvent used was hexane rather than ethanol. The choice of extraction solvent plays an important role in the subsequent activity of plant extracts (Abubakar and Haque, 2020). Plants are a source of a wide variety of compounds that can be assessed for their antifungal properties and the solvent is necessary to aid in the separation of these bioactive compounds from other plant material (Abubakar and Haque, 2020). Additionally, the extraction medium utilised can influence the activity of the extract. Solvents such as water and alcohols like ethanol are more polar while solvents such as acetone and hexane are non-polar (Abubakar and Haque, 2020). As such the extraction solvent can influence the compounds that are extracted from the plant. Polar molecules generally dissolve other polar substances which includes compounds such as secondary metabolites, phenolics and membrane associated lipids (Abubakar and Haque, 2020; Amaro *et al.*, 2015). Non-polar solvents can be useful for the extraction of alkaloids, terpenes, and fats (Abubakar and Haque, 2020). Thus, the lack of activity seen may also be that the extracted compounds from those plants did not possess antifungal activity.

There are a variety of other factors that may account for the lack of antifungal activity of the plant extracts. Firstly, the compounds were only tested at a single concentration of 50 mg/mL and as such the effective concentration may have been too low to inhibit the growth of the test organisms. Additionally, the solvent used to dissolve the test compounds is also of importance (Abubakar and Haque, 2020). This study suspended the compounds in distilled water. The advantage of this is that there is no external influence from the solvent that may cause any adverse effects to the test organisms as it is non-toxic (Das *et al.*, 2010). Additionally, it is cheap, widely available, and acts as an appropriate indicator to assess the relative solubility of the extracts (Das *et al.*, 2010). However, it does come with the downside that the range of compounds tested within the extracts are limited to those that are polar.

The extracts *A. betulina, C. longa, H. procumbens* and *K. africana* showed some activity against fungal species in the *Cryptococcus* genera but not those in the *Candida* genera at the test concentrations (Table 2.3). This difference in activity may be attributed to the differences in the cell structure between the two genera. This may suggest that these extracts contain a compound which inhibits a *Cryptococcus* specific structure or process. There are a few key differences between the two genera, however of note is the difference in cell wall structure between the two. This is a likely target as fungal cell walls contain molecules that are distinct

and absent from the human body, thus targeting these factors reduces the risk of interrupting human processes. While there may be slight variations in the organisation and composition of the cell wall between species the cell walls generally have a fixed structure. Both *Candida* and *Cryptococcus* cell walls contain chitin and β-glucans including β-1,3-glucan and β-1,6-glucan which maintain the integrity and structure of the fungal cells (Garcia-Rubio *et al.*, 2020). In addition to these components, *Cryptococcus* also contain a deacetylated version of chitin, known as chitosan (Garcia-Rubio *et al.*, 2020). *Cryptococcus* also have the presence of an exopolysaccharide capsule which is also a virulence factor and aids in biofilm formation (Martinez and Casadevall, 2015).Thus, it is possible that the plant extracts target the production or interfere with the biochemical pathways involved in the proper functioning of one of these components. The literature shows that *C. longa* and *K. africana* do possess some antifungal activity against other *Candida* species such as *C. albicans* (Murugesh *et al.*, 2019; Zorofchian *et al.*, 2014). However, there is not much research investigating their role against *C. auris* strains in particular. It is well known that *C. auris* generally displays increased resistance towards antifungal treatments compared to other members of the *Candida* genera (Ahmad and Alfouzan, 2021; Bhattacharya *et al.*, 2019). Thus, the plant extracts may not have displayed activity at the concentration tested.

The extracts *C. longa, H. hemerocallidea, H. procumbens, K. africana* and *P. zonale* showed varying degrees of antifungal activity (Table 2.3). Extracts of *C. longa* have been widely studied for their medicinal and antimicrobial activities. Although this study did not find *C. longa* to have antifungal activity against the *C. auris* strains at the tested concentration, there have been studies that agree that these extracts can be effective against *Cryptococcus* (Apisariyakul *et al.*, 1995; Da Silva *et al.*, 2016). The results from Table 2.3 show that *C. longa* was effective against all the *Cryptococcus* strains except for *C. neoformans*. This contrasts with other studies where *C. longa* shows good activity against *C. neoformans* in particular (Rotchanasathian *et al.*, 2000). A study also using ethanolic *C. longa* extracts also found *C. neoformans* to be susceptible to *C. longa* (Sagarika and Pratima, 2012). This suggests that the concentration of *C. longa* may have been inadequate for eliciting a result.

The *H. procumbens* extract showed to be effective against all the *Cryptococcus* species except for *C. deneoformans*. A large ZOI of 20.5 (± 0.7) mm was observed against *C. neoformans*

however the *C. gattii* and *C. tetragattii* showed to be considerably less susceptible with ZOIs of 2 (± 0) and 0.5 (± 0) mm respectively. Previous research has shown that non-neoformans species such *C. gattii* tend to be less susceptible to available antifungal treatments such as amphotericin B and fluconazole (Bernal-Martinez *et al.*, 2010). At present, there has not been much research done exploring the antifungal properties of *H. procumbens* against human pathogens however it has shown mild activity against some food fungi such as *Botrytis cinerea* and *Penicillium digitatum* (Brendler, 2021; Guerin and Reveillere, 1985).

Research has been conducted to evaluate not only the antimicrobial properties of *K. africana* but also various other pharmacological activities such as its role as an analgesic, antimalarial and anticancer drug (Omonkhelin and Eric, 2007; Kamau *et al.*, 2016; Oketch-Rabah *et al.*, 1999). The *K. africana* extract showed promising results against the *Cryptococcus* species. The ZOIs were seen in all species except *C. deneoformans* (Table 2.3). Studies have shown that ethanolic and methanolic *K. africana* extracts possess antifungal activity against *C. neoformans* as well as some *Candida* species (Hamza *et al.*, 2006; Omonkhelin and Eric, 2007; Osman *et al.*, 2017).

Interestingly, both *H. procumbens* and *K. africana* showed to have some degree of antifungal activity against all other *Cryptococcal* isolates except *C. deneoformans*. This species is not as well studied however it is has been found to have global distribution and be implicated in over 30% of infections in Europe (Hagen *et al.*, 2015). In a study conducted by Gago and colleagues (2017), the susceptibility profiles of several *C. deneoformans* and *C. neoformans* patient-derived isolates were examined (Gago *et al.*, 2017). The results showed that in general, *C. deneoformans* was inhibited by lower or the same concentrations of common antifungals such as amphotericin B and fluconazole (Gago *et al.*, 2017). Thus, the results obtained by the diffusion assay for *C. deneoformans* do not align with the trends set out in the literature.

The extract of *H. hemerocallidea* was effective against the *Cryptococcus* species as well as *C. auris* (eABCC239). Previous research has found *H. hemerocallidea* to have mild antifungal effects against *C. neoformans* (Zulfiqar *et al.*, 2022) and against other *Candida* species such as *C. glabrata* and *C. krusei* (Mwinga *et al.*, 2019). When this extract was applied to the discs, it appeared to have migrated well through the agar as is seen by the large reddish-brown halos

around the discs in Figure 2.1 (yellow stars). This suggests that the extracted compounds are very soluble in aqueous conditions (Hudzicki, 2009). Additionally, the largest ZOIs were observed suggesting that at the tested concentration (50 mg/mL) the extract displayed the highest potency (Hudzicki, 2009).

Lastly, from the diffusion assays the *P. zonale* extracts performed the best causing ZOIs in all seven test organisms (Table 2.3). This is in line with other researchers who have found there to be against *C. neoformans* (Samie *et al.*, 2019; Rath *et al*., 2005) *C. albicans* (Gucwa *et al.*, 2018) and even *C. auris* (Parker *et al.*, 2022).

2.6.1.2 Silver (I)-phosphine Complexes (SPCs)

Of all the SPCs, SPC 7 was determined to be the best performing compound (Table 2.4). When SPCs 1 - 6 were added to the discs the results show that no zones of inhibition formed (Figure 2.2a). This would suggest that none of these compounds possess antifungal activity. However, when the compounds were added directly to the surface of a pre-inoculated agar plate localised ZOIs were observed (Figure 2.2b). This suggests that the compounds have poor solubility rather than poor antifungal activity. Silver is known to be a hydrophobic metal (Cataliotti *et al.*, 2009) and despite the addition of the phosphine ligands this may not have been enough to overcome these solubility issues (Sani and Baba, 2016). As such it is possible that the SPCs failed to diffuse from the paper discs and from where they were spotted directly onto the agar, which would account for the localised ZOI seen in Figure 2.2b.

Only SPC 7 resulted in the formation of ZOIs among the SPC molecules (Figure 2.2 and Table 2.4). The SPC 7 is a reformulated version of SPC 6 with the aim of increasing solubility. This reformulation proved to be more effective as small ZOIs could be observed (Figure 2.2c - d) when it was resuspended in both distilled water and DMSO. On average, larger ZOIs were observed when SPC 7 was resuspended in DMSO than in distilled water (Table 2.4). The compound DMSO is a polar solvent that is often used to dissolve both polar and non-polar compounds and can be utilised for a variety of functions (Rammler and Zaffaroni, 1967). As such, it is commonly used as a solvent for novel antimicrobial compounds as well as test compounds with poor solubility for antimicrobial screening (Cushnie *et al.*, 2020; Kirkwood *et al.*, 2018). This is demonstrated with SPC 7 whereby the compound dissolved in DMSO

showed to diffuse further into the agar than when it was dissolved in water (Figure 2.2). Although the solubility of the compounds was increased it was decided to continue with SPC 7 that was dissolved in water. While DMSO is considered to be generally safe and non-toxic to humans, some research has shown that it can cause adverse effects against other organisms (Rammler and Zaffaroni, 1967). Some studies show that even at low concentrations of 2 - 10% (v/v) DMSO can inhibit the growth of some *Candida* and *Cryptococcus* isolates (Rodríguez-Tudela *et al.*, 2001; Randhawa, 2006; Zgoda and Porter, 2001), while at concentrations higher than 0.5% DMSO can have adverse effects on the lifespan and physiology of *Caenorhabditis elegans* (AlOkda and Van Raamsdonk, 2022).

2.6.2 Minimum Inhibitory Concentrations (MICs)

The MIC is an important parameter that determines the lowest concentration of an antimicrobial that will inhibit the growth of a microorganism (Andrews, 2001). This value is of great clinical relevance. Firstly, it is of great importance to know whether a fungal pathogen is susceptible to a specific drug when implementing treatment strategies (Andes *et al.*, 2012; Scorzoni *et al.*, 2017). For example, although echinocandins and azoles are often prescribed for the treatment of candidiasis, cryptococcal infections usually require the use of polyenes as they exhibit some level of resistance to other treatments (Iyer *et al.*, 2021). In addition, appropriate dosages should be established to ensure the elimination of the pathogen to prevent the emergence of resistance mechanisms (Cantón and Morosini, 2011). The MIC values also provide valuable information for the monitoring of a pathogen's susceptibility to known antifungals. In doing so, trends towards a pathogen becoming resistant may be evaluated (Govender *et al.*, 2011).

The MICs were determined using two different methodologies. The plant extract MICs were determined by the lowest concentration that resulted in optically clear wells while the controls and SPCs were determined with the use of a colourimetric assay using INT dye. The difference in technique was due to the difference in the properties of the plant extracts and the SPCs. The plant compounds possessed pigments which were likely the result of chlorophyll, carotenoids and flavonoids being isolated during the extraction process (Hu *et al*., 2013). As such, the use of a colorimetric assay would be inappropriate as a distinct colour change would be difficult to assess. Thus, the method of assessing wells which lack growth is more appropriate as is

outlined by the CLSI guidelines (Berkow *et al*., 2020; Wayne, 2002). In contrast, the SPCs are cloudy when in solution which does not allow for one to see a distinct change in the turbidity in the wells. As such the INT assay was utilised to assess the metabolic activity of the fungal cells when treated with the compounds (Zalazar *et al.*, 2018). The tetrazolium salt acts as an artificial electron acceptor for cellular dehydrogenases (Berridge and Patries, 2005). In the presence of dehydrogenases the INT salt is reduced to formazan and subsequently changes from colourless to a brightly coloured product (Berridge and Patries, 2005). Dehydrogenases are enzymes that catalyse the removal of hydrogen atoms from molecules and are typically involved in vital metabolic processes of viable and metabolically active organisms (Huet *et al.*, 1992). In the case of a viability assay, the more metabolically active cells there are, the more dehydrogenase enzymes will be available thus more of the INT salt will be reduced leading to the formation of formazan which shows as a bright pink colour (Figure 2.3). As such, wells that remained colourless indicate that those cells show a loss of metabolic activity and were affected by the treatment at that specific concentration.

The MICS of the best performing plant extracts from the diffusion assays were determined. Overall, *P. zonale* was the best performing plant extract, inhibiting all isolates at concentrations between 20 – 40 mg/mL (Table 2.5). These results are in line with those displayed in the diffusion assays. The *K. africana* extract was able to inhibit the growth of all the *Cryptococcus* species between 20 – 40 mg/mL, and in contrast to the diffusion assays, displayed that it could inhibit *C. auris*. Surprisingly, the *H. hemerocallidea* extract was the least effective of the extracts despite showing potency in the diffusion assays. The extract was unable to inhibit the growth of *C. gattii* as well as *C. auris* and only inhibited the growth of the other species at the highest concentration tested (80 mg/mL), except for *C. deneoformans* which was inhibited at 20 mg/mL. The disconnect between results observed in the diffusion assay and those obtained in the MIC determination may be as a result of a difference in cell concentration between the two experiments. The MICs were performed with a set cell concentration of 1×10^6 cells/mL however the diffusion assays utilise overnight cultures and are dispensed into the agar using a sterile cotton bud. This allows room for variation in the number of cells inoculated into each agar plate which may explain the variations observed (Espinel-Ingroff *et al*., 2002). Thus, a lower cell concentration may account for the reason *H. hemerocallidea* seemed to perform so well according to the diffusion assays. Additionally, the difference in media utilised may play a role. The Mueller-Hinton agar (MHA) was utilised for the diffusion assay and a MM for the

MICs. While the MM contains only the minimum nutrients necessary for the growth of the fungi, the MHA is a nutrient rich agar, thus the MHA may promote the overgrowth of some organisms (Espinel-Ingroff *et al*., 2002). This could explain the disparities seen in the activity of the compounds against *C. deneoformans* and the *C. auris* strains.

While further studies would be required to elucidate the mechanism of action employed by the plant extracts, one study has shown that certain compounds found within *Phytolacca tetramera* plant extracts induce damage to *C. albicans* by binding to one of the main constituents of the plasma membrane, ergosterol (Butassi *et al.*, 2019). Similarly, *Myrtus communis* extracts were shown to alter the morphology of some *Candida* species by causing a loss of cell integrity (Alyousef, 2021).

The SPC 7 was seen to inhibit the growth of both the *Candida* and *Cryptococcus* test organisms at all concentrations tested (Table 2.5). This suggests its mechanism of action is not directed towards a genera-specific process. While the mechanisms by which SPC 7 work should be studied further, previous studies have shown that other silver-based treatments are able to bind to many cellular components including lipids, nucleic acids and other proteins, but are generally seen to affect the cell wall and cell membranes of microbes (Clement and Jarrett, 1994; Coward *et al*., 1973). It is also thought to intracellularly affect cell metabolism, play a role in nucleic acid damage by the formation of reactive oxygen species (ROS) which leads to mitochondrial dysfunction (Thurman *et al*., 1989; Berners-Price *et al.*, 1994).

2.6.3 Minimum Fungicidal Concentrations (MFCs)

Like the MIC, the MFC is an important parameter to determine if a compound has fungicidal properties (Espinel-Ingroff *et al.*, 2002). This is important to establish as a compound may exhibit inhibitory activity but might not necessarily kill the microorganism (Espinel-Ingroff *et al.*, 2005). There are many compounds which function mainly as fungistatic compounds such as the azoles, however fungistatic compounds are not always ideal as a therapeutic, especially in the case of those who are immunocompromised (Graybill *et al*., 1997; Como and Dismukes, 1994). Studies show that the treatment of fungal infections with fungicidal therapies is associated with increased probability of therapeutic success and a decreased probability of persistent and recurring infections (Kumar *et al.*, 2018). Thus, it would be desirable to develop

a compound with fungicidal properties. The results of this study show that only *K. africana* extract possessed fungicidal activity against the *Cryptococcus* species (Table 2.6). Additionally, *H. hemerocallidea* only showed fungicidal activity against *C. deneoformans*. Despite the inhibitory activity exhibited against the test strains with the *P. zonale* extract, it did not possess fungicidal properties suggesting it may be fungistatic rather than fungicidal. This may suggest that the *P. zonale* extract functions by inhibiting processes that are necessary for proliferation of the test organisms. Some research shows that fungistatic agents, such as fluconazole, can exert fungicidal activity in a dose-dependant manner (Lee and Lee, 2018). Fluconazole is a commonly used fungistatic compound that inhibits ergosterol biosynthesis (Vasicek *et al.*, 2014). Although effects such as cell shrinkage and apoptotic responses were not observed in *C. albicans* when treated with low dosages of fluconazole, they were present with a 4-fold increase in the concentration (Lee and Lee, 2018). As such, a higher concentration of the plant extracts may be necessary to induce the killing of test organisms. However, it must be noted that this increase in concentration may also be associated with an increase in host-toxicity of the compounds (Lepak and Andes, 2015).

The SPC 7 displayed fungicidal activity against all the test organisms (Table 2.6). This is in line with other silver based molecules and silver nanoparticles which also show potent fungicidal activity (Panáček *et al.*, 2009; Mussin *et al.*, 2019). As discussed, the mechanisms by which the SPC 7 works must be further studied, however previous studies have shown silver to play a role in disrupting several important processes involved in membrane integrity and mitochondrial function, which can account for the fungicidal activity observed (Clement and Jarrett, 1994; Berners-Price *et al.*, 1994).

2.7 Conclusions

The initial screening of compounds found *H. hemerocallidea, K. africana, P. zonale* and SPC 7 to be the best performing compounds. Further experiments proved *K. africana, P. zonale* and SPC 7 to have inhibitory effects against the test organisms. Of the plant extracts, *K. africana* showed fungicidal effects against the *Cryptococcus* species while SPC 7 showed fungicidal effects against all the test organisms. While the mechanisms by which the compounds work must be further studied, evidence from previous studies suggest both

K. africana and SPC 7 may work by disrupting plasma membrane activity and mitochondrial function while. Further studies should be conducted with *K. africana* and SPC 7 to elucidate the extent of their antifungal properties as well as their suitability as therapeutic treatments.

2.8 References

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CHAPTER 3
Evaluating the antibiofilm activity of novel natural and synthetic compounds against select pathogenic yeast

3.1 Abstract

Biofilms have been shown to play a key role involved in fungal resistance in some clinically important fungi including *Candida* and *Cryptococcus* species. Furthermore, biofilm formation has been linked the establishment of persistent infections and increased severity of disease, which leads to poorer outcomes in patients. Biofilms are notoriously difficult to treat and at current, there are limited therapeutic options available for the treatment of biofilms. As such this study investigated the antibiofilm properties of a novel silver (I)-phosphine complex (SPC) and an ethanolic *Kigelia africana* extract for their ability to disrupt and prevent the formation of biofilms. When pre-formed biofilms were exposed to SPC 7, significant decreases in the metabolic activity in all but one of the biofilms was observed ($p < 0.05$). Addition of SPC 7 during biofilm formation also resulted in significant reductions in the metabolic activity of all test strains ($p < 0.05$). Interestingly, the addition of *K. africana* to the *Cryptococcus* biofilms resulted in an increase in the metabolic activity. This is speculated to be due to the extract causing an increase in the oxidative stress of the biofilms. The results showed that neither of the compounds significantly decreased the biomass of the biofilms, with higher concentrations of the compounds possibly being needed to see biomass reductions. Overall SPC 7 shows promise as a potential antibiofilm compound and should be researched further.

3.2 Introduction

Antimicrobial resistance has been deemed as one of the major threats to human health this century as microorganisms become less susceptible to the antimicrobials available (Prestinaci *et al.*, 2015). One of the main drivers of this resistance is the overuse and misuse of antimicrobial agents through inappropriate treatment choice and inadequate treatment dosages (Prestinaci *et al.*, 2015). This phenomenon is further exacerbated in the case of fungal resistance, where the arsenal of treatments is considerably more limited with only a few classes of molecules that can be utilised (Roemer and Krysan, 2014). This has led to the development of novel emerging multidrug resistant pathogens such as *Candida auris* (Navalkele *et al.*, 2017).

One of the difficulties associated with developing new antifungals lies in the fact that fungi are eukaryotic thus, there are some shared processes between the pathogen and host which may be

targeted, causing host toxicity (Kathiravan *et al.*, 2012). Many treatments are developed to target and disrupt the growth of fungal pathogens, however there are limited processes which are distinctly fungal which will not have adverse effects for a patient (Kathiravan *et al.*, 2012). Furthermore, studies have shown that this places a high degree of selective pressure on the pathogen which further leads to the emergence of resistant strains (Clatworthy *et al.*, 2007). Thus, with the innate problems associated with targeting pathogen specific processes, an alternative approach lies in that of targeting the pathogen specific virulence factors (Ahmad *et al.*, 2020). Virulence factors are defined as the structures, molecules or systems utilised by a pathogen to infect and colonise a host (Sharma *et al.*, 2017). While targeting the activity of virulence factors may not directly lead to the eradication of the pathogen, it may influence the pathogenesis which can play a part in lessening the severity of disease (Clatworthy *et al.*, 2007). One such virulence factor is the ability of microorganisms to form biofilms.

Biofilms are defined as structured communities of microorganisms that have the ability to stick not only to surfaces, but also to each other (Ramage *et al.*, 2012). An important feature of biofilms is the ability of a microbial community to produce a protective extracellular matrix (ECM) that allows for several advantages, including protection from the environment and physical or chemical stressors (López *et al.*, 2010; Ramage *et al.*, 2012). As such, biofilms have been associated with playing a key role in fungal resistance for some clinically important fungi including *Candida* and *Cryptococcus* species (Kernien *et al.*, 2018). Additionally, some studies have demonstrated that biofilm formation is a crucial factor involved with persistent fungal infections and that it even plays a critical role in the shifting of superficial infections to invasive or systemic infections (Andes *et al.*, 2012; Ala-Houhala and Anttila, 2020).

Biofilms pose such a challenge as they demonstrate increased antimicrobial tolerance and are able to evade host immune responses (Roilides *et al.*, 2015). Studies show that biofilms exhibit much higher rates of resistance when compared to their planktonic counterparts (Sherry *et al.*, 2017; Hawser and Douglas, 1995). Often, higher concentrations of compounds are required to be effective against biofilms when compared to that of planktonic cells (Melo *et al.*, 2011; Ramage *et al.*, 2012). This increased tolerance is in large part due to the ECM which is able to act as a physical barrier between the antimicrobial and the microorganisms, but also the resistance strategies that would be implemented by planktonic cells are amplified due to the increased cell density of the biofilm structure (Ramage *et al.*, 2012; Perumal *et al.*, 2007). The

increased resistance of the biofilms results in higher concentrations of the antifungal drugs being required for effective activity, however, treatment at the increased concentrations can be unsafe (Ramage *et al.*, 2001; Ramage *et al.*, 2002; Pierce *et al.*, 2013).

Not only do biofilms play a role in the infection process but they also have a propensity to colonise abiotic surfaces, including medical equipment and devices routinely used in hospital settings (Harriott *et al.*, 2010; Lebeaux *et al.*, 2014). This poses a problem as the formation of biofilms on these devices is highly correlated with dissemination of the pathogen leading to bloodstream infections with high associated mortality rates (Donlan, 2001; Tumbarello *et al.*, 2007). In fact, estimates show almost 80% of patients with invasive candidiasis to have had indwelling medical devices, thus a high correlation exists between biofilm formation and increased infection severity (Andes *et al.*, 2012). The treatment of fungal infections becomes incredibly difficult once a mature biofilm has formed. For indwelling devices, treatment opportunities are almost non-existent with the only course of action being removal of the affected device (Kojic and Darouiche, 2004).

Considering the important role that biofilms play in establishing and increasing the severity of disease, relatively little research has been done to elucidate treatments with antibiofilm properties. Much of the research focuses on assessing the activity of developing treatments against planktonic cells and does not further evaluate how and if they would also be effective against biofilms. With the increasing number of immunocompromised individuals who are required to be in a hospital setting or those who will require the aid of medical devices, it is important to reduce the risk of adverse outcomes by having effective therapeutic treatments readily available to treat biofilms. With the current opportunities to treat biofilms severely lacking, it is imperative to search for novel treatments that can either disrupt biofilm formation or prevent it altogether.

Silver and silver-based compounds were utilised as effective antimicrobials before the introduction of antibiotics (Wlodkowski and Rosenkranz, 1973; Clement and Jarrett, 1994). With the rise of microbial resistance, interest in their activity has been renewed in the search for novel treatments and several studies have shown that they possess promising antifungal activity (Gandra *et al.*, 2017; Panáček *et al.*, 2009; Berners-Price *et al.*, 1988). There has been

some research with silver-based compounds that suggests they possess a degree of antibiofilm activity, however most of this research was performed against bacterial biofilms (Mohanta *et al.*, 2020; Kostenko *et al.*, 2010; Markowska *et al.*, 2013). Far less research has been done to determine the role of silver against a range of fungal biofilms with most studies focussing on *C. albicans* biofilms (Ahamad *et al.*, 2022; Lara *et al.*, 2015). Similarly, plant based compounds once acted as a primary source of medicines for treatment and are still utilised today in developing and rural areas (Salim *et al.*, 2008). There is great abundance in the variety of phytochemicals and compounds produced by plants which have already exhibited antifungal activity and promise as a possible treatment of fungal infections (Olawuwo *et al.*, 2022; Andriani *et al.*, 2021; Bonifacio *et al.*, 2019; Suurbaar *et al.*, 2017). As is the case with silver, few plant extracts have been shown to exhibit antibiofilm activity against fungal biofilms (Guimarães *et al.*, 2021; Meccatti *et al.*, 2021) however, the majority of research investigates bacterial and *C. albicans* biofilms (Famuyide *et al.*, 2019; Olawuwo *et al.*, 2022).

As such, there is a need to not only screen compounds for their antibiofilm activity, but also screen their activity across a wider range of fungal pathogens. To aid in the endeavour, the use of a variety of synthetic and natural products will be tested against various *Candida* and *Cryptococcus* biofilms. This will be done by testing a novel silver (I)-phosphine complex as well the ethanolic *Kigelia africana* extracts for their ability to disrupt and prevent the formation of biofilms. With changes to the overall fungal mass of the biofilms as well as the metabolic activity of the cells in the biofilms being assessed.

3.3 Aims and Objectives

To determine the antibiofilm activity of the novel silver (I)-phosphine complex 7 (SPC 7) and *Kigelia africana* extract against fungal pathogens within the *Candida* and *Cryptococcus* genera.

1. Determine if the compounds inhibit or degrade biofilms by assessing the density of the treated fungal cells using the crystal violet assay.
2. Determine if the compounds inhibit or degrade biofilms by assessing the metabolic activity of the treated fungal cells using the XTT assay.

3.4 Methods and Materials

3.4.1 Maintenance of Yeast Isolates

Seven test organisms from the fungal genera *Candida* and *Cryptococcus* (Table 3.1) were utilised during this study to determine the antifungal effects of the compounds. The *Candida auris* strains were kindly provided by Dr Aijaz Ahmad (Department of Clinical Microbiology and Infectious Diseases; The University of the Witwatersrand) (NICD ethics approval number M140159) and the remaining isolates were retrieved from the culture collection of Dr Angela Botes (ABCC; School of Molecular and Cell Biology; The University of the Witwatersrand). All test organisms were sub-cultured on Sabouraud dextrose agar (SDA) and incubated at 37°C before use. *Candida* strains were grown for 48 hours while *Cryptococcus* strains were grown for 72 hours.

Table 3.2: Pathogenic Fungal Strains. A range of pathogenic fungal isolates chosen for their clinical relevance from the *Candida* and *Cryptococcus* genera.

Culture collection number	Fungal Strain Name	Source
eABCC239	*Candida auris* (intermediate resistance to AmB – 1 µg/mL)	Dr. Aijaz Ahmad
eABCC240	*Candida auris* (low resistance to AmB – 0.25 µg/mL)	Dr. Aijaz Ahmad
eABCC241	*Candida auris* (high resistance to AmB – 4 µg/mL)	Dr. Aijaz Ahmad
eABCC15	*Cryptococcus deneoformans*	Dr. Angela Botes
eABCC50	*Cryptococcus gattii*	Dr. Angela Botes
eABCC16	*Cryptococcus neoformans*	Dr. Angela Botes
eABCC51	*Cryptococcus tetragattii*	Dr. Angela Botes

AmB – Amphotericin B. AmB resistance values calculated by the NICD. NICD ethics approval number for *Candida auris* strains: M140159.

3.4.2 Preparation of Silver (I)-phosphine Complex 7

The silver-phosphine complex 7 (SPC 7) was synthesised at The University of Johannesburg and was kindly provided by Dr. Zelinda Engelbrecht and Prof. Marianne Cronjé (School of Molecular and Cell Biology; The University of the Witwatersrand). The silver compound was dissolved in sterile distilled water and was stored at 4°C in the dark until needed. When required, it was heated to 70°C for 30 minutes before use.

3.4.3 Preparation of Plant Extracts

Dried plant material of *Kigelia africana* was obtained from Medico Herbs (https://medicoherbs.co.za/). The plant material was ground into a fine powder using a standard mortar and pestle. The extracts were prepared by combining the ground plant material with ethanol in a 1:10 ratio whereby 10 mL of solvent was mixed with 1 g of plant material (Ahmad *et al.*, 2020). The mixture was vortexed vigorously for five minutes and left to stand undisturbed for one hour at room temperature to allow for extraction. The mixture was then centrifuged for 10 minutes at 1400 × g at room temperature. The supernatants of the mixture were filtered through Whatman No. 1 filter paper into pre-weighed 50 mL Falcon tubes. The filtrate was left to air-dry at room temperature with the aid of a fan to expedite the evaporation process. Once the extract was dry, the Falcon tubes were weighed, and the extract was resuspended in distilled sterile water to a concentration between 150 – 200 mg/mL. The *K. africana* extracts were stored at 4°C in the dark until needed.

3.4.4 Preparation of Test Compounds

The test compounds were diluted in fresh minimal media (MM) (30 mM glucose, 20 mM magnesium sulphate, 58.8 mM potassium dihydrogen phosphate, 26 mM glycine, 330 mM 3-(N-morpholino)propanesulfonic acid and 6 μM thiamine hydrochloride) with a range of concentrations for each compound being evaluated (Table 3.2). The concentration ranges were chosen based on the minimum fungicidal concentrations (MFC) that had previously been determined (Chapter 2). The MFC and two sub-minimum fungicidal concentrations for each fungal strain were evaluated to assess for a dose-dependant relationship. Of the plant extracts, only *K. africana* was utilised in this experiment as it was the only extract to exhibit fungicidal

activity against the *Cryptococcal* fungal isolates. No plant extracts were tested against the *Candida* species as no MFCs could be determined. Amphotericin B was used a positive control.

Table 3.2: Antifungal compound concentrations. A summary of the concentration ranges tested against the biofilms based on the minimum fungicidal concentrations determined in Chapter 2.

	Treatment								
Fungal Strain	Amphotericin B (µg/mL)			Silver (I)-phosphine complex 7 (mg/mL)			*Kigelia africana* (mg/mL)		
Candida auris (eABCC239)	8	4	2	2	1	0.5	ND	ND	ND
Candida auris (eABCC240)	8	4	2	2	1	0.5	ND	ND	ND
Candida auris (eABCC241)	8	4	2	2	1	0.5	ND	ND	ND
Cryptococcus deneoformans (eABCC15)	4	2	1	4	2	1	40	20	10
Cryptococcus gattii (eABCC50)	4	2	1	2	1	0.5	80	40	20
Cryptococcus neoformans (eABCC16)	2	1	0.5	1	0.5	0.25	40	20	10
Cryptococcus tetragattii (eABCC51)	4	2	1	8	4	2	80	40	20

ND – Not Determined.

3.4.5 Biofilm Formation

Biofilm formation was performed according to work that was previously done with some modifications (Martinez and Casadevall, 2015; Melo *et al.*, 2011). The fungal isolates were grown as previously described in section 3.4.1. A colony of each culture was added to 5 mL of yeast peptone dextrose (YPD) broth and was left to incubate on a rotary wheel for 24 hours at 37°C. The cells were harvested by centrifugation at 5000 × g for five minutes at room temperature. The supernatant was discarded, and the pellet was washed twice with phosphate buffered saline (PBS)(pH 7). The pellet was resuspended in MM and a working solution was prepared by adjusting the cell concentration to 1×10^7 cells/mL using a haemocytometer and diluting the cells in MM. An aliquot of 100 µL of the adjusted cell suspension was added to the wells of a flat bottomed 96-well plate. The negative control wells contained an aliquot of only MM, while the positive control wells contained MM and the fungal cells. The 96-well plates were sealed with parafilm, wrapped in foil and left to incubate at 37°C with shaking at 80 rpm for 24 hours. Following the incubation period, the unattached cells were aspirated and each well was washed with 100 µL of PBS. An aliquot of 100 µL of fresh MM was added to the wells and the plate was resealed and left to incubate at 37°C with shaking at 80 rpm for 72 hours to allow for biofilm formation.

3.4.5.1 Disrupting Biofilm Formation

Following the successful formation of the biofilm, the compounds were tested for their ability to disrupt the pre-formed biofilm. After the incubation of the biofilms for the 72 hour period, the wells were aspirated to remove the unattached cells and washed with 100 µL of PBS. The test compounds were prepared as previously described (section 3.4.4) and 100 µL of each was added to the wells. The plates were resealed with parafilm, wrapped in foil and were incubated at 37°C with shaking at 80 rpm for a further 72 hours (Melo *et al.*, 2011).

3.4.5.2 Inhibiting Biofilm Formation

In addition to assessing if the compounds can disrupt a pre-formed biofilm, the ability of the compounds to inhibit the formation of a biofilm was also tested. In this assay the test compounds (prepared as described in section 3.4.4) were added at the same time as the initial cell suspension and not to a pre-formed biofilm. Following a 24 hour incubation the unattached

cells were aspirated from each well. A 100 µL aliquot of each compound was added to the wells once again. The plates were resealed with parafilm and covered in foil and left to incubate at 37°C with shaking at 80 rpm for a further 72 hours (Melo *et al.*, 2011).

3.4.6 Crystal Violet Assay

Crystal violet (CV) is a purple-coloured stain commonly used for biofilm quantification (Feoktistova *et al.*, 2016). It can be used to stain negatively charged molecules and cells present in the biofilm matrix, to indirectly determine the optical density (OD) of the biofilm (Feoktistova *et al.*, 2016). The CV assay was performed according to work previously done by Melo and colleagues (2011) with some modifications (Melo *et al.*, 2011). Following biofilm formation, the wells were aspirated and washed with 100 µL of PBS. The plate was left to dry for 20-30 minutes before it was stained with a 0.04% (w/v) CV solution (Sigma-Aldrich, United States). The CV solution was freshly prepared and a 110 µL aliquot was added to each well. The plates were incubated for 45 minutes at room temperature to allow for staining of the biofilms. Following staining, the CV solution was removed from the wells and each well was thoroughly washed with 200 µL of PBS at least three times or until no trace of the CV stain could be observed washing off the biofilm. The biofilms were then destained for 45 minutes at room temperature by adding 100 µL of 95% (v/v) ethanol to each well. Subsequently, the ethanol was transferred into new wells after the destaining period. The absorbance of the ethanol solution was measured at a wavelength of 595 nm using a Victor Novo plate reader (PerkinElmer, United States of America). The absorbance readings of the wells treated with the compounds was compared to that of the untreated wells as well as the controls to determine if the extracts inhibited biofilm formation.

3.4.7 XTT Assay

The XTT (sodium 3´-[1- (phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate) assay can be used as a method of measuring the metabolic activity of fungal cells within biofilms (Kuhn *et al.*, 2003). This reagent is a tetrazolium salt that is converted to a coloured formazan product in the presence of metabolically active fungal cells (Kuhn *et al.*, 2003). Following biofilm formation, the wells were aspirated to remove any unattached cells and washed with 100 µL of PBS. After leaving the plate to dry for

20 - 30 minutes, a 50 µL aliquot of fresh MM was added to the wells. The XTT reagent was prepared as per the manufacturer's instructions (Thermo Fisher Scientific, United States of America). Briefly, the XTT component and the electron coupling reagent (N-methyl dibenzopyrazine methyl sulfate) were thawed from -20°C to room temperature and then vortexed. A 1 mL aliquot of the electron coupling reagent was added to 6 mL of the XTT solution and vortexed vigorously. The solution was used immediately after preparation with a 70 µL aliquot being added to each well. The plates were incubated at 37°C with shaking at 80 rpm for 4 hours. Following the incubation period, the absorbance of the wells was measured using a Victor Novo plate reader (PerkinElmer, United States of America). The absorbance was measured at 450 nm as well as 660 nm to account for background interference. The following formula was used to determine the specific absorbance for each well:
Specific absorbance [[Abs_{450nm} (Test) - Abs_{450nm} (Blank)] - Abs_{660nm} (Test)].

3.4.8 Statistical Analysis

All statistical analyses were performed using Microsoft Office Excel 365. The standard error of the mean (SEM) was calculated for all samples. A Student's T-test was used to determine if the biofilm growth of the untreated control samples was statistically different when compared to that of the treated samples (De Winter, 2013). Samples that included P-values < 0.05 were considered statistically significant in determining whether treatment with compounds was effective.

3.5 Results

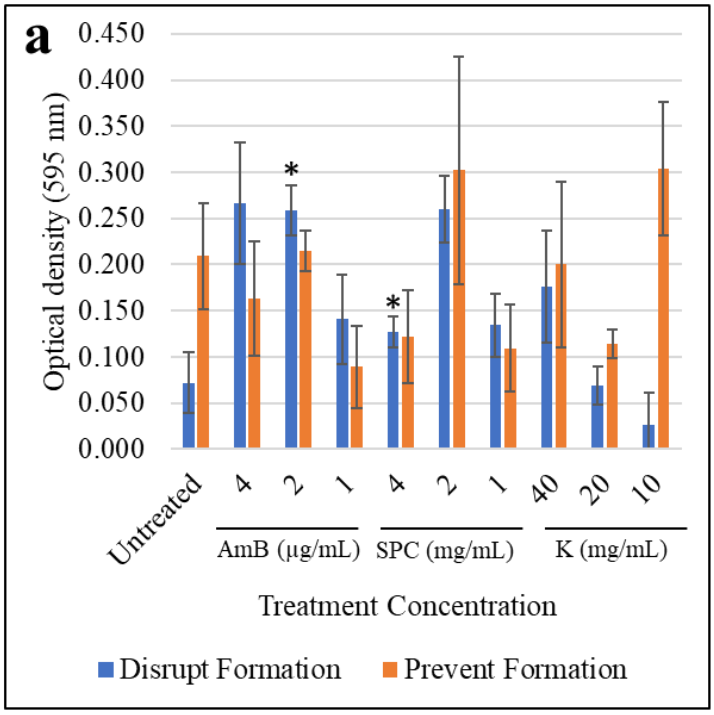
Biofilms play an important role in the pathogenesis of fungal infections (Clatworthy *et al.*, 2007). Thus, it would be beneficial to develop treatments that have antibiofilm properties. Various methods were implemented to determine if the test compounds were able to disrupt a pre-formed biofilm as well as their ability to prevent the formation of the biofilm altogether. The CV assay was used to quantify the relative biomass of the treated and untreated biofilms (Feoktistova *et al.*, 2016) while the XTT assay was used to determine the relative metabolic activity of the fungal cells that made up the biofilms (Kuhn *et al.*, 2003).

3.5.1 CV Assay

The CV assay serves as a way to quantify the relative biomass of each biofilm following exposure to the compounds (Feoktistova *et al.*, 2016). The more cells present in each biofilm, the higher the degree of cell staining which results in an increased absorbance reading (Melo *et al.*, 2011). The results showed that none of the test compounds were able to significantly decrease the biomass of the biofilms when compared to the untreated control (Figure 3.1). This was also observed with amphotericin B (Figure 3.1).

The results show that treatment with the *K. africana* extract did not significantly decrease the biomass of the *Cryptococcus* biofilms (Figure 3.1a – d). Notably, it can be seen that at concentrations of 40 and 80 mg/mL, the *K. africana* extract caused a significant increase, rather than decrease, in the biofilm formation of *C. neoformans, C. gattii*, and *C. tetragattii* when compared to the untreated control (Figure 3.1b - c).

In the *Cryptococcus* biofilms, treatment with the SPC 7 is not seen to significantly decrease the overall biomass of the biofilms (Figure 3.1a – d). For the *Candida* biofilms, the SPC 7 is seen to slightly decrease the number of cells in the treated biofilms when compared to the untreated control (Figure 3.1e, g) however it was not a significant decrease ($p < 0.05$). Additionally, treatment of some of the *Candida* biofilms with SPC 7 showed that lower concentrations of SPC 7 better inhibited the formation of biofilms (Figure 3.1f, g). From these results, the compounds are not able to significantly disrupt biofilms or prevent biofilm formation at the tested concentrations.


a
Optical density (595 nm)
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
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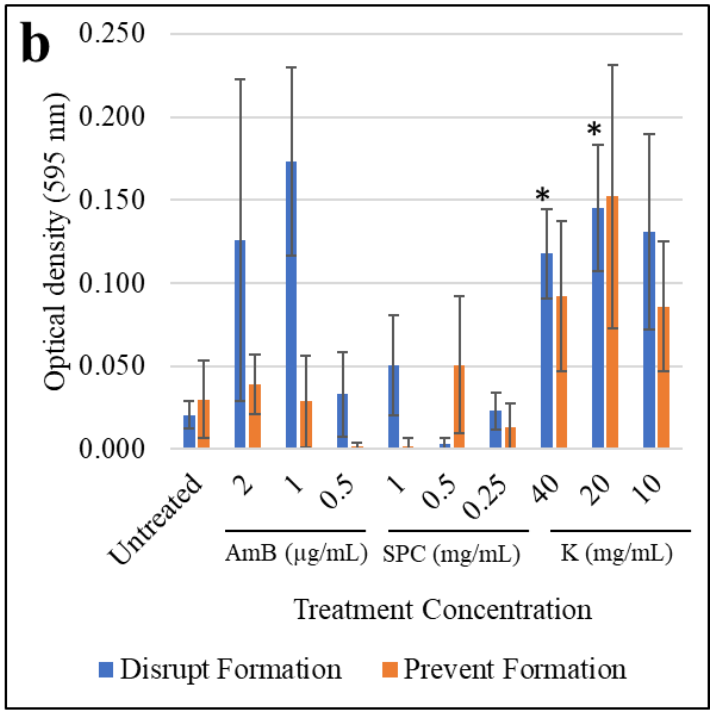
Untreated
4
2
1
4
2
1
40
20
10
AmB (µg/mL)
SPC (mg/mL)
K (mg/mL)
Treatment Concentration
Disrupt Formation
Prevent Formation


b
Optical density (595 nm)
0.250
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Untreated
2
1
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AmB (µg/mL)
SPC (mg/mL)
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Treatment Concentration
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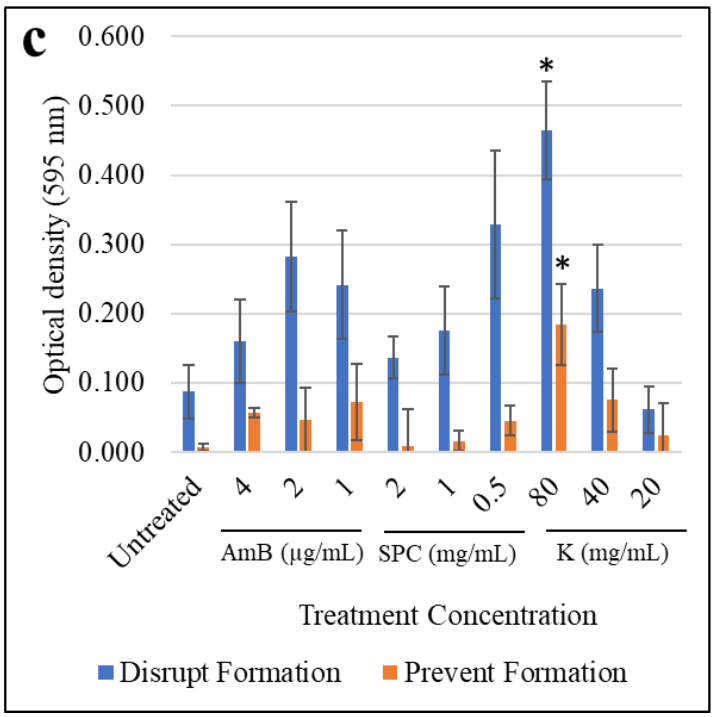
Prevent Formation


c
Optical density (595 nm)
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0.400
0.300
0.200
0.100
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Untreated
4
2
1
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1
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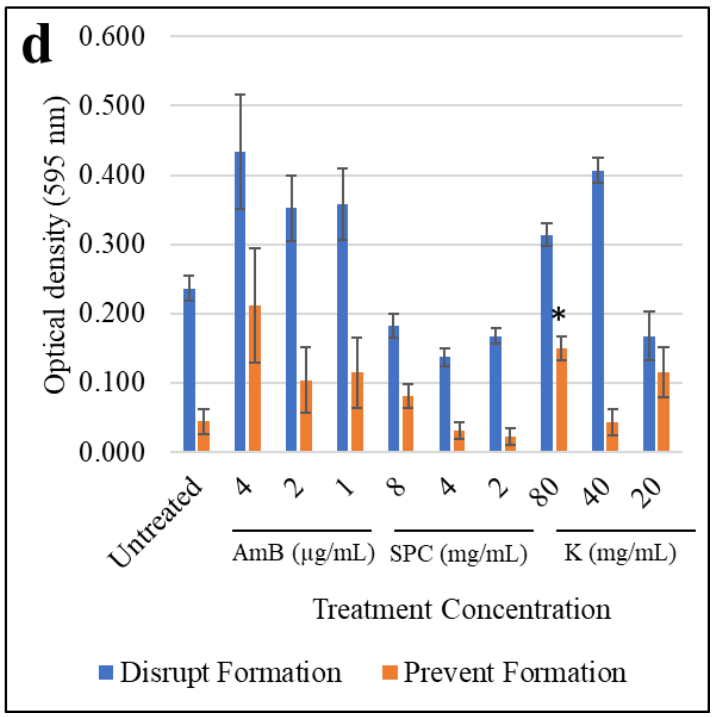
80
40
20
AmB (µg/mL)
SPC (mg/mL)
K (mg/mL)
Treatment Concentration
Disrupt Formation
Prevent Formation


d
Optical density (595 nm)
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Untreated
4
2
1
8
4
2
80
40
20
AmB (µg/mL)
SPC (mg/mL)
K (mg/mL)
Treatment Concentration
Disrupt Formation
Prevent Formation

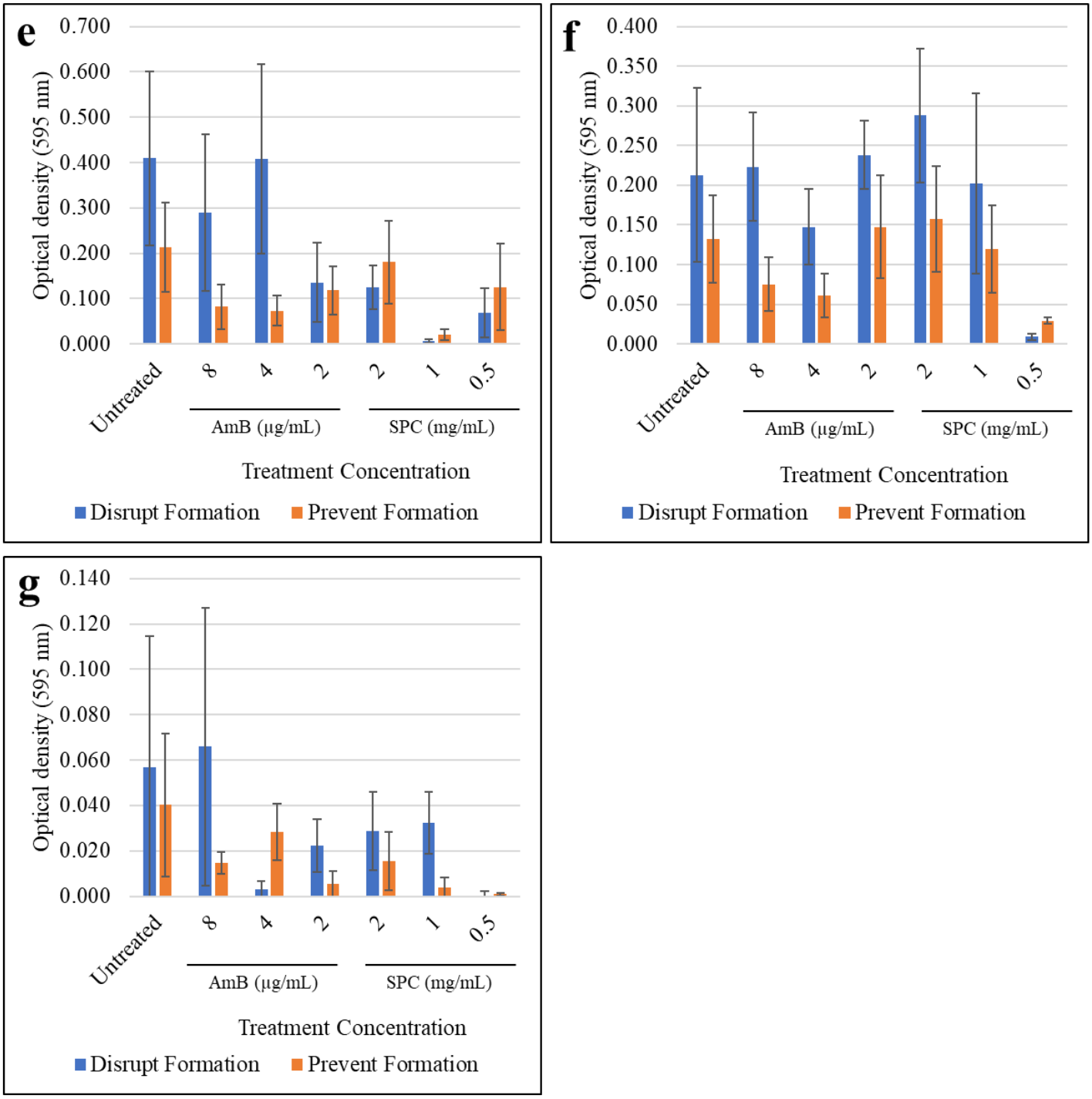


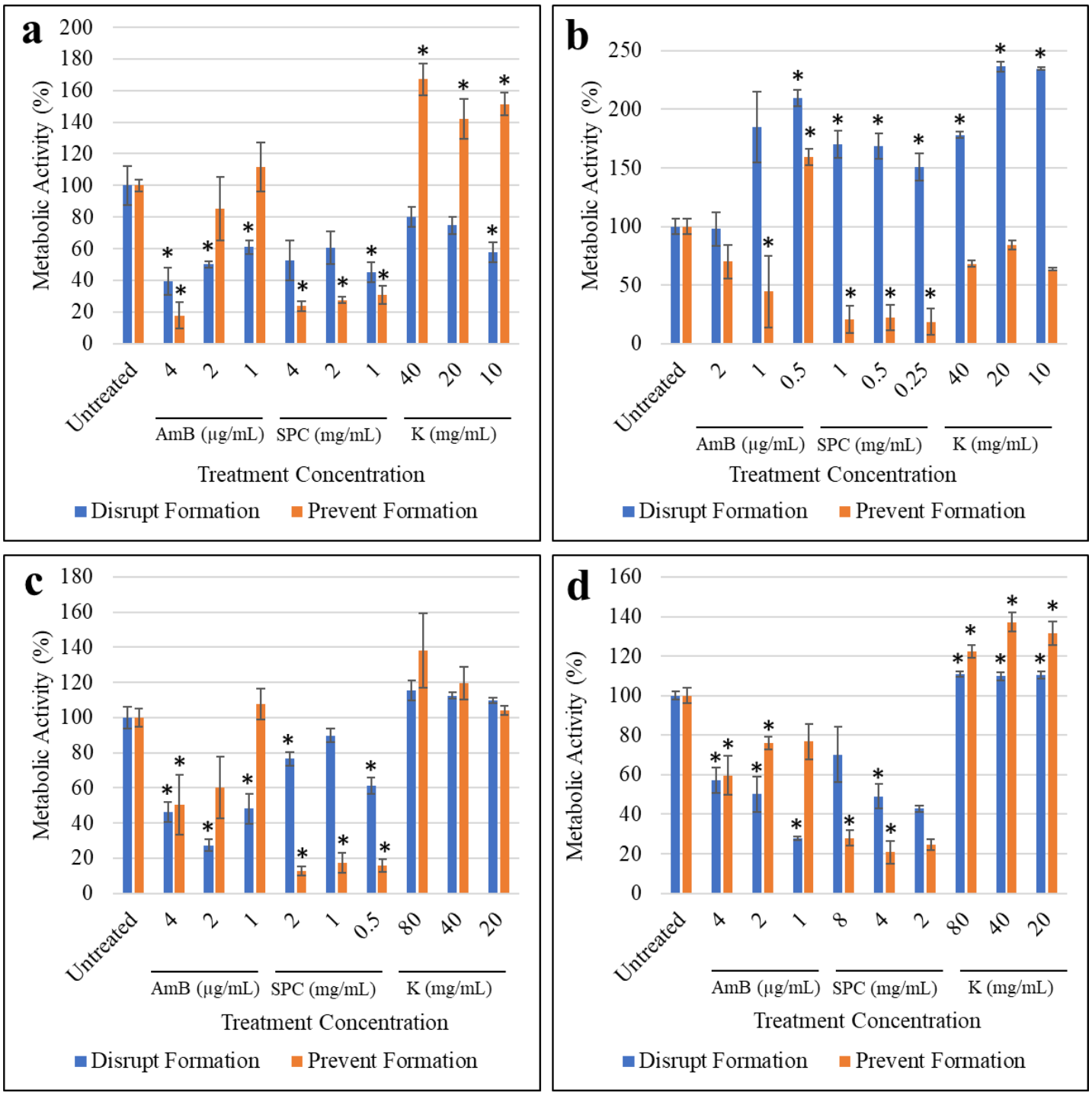
Figure 3.1: Crystal violet (CV) assay. The compounds were tested for their ability to disrupt preformed biofilms (blue) and prevent their formation (orange). Following the formation of the biofilms under the various conditions, the biofilms were stained with 0.04% CV solution and destained with 95% ethanol. The absorbance of the ethanol solution was measured using the spectrophotometer at a wavelength of 595 nm. The higher the absorbance reading, the higher the number of cells present in the biofilm. AmB – Amphotericin B; SPC – Silver (I)-phosphine complex 7; K – *Kigelia africana*. (a) *C. deoneoformans* (eABCC15); (b) *C. neoformans* (eABCC16); (c) *C. gattii* (eABCC50); (d) *C. tetragattiii* (eABCC51); (e) *C. auris* (eABCC239); (f) *C. auris* (eABCC240); (g) *C. auris* (eABCC241). (*) indicates significant difference $p < 0.05$. The error bars represent the standard error from of mean (n = 3).

3.5.2 XTT Assay

The XTT assay gives insight into how the compounds affect the metabolic activity of the treated fungal cells (Kuhn *et al.*, 2003). The results demonstrated that when SPC 7 is used to prevent the formation of the biofilms, there is a significant decrease in the metabolic activity of all the test species ($p < 0.05$) (Figure 3.2). The SPC 7 is also seen to cause decreased metabolic activity across the various strains when used to treat pre-formed biofilms (Figure 3.2), except in *C. neoformans* (Figure 3.2b) where an increase in the activity is observed.

The *K. africana* extract had varying effects on the metabolic activity of the *Cryptococcus* species (Figure 3.2a-d). When the extract was used to treat the pre-formed biofilms, an increase in the metabolic activity was observed in all the *Cryptococcus* species (Figure 3.2b - d) except for *C. deneoformans* (Figure 3.2a). Significant increases in activity were observed in *C. neoformans* as well as *C. tetragattii* across their relevant concentration ranges ($p < 0.05$). When treated with 10 mg/mL of the extract a significant decrease in the activity of *C. deneoformans* is observed ($p < 0.05$). Additionally, when the extract was used to prevent the formation of the biofilms, an increase in the metabolic activity is seen in all the strains except for *C. neoformans* (Figure 3.2b). Significant increases in metabolic activity are seen in *C. deneoformans* and *C. tetragattii* across the concentration ranges ($p < 0.05$). The *K. africana* extract showed an overall trend of increasing the metabolic activity of the biofilms when both disrupting pre-formed biofilms as well as preventing the formation of biofilms.

Treatment of the pre-formed *Cryptococcus* biofilms with amphotericin B showed there to be significant decreases in the metabolic activity of *C. deneoformans*, *C. gattii* and *C. tetragattii* across the concentration range (1 – 4 µg/mL) ($p < 0.05$) (Figure 3.2a, c, d). When amphotericin B was used to prevent the formation of the biofilms, decreases in the metabolic activity were observed for all the *Cryptococcus* strains (Figure 3.2a – d). Amphotericin B was also seen to cause significant decreases in the metabolic activity when added before and after the formation of the biofilms in *C. auris* (eABCC239) ($p < 0.05$) (Figure 3.2e). The other *Candida* strains showed that when amphotericin B is used to prevent biofilm formation, significant decreases in metabolic activity are observed at a concentration of 8 µg/mL (Figure 3.2f, g).


a
Metabolic Activity (%)
200
180
160
140
120
100
80
60
40
20
0
Untreated
4
2
1
4
2
1
40
20
10
AmB (µg/mL)
SPC (mg/mL)
K (mg/mL)
Treatment Concentration
Disrupt Formation
Prevent Formation
b
Metabolic Activity (%)
250
200
150
100
50
0
Untreated
2
1
0.5
1
0.5
0.25
40
20
10
AmB (µg/mL)
SPC (mg/mL)
K (mg/mL)
Treatment Concentration
Disrupt Formation
Prevent Formation
c
Metabolic Activity (%)
180
160
140
120
100
80
60
40
20
0
Untreated
4
2
1
2
1
0.5
80
40
20
AmB (µg/mL)
SPC (mg/mL)
K (mg/mL)
Treatment Concentration
Disrupt Formation
Prevent Formation
d
Metabolic Activity (%)
160
140
120
100
80
60
40
20
0
Untreated
4
2
1
8
4
2
80
40
20
AmB (µg/mL)
SPC (mg/mL)
K (mg/mL)
Treatment Concentration
Disrupt Formation
Prevent Formation

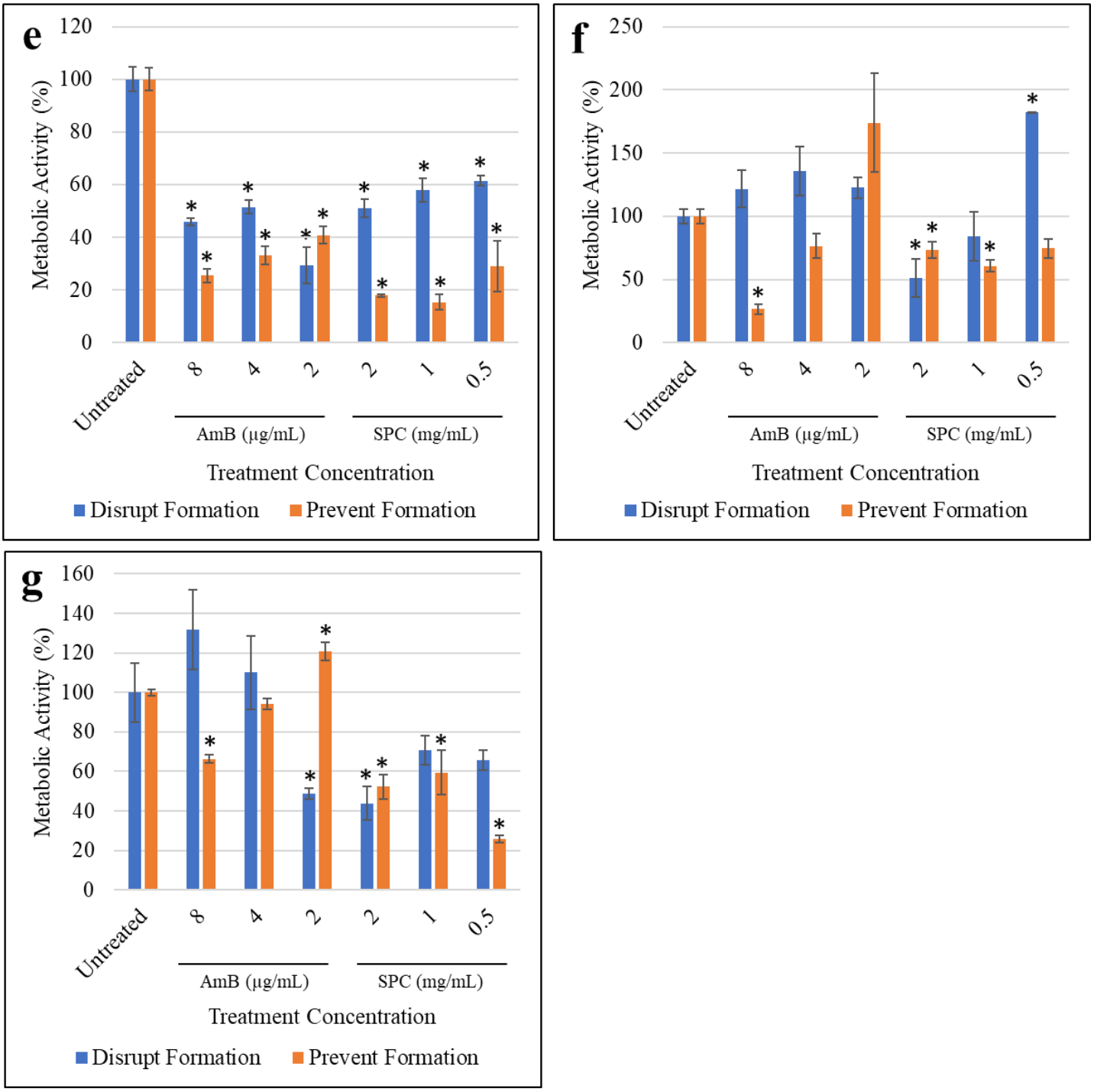


Figure 3.2: XTT assay. The compounds were tested for their ability to disrupt preformed biofilms (blue) and prevent their formation (orange). Following the formation of the biofilms under the various conditions, the XTT reagent was added to each well and incubated for 4 hours. The absorbance was measured at 450 nm and 660 nm to account for interference. The metabolic activity (%) was adjusted in relation to the untreated control. AmB – Amphotericin B; SPC – Silver (I)-phosphine complex 7; K – *Kigelia africana*. (a) *C. deoneoformans* (eABCC15); (b) *C. neoformans* (eABCC16); (c) *C. gattii* (eABCC50); (d) *C. tetragattiii* (eABCC51); (e) *C. auris* (eABCC239); (f) *C. auris* (eABCC240); (g) *C. auris* (eABCC241). (*) indicates significant difference $p < 0.05$. The error bars represent the standard error of the mean (n = 3).

In the CV assays, the varying compound concentrations did not influence the results in a dose dependant manner as expected. That being that the highest concentration of each compound would result in the formation of a biofilm with the lowest biomass and thus lowest optical density. Treatment with SPC 7 showed this trend in *C. gattii* (Figure 3.1c) when disrupting the pre-formed biofilm however the opposite effect was seen in *C. auris* (eABCC240), where the highest concentration of the compound resulted in a higher biomass (Figure 3.1f). Similarly, when preventing the formation of the biofilms in some *Candida* strains, the higher the concentration of SPC 7, the higher the biomass. The concentration for treatment with the *K. africana* extract did not show any patterns except for *C. gattii* which also showed that a higher biofilm biomass formed in the presence of a high extract concentration (Figure 3.1c). Lastly, amphotericin B treatment concentration did not seem to influence the resulting biomass of any of the strains.

Similarly, no significant concentration dependant trends were observed in the metabolic activity assays. However of note, treatment with amphotericin B did show to effect metabolic activity in a dose dependent manner when preventing biofilm formation in all the strains (Figure 3.2). Whereby the highest concentration of amphotericin B resulted in the lowest degree of metabolic activity. This was expected as amphotericin B is a known antifungal.

In general, the compounds did not seem to decrease the relative biomass of the biofilms however, the XTT assays showed that amphotericin B and SPC 7 were able to decrease the metabolic activity of most of the biofilms. The *K. africana* extract displayed mixed effects with a general trend towards increasing the metabolic activity of the *Cryptococcus* biofilms following treatment.

3.6 Discussion

The development of biofilms plays a critical role in establishing persistent and invasive fungal infections (Andes *et al.*, 2012; Ala-Houhala and Anttila, 2020). Many studies have shown that biofilms are often more resistant to common antifungals than their planktonic counterparts (Sherry *et al.*, 2017; Hawser and Douglas, 1995). With the small arsenal of antifungal drugs available this highlights the need for finding novel treatments which not only possess inhibitory properties but also antibiofilm properties.

3.6.1 Antibiofilm Activity of Amphotericin B

Amphotericin B has long been considered the gold standard of antifungal therapies due to its broad-spectrum fungicidal activity (Sarosi, 1990). Previous studies have shown that amphotericin B does possess antibiofilm properties against both *Candida* (Mahmoudabadi *et al.*, 2014) and *Cryptococcus* (Martinez and Casadevall, 2006) species. In a study done by Martinez and Casadevall (2006) it was found that *C. neoformans* biofilms were susceptible to amphotericin B, showing reductions in the metabolic activity of the treated biofilms (Martinez and Casadevall, 2006). Similarly, most of the *Cryptococcus* biofilms used in this study demonstrated that treatment with amphotericin B also decreases the metabolic activity of pre-formed *C. deneoformans*, *C. gattii* and *C. tetragattii* biofilms at concentrations of 1 – 4 µg/mL (Figure 3.2a, c, d). Notably however, reductions in the metabolic activity of treated pre-formed *C. neoformans* biofilms was not observed in this study, but rather an increase in metabolic activity (Figure 3.2b).

In the same study by Martinez and Casadevall (2006), it was seen that amphotericin B treated *C. neoformans* biofilms also showed significant reductions in biofilm formation at a concentration of 0.5 µg/mL (Martinez and Casadevall, 2006). In contrast, this study found no significant decreases in the formation of biofilms even at the highest concentration assessed of 4 µg/mL (Figure 3.1a – d). However, a study by Brilhante and colleagues (2020) found amphotericin B to only cause significant decreases in both the biomass and metabolic activity of *C. neoformans* and *C. gattii* species at around 32 µg/mL (Brilhante *et al.*, 2020). Thus, it may be possible that higher concentrations of amphotericin B should have been utilised in this study in order to see significant reductions in the biomass of the *Cryptococcus* isolates.

In general, amphotericin B showed to decrease the biomass of the *Candida* biofilms when preventing formation, however it did not work well to disrupt the pre-formed biofilms (Figure 3.1e - g). Additionally, amphotericin B was seen to significantly decrease the metabolic activity when preventing biofilm formation (Figure 3.2e - g) however it only significantly decreased the metabolic activity of the pre-formed biofilm of *C. auris* (eABCC239) (Figure 3.2e). This is in line with studies that show amphotericn B is able prevent biofilm formation in *Candida* isolates such as *C. albicans* (Mahmoudabadi *et al.*, 2014) and *C. auris* (Chatzimoschou *et al.*, 2021). Previous studies have found that *C. auris* biofilms were inhibited by amphotericin B at concentrations between 4 – 2048 µg/mL when also using the XTT assay (Chatzimoschou *et al.*, 2021). This correlates with the data observed in this study, where reductions in the metabolic activity of the biofilms were seen at 8 µg/mL (Figure 3.2e - g). Amphotericin B was not seen to significantly decrease the overall biomass of the *Candida* biofilms in this study however, other studies show significant decreases in the biofilm biomass from around 4 µg/mL (Sherry *et al.*, 2017).

For both *Candida* and *Cryptococcus*, significant reductions in the biomass of the amphotericin B treated biofilms were not observed despite the literature suggesting otherwise (Sherry *et al.*, 2017; Brilhante *et al.*, 2020). It is well known that biofilms have higher levels of resistance than their planktonic counterparts (Sherry *et al.*, 2017; Hawser and Douglas, 1995). As such, higher concentrations of compounds are required to be effective against biofilms when compared to that of the same planktonic cells (Melo *et al.*, 2011; Ramage *et al.*, 2012). This increased tolerance is in large part due to the ECM which is able to act as a physical barrier between the antimicrobial and the microorganisms (Ramage *et al.*, 2012). Additionally, resistance strategies that would be implemented by planktonic cells such as altering drug-target affinity, overexpression of membrane transport proteins for drug efflux, increased stress responses, and inducing fungal cell wall changes to reduce absorption of drugs are amplified due to the increased cell density of the biofilm structure are amplified due to the increased cell density of the biofilm structure (Ramage *et al.*, 2012; Perumal *et al.*, 2007; Íñigo and Del Pozo, 2018). This study evaluated the antibiofilm activity using sub-fungicidal concentrations that were calculated in Chapter 2 (Table 3.2). However, it may be possible that higher concentrations of amphotericin B should have been utilised in this study to see significant inhibition of biofilm formation in the *Candida* and *Cryptococcus* isolates.

3.6.2 Antibiofilm Activity of SPC 7

Silver and silver nanoparticles have been shown to have some degree of antibiofilm activity against bacterial strains such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Mohanta *et al.*, 2020; Bottero *et al.*, 2013; de Lacerda Coriolano *et al.*, 2021). There have been studies that also show the antibiofilm properties of silver-based molecules against *Candida* biofilms such as *C. albicans* and *C. glabrata* (de Lacerda Coriolano *et al.*, 2021; Monteiro *et al.*, 2012). Despite the prevalence of *Cryptococcal* and *C. auris* biofilms there is much less research investigating the antibiofilm role of silver-based products against them.

There has been limited research done with *C. auris* biofilms that shows some silver-based compounds can prevent the formation of biofilms (AlJindan and AlEraky, 2022; Lara *et al.*, 2020). Some studies demonstrate biofilm inhibition and degradation in the range of 20 – 25 µg/mL using a variety of silver nanoparticles (Ahamad *et al.*, 2022; Miškovská *et al.*, 2022). Even though a higher concentration range of 0.5 – 2 mg/mL was utilised in this study, the compound did not significantly disrupt or prevent the formation the *C. auris* biofilms (Figure 3.1e – g). This was also seen in the case of the *Cryptococcus* biofilms where SPC 7 did not significantly disrupt or prevent the formation of the biofilms (Figure 3.1a – d). As was the case with amphotericin B, this may be as a result of the concentration range that was used in this study being too low to inhibit biofilm formation.

The metabolic activity of all *Cryptococcus* strains is seen to be reduced when SPC 7 is used to prevent biofilm formation (Figure 3.2a – d). Although research evaluating the antibiofilm effects of silver against *Cryptococcus* biofilms is limited, research has found other metal-based molecules such as gold nanoparticles are effective at reducing the activity of some *Cryptococcus* biofilms (Ghosh *et al.*, 2021). Additionally, in this study the treatment of *C. auris* biofilms with the novel SPC 7 at a concentration of 2 mg/mL is seen to cause a significant decrease in the metabolic activity of all the biofilms when used to both disrupt pre-formed biofilms and prevent formation ($p < 0.05$) (Figure 3.2e – g). This is in line with previous studies which also demonstrate a reduction in the metabolic activity of various *Candida* biofilms after treatment with silver-based compounds (Hamid *et al.*, 2018; Vazquez-Munoz *et al.*, 2020). Although the mechanisms by which silver compounds work has not been fully elucidated, some research has found that silver is found to intracellularly affect cell metabolism and play a role

in nucleic acid damage through the formation of reactive oxygen species (ROS) which leads to mitochondrial dysfunction (Thurman *et al.*, 1989; Berners-Price *et al.*, 1994). The XTT assay was utilised in this study to assess the metabolic activity of the treated biofilms which works by measuring the activity of some mitochondrial enzymes (Kuhn *et al.*, 2003). Since silver is seen to cause mitochondrial dysfunction, this would affect the production and activity of these enzymes resulting in lowered metabolic activity, which is confirmed by the results presented in this study (Figure 3.2e – g).

3.6.3 Antibiofilm Activity of *K. africana*

In the *Cryptococcus* biofilms a general trend can be seen of an increase in the biomass (Figure 3.1a - d) and metabolic activity (Figure 3.2a - d) of the biofilms treated with the *K. africana* extract. However, studies that examine the effects of a variety of plant extracts and plant products find that they often have substantial inhibitory effects to prevent and disrupt the formation of a variety of *Cryptococcus* biofilms (Almeida-Apolonio *et al.*, 2018; Kumari *et al.*, 2017; Kumari *et al.*, 2019). Similarly, previous studies have shown that treatment of various *Cryptococcus* biofilms with a variety of plant extracts resulted in reduced metabolic activity (Almeida-Apolonio *et al.*, 2018). Thus, the findings of this study do not agree with those presented in the literature.

The mechanisms that plant extracts utilise is not well understood however there is some research to suggest that the extracts exert their activity through the induction of apoptosis of fungal cells (Da *et al.*, 2019). A study by Da and colleagues (2019) showed that when *C. albicans* was exposed to *Scutellaria baicalensis* root extracts, the fungal cells produced high levels of ROS which subsequently accumulated and caused cell damage (Da *et al.*, 2019). Another study performed by Kim and colleagues (2019) also found that the production of ROS was a key process by which a variety of plant extracts caused cell death of fungal species (Kim *et al.*, 2019). The production of ROS leads to oxidative stress which increases the metabolic activity of the cells, however when the ROS accumulation is too high abnormal fungal growth, mitochondrial dysfunction, and apoptotic pathways are activated in fungal cells which ultimately results in cell death (Da *et al.*, 2019). Considering the significant increases in the metabolic activity of the *Cryptococcus* biofilms after treatment with the *K. africana* extract (Figure 3.2a – d), it may be that the extract causes oxidative stress to the fungal cells, however

at the tested concentrations it is not sufficient to induce significant inhibitory effects. As such, treatment with a higher concentration of the extract may be necessary for reductions in the formation and metabolic activity of the biofilms.

There has not been much research done to evaluate the role of *K. africana* against *Cryptococcus* biofilms however research has been done that establishes anti-quorum sensing properties of the *K. africana* plant against some bacterial biofilms (Chenia, 2013). Quorum sensing defines the cell-to-cell communication of microorganisms whereby they can share information about cell density and adjust their gene expression accordingly (Rutherford and Bassler, 2012). This process often influences and controls the genes that are beneficial when performed by the group of microorganisms as is the case with biofilm production (Rutherford and Bassler, 2012). In a study done by Chenia (2013), *K. africana* crude extracts were seen to impact the production of the pigment violacein in bacterial biosensor systems (Chenia, 2013). This pigment is under the control of quorum sensing systems as well the signalling molecules that are used for cell communication (Chenia, 2013). Since quorum sensing plays an important role in the production and maintenance of biofilms it follows that compounds that affect microbial communication might impact the formation of these biofilms. Although the study by Chenia (2013) was conducted using bacterial biosensor systems, quorum sensing has also been found to play a role in the formation of a variety of fungal biofilms (Albuquerque *et al.*, 2014; Kovács and Majoros, 2020; Mehmood *et al.*, 2019). Although the *K. africana* extract did not seem to have any inhibitory effects on the *Cryptococcus* biofilms in this study at the tested concentrations, it would be interesting to assess if the extract would also affect quorum sensing molecules of fungal biofilms in the same manner as bacterial biofilms.

3.6.4 Increased Biofilm Formation After Antifungal Treatment

Interestingly, this study found that in some cases treatment with the compounds led to an increase in biofilm formation, as opposed to a decrease as was expected (Figure 3.1). This apparent stimulation of biofilm formation may be explained by the exposure to the antifungals themselves. In bacterial biofilms formed by *P. aeruginosa* and *Escherichia coli*, exposure to the antibiotic, tobramycin, at sub-inhibitory concentrations has been seen to increase biofilm formation (Römling and Balsalobre, 2012; Hoffman *et al.*, 2005). Additionally, some antibiotics that target translation processes have been shown to increase bacterial biofilm

formation by upregulating off target biofilm-specific processes (Römling and Balsalobre, 2012; Hamza *et al.*, 2006). And importantly, oxidative stress has been shown to play a role in promoting biofilm formation in both bacterial (Oh *et al.*, 2016; Boles and Singh, 2008) and fungal biofilms (Pemmaraju *et al.*, 2016). As previously discussed, the production of ROS was seen to be a potential mechanism by which both the silver compounds and plant extracts disrupt the metabolic activity of biofilms. As such, it may be that the production of ROS is the reason for the increase in biofilm production as a result of exposure to the test compounds.

This may also account for the trend observed where treatment of the biofilms with higher concentrations of the compounds resulted in the highest degree of biofilm formation (Figure 3.1). This was demonstrated in the *C. gattii* biofilm after exposure to the *K. africana* extract (Figure 3.1c) as well as in the *C. auris* biofilms after exposure to SPC 7 (Figure 3.1f, g). To this end, the higher concentrations of the compounds may have stimulated biofilm formation by exerting a higher degree of oxidative stress which in turn promoted biofilm formation. This may explain why lower concentrations of the compounds did not result in higher levels of biofilm production, as there was a lower degree of oxidative stress put on the fungal cells following exposure to the test compounds, thus less pressure to proliferate.

In addition to the stressors that the antifungal treatments have placed on the biofilms, it is also possible that the environmental conditions may have also played a role in amplifying the formation of the biofilms. The static nature of the method used to grow the biofilms comes with nutrient and aeration limitations that are not necessarily indicative of how the biofilms may form naturally (Merritt *et al.*, 2011). This results in environmental conditions such as increased nitric oxide, glucose starvation, and high cell densities in the biofilms which are factors which have been found to encourage proliferation of biofilms (Rode *et al.*, 2020; Barraud *et al.*, 2009). Thus, the increased biofilm formation may be as a result of the biofilms reacting to the stressors of the antifungal compounds as well as the environmental conditions.

3.6.5 Limitations

The major limitation of this study included the limited concentration range utilised. This hindered the ability to accurately assess the efficacy of the compounds against the biofilms. As such, future work should include an expanded concentration range and should consider testing for the minimum biofilm inhibition and eradication concentrations of the compounds (Melo *et al.*, 2011). This however is labour intensive as is the nature of these assays. Additionally, the sample size included in the experiment was quite small. Only three biological repeats were performed for each treatment condition, however, there was a fair amount of variation exhibited in the results. As discussed, there were a variety of factors that influenced the growth of the biofilms in each well however there were also some technical issues with the method itself that may have influenced the results. The assays employ the use of multiple washes to remove the unattached fungal cells as well as excess dyes, in the case of the CV assay. This led to instances where poorly attached biofilms were sometimes washed away, which influenced the resulting biofilm readings.

The CV assay showed little or no decrease in the treated biofilms and often tended to skew towards an increase in the biomass of the biofilms (Figure 3.1). As explained, this may have been as a result of environmental stressors that force the biofilms to proliferate. This theory is further supported by the increases in metabolic activity observed (Figure 3.2). However, in the case of the CV assays, a limitation of the method is that even if the pre-formed biofilms are susceptible to the compounds, the associated matrix may not be removed and subsequently may be stained (Latka and Drulis-Kawa, 2020). In short, the CV assay works on the principle that the CV dye is positively charged and can non-discriminately bind to negatively charged molecules (Feoktistova *et al.*, 2016). This includes cell membranes, nucleic acids, polysaccharides and proteins which can be found in the biofilm matrix following matrix overproduction or even release of negatively charged components into the matrix following cell death (Latka and Drulis-Kawa, 2020; Feoktistova *et al.*, 2016). Thus, staining of matrix molecules may inflate staining and subsequent readings. It should also be noted that external growth factors such as differing biofilm formation rates, structure and matrix composition may be influenced by the fact that the cultures are not continuously supplied with fresh media, nor are they adequately aerated (Latka and Drulis-Kawa, 2020; Merritt *et al.*, 2011). These limitations may hinder the ability of biofilms to form uniformly, also accounting for differences in the biomass levels.

The XTT metabolic activity assay has been largely utilised as a way to examine and measure qand compare the activity of biofilms (Kuhn *et al.*, 2003). Briefly, actively respirating cells are able to convert the XTT tetrazolium salt to a water-soluble, orange coloured formazan product (Kuhn *et al.*, 2003). A direct relationship exists between the number of fungal cells present and the resulting colourimetric signal which is useful for comparing the effects of a treatment on biofilms (Hawser, 1996). However, there is some research that suggests XTT can be retained as intracellular product in some *Candida* strains, which only becomes soluble after treatment with dimethyl sulphoxide (Kuhn *et al.*, 2003). This would confound the signal intensity readings observed and might affect interpretation of results. Despite this, XTT remains the most reproducible and accurate method to quantitatively evaluate biofilms (Taff *et al.*, 2012).

3.7 Conclusions

None of the compounds showed to significantly decrease the biomass of the biofilms. Instead, a general trend of an increase in the biomass of the treated biofilms was observed. This increase was likely attributed to the addition of the compounds causing oxidative stress that promoted the over proliferation of the biofilms. Similarly, upon exposure of the *K. africana* extract to the *Cryptococcus* biofilms, an increase in the metabolic activity of the biofilms was observed. This is also speculated to be as a result of the extract causing an increase in the oxidative stress of the biofilms. The novel SPC 7 compound showed to decrease the metabolic activity of all the biofilms when both preventing formation and acting against a pre-formed biofilm, except in the case of *C. neoformans* where the metabolic activity increased when disrupting the pre-formed biofilm. This experiment was hindered by the use of a low concentration range and thus the true antibiofilm properties of the compounds could not be ascertained. Future work should focus on establishing minimum biofilm inhibitory and eradication concentrations before conclusions are made about the efficacy of the compounds. However, overall SPC 7 shows promise as a potential antibiofilm compound and should be researched further.

3.8 References

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CHAPTER 4

Assessing the toxicity and efficacy of plant extracts and a novel silver (I)-phosphine complex using *Caenorhabditis elegans* as a model organism

4.1 Abstract

A limited arsenal of antifungal drugs is available for the treatment of fungal infections. One reason for this lies in the fact that fungal treatments come with the inherent caveat that they must target processes that are distinctly fungal, as fungi are eukaryotic and thus more closely related to their human hosts than other microbial pathogens. This limits the molecules that can be used, as non-specific compounds can cause toxicity in the patient if host related processes are targeted. As such, there is a need for new drugs that are not only effective antifungals, but also non-toxic. In this study, a novel silver (I)-phosphine complex as well as a variety of ethanolic native plant extracts were assessed for their toxicity using *Caenorhabditis elegans* as a model organism. It was found that of the plant extracts, the *Kigelia africana* extract displayed the lowest degree of toxicity towards the *C. elegans* models, with 50% population mortality (LC50) observed at 60 mg/mL after 48 hours. Additionally, SPC 7 displayed low levels of toxicity as was seen by a population mortality of less than 10%, even at the highest concentration tested (4 mg/mL) after 48 hours. Additionally, the efficacy of SPC 7 and *K. africana* was assessed *in vivo* against *Candida auris* and *Cryptococcus neoformans* infected nematodes. Only slight reductions in mortality were seen in the *K. africana* treated nematodes. However, the *C. auris* and *C. neoformans* infected nematodes showed a 40 and 48% ($p < 0.05$) decrease in mortality respectively after seven days when treated with SPC 7. The compound SPC 7 shows potential as a novel antifungal treatment, showing both low toxicity and the ability to significantly decrease the mortality of *C. auris* and *C. neoformans* infected nematodes.

4.2 Introduction

Fungal infections are believed to affect over a billion people worldwide, with more than 150 million people estimated to be experiencing serious or fatal fungal diseases annually (Bongomin *et al.,* 2017). Many of these infections are as a result of opportunistic fungal pathogens within the *Candida* and *Cryptococcus* genera (de Pauw, 2011). *Cryptococcus* related diseases are estimated to affect over a million people each year, with infection often manifesting as severe diseases such as cryptococcal meningitis and pulmonary cryptococcosis (Park *et al.,* 2009). Studies have shown that *Cryptococcus* infections are one of the leading causes of death for people living with human immunodeficiency virus (HIV) or acquired

immunodeficiency syndrome (AIDS), accounting for 15% of AIDS-related deaths globally (Rajasingham *et al.*, 2022). The causative agent behind many of these infections being *C. neoformans* (Maziarz and Perfect, 2016). Many *Candida* infections are often superficial and easily treatable, primarily affecting the skin and mucosal membranes, however inadequate treatment can lead to the development of severe, systemic infections (Kullberg and Arendrup, 2015). These infections are much harder to treat and are often fatal, with estimates showing the crude mortality rate of various *Candida* related infections to be between 19 – 60% depending on the species (Morgan *et al.*, 2005; Hirano *et al.*, 2015). The main culprit responsible for many of these infections is *C. albicans*, however recently there has been an emergence of a number of invasive infections caused by a new isolate called *Candida auris* (Ademe and Girma, 2020; Hajjeh *et al.*, 2004). This species is particularly worrisome due to its reduced susceptibility to the main classes of antifungal drugs, which makes these infections difficult to treat (Navalkele *et al.*, 2017). As such, global crude mortality rates as a result of *C. auris* are high at around 30 - 60% depending on the region (Lockhart *et al.*, 2017; Al-Rashdi *et al.*, 2021).

Perceptions of fungal infections being a less significant burden on society compared to other microbes, such as bacteria and viruses, have resulted in far less research inputs being directed towards the development of new antifungal treatments (Lange, 2014). Additionally, fungal treatments come with the inherent caveat that they must target processes that are distinctly fungal, as fungi are eukaryotic and thus more closely related to their human hosts than other microbial pathogens (Kathiravan *et al.*, 2012). Not only does this limit the molecules that can be used, but non-specific compounds can be catastrophic if host related processes are targeted as well. For example, amphotericin B is popular and effective antifungal which works by forming complexes with ergosterol in the fungal membrane, causing increased membrane permeability and cytoplasmic leakage (Kathiravan *et al.*, 2012). However, it has also been seen to cause cellular toxicity in the host (as it interacts with mammalian sterols) thus causing serious side effects (Kischkel *et al.*, 2020). These factors have resulted in a limited collection of antifungal molecules and drugs that can be utilised in clinical settings. This problem is further compounded by the rise of drug-resistant and novel fungal pathogens such as *C. auris* (Fisher *et al.*, 2018). As such, it is vital that efforts are made to aid in the search for novel antifungal treatments that do not only possess antifungal properties but ones that are also non-toxic.

To this end, the use of both silver and plant-based treatments have been proposed as promising treatment opportunities. Silver based treatments have shown to have potent antimicrobial properties against a variety of microbes including fungi (Gandra *et al.*, 2017; Panáček *et al.*, 2009). Additionally silver is not known to interfere with mammalian biological processes and has demonstrated low toxicity in mammalian cells, making it a good treatment option (Lansdown, 2006). Similarly, plants have been utilised for centuries as a part of traditional based medicine as well as in modern medicine with 40 – 50% of all drugs currently in circuit believed to be derived from plants (Veeresham, 2012). A variety of plant extracts have been shown to possess potent antifungal properties. Additionally, many natural products are also generally deemed to be safer or less toxic than their synthetic counterparts (Upadhyay *et al.*, 2014). As such the use of synthetic and natural compounds shows potential in the search for novel antifungal treatments.

As discussed, a problem with antifungal treatments is the possibility of host toxicity as a result of the treatment targeting similar biochemical processes between the fungal pathogen and host (Kathiravan *et al.*, 2012). Toxicity testing is an important step in the drug discovery process and is necessary to assess a potential treatment's properties when introduced to a living organism. This is usually achieved by means of the mammalian model organisms, such as murine models, however the use of these models comes with significant resource, cost and ethical challenges (Qiu and Su, 2013). As such the use of lower order model organisms such as *Caenorhabditis elegans* have been studied as an alternative way to study the toxicity of bioactive compounds (Ahamefule *et al.*, 2021). Studies have found that *C. elegans* function as powerful predictors of compounds that are not only lethal, but those that can also cause reproductive and developmental anomalies in mammals (Hunt, 2017). They also come with several advantages including their small size, minimal growth and maintenance requirements, high number of offspring, quick generation time, and fewer ethical concerns that makes them advantageous for toxicity testing (Stiernagle, 1999). Although mammalian models remain the golden standard for toxicity testing, *C. elegans* serves as a suitable intermediary between *in vivo* and mammalian models to screen potential compounds. Additionally, nematodes have previously been used to study a variety of human diseases including neurodegenerative diseases, and importantly, the pathogenesis of a variety of microbial pathogens (Markaki and Tavernarakis, 2020; Ahamefule *et al.*, 2020).

While *in vitro* studies can elucidate a variety of properties about potential drug candidates these methods cannot infer the effects the drug will have when introduced to a complex living organism. This includes issues of compound solubility, absorption and pathogen-host interactions including immunity to name a few (O'Reilly *et al.*, 2014). It has been found that *C. elegans* are highly susceptible to a variety of human pathogens across a range of genera including *Candida* and *Cryptococcus* (Tan *et al.*, 1999). A variety of similar innate immune system signal cascades are found to be present between *C. elegans* and humans (Kim *et al.*, 2002). Thus, the nematode is an ideal candidate to not only study the pathogenesis of microbes but can also act as an initial screening tool to assess the efficacy of novel bioactive compounds.

As such, this study aims to evaluate the toxicity and efficacy of a novel silver (I)-phosphine complex as well as a variety of ethanolic native plant extracts using *C. elegans* as a model organism to aid in the search for novel antifungal compounds.

4.3 Aims and Objectives

Assess the cell toxicity and efficacy of the novel silver (I)-phosphine complex 7 (SPC 7) and native plant extracts using *Caenorhabditis elegans* as a model organism.

1. *Caenorhabditis elegans* will be used to assess the toxicity of the test compounds.
2. *Caenorhabditis elegans* will be used as a model of pathogenesis to assess the efficacy of the compounds *in vivo*.

4.4 Methods and Materials

4.4.1 Preparation of Plant Extracts

Dried plant material of *Hypoxis hemerocallidea, Kigelia africana* and *Pelargonium zonale* was obtained from Medico Herbs (https://medicoherbs.co.za/). The plant material was ground into a fine powder using a standard mortar and pestle. Extracts were prepared by combining the ground plant material with 95% (v/v) ethanol in a 1:10 ratio whereby 10 mL of solvent was mixed with 1 g of plant material (Ahmad and Beg, 2001). The mixtures were vortexed vigorously for five minutes and left to stand undisturbed for one hour at room temperature to allow for extraction. The mixtures were centrifuged for 10 minutes at 1400 × g at room temperature. The supernatants of each mixture were filtered through Whatman No. 1 filter paper into pre-weighed 50 mL Falcon tubes. The filtrate was left to air-dry at room temperature with the aid of a fan to expedite the evaporation process. Once the extracts were dry, the Falcon tubes were weighed, and the extract was resuspended in distilled sterile water to a concentration of 150 – 200 mg/mL. The extracts were stored at 4°C in the dark until needed.

4.4.2 Preparation of Silver (I)-phosphine complex 7

The silver-phosphine complex 7 (SPC 7) was synthesised at The University of Johannesburg and was kindly provided by Dr. Zelinda Engelbrecht and Prof. Marianne Cronjé (School of Molecular and Cell Biology; The University of the Witwatersrand). The silver compound was dissolved in sterile distilled water and was stored at 4°C in the dark until needed. When required, it was heated to 70°C for 30 minutes before use.

4.4.3 Strains

Caenorhabditis elegans glp-4; sek-1 mutant nematodes were kindly provided by Prof Carolina Pohl-Albertyn (Department of Microbial Biochemical and Food Biotechnology, The University of the Free State). This mutant strain has properties that make it more susceptible to infection by pathogens than the wild type (Hoyt *et al.*, 2017). This is due to the *sek-1* mutation which inhibits the correct functioning of protein kinase activity that plays a vital role in the expression of immunity genes (Kim *et al.*, 2002; Hoyt *et al.*, 2017). Additionally, these nematodes are temperature sensitive such that the production of offspring may be controlled at different stages of the study (Miyata *et al.*, 2008). At a temperature of 25°C, the *glp-4* mutation

causes the nematodes to stop producing gonads (Miyata *et al.*, 2008). This stops the production of offspring which can interfere when studying population of the nematodes over a period of time.

4.4.4 Growth and Maintenance of Nematodes

The nematodes were grown and maintained on nematode growth medium (NGM; 17.5 g/L agar, 3.0 g/L NaCl, 2.5 g/L peptone, 0.0005 g/L cholesterol, 0.5 M potassium phosphate buffer (pH 6.0), 1M $CaCl_2$ and 1M $MgSO_4$). The *Escherichia coli* OP50 strain (kindly provided by Prof Carolina Pohl-Albertyn, Department of Microbial Biochemical and Food Biotechnology, The University of the Free State) was used as the primary food source for the nematodes. The OP50 was grown on Luria-Bertani (LB) agar (12 g/L agar, 10 g/L peptone, 5 g/L yeast extract, 5 g/L sodium chloride) plates for 24 hours at 37°C. A single colony was picked and inoculated into LB broth (10 g/L peptone, 5 g/ yeast extract, 5 g/L sodium chloride) and incubated for 24 hours on a rotary wheel at 37°C. Following incubation, a 100 µL aliquot of the fresh bacterial culture was dispensed onto the NGM plate and spread only on the middle of the plate, to ensure that the nematodes stayed in the middle of the agar plate and deter them from crawling up the sides (Stiernagle, 1999). The seeded plates were sealed and left to incubate overnight at 37°C to allow for the growth of the bacterial lawn. Following incubation, the plates were left to cool and then were stored at 4°C until needed. Nematodes from a starved plate were sterilely added to the fresh plate using the 'chunking' method (Stiernagle, 1999); briefly a small chunk of the agar containing nematodes is cut out and added to a fresh seeded NGM plate. The nematodes were incubated at 16°C until further use. Nematode transfers were performed every six to seven days to prevent starvation.

4.4.5 Preparation of Nematodes for Assays

The nematodes were incubated at 16°C for five to six days until the majority of the nematodes were in the L4 stage of their development. The L4 nematodes represent those in the last stage of larval development before the nematode enters the adult stage (Altun and Hall, 2009). It is beneficial to use the nematodes at this stage of development as they have not yet completed gonadogenesis (and this process is further halted at 25°C) and thus cannot produce offspring that will interfere with the population studies and counts during the course of the experiment. The nematodes were harvested by washing the populated NGM plates with 5 mL of M9 buffer

(3 g/L KH_2PO_4, 6 g/L Na2HPO_4, 5 g/L NaCl, 1M $MgSO_4$) to remove the nematodes from the surface of the agar. This M9 medium was aspirated from each plate and added to a sterile 15 mL Falcon tube. The M9 media containing the nematodes was left at room temperature for 10 - 15 minutes so that the nematodes could settle at the bottom of the tube and form a pellet. The remaining M9 media was aspirated from the tube, 5 mL of fresh M9 media was added to the tube and the nematodes were allowed to settle again. This step was repeated twice more to remove any traces of the OP50 food medium that remained from the initial wash step.

Following the wash steps, the nematode pellet was added to a 90 mm Petri dish containing M9 liquid media. The nematodes were viewed using an inverted microscope (Wincom Company NIB-100, China) at 400X magnification. Nematodes that were in the L4 stage were selected and added to the wells of the 48-well plate containing the screening media (80% (v/v) M9 buffer and 20% (v/v) brain heart infusion (BHI) media (5 g/L beef heart, 12.5 g/L calf brains, 2.5 g/L disodium hydrogen phosphate, 2 g/L D(+)-glucose, 10 g/L peptone and 5 g/L sodium chloride)) and the antifungal agents. The L4 nematodes could be identified by their size (900 – 1150 µm) as well as the presence of a clear half circle on their ventral side (Chaudhuri *et al.*, 2011). The 48-well plates contained no more than 30 - 40 nematodes per well while six-well plates contained no more than 60 - 80 nematodes per plate.

4.4.6 Toxicity Assays

The toxicity assays were performed to determine the concentration of the extracts that resulted in the death of the nematodes. Screening media was added to 48-well plates followed by the addition of the antifungal compounds at various concentrations. The *K. africana, H. hemerocallidea,* and *P. zonale* plant extracts were used at a concentration range of 1 – 80 mg/mL and the silver (I)-phosphine complex 7 was tested in the range of 0.25 – 8 mg/mL. In addition, amphotericin B was tested at a range of 10 – 80 µg/mL. Control wells contained only the screening media. Following the addition of the nematodes to the wells containing the screening media and antifungal treatments, the nematodes were observed under the inverted microscope and counted. Any nematodes that were in the incorrect stage; or nematodes that had died during handling; were removed from the wells. The culture plate was sealed with parafilm and placed into a polystyrene box containing damp paper towels. This was incubated in the dark at 25°C for 48 hours. After 24 and 48 hours of incubation, the nematodes were viewed using the inverted microscope and the number of nematodes that had died in each

well was noted (Desalermos *et al.*, 2011). This experiment was conducted in triplicate with 30 – 40 nematodes per well. The average percentage mortality of the nematodes was calculated to determine the toxicity of the compounds over the 48 hour period (Dengg and van Meel, 2004). The number of nematodes that had died in the treated population was compared to that of the nematodes in the control wells to determine if the antifungal compounds caused a significant decrease in viability.

4.4.7 Infection Assay

The infection assay was performed to assess the efficacy of the test compounds *in vivo* using *C. elegans* as a model of pathogenicity. The nematodes were infected with the various fungal pathogens and were subsequently incubated with the antifungal agents to determine if the treated populations show decreased percentage population mortality when compared with the untreated control. Following the toxicity assays, the *K. africana* extract and SPC 7 compound were chosen to be utilised in this experiment due to their low toxicity.

4.4.7.1 Maintenance of Yeast Isolates

The fungal pathogens *Candida auris* (eABCC240) and *Cryptococcus neoformans* (eABCC16) were utilized during this study to infect the *C. elegans* nematodes. The *C. auris* strain was kindly provided by Dr Aijaz Ahmad (Department of Clinical Microbiology and Infectious Diseases; The University of the Witwatersrand) (NICD ethics approval number M140159) and the *C. neoformans* strain was retrieved from the culture collection of Dr Angela Botes (ABCC; School of Molecular and Cell Biology; The University of the Witwatersrand). Both test organisms were sub-cultured on Sabouraud dextrose agar (SDA) and incubated at 37°C before use. The *Candida* strain was grown for 48 hours while *Cryptococcus* strain was grown for 72 hours.

4.4.7.2 Preparation of Yeast Lawns

Fresh cultures of the fungal strains were used to inoculate Yeast Peptone Dextrose (YPD) broth (3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose). The inoculated media was incubated at 37°C for 24 hours on a rotary wheel. Yeast lawns were prepared by spreading 100 µl of the inoculated medium onto BHI culture plates supplemented with antibiotics (100 µg/mL of

ampicillin) (Elkabti *et al.*, 2018). The addition of the antibiotics to the BHI creates a selective media to prevent the growth of the *E. coli* OP50 food source being carried over with the nematodes (Elkabti *et al.*, 2018). These plates were grown for 24 hours at 37°C until a visible lawn of yeast formed. These plates were prepared as needed.

4.4.7.3 Infection of *C. elegans*

The nematodes were prepared as was described previously in section 4.4.5. Following the collection and subsequent wash steps, the pelleted nematodes were added to the middle of the yeast seeded BHI plates. The plate was sealed with parafilm, incubated at 25°C and the nematodes were left to feed for four hours. The uninfected control nematodes were added to a new NGM plate seeded with OP50. Following the incubation time, the nematodes were washed off the plate using sterile M9 buffer and pipetted into a sterile 15 mL Falcon tube. These were then centrifuged at 1500 × g for 5 minutes. The nematodes formed a clear layer above a pellet of yeast. The nematodes were collected and resuspended in 10 mL of sterile M9 buffer and centrifuged again with the same conditions. This step was repeated twice more to ensure that as much of the pathogenic yeast was removed from the nematodes as possible before continuing with further steps (Breger *et al.*, 2007). Following the final wash step, 60 - 80 nematodes in the L4 stage were picked and added to the wells of a six-well culture plate. Each condition was tested in duplicate. Control wells contained only screening media while experimental wells contained the test antifungals diluted with screening media. The antifungal compounds were tested at non-toxic, fungicidal concentrations that were previously established: *K. africana* (20 mg/mL), SPC 7 (2 mg/mL) and amphotericin B (2 µg/mL).

The culture plates were sealed with parafilm and placed into a polystyrene box containing damp paper towels. They were incubated in the dark at 25°C for a 7 day period. The plates were examined every 24 hours to check the viability of the nematodes with those that were dead being removed. Dead nematodes appeared straight, or rod shaped while live nematodes displayed a characteristic sinusoidal shape and also displayed movement when touched with a pipette (Tampakakis *et al.*, 2008). Following the incubation period the percentage mortality of the treated populations was compared to that of the untreated populations. This was done to assess if the antifungal compounds were effective at reducing the mortality rate of the infected nematodes.

4.4.8 Statistical Analysis

All statistical analyses were performed using Microsoft Office Excel 365. The standard error of the mean (SEM) was calculated for all samples. A Student's T-test was used to determine if the percentage mortality of the infected, untreated populations was statistically different when compared to the infected, treated populations (De Winter, 2013). Samples that included P-values < 0.05 were considered statistically significant in determining whether treatment with compounds was effective at reducing the mortality of the infected nematodes.

4.5 Results

Once a potential antifungal drug has displayed promising activity against fungal cells *in vitro*, another important step is to establish its toxicity. The toxicity assay using *C. elegans* as a model organism is an efficient way to easily screen potential drugs for their suitability as a non-toxic treatment (Dengg and van Meel, 2004). This can also be a good predictor if a treatment is going to be safe to use in other organisms such as mammalian models (Hunt, 2017).

4.5.1 Toxicity Assay

The nematodes were incubated with various concentrations of the test compounds over 48 hours and the number of nematodes that died each day was recorded. The percentage of the population that died was calculated (Figure 4.1). In general, the mortality of the SPC 7 and amphotericin B treated populations remained the same or slightly increased between the 24 to 48 hour interval (Figure 4.1d, e). Some significant increases in the mortality rate can be observed in the plant extracts between the time intervals (Figure 4.1a - c). Additionally, a dose-dependent effect can be observed between the concentration of the compounds and the population mortality observed. This is especially pronounced in the plant extracts (Figure 4.1a - c) where increasing compound concentration results in higher population mortality. This trend is not seen to be as defined in the SPC and amphotericin B treated populations.

Of the plant extracts, it can be seen that *P. zonale* (Figure 4.1b) exhibits the highest degree of toxicity with high rates of population mortality even at lower concentrations. The

H. hemerocallidea extract shows relatively low toxicity at concentrations lower than 20 mg/mL, however the mortality of the nematodes significantly increases when the concentration reaches 40 mg/mL (Figure 4.1a). The *K. africana* extract appears to have the lowest toxicity of the plant extracts with the population mortality remaining under 10% even at 20 mg/mL (Figure 4.1c). This is confirmed when the LC50 of the populations are calculated after 48 hours - *H. hemerocallidea* (20 – 40 mg/mL), *K. africana* (40 – 60 mg/mL), and *P. zonale* (5 – 10 mg/mL). Interestingly, the SPC 7 compound shows incredibly low toxicity within the concentration range tested, with the peak mortality observed at just over 10% at 4 mg/mL (Figure 4.1d). It should be noted that at higher concentrations, the SPC 7 compound is seen to precipitate out of solution, obstructing the field of vision and preventing the accurate observation and counting of nematodes. Lastly, the toxicity of amphotericin B was tested as a control which showed low population mortality of under 10% even at the highest concentration tested (Figure 4.1e). As such, the LC50 could not be determined for SPC 7 and amphotericin B as the population mortality did not exceed 50% even at the highest tested concentrations.

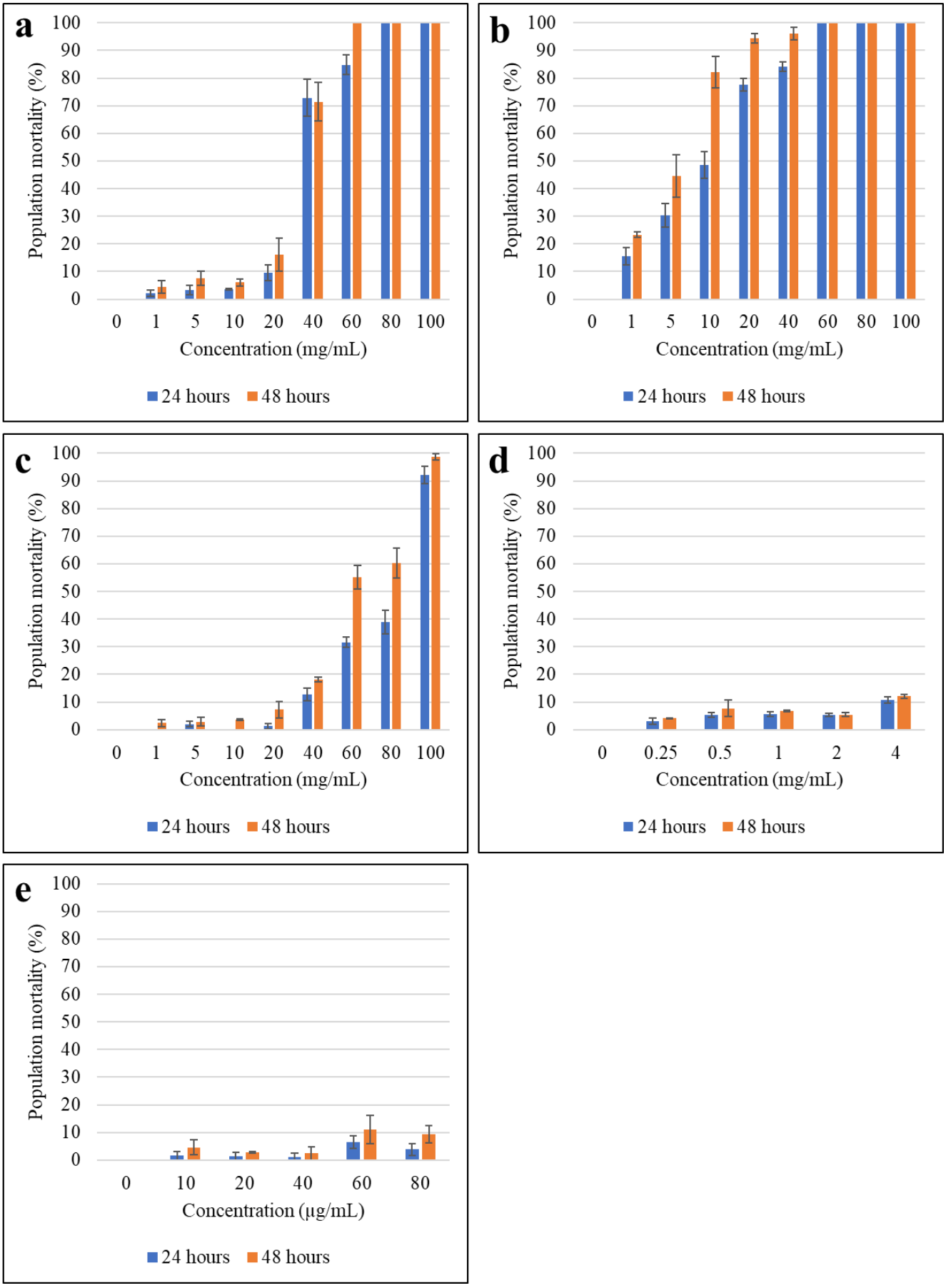

Figure 4.1. Toxicity assay using *Caenorhabditis elegans*. The antifungal test compounds were tested for their toxicity against *C. elegans* nematodes over a 24 and 48 hour period at a range of concentrations. The nematodes were incubated with the antifungal agents and the number that died at each time period was recorded. The error bars represent the standard error from the mean of three replicates (n = 3). a) *Hypoxis hemerocallidea*; b) *Pelargonium zonale*; c) *Kigelia africana;* d) Silver (I)-phosphine complex 7; e) Amphotericin B.

4.5.2 Infection Assays

Once the antifungal activity of the compounds has been established, it is important to have some understanding of how a potential treatment will perform when in a complex biological setting. The infection assay using *C. elegans* as a model organism allows one some insight into the efficacy of the compounds *in vivo* (Ahamefule *et al.*, 2021; Hernando-Ortiz *et al*., 2020).

In both the *Candida* and *Cryptococcus* cohorts, the infected, untreated populations have close to a 100% mortality rate seven days post infection (Figure 4.2). In contrast, the uninfected, untreated control shows a mortality rate of 66% after the incubation period. This increase in the mortality rate of the infected population confirms that the infection protocol was successful. The *Candida* infected population (Figure 4.2a) shows a decrease in the population mortality when treated with all the treatments as compared to the uninfected population ($p < 0.05$). The amphotericin B, *K. africana* and SPC 7 decreased the mortality of the infected populations by 37, 14 and 40% respectively after seven days ($p < 0.05$). Interestingly, the amphotericin B and SPC 7 treated populations showed slightly reduced mortality even when compared to the uninfected control (Figure 4.2a). The *Cryptococcus* infected populations (Figure 4.2b) also show an overall trend of the treatments decreasing the percentage population mortality of the infected nematodes. The amphotericin B, *K. africana* and SPC 7 decreased the mortality rate of the infected populations by 4, 8 and 48% respectively after seven days ($p < 0.05$). Again, the SPC 7 showed to decrease the mortality of the infected nematodes to below that of even the uninfected control. When amphotericin B was used as a treatment it was seen to considerably decrease the mortality rate of the *Candida* infected nematodes however it was not as successful at decreasing the overall mortality of the *Cryptococcus* infected nematodes.

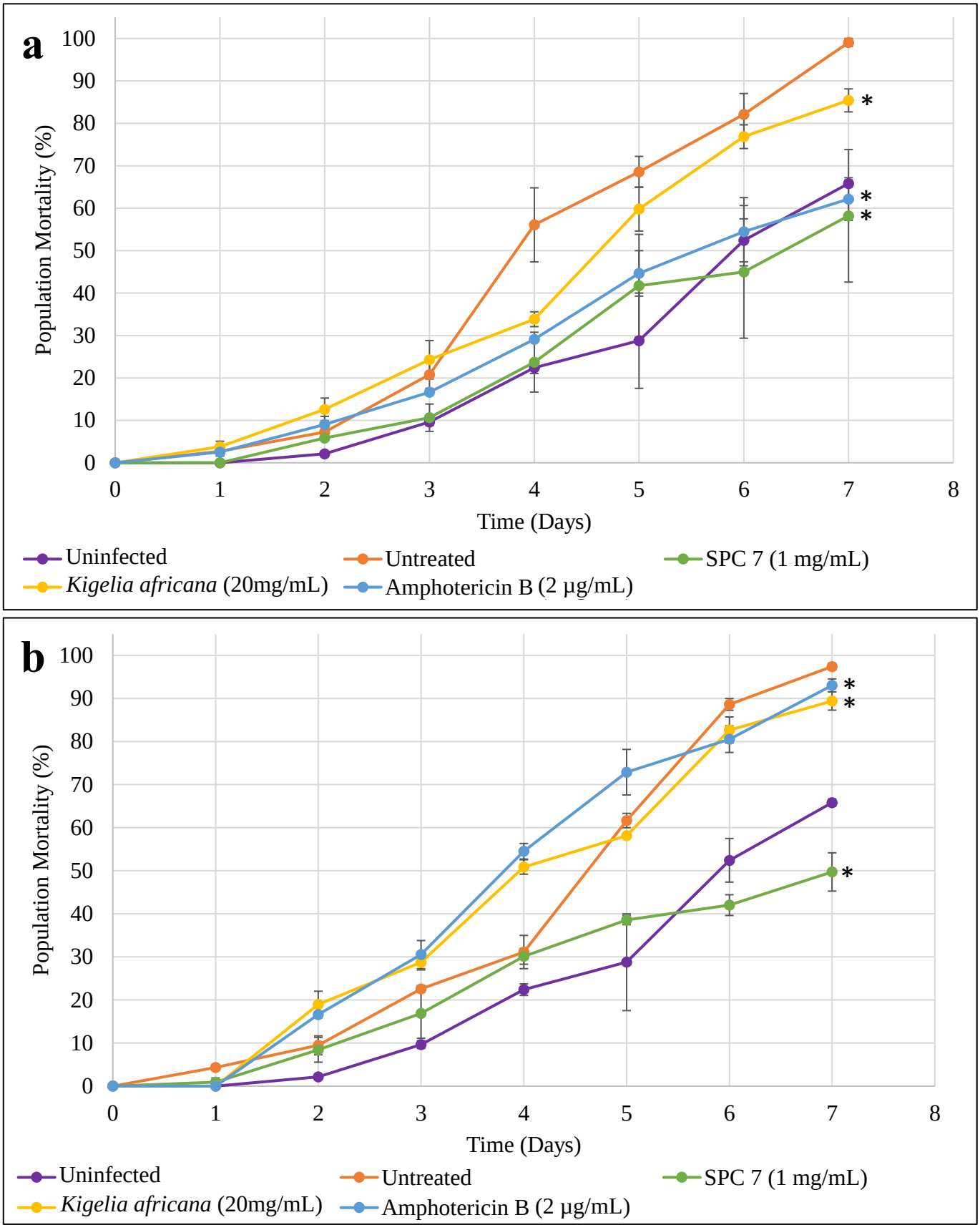

Figure 4.2. Infection assays using *Caenorhabditis elegans* as a model of pathogenesis. The nematodes were infected with a) *Candida auris* (eABCC240) or b) *Cryptococcus neoformans* (eABCC16) then incubated with the antifungal treatments. The number of nematodes that died each day was recorded. The experiment was run over the course of seven days. The control group was uninfected and was fed *Escherichia coli* (OP50). SPC – Silver (I)-phosphine complex 7. The compounds were tested at non-toxic and inhibitory concentrations: amphotericin B (2 µg/mL); *Kigelia africana* (20 mg/mL); and SPC 7 (2 mg/mL). (*) indicates significant difference $p < 0.05$. The error bars represent the standard error from the mean of two replicates (n = 2).

4.6 Discussion

4.6.1 Toxicity Assays

Plant-based treatments are attractive treatment options for a variety of reasons including their widespread accessibility and affordability compared to modern medicines (Dekebo, 2019). Notably, there is also the perception that they are safe and possess low toxicity since they are natural products (Anywar *et al.*, 2021; Mounanga *et al.*, 2015). However, there is great variation in the toxicity of the plant extracts (Ononamadu *et al.*, 2020). Plants contain a plethora of secondary metabolites and phytochemicals which mainly act to improve the survivability of the plant by playing a role in plant defence (Hussein and El-Anssary, 2019; Teoh, 2016). These metabolites can play a role by aiding in the protection of the plant against herbivory, other plants, pests and pathogens (Teoh, 2016). For this reason, the plant secondary metabolites are a good candidate as a source of antimicrobial compounds however their activity can also extend to other living organisms such as pests. While *C. elegans* is not a plant parasite, there are a variety of other plant-parasitic nematodes (Ndjonka *et al.*, 2011). In fact, various studies have investigated the nematocidal role of plant extracts as a potential control approach and treatment for parasitic nematode infections (Klimpel *et al.*, 2011; Zaidat *et al.*, 2020; Spiegler *et al.*, 2017; Abadu *et al.*, 2021; Ndjonka *et al.*, 2011). A variety of plant metabolites including alkaloids, flavonoids, glucosinolates, phenolic compounds, and terpenoids as well as many others have been identified to possess anti-nematode properties (Desmedt *et al.*, 2020). Many of these molecules are also known to possess antimicrobial activity which is believed to account for the antifungal activity exhibited by the plant extracts in the previous chapters (Pusztahelyi *et al*., 2015). As such, the toxicity displayed by the plant extracts towards the nematodes (Figure 4.1) may be as a result of the inherent anti-nematode properties of the metabolites found in the plant extracts.

Additionally, the toxicity assays showed that the plant extracts displayed varying levels of toxicity with the *P. zonale* extract being the most toxic (LC50 5 – 10 mg/mL), followed by *H. hemerocallidea* (LC50 20 – 40 mg/mL), and lastly *K. africana* displaying the lowest toxicity (LC50 40 – 60 mg/mL) (Figure 4.1a - c). While the extracts utilised in this study have not previously been studied for their toxicity, other crude plant extracts have been evaluated (Anywar *et al.*, 2021; Seremet *et al.*, 2018; Ndjonka *et al.*, 2011; Abadu *et al.*, 2021). These studies display a wide range of results with some aqueous and plant extracts displaying potent toxicity and causing high rates of mortality in *C. elegans* nematodes (Abadu *et al.*, 2021) while

others showed moderate to low toxicity at similar concentrations to what was tested in this study (Ndjonka *et al.*, 2011). For example Abadu and colleagues (2021) found that aqueous extracts (10 mg/mL) of *Leucaena leucocephala* and *Moringa oleifera* leaves caused 100 and 97% mortality respectively in *C. elegans* after 40 hours (Abadu *et al.*, 2021). However Ndjonka and colleagues (2010) found that ethanolic extracts of *Anogeissus leiocarpus*, *Euphorbia hirta and Khaya senegalensis* (4 mg/mL) saw only 50% population mortality (LC50) after 72 hours (Ndjonka *et al.*, 2011). This variation may be attributed to differences in the metabolites produced by the different plant types (Hussein and El-Anssary, 2019). For example, there may be a molecule, or a higher concentration of a molecule, present in the *P. zonale* extract (Figure 4.1b) that makes it more toxic than the other plant extracts (Mounanga *et al.*, 2015). For example, chemical profiling of some *Pelargonium* species such as *P. graveolens* has shown extracts to contain high concentrations of menthol which has been linked with toxicity in *C. elegans* (Al-Mijalli *et al.*, 2022; Nikitin *et al.*, 2021).

The results of this study demonstrated that the novel SPC 7 showed low toxicity towards the nematodes at the effective concentration range tested (Figure 4.1d). Silver and silver-based treatments have gained popularity in recent years due to the development of nanomaterials such as silver nanoparticles (AlJindan and AlEraky, 2022). As such there has been considerable research on the toxicity of silver nanoparticles. These studies show that the silver nanoparticles do possess a degree of toxicity towards *C. elegans* (Luo *et al.*, 2016; Yang *et al.*, 2014; Kleiven *et al.*, 2018). Notably, many of these studies do not only assess the mortality of the nematode population as a measure of toxicity but other factors such as germ cell death, brood size assays over multiple generations, growth and reproduction (Moon *et al.*, 2019; Luo *et al.*, 2016). It should be noted that while there is far less research investigating the toxicity of silver compounds, some studies show that compounds such as silver nitrate exhibit lower levels of toxicity when compared to their silver nanoparticle counterparts (Kleiven *et al.*, 2018). This difference is largely thought to be attributed to the size difference of the molecules, where smaller nanoparticles can more easily accumulate in tissues resulting in a higher degree of toxicity (Moon *et al.*, 2019; Luo *et al.*, 2016). Although SPC 7 does not appear to have nematocidal properties at these concentrations, further experiments should be performed using other measures of toxicity before fully classifying it as non-toxic.

Lastly, amphotericin B also showed relatively low toxicity with under 10% population mortality even at the highest concentration tested of 80 µg/mL (Figure 4.1e). This is in agreement with previous studies that show amphotericin B to be non-toxic towards *C. elegans* (Breger *et al.*, 2007).

4.6.2 Infection Assays

The *C. elegans* nematodes were successfully infected by both the *Candida* and *Cryptococcus* strains as is seen by the increased mortality rate of the infected cohort as compared to the uninfected control over the incubation period (Figure 4.2). This is in agreement with previous studies that show *C. auris* and *C. neoformans* strains are able to infect *C. elegans* models (Hernando-Ortiz *et al.*, 2021; Lima *et al.*, 2020; Mylonakis *et al.*, 2002). For both *Candida* and *Cryptococcus* strains, the mechanism of infection is seen to occur through the digestion of the fungal pathogens. Various studies have demonstrated that these pathogens are able to pass through and survive the pharyngeal grinder of the nematode, which provides mechanical crushing to destroy ingested organisms (Mylonakis *et al.*, 2002; Elkabti *et al.*,2018). Subsequently, the fungal pathogens are then seen to accumulate in the gastrointestinal tract however, the exact method by which they cause mortality is not well defined. There are studies that investigate a variety of virulence related factors that subsequently lead to a decrease in viability of *C. elegans* (Mylonakis *et al.*, 2002)). For *Cryptococcus* some of the associated factors involved in *C. elegans* pathogenesis include: the presence of the cryptococcal polysaccharide capsule (Chang and Kwon-Chung, 1994), laccase production (Salas *et al.*, 1996), modulation of the insulin-like growth factor signalling pathways in *C. elegans* which are known to play a vital role during cryptococcal infections (Kitisin *et al.*, 2022a), as well as inducing the degeneration of dopaminergic neurons in *C. elegans* (Kitisin *et al.*, 2022b). For *Candida* based infections, factors such as cell adherence and invasion and biofilm formation play an important role in the infection of *C. elegans* (Elkabti *et al.*, 2018; Pukkila-Worley *et al.*, 2011). Additionally, hyphae formation is also known to play a significant role in the killing of *C. elegans* as it pierces through their cuticle (Pukkila-Worley *et al.*, 2011).

Amphotericin B has been shown to successfully rescue some infected *C. elegans* nematodes from infection by fungal pathogens (Ahamefule *et al.*, 2020; Hernando-Ortiz *et al.*, 2020). One study showed that increased life expectancy and reduced mortality was observed in rescue *Aspergillus fumigatus* infected *C. elegans* when treated with amphotericin B at concentrations

of 1 µg/mL and higher (Ahamefule *et al.*, 2020). Additionally, a study done by Hernando-Ortiz and colleagues demonstrated that when *C. elegans* was infected with various *Candida* pathogens and subsequently treated with amphotericin B at a concentration of 1 – 2 µg/mL, improved survival rates of 57 - 95% in the treated populations was observed (Hernando-Ortiz *et al.*, 2020). Similarly, in this study, *C. auris* infected *C. elegans* that was treated with 2 µg/mL of amphotericin B saw a 37% decrease in the population mortality compared to the untreated controls ($p < 0.05$) (Figure 4.2a).

Interestingly, this study demonstrated only a 4% decrease in the mortality of the *Cryptococcus* infected nematodes that were treated with amphotericin B (2 µg/mL) compared to the untreated control ($p < 0.05$) (Figure 4.2b). While there is not much research that investigates the role of amphotericin B during *C. elegans* infection with *Cryptococcus* species, amphotericin B has shown to prolong the survival of other *Cryptococcus* infected model organisms such as *Galleria mellonella* larva (Mylonakis *et al.*, 2005). Additionally, amphotericin B has already been established as an effective treatment for *C. neoformans* infections in a variety of model organisms including mammalian models (Dromer and Charreire, 1991) and in humans (Abassi *et al.*, 2015). Thus, the apparent lack of activity from amphotericin B demonstrated in this study is likely the result of an unsuitable concentration that was too low to inhibit the microbial load present. This is confirmed when the minimum inhibitory concentrations (MIC) determined in Chapter 2 are considered. Those results found that the *C. neoformans* strain had an MIC of 2 µg/mL for amphotericin B. This study also utilised an concentration of 2 µg/mL of amphotericin B in the infection assay which can account for the relatively small decrease observed in the population mortality of the *Cryptococcus* infected nematodes. In contrast, the MIC determined for the *C. auris* strain was 0.5 µg/mL for amphotericin B, which would explain why a more significant decrease in mortality was observed.

The *K. africana* extract was seen to reduce the mortality of the *Candida* and *Cryptococcus* infected populations by 14 and 8% respectively ($p < 0.05$) (Figure 4.2). The use of plant extracts has not previously been evaluated for their efficacy against *C. auris* and *C. neoformans* infected nematodes. However, a variety of other plant-based extracts (Vediyappan *et al.*, 2013), essential oils (Pedroso *et al.*, 2019) and other natural products (Lu *et al.*, 2021) have demonstrated their ability to decrease the mortality of a variety of *Candida*-infected *C. elegans* populations. In addition to possessing antifungal properties, a variety of plant extracts have

also been linked with the ability to extend the lifespan of *C. elegans* (Martel *et al.*, 2020). At low to moderate concentrations these plant-based compounds have been found to modulate a variety of aging pathways and genes including the activation of autophagy and expression of antioxidant enzymes (Martel *et al.*, 2019; Martel *et al.*, 2020). These processes have also been implicated in playing a role in host antifungal immunity in *C. elegans* (Kanayama and Shinohara, 2016; Jain *et al.*, 2009; Meléndez and Levine, 2009). As such, it is possible that the *K. africana* extract is activating these lifespan extending pathways that consequently also aid in the host defence against infection from *C. auris* and *C. neoformans,* which may account for the small decrease in mortality observed in the treated populations. As was the case with the amphotericin B treated populations, a more significant decrease in mortality may not have been observed due to the concentration being too low. However, it should be noted that the use of higher concentrations of *K. africana* are also associated with higher levels of toxicity (Figure 4.1c)

The SPC 7 proved to be the most effective of the tested compounds, showing a 40 and 48% reduction in the mortality rate of the *C. auris* and *C. neoformans* infected populations respectively ($p < 0.05$) (Figure 4.2). Previous studies studying bacterial infection of *C. elegans* including *Pseudomonas aeruginosa* (Richter *et al.*, 2017) and *Listeria monocytogenes* (Muthulakshmi *et al.*, 2022) have also noted significantly reduced mortality in infected *C. elegans* models that were treated with silver-based compounds and nanoparticles. Again, although there has not been much research done as yet using *C. auris* and *C. neoformans* as infection models for the discovery of novel antifungals, the use of other *Candida* species have been investigated. One such study by Muthamil and colleagues (2018) found that when *C. elegans* was infected with various *Candida* species including *C. albicans, C. glabrata* and *C. tropicalis,* those nematodes treated with the silver nanoparticles displayed reduced colonisation of *Candida* cells in the gastrointestinal tract (Muthamil *et al.*, 2018). Some studies have shown that silver molecules can cause the formation of reactive oxygen species in *C. elegans* which is suspected to be a potential cause of their toxicity (Lim *et al.*, 2012; Roh *et al.*, 2012). In turn, *C. elegans* increases the activity of mitogen-activated protein kinases (MAPK) as a protection mechanism against the compounds (Lim *et al.*, 2012; Roh *et al.*, 2012). The MAPK pathway is known to regulate the expression of immunity factors in *C. elegans* in response to harsh environmental stressors as well as pathogens (JebaMercy *et al.*, 2013; Li *et al.*, 2018). As such, it may be possible that the presence of SPC 7 increases the nematode's

innate immune response through the MAPK pathway which subsequently leads to the more effective host defence and immunity against the pathogenic fungi.

Interestingly, the infected nematodes that were treated SPC 7 had a lower percentage mortality at the end of the incubation period than even the uninfected control. The literature does not demonstrate that silver improves the lifespan of *C. elegans* but rather has the opposite effect (Piechulek and von Mikecz, 2018; Luo *et al.*, 2016; Contreras *et al.*, 2014). However, as was noted in the mortality assays, many of these studies investigate nanoparticles and not the role of the silver compounds themselves. Similarly, the disparity may be as result of SPC 7 being a compound, rather than a nanoparticle and larger silver-based molecules are seen to induce lower levels of toxicity (Contreras *et al.*, 2014; Hunt *et al.*, 2014). As was discussed earlier, silver has been shown to increase the activity of the MAPK pathways which in addition to immunity, also govern several processes linked to the longevity of *C. elegans* (Okuyama *et al.*, 2010; Chamoli *et al.*, 2020). The activation of the MAPK pathway in *C. elegans* is shown to upregulate cytoprotective genes which have been linked to longevity (Chamoli *et al.*, 2020). As such, SPC 7 may also be activating these pathways which may account for the decrease in mortality observed compared to that of the uninfected controls.

4.6.3 Limitations and Future Work

There are several limitations associated with the use of *C. elegans* as model organisms (Hunt, 2017; Golden and Riddle, 1984). Nematodes are sensitive to small environmental changes including temperature fluctuations, nutrient requirements, and nematode density (Hunt, 2017). Changes to these requirements can affect a variety of factors such as their growth rate, motility, reproduction, and gene expression which can sometimes be modified for multiple generations (Hunt, 2017). These factors can alter the nematode's response to potential toxic compounds as well as their ability to become infected with the pathogens, so it is imperative that the stocks are handled and maintained correctly to prevent stress responses (Ahamefule *et al.*, 2021). Of note, it is imperative that the formation of Dauer larvae is prevented (Golden and Riddle, 1984). This describes an alternative developmental stage of the nematodes in response to unfavourable conditions which allows them to survive in harsh environmental conditions and to live for extended periods of time (Golden and Riddle, 1984). This change might result in increased viability when incubated with the compounds, where they might otherwise have been affected.

Additionally, these assays can be both time-consuming and labour intensive since high-throughput systems involve the use of expensive, specialised equipment (Hunt, 2017).

An inherent limitation of utilising *C. elegans* for toxicity testing is the presence of their robust cuticle which acts as a barrier against chemicals and toxins (Johnstone, 1994). Some studies have shown that the uptake of compounds is limited to varying degrees which can mean that the toxic effects of compounds may be underestimated when utilising *C. elegans* models (Boyd *et al.*, 2010; Xiong *et al*., 2017). Additionally, the use of a liquid medium limits the compounds to those that are water soluble, and a degree of insolubility can affect the dispersion and exposure of the compounds in the medium (Hunt, 2017). As discussed, this study only evaluated mortality as an endpoint of toxicity however measures of toxicity such as brood size, egg hatching, motility, and reproduction should also be elucidated before definitively stating if a compound is non-toxic towards *C. elegans*.

Although the *C. elegans* infection model is a good option for the discovery of new antifungal treatments, the assay does come with some limitations. Firstly, the experiment takes place at 25°C as the nematodes are unable to survive at higher temperatures (Zhang *et al.*, 2015). This presents a challenge as both *C. auris* and *C. neoformans* are known to grow optimally at around 37°C (Rossato and Colombo, 2018; Kraus *et al.*, 2004). The lower incubation temperature might not allow for the true antifungal properties of the compounds to be elucidated as the pathogens are not at their optimal growing temperature. Secondly, the method itself relies on the nematodes ingesting the fungal pathogens, and although there is an effort to standardise the concentration of the fungal pathogen the nematodes are exposed to, it cannot be confirmed that all the nematodes ingested the pathogen and if they consumed the same amount. This may also have affected the degree of infection caused for different members of the population. Lastly, even though there is significant homology between *C. elegans* and mammalian models, *C. elegans* does lack systems such as an adaptive immune system and organs, for example lungs, which play a role in the dissemination and pathogenesis of some fungal pathogens in humans (Lai *et al.*, 2000; Hunt, 2017). As such the method of pathogenesis is not the same which can also influence the perceived efficacy of the compounds. However, despite these limitations the *C. elegans* model remains suited for its role as a way to preliminarily identify new antifungal compounds. The use of *C. elegans* as a toxicity and infection model in

combination with other standard testing procedures may drive the search for a variety of new antifungal treatments.

This study could be expanded to assess the efficacy of the compounds in combination with existing treatments as well as to reassess pathogenicity using a larger concentration range. The toxicity testing performed was also done with crude plant extracts in which the active components themselves may not be toxic but rather another constituent. As such, more detailed phytochemical analysis could be done first to establish the active compounds before reperforming the toxicity testing. As discussed, the use of *C. elegans* should be used as a preliminary tool to screen and identify compounds which show promise as potential treatments. As such, further tests should be done to confirm the toxicity and infection results using mammalian cell lines and potentially higher order model organisms. Studies have also shown that many of the genes required for virulence in murine models of infection are also required for virulence in nematodes (Elkabti ***et al.***, 2018). As such, future experiments should also examine the effect that the compounds have on these virulence factors *in vitro*. This can give further insights into the mechanism of action by which the compounds function against the pathogens. Additionally, it would be interesting to conduct gene-expression studies that compare the treated and untreated infected *C. elegans* models to elucidate how the presence of the compounds alters the nematode's response towards infection. This could also further elucidate and validate the mechanisms by which the compounds work in host-pathogen interactions and can give insight into how they may perform in mammalian models.

4.7 Conclusions

This study demonstrated that of the plant extracts, the *K. africana* extract (LC50 40 – 60 mg/mL) displayed the lowest degree of toxicity towards the *C. elegans* models when compared to the *H. hemerocallidea* (LC50 20 – 40 mg/mL), and *P. zonale* extracts (LC50 5 – 10 mg/mL). Additionally, SPC 7 and amphotericin B displayed very low levels of toxicity as seen by a population mortality of less than 10%, even at the highest concentrations tested for both compounds. As such SPC 7 and *K.* africana were further tested for their efficacy *in vivo* using a *C. elegans* infection model. These results showed that the *C. auris* and *C. neoformans* infected nematodes had a 14 and 4% decrease in mortality respectively when treated with *K. africana* compared to the untreated control. The small decrease in mortality is promising and more significant results might be achieved with treatment at higher concentrations of *K. africana,* however it must be noted that significant toxicity is observed in *C. elegans* at concentrations above 40 mg/mL. In addition, the *C. auris* and *C. neoformans* infected nematodes showed a 40 and 48% decrease in mortality respectively when treated with SPC 7. The compound SPC 7 shows potential as a novel antifungal treatment, showing both low toxicity and the ability to significantly decrease the mortality of *C. auris* and *C. neoformans* infected nematodes.

4.8 References

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CHAPTER 5
Summary

This study ultimately aimed to aid in the search for novel antifungal compounds to widen the arsenal of antifungal drugs that can be used to combat the issue of fungal resistance. As such seven novel silver (I)-phosphine complexes (SPC) as well as eleven native plant extracts were screened for their antifungal activity against the emerging, multidrug resistant pathogen, *Candida auris* as well as clinically important pathogens within the *Cryptococcus* genus. This initial screening was conducted using disc diffusion assays and the results of this showed that ethanolic extracts of *Hypoxis hemerocallidea, Kigelia africana* and *Pelargonium zonale* as well as SPC 7 had promising antifungal activity. As such the minimum inhibitory and fungicidal concentrations of these compounds was assessed further. Of the plant extracts, it was found that the *P. zonale* extract had the best inhibitory effects against the pathogens, followed by *K. africana* and H. *hemerocallidea.* However, only *K. africana* displayed fungicidal properties, against the *Cryptococcus* species. None of the extracts possessed fungicidal activity against the *Candida* strains. The SPC 7 showed to be the most effective of the compounds, exhibiting both fungistatic and fungicidal properties.

Biofilm formation plays a key role in the establishment, pathogenicity and severity of fungal diseases. As such the ethanolic *K. africana* extract as well as SPC 7 was used to assess the ability of the compounds to disrupt and prevent the formation of *Candida* and *Cryptococcus* biofilms. The use of the crystal violet assay was used to determine the biofilm biomass following treatment with the compounds however it was found that neither of the compounds were able to significantly decrease the biomass of the biofilms. Additionally, the use of the XTT assay revealed that the novel SPC 7 compound was able to significantly decrease the metabolic activity of the biofilms except for that of *C. neoformans*. Interestingly, the *K. africana* extract showed to increase the metabolic activity of the *Cryptococcus* biofilms.

Lastly, the toxicity and efficacy of the compounds was assessed using *Caenorhabditis elegans* as a model organism. It was found that SPC 7 exhibited the lowest level of toxicity towards the nematodes. Of the plant extracts, *K. africana* (LC50 40 – 60 mg/mL) was found to have the lowest toxicity followed by *H. hemerocallidea* (LC50 20 – 40 mg/mL) and *P. zonale* (LC50 5 – 10 mg/mL). The nematodes were infected with *C. auris* and *C. neoformans* and were subsequently treated with *K. africana* extract and SPC 7. The *C. auris* and *C. neoformans* infected nematodes showed significant reductions in mortality (40 and 48% respectively) when treated with SPC 7.

As such, this study identified SPC 7 as a promising novel antifungal treatment that should be studied further. It possesses potent fungistatic, fungicidal and antibiofilm properties while remaining non-toxic and improving the lifespan of both *Candida* and *Cryptococcus* infected nematodes. Further studies should be done to examine SPC 7's effect on other fungal virulence factors to elucidate the mechanism of action by which it works. Additionally, toxicity studies should be expanded to include mammalian cell lines and models to further assess its suitability as a treatment.

ETHICS

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

02 October 2017

Dr Vanessa Quan
Division of Public Health Surveillance and Response
National Institute for Communicable Diseases
1 Modderfontein Road
Sandringham
2031
Sent by email to: vanessaq@nicd.ac.za

Dear Dr Quan

Re: **Protocol Ref no: M140159 (Previously M081117)**
Protocol Title: GERMS-SA: Laboratory-based Surveillance for Pathogens of Public Health Importance in South Africa
Principal Investigator: Dr Vanessa Quan et al
Protocol Amendments

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has approved the protocol amendments for the abovementioned protocol, as detailed in your letter dated 20 September 2017.

The following documents were received:

- Cover Letter dated 20 September 2017.
- Study Proposal version 1.4 for 2018.
- Appendix C-1 version 1.4 for 2018.
- GERMS-SA: CRF for version 1.4 for 2018 dated 18 September 2017.
- GERMS-SA: Instruction Sheet for Case Report Form 2018.
- GERMS-SA: Questionnaire version 1.4 for 2018.
- Appendix D-4 Instruction Sheet version 1.4 for 2018.
- GERMS-SA: Case Report Form 2018 version 1.4 for 2018.
- GERMS-SA: Clinical based Surveillance version 1.4 for 2018.
- Candida Case Report Form for NICD/GERMS Lab-based Surveys.

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Thank you for keeping us informed and updated.

Yours Sincerely,

Mr Lebohang Moeng
Administrative Assistant
Human Research Ethics Committee (Medical)

UNIVERSITY OF THE WITWATERSRAND
OFFICE OF THE DEPUTY VICE CHANCELLOR RESEARCH
02 OCT 2017
RESEARCH OFFICE HREC (Medical)
JOHANNESBURG

Research Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 302, Corner York Road and 29 Princess of Wales Terrace, Parktown, 2193 Private Bag 3, Wits 2050 | **T** +27 (0)11-717-1234/2656/2700/1252 **E** Lebo.Moeng@wits.ac.za | **Office E** HREC-MedicalResearchOffice@wits.ac.za | **Website:** www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE
(R 14/16)

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20200503Lab**

BRIEF DESCRIPTION OF APPLICATION:

Laboratory situated in room 216 Biology Building, School of Molecular and Cell Biology

APPLICANT: Dr A Botes

HEAD OF LABORATORY:

SCHOOL/DEPARTMENT : School of Molecular and Cell Biology

DATE CONSIDERED: By circulation 25 May 2020

DECISION OF COMMITTEE: Approved unconditionally

This laboratory is considered to meet the standards of the Institutional Biosafety Committee (IBC) for BSL2 approval, as specified in the IBC's published Standard Operating procedures.
Any change to the type of biohazardous agents being handled should be reported to the IBC
DAFF - Reg no 39.2/University of the Witwatersrand - 20/165 expiry date 03 June 2020

1. This clearance certificate expires on 25 May 2025 and may be renewed on application.

DATE OF APPROVAL: 11 September 2020

CHAIRPERSON: _____

(Professor J Larkin)

DECLARATION OF APPLICANT:

To be completed in duplicate and **one copy** returned to the University of the Witwatersrand, Faculty of Health Sciences, Research Office, Office 301, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown,2193.

1. I have read, understood and accepted the approval conditions above
2. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
3. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://intranet.wits.ac.za/academic/uro/Pages/Institutional-Biosafety-Committee-(IBC).aspx

Signed: Botes Date: 22 Sept 2020

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE (IBC)
(R 14/16)

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20171001**

BRIEF DESCRIPTION OF APPLICATION:

Antifungal activity of indigenous herbal remedies against the opportunistic pathogen Cryptococcus neoformans

APPLICANT: Dr A Botes

SCHOOL/DEPARTMENT : Molecular and Cell Biology

DATE CONSIDERED: 02 October 2017

EXPIRY DATE: 02 October 2022

DECISION OF COMMITTEE: Approved unconditionally

1. This clearance certificate expires on 02 October 2022 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form
4. Unreported changes to the application may invalidate the clearance given by the IBC

DATE: 02 October 2017

CHAIRPERSON: _____
(Professor J Larkin)

DECLARATION OF APPLICANT:

To be completed in duplicate and **one copy** returned to the Research Office, Phillip Tobias Building, Room 301, 3rd Floor, 29 Princess of Wales Terrace, Parktown, Faculty of Health Sciences, University.

1. I have read, understood and accepted the approval conditions above
2. I agree to submit a yearly progress report to the Committee and to submit an interim report on the form provided, in the event of any significant unforeseen event, *e.g.* suspension of a drug trial, temporary closure or relocation of my laboratory, etc
3. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
4. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://web.wits.ac.za/Academic/Research/Biosafety.htm
5. I declare (delete as appropriate) that:
    a. I have all the approvals required by statute or regulation and by the funding agencies supporting this work, or
    b. that I will not begin work until such approvals are obtained

Signed: Botes **Date:** 2 Oct 2017

MSWorks2000/Iain0015/IBCClear.wps

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES