Evaluating the anti-fungal properties of indigenous plant species

by

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DECLARATION

I, Cody Frazer van Wyk (1728114), am a student registered for the degree of Master of Science (by dissertation) in the academic year 2021.

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PREFACE

Cryptococcosis, a common AIDS-defining illness, is diagnosed annually in approximately 215,000 people across the globe and has a mortality rate as high as 70%. Until recently, early diagnosis and the administration of antifungals had resulted in successful treatment; however, reports of increased resistance of these pathogens against standard azole therapies have begun to surface. This resistance shift has prompted the investigation into the use of medicinal plants as an alternative antifungal therapy, particularly those that are able to target known virulence factors and inhibit disease progression.

In **Chapter 1** the increase in cryptococcal-related diseases and the therapeuticapproaches currently employed to treat them is discussed. This is followed by outlining the resistance mechanisms used by pathogenic *Cryptococcus* species and the potential of medicinal plants to serve as novel antifungal alternatives against them.

In **Chapter 2** three indigenous medicinal plants were screened, namely *Agathosma betulina* (Buchu), *Hypoxis hemerocallidea* (African potato) and *Kigelia africana* (Sausage tree), for antifungal activity (fungistatic and fungicidal) and their ability to inhibit ergosterol production. Data indicated thatethanolic *K. africana* extracts yielded the most effective growth inhibition overall with *C. gattii* being the most susceptible to this extract.

In **Chapter 3** the antivirulence property of ethanolic *K. africana* extract against all four pathogenic *Cryptococcus* species was assessed. Laccase activity was significantly inhibited in *C. deneoformans* and *C. gattii*, while urease activity in *C. neoformans* and *C. tetragattii* was significantly inhibited by plant bioactives.

Overall, the application of ethanolic *K. africana* was identified as a promising alternative therapeutic with strong antifungal and antivirulence activity. Future work identifying specific metabolites and mechanisms of action are recommended.

DEDICATION

I dedicate the work done over the last two years to the most important people in my life:

My mother, Michelle van Wyk

And

My father, Frazer van Wyk

Simply for always striving to give me everything that they never had.

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You all have been a massive pillar of support and provided a space to build one another up.
Thank you. Love you.

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List of symbols and abbreviations

α : alpha

β : beta

± : plus minus

°C : Degrees Celsius

μg : microgram

μl : microliter

% : percent

< : less than

~ : approximately

10x : ten times

5-FC : Flucytosine

ABC : ATP-binding cassette

AIDS : acquired immunodeficiency syndrome

AmBd : amphotericin B deoxycholate

CNS : central nervous system

et al : and others

Fe : Iron

g : acceleration

g/l : grams per litre

GXM : glucuronoxylomannan

H. hemerocallidea : *Hypoxis hemerocallidea*

HIV : human immunodeficiency virus

K. africana Kigelia africana

kb : kilobase

LAMB : liposomal amphotericin B

L-DOPA : L-3,4-dihydroxyphenylalanine

MFC : minimum fungicidal concentration

mg : milligram

mg/kg/d : milligrams per kilogram per dose

mg/ml : milligram per milliliter

MIC : minimum inhibitory concentration

mM : millimolar

MM : minimal media

nm : nanometer

OD : optical density

PAMPS : pathogen-associated molecular patterns

PRRs : pattern recognition receptors

rpm : revolution per minute

SEM : standard error of the mean

TNF-α : tumor-necrosis factor alpha

w/v : weight by volume

YNB : Yeast Nitrogen Base

YSS : Yeast Stock Solutions

YWS : Yeast Working Solution

ZOI : zones of inhibition

CHAPTER ONE: Literature review

1.1 General fungal infections

Fungi are microorganisms that are the cause of a broad variety of illnesses, ranging from minor skin conditions to life threatening systemic diseases (Mbunde *et al.*, 2019). Of the types of infections that are initiated by fungi on a global scale, invasive fungal infections have been estimated to be the cause of over one million deaths each year (Brown *et al*., 2012). Fungal infections become fatal in people who are immunocompromised as a result of infections such as human immunodeficiency virus (HIV), the onset of acquired immunodeficiency syndrome (AIDS), and in cancer patients that are undergoing treatment (Kwon-Chung *et al*., 2014). Additional underlying conditions that may predispose patients to fungal infections include extended corticosteroid treatment, the extended use of immune-suppressive drugs during organ transplantation and diabetes (Perfect, 2010). Primary defense systems of animmunocompromised individual, which include the capacity to phagocytose alveolar macrophages and neutrophils, are often unable to function in the presence of immuno-suppressive treatment and/or corticosteroids (Maertens, 2019).

Literature has shown that approximately 90% of the reported invasive fungal related cases/deaths are due to species categorized into one of four genera: *Aspergillus, Candida, Cryptococcus,* and *Pneumocystis* (Masoko *et al.*, 2007; Brown *et al*., 2012). Prior to the introduction of antifungal prophylaxis, *Candida*-related infections were prevalent in 18-20% of all hematopoietic stem cell transplant receivers (Maertens, 2019). However, pervasive use of fluconazole prophylaxis has significantly lowered the occurrence of systemic *Candida* infections since the mid to late 1990s (Maertens, 2019). Similarly, cryptococcosis, caused by *Cryptococcus* species, is responsible for the global infection of 215 000 people annually, leading to roughly 180 000 deaths (Kwon- Chung *et al*., 2014; Rajasingham *et al*., 2017). Before the HIV/AIDS pandemic, less than 200 cases of cryptococcosis were reported in the literature (Hajjeh *et al*., 1995); currently, however, cryptococcosis is regarded as an AIDS-defining illness and mirrors the rate of the AIDS pandemic (Kwon-Chung *et al*., 2014; Esher *et al*., 2018).

The parallel between infection rates of cryptococcosis and HIV/AIDS emphasises the fact that invasive fungal infections mainly affect immunocompromised individuals (Park *et al*., 2009). As a counter-acting measure, fluconazole therapy was introduced in immunocompromised patients,

which led to an evident decrease in cryptococcosis-related cases (Bicanic and Harrison, 2004). Despite the initiation of azole and antiretroviral therapy, cryptococcosis remains problematic in developing regions such as sub-Saharan Africa, where more than 25 million people live with HIV/AIDS (Park *et al*., 2011; Maziarz and Perfect, 2016; Esher *et al*., 2018).

Preliminary work has further shown that other *Cryptococcus* species that were previously considered non-pathogenic, including *Cryptococcus albidus*, *C. curvatus* and *C. laurentii*, have also become prevalent in opportunistic infections (Shankar *et al*., 2006; Banerjee *et al*., 2013; Smith *et a*l., 2017). *Cryptococcus albidus* and *C. laurentii* are recorded as being responsible for approximately 80% of these infections (Arendrup *et al*., 2014; Smith *et al*., 2017). Khawcharoenporn and co-workers (2007) further recorded, after a systemic review of 38 articles, that non-*neoformans* cryptococcal infections generally presented as fungemia (39%), central nervous system (CNS) infections (32%), or as pulmonary, gastrointestinal and dermatological infections. These findings highlight that fungal infections are complex and that the number of systemic cases involving yeasts previously considered non-pathogenic is increasing.

1.2 Taxonomy and Ecology

Traditionally, pathogenic cryptococci were classified into three varieties, with five serotypes and eight molecular subtypes; on the basis of the structural distinctions recorded in thepolysaccharide capsule (Maziarz and Perfect, 2016). As a result of improved molecular techniques, pathogenic cryptococcal species are currently categorised into molecular divisions, which recognises seven species capable of initiating disease in humans (Hagen *et al*., 2015). Thus, the recent taxonomic revision of the *Cryptococcus neoformans/C. gattii* species clade contains the following divisions; *C. neoformans* with three genotypes (VNI, VNII, VNIII), *C. deneoformans* (VNIV); and five other cryptic species, *C. gattii* (VGI), *C. bacillisporus* (VGIII), *C. deuterogattii* (VGII), *C. tetragattii* (VGIV), and *C. decagattii* (VGIV/VGIIIc) (Hagen *et al*., 2015). Hybrid species have also been described; however, their degree of pathogenicity remains unknown (Bovers *et al*., 2008).

Focusing research on *C. neoformans* can be deemed vital due to the fact that close to 95% of cryptococcal infections are initiated by this particular species (Maziarz and Perfect, 2016). *Cryptococcus neoformans* is known to have a global distribution and is capable of infecting a broad range of hosts, including cats, dogs, dolphins, as well as plants (Warpeha *et al*., 2013). Globally, weathered droppings from pigeons, soil and guano from a range of bird species have been recorded as necessary saprophytic sources for survival (Mitchell *et al*., 2011; Kwon-Chung *et al*., 2014). Interestingly, pigeons and other avians are rarely infected as a result of their higher body temperatures which range between 41°C to 42°C, exceeding favourable growthtemperatures of *C. neoformans* (Kwon-Chung *et al*., 2014). However, these avian sources are suspected of serving as a reservoir contributing to the global dispersal of this fungal species (Litvintseva *et al*., 2011). Arboreal regions such as the bark of trees, tree hollows, and decaying wood have been recorded as sources for *C. neoformans* growth as well (Mitchell *et al*., 2011).

Cryptococcus deneoformans in immunocompromised individuals recorded as the fungal species responsible for the remaining 4 to 5% of cryptococcal infections on a global scale (Maziarz and Perfect, 2016; Esher *et al*., 2018). The occurrence of this fungal species is more evident in subtropical to tropical regions, with modern research highlighting species expansion totemperate zones, potentially as a result of environmental moisture and temperature shifts due to climate change (Kwon-Chung *et al*., 2014; Maziarz and Perfect, 2016). In contrast to *C. neoformans*, *C. deneoformans* has not been isolated from avian guano; however, an increasing number of tree species are being recorded as reservoirs of this fungal species (Stephen *et al*., 2002; Mitchell *et al*., 2011).

1.3 Cryptococcosis

Cryptococcosis is described as an opportunistic, disseminated disease that is characterised by the occurrence of meningoencephalitis and fungemia, while also being able to induce pneumonia and skin lesions in infected individuals (Kwon-Chung *et al*., 2014; Rohatgi and Pirofski, 2015; Gago *et al*., 2017; Esher *et al*., 2018). Cryptococcosis is initiated by encapsulated pathogenic yeasts in the polyphyletic genus *Cryptococcus* and has a worldwide distribution while also having a broad array of clinical presentations (Maziarz and Perfect, 2016; Gago *et al*., 2017). The overall management of fungal infections, specifically cryptococcosis, has become highly

complex due to several reasons. The high cost and limited number of available antifungals, coupled with increasing resistance of pathogens to these antifungals, has contributed to the complex management of infections (Mbunde *et al*., 2019).

Cryptococcosis is initiated through the inhalation of desiccated, airborne yeast cells or from basidiospores that are naturally present in the environment (Kwon-Chung *et al*., 2014; Rohatgi and Pirofski, 2015; Bielska and May, 2016). The latter scenario is common in early childhood during the period of acquisition of other encapsulated microbes (Goldman *et al*., 2001). Upon analysis of serological evidence, Goldman and co-workers (2001) indicated that the overall prevalence of cryptococcal infection is high, but that the onset of disease is rare. At the point of yeast/basidiospore inhalation, recruitment of immune cells by activated alveolar macrophages through cytokines and chemokines is triggered in order to express the appropriate Th1 response and/or granulomatous inflammation (Kwon-Chung *et al*., 2014). In healthy hosts, the expressed immune response functions successfully to eradicate the cryptococcal cells.

However, when immunocompromised hosts are infected, two possible pathogenic pathways are activated. The first path that may be taken upon cryptococcal infection is that small lung-lymph complexes may develop where yeasts persist in a dormant state, resulting in patients that remain clinically asymptomatic (Perfect, 2010; Bielska and May, 2016). As the condition of an individual deteriorates, due to, for example, HIV infection progression, corticosteroid treatment orvarious immuno-suppressing conditions, dormant yeasts are activated and proliferation withinthe pulmonary-lymph node occurs. Dormant yeast activation is followed by dissemination of cells into extrapulmonary sites (Perfect, 2010; Dromer *et al.*, 2011). The second pathway that may be initiated results in the proliferation of cryptococcal cells, followed by hematogenic dissemination to the brain across the blood-brain barrier (Shi *et al.*, 2010). The human brain is noted as having an environment with decreased levels of oxygen and nutrients; however, upon dissemination of yeast cells into the brain, cell proliferation begins and leads to meningoencephalitis (Perfect, 2010; Shi *et al*., 2010). Infection of the CNS is most common during clinical colonization of cryptococcosis, often causing death in infected patients despite treatment (Perfect, 2010). *Cryptococcus deneoformans* follows a similar pathogenic route, however pulmonary infection is more common when compared to CNS infection, as seen with *C. neoformans* (Galanis *et al*., 2010).

1.4 Antifungal treatment

An important factor for determining the type of treatment to administer to a patient is the category of risk group to which they belong (Perfect *et al*., 2010). These categories include HIV- infected patients, organ transplant receivers, non-HIV-infected and non-transplant patients (Kwon-Chung *et al*., 2014). The site of infection, state of renal functioning and specific contraindications for specific patients, should also be accounted for when determining the necessary treatment procedure (Whitney and Bicanic, 2015).

The treatment of severe cryptococcal infections has generally been orientated aroundamphotericin B deoxycholate (AmBd), with a standard dose of 0.7-1 mg/kg/d being the recommended quantity (Maziarz and Perfect, 2016). This method of treatment focuses on eliminating ergosterol in the yeast cell membrane and may cause cell lesions through pore formation, resulting in intracellular cation leakage (Mesa-Arango *et al*., 2014). However, due to the high levels of nephrotoxicity of AmBd and the association with infusion-related toxicity, long-term treatment may result in detrimental effects on the body of patients (Lestner *et al*., 2017). For this reason, AmBd treatments are closely monitored when administered and occur over short periods (Maziarz and Perfect, 2016). Flucytosine (5-FC) has been utilized as a combination therapy with AmBd for cryptococcal meningitis and/or pulmonary cryptococcosis, with a recommended dosage of 100 mg/kg/d in divided doses (Whitney and Bicanic, 2015; Maziarz and Perfect, 2016;Limper *et al*., 2017). The second treatment that is also used with AmBd and 5-FC, is fluconazole over an eight-week course and is administered in doses between 400 – 800 mg per day (Maziarz and Perfect, 2016). Treatment occurs over one year until normal function of the immune system is restored (Kwon-Chung *et al*., 2014; Whitney and Bicanic, 2015; Rajasingham *et al*., 2017). A recent alternative, liposomal amphotericin B (LAMB), yields similar outcomes as AmBd but with lowered nephrotoxicity (Lestner *et al*., 2017; Jarvis *et al*., 2018). Treatment with LAMB is associated with the clearance of cryptococcal cells in the cerebrospinal fluid of patients (Lestner *et al*., 2017).

1.5 Virulence factors

Cryptococcus neoformans is a free-living species that has no requirement for a host for successful reproduction and survival (Rohatgi and Pirofski, 20l5). It is characteristically knownto possess features that allow it to adapt within the lung environment and induce evasion of host immune responses (Trevijano-Contador *et al*., 2018). Melachowski and colleagues (2016) have reported more than 150 virulence factors associated with *C. neoformans*. Key factors thatcontribute to efficient adaptation capabilities include the development of a polysaccharide capsule, production of cell wall-associated melanin, the activity of certain enzymes, and the ability to grow at 37 °C (Cox *et al.*, 2000; Rohatgi and Pirofski, 2015).

The host immune system typically responds to cryptococcal infections by recognizing antigens present on fungal cell walls or through detecting pathogen-associated molecular patterns (PAMPS) by pattern recognition receptors (PRRs) found on the host immune cells (Hueng, 2017). Phagocytosis and the production of cytokines may be triggered by the engagement of PRRs that stimulate a signal transduction sequence. However, the polysaccharide capsule confers a protective shield that can mask cell wall components of PAMPS, including β-glucans and chitin, leading to the initial infection stages of cryptococcosis (Hueng, 2017). Through thepossession of a polysaccharide glucuronoxylomannan (GXM) component, the capsule may exert a deleterious effect on the host through the interference of T-cell functioning. This interference leads to the disruption of cell-mediated immunity (Liu *et al*., 2012; O'Meara and Alspaugh, 2012; Vecchiarelli and Monari, 2012). Additionally, GXM may be shed which results in circulation of these components through the bloodstream and cerebral spinal fluid (Yauch *et al*., 2004). In this case the circulated GXM has the ability to alter the production of cytokines by leukocytes, inhibit the anticryptococcal activity of neutrophils and may interfere with the maturity rate of dendritic cells (Yauch *et al*., 2004).

In the human body, the pathogenic *Cryptococcus* species are able to produce melanin from compounds including dopamine and L-DOPA (Eisenman *et al*., 2011). Melanin production has been shown to be responsible for contributing to colour changes in *C. neoformans* that result in a brown-to-black pigmentation (Zhu *et al*., 2001; Zhu and Williamson, 2004). Molecular characterisation has further highlighted the importance of laccase in melanin production as the

construction of targeted gene knockout trials can be performed (Zhu and Williamson, 2004). Research was conducted by Zhu and Williamson (2004) using a constructed strain containing a 4kb deletion of the 5' end of a laccase encoding gene (*CNLAC1*) revealed the formation of white colonies when cultured on Niger seed agar (Noverr *et al*., 2004; Zhu and Williamson, 2004). Moreover, disruption of the *C. neoformans* laccase genes, *CNLAC1* and *CNLAC2*, recorded a significant increase in the survival rate of infected mice, serving as evidence to infer that melanin plays a role in overall virulence (Thammasit *et al*., 2018). The cryptococcal laccase enzyme has been found to be responsible for catalysing the production of melanin, further supporting the multi-functional characteristics of this enzyme (Brown *et al*., 2007; Idnurm, 2011). Additionally, *in vitro* studies have highlighted that upon use of antifungal agents such as amphotericin B and caspofungin, melanised *C. neoformans* cells are less susceptible to killing when compared to non-melanised cells (Casadevall *et al*., 2000; Dalisay *et al*., 2011).

The utilisation of enzymes to enhance virulence is also well documented (Samie *et al*., 2019). Enzymes involved in successful host colonization play an important role in the virulence of fungal pathogens, as host defence mechanisms are targeted by specific molecular factors (Wozniak *et al*., 2015). In response, host protection is maintained/primed through various factors by both the host and additional effector functions triggered by immune cells that manipulate enzyme expression, consequently impacting/altering the infection process (Almeida *et al*., 2015). This complex association between pathogen-derived factors and host defense mechanisms determine the probability of a cryptococcal infection being resolved, progressed or dormant (Wozniak *et al*., 2015).

Laccases are multicopper oxidoreductases that utilise molecular oxygen as an electron acceptor to function on a range of diphenols and aromatic substrates (Singh *et al*., 2016). *In vitro* studies conducted by Liu and colleagues (2012) on *C. neoformans* revealed iron oxidase activity in laccase that converts phagosomal Fe(II) to Fe(III), leading to a reduction in the formation of hydroxyl radicals. The presence of these toxic hydroxyl radicals is unfavoured as studies have identified their contribution towards antimicrobial and anticryptococcal activity of phagocytic cells (Liu *et al*, 1999). This finding suggests that laccase-iron activity can protect *C. neoformans* through the maintenance of iron in an oxidised state. Furthermore, laccase can be deemed a multi-functional enzyme that contributes to virulence independent of its contribution to

iron-oxidase activity. When *C. neoformans* is found in an environment containing appropriate substrates, the pigment melanin is synthesised by laccase through the process of oxidising catecholamines. (Casadevall *et al*., 2000; Sharma *et al*., 2018).

Research investigating the importance of urease in virulence supports the notion that it contributes to pathogenesis and invasion of the CNS (Morrow and Fraser, 2013). Urease is a metalloenzyme that functions in catalyzing the hydrolysis of urea to form ammonia and carbon dioxide (Cox *et al*., 2000; Feder *et al*., 2015). Previous studies have confirmed that the *URE1* gene encodes the urease apoenzyme in *C. neoformans*, which has consequently led to virulence research where deletion of the *URE1* gene was documented/analysed (Singh *et al*., 2013; Feder *et al*., 2015). In a mouse model, urease-positive strains recorded a higher degree of severity of infection, leading to more mortalities and a higher transmission to brain tissue when compared to deletion strains. It was also hypothesised that the ammonia production by urease altered the pH within the brain environment, preventing the immunity action of phagolysosomes in phagocytes. Additionally, host epithelial tissue would be damaged, causing cell permeability to increase, ultimately providing simpler means for pathogen dissemination from lungs to the bloodstream (Brown *et al*., 2007; Liu *et* al., 2012; Morrow and Fraser, 2013; Feder *et al*., 2015).

The capacity to grow at 37 °C is a virulence factor that contributes to the success of highly pathogenic *Cryptococcus* species (Perfect *et al*., 2006; Kwon-Chung *et al*., 2015). Amongst the more than 70 species of the genus *Cryptococcus; C. neoformans* and *C. gattii* are identified as species that tolerate and adapt to physiological temperatures efficiently, while remaining *Cryptococcus* species such as *C. deneoformans* and *C. tetragattii* experience suboptimal growth at 37 °C (Petter *et al*., 2001; Perfect *et al*., 2006; Fonseca *et al*., 2011). Variability in thermotolerance is further evidenced in *C. podzolicus* as this strain possesses a capsule and is capable of melanin production, virulence factors associated with pathogenicity, but is unable to sufficiently grow at temperatures ranging between 35 to 40 °C (Petter *et al*., 2001). Interestingly,research conducted by Oliveira and colleagues (2020) has confirmed that *C. neoformans erg6Δ* strains display significant decreases in membrane ergosterol content, major growth impairments at 37 °C, slowed growth rates at both 35 and 30 °C and reduced survival rates after macrophage phagocytosis. The *ERG6* gene encodes the enzyme sterol 24- C-methyltransferase, which is

responsible for the conversion of zymosterol to fecosterol in the ergosterol biosynthetic pathway. This study suggests that targeting the *ERG6* gene and/or sterol 24-C-methyltransferase would impact the pathogen's ability to adapt to and withstand high temperatures thereby decreasing its pathogenicity.

1.6 Antifungal resistance mechanisms

Widespread use of antifungal treatment is recognized as a contributor that has led to an increase in drug resistance (Cowen, 2008). As a result, the management of infected individuals has changed as resistance among fungal pathogens has limited treatment alternatives. Ergosterol is a crucial target sterol for azole therapies, with the interruption of ergosterol synthesis leading tothe accumulation of 14α-methyl sterols. The interruption modifies various aspects of the cell membrane, such as overall stability, permeability and the action of membrane-bound enzymes (Cowen *et al*., 2015). Azole treatments, therefore, target the ergosterol biosynthetic pathway, specifically the cytochrome p450-dependent enzyme lanosterol 14α-demethylase encoded by *ERG11*. Lanosterol 14α-demethylase functions to catalyze the oxidative removal of the 14α- methyl group from lanosterol. Additionally, the biosynthetic pathway is disrupted due to the removal of the 14α-methyl group (Cowen *et al*., 2015). Resistant *C. neoformans* strains have been recorded to possess a limited number of single point mutations within the *ERG11* gene conferring resistance to common azoles, including fluconazole (Rodero *et al*., 2003).

Azole resistance may also be conferred through the over-expression of *ERG11*. Over-expression leads to the need for higher treatment doses for inhibition as the target abundance is increased. Thus, fungal pathogen susceptibility is reduced (Cowen *et al*., 2015). The duplication of the *ERG11* gene may occur through the formation of an isochromosome with two copies of the left arm of chromosome 5 (i(5L), which is the region where *ERG11* resides. Alternatively, duplication of the whole chromosome may also occur (Salmecki *et al*., 2006).

The recognition of diverse chemicals that facilitate multi-drug resistance is activated through membrane-associated efflux pumps, which have been identified as a ubiquitous resistance mechanism (Cowen *et al*., 2015). In fungi, a drug efflux system that regulates azole resistance is known as the ATP-binding cassette (ABC). ATP-binding cassette proteins are ATP-dependent

transporters that are organized in a duplicated topology, consisting of two transmembrane spanning domains and two cytoplasmic nucleotide-binding domains that catalyze ATP hydrolysis (Cowen *et al*., 2015). In *C. neoformans*, a specific ABC transporter, namely AnfiFungal resistance (encoded by *CnAFR1*), is responsible for azole resistance (Posteraro *et al*.,2003; Coleman and Mylonakis, 2009). The *CnAFR1* gene is well documented, with preceding literature indicating that the gene has a high number of introns that are required for geneexpression (Cox *et al*., 1995; Posteraro *et al*., 2003). Introns are also multi-functional due to their ability to block transposon dispersal, promote genome stability and regulate gene expression through alternative splicing to respond appropriately to environmental changes (Kwon-Chung, etal., 2015; Janbon, 2018). When investigating the influence of the *CnAFR1* gene, Posteraro and researchers (2003) found that deletion of the gene had a significant impact in increasing susceptibility to azoles.

Along with mechanisms employed to decrease their susceptibility to antifungal therapeutics, *Cryptococcus* species possess virulence factors that further enhance the probability to successfully induce host colonization and disease (Vu *et al*., 2019). The development of agents that target these virulence factors has become the focus of recent research efforts as these therapeutics target factors that are critical in causing disease instead of factors necessary for growth (Vu *et al*., 2019; Iyer, 2021). As a result, these agents reduce the potential of pathogensto induce antifungal drug resistance by imposing minimal selective pressure, which may preserve the long-term use of antifungals (Dickey *et al*., 2017). Moreover, the potential use of anti-virulence agents is beneficial as toxicity risks associated with antifungal therapeutics may be subverted as valuable gut commensal populations will not be eliminated (Cui *et al*.,2015; Vila *et al*., 2017).

1.7 Identification of novel antifungal agents

Across the African continent, a diverse range of indigenous flora is collected and utilised as herbal remedies in various form, thus playing an important role in healthcare measures indeveloping communities (Katerere and Eloff, 2008; Ncube *et al*., 2013). The use of medicinal plants as a source of effective antimicrobial therapy has been reported as a new avenue for research across different countries (Singh and Jain, 2011). South Africa has observed an increasing interest in natural-based remedies, with approximately 80% of the population making use of traditional medicines to meet their primary health care requirements (Louw *et al*., 2002; Street and Prinsloo,

2013). The South African landscape is documented as having more than 30,000 flowering species, favouring the prospect of research aimed at discovering novel medicinal agents for commercialization (Street and Prinsloo, 2013).

Agathosma betulina is commonly known as "Buchu" and is an aromatic flowering plant belonging to the family *Rutaceae*. It is endemic to the fynbos habitat and can be found amongst the lower regions of mountains in the Western Cape Province, South Africa (Witbooi *et al*., 2017). This plant has gained an economic reputation as it has an extensive history of being involved in traditional medicine. It contains highly sought-after essential oils which have a high diosphenol content (Moolla *et al*., 2007; Mavimbela *et al*., 2014). Analysis of these essential oils has shown that two compounds, limonene and menthone, were the most abundant. *In vitro* studies performed using these compounds documented antifungal activity against *C. albicans* and *Aspergillus niger* (Omran *et al*., 2011; Fajinmi *et al*., 2019). A second plant recognised for its beneficial characteristics is the endemic fynbos species *Aspalathus linearis,* commonly known as "Rooibos" (Street and Prisnloo, 2013). Due to the absence of caffeine in *A. linearis*, popularity has increased as the plant may be conveniently prepared as a beverage andexhibits capacity to alleviate digestive-related problems (Grunewald, 2009). These health benefitshave previously been hypothesised to be associated with the phenolic content of *A. linearis*;however, more data on dietary exposure to rooibos phenolic compounds is still required (Joubert *et al*., 2009).

Similarly, *Kigelia africana* (Lam.) Benth. has been found to possess antimicrobial properties (Akunyili *et al*, 1991; Binutu *et al.*, 1996). Belonging to the *Bignoniaceae* family with a geographical distribution that spans across west and central Africa (Sidjui *et al*., 2015; Singh *et al*., 2018), it is commonly named the "sausage tree" due to the shape of the fruit that the plant produces (Bello *et al*., 2016). *Kigelia africana* is a known hydrophile and is therefore most commonly found near sources of water such as the floodplains in Cameroon, Guinea, Kenya, Nigeria and Senegal (Sidjui *et al*., 2015). Different sections of the plant are used to treat varying types of infections/diseases; however, the predominant use is in the treatment of fungal-associated skin diseases (Oyedeji and Bankole, 2012). Studies have isolated approximately 145 phytoconstituents from different sections of the plant, with flavanoids, iroids and naphthoquinones serving as the predominant class of compounds (Bello *et al*., 2016). Theantimicrobial activity of the stembark against *Bacillus subtilis, C. albicans, Escherichia coli, Pseudomonas aeruginosa* and

Staphylococcus aureus has also been established (Akunyili *et al*, 1991; Kwo and Cracker, 1996), however, further research is required in order to determine the full extent of this plant's antifungal properties.

Using herbal treatments to control opportunistic fungal infections is a promising and innovative alternative to commercial antifungals (Mbunde *et al*., 2019). Therefore, with the above literature serving as background, research emphasis was placed on assessing the antifungal properties of indigenous medicinal plants for antifungal activity against pathogenic *Cryptococcus* species. Findings obtained from this research will look to serve as information/data to assist in selecting particular indigenous medicinal plants that are suitable for future commercialization, while also ensuring effective antifungal treatments are more readily available to reduce immunocompromised fungal fatalities in developing geographical regions.

1.8 Aims and objectives

This study aims to screen compounds extracted from various indigenous medicinal plants for antifungal / anti-virulence activity against pathogenic *Cryptococcus* species.

Objectives

Crude extraction of plant bioactive compounds from selected indigenous medicinal plants using ethanol, methanol, and hexane as extraction solvents.

1. Assess the antifungal efficacy of each crude extract by means of disc diffusion assays.
2. Determine the minimum fungicidal concentration (MFC) of each crude extract.
3. Screen select crude extracts for their potential to inhibit ergosterol biosynthesis.
4. Screen select crude extracts for potential antivirulence activity against specific cryptococcal virulence factors, specifically the enzymes laccase and urease.

CHAPTER TWO: Antifungal properties of select indigenous plants against pathogenic *Cryptococcus* species

2.1 Introduction

The influence of fungal diseases on human health has been largely overlooked despite infection rates increasing at an alarming rate (Rodrigues and Albuquerque, 2018). This under-estimation of fungi as influential pathogens is seen in the fact that illnesses and diseases initiated by bacteria, protozoa and viruses have been treated as high priority research projects for more than one hundred years (Brachman, 2003). In contrast, opportunistic and invasive mycoses have only been acknowledged as medically significant pathogens since the 1980s (Nucci and Marr, 2005). This imbalance in research priority is still observed when we look at the number of scientific articles on PubMed. In the last year alone, a total of 12,315 and 6,161 articles centred on tuberculosis and malaria respectively, while only 344 and 496 articles about candidemia and cryptococcosis, respectively, were published. One reason for this delay in acknowledging the importance of fungal pathogens as a threat to human health may be attributed to the low impact and rare occurrence of fungal diseases in the past (Rodrigues and Nosanchuk, 2020).

In 2014, approximately 150 million new serious fungal infections were reported, of which 1.5 million were fatal (Bongomin *et al*., 2017). Cryptococcal meningitis alone accounted for 223,100 new cases, with sub-Saharan Africa carrying the greatest burden reporting 162,500 new cases (Bongomin *et al*. 2017; Nyakiza et al., 2017; Rajasingham *et al*., 2017). These numbers can be largely attributed to a lack of available resources. Limited treatment options are available due to antifungal therapeutics being costly and the possible introduction of severe side effects as a result of the toxicity of these resources (Masoko *et al*., 2007; Wickes and Wiederhold, 2018). In addition, diagnosis can be laborious or require specialized equipment that is not readily available (Perfect and Bicanic, 2015). This has resulted in a concentrated search for new antifungal agents, such as those produced by plants. Alternative, readily available, cheap and less harmful treatment options would greatly benefit the African continent.

South Africa is a country that hosts approximately 30,000 flowering plant species. This is justified by the fact that the Cape Floristic Region, the Succulent Karoo and the Albany-Maputaland corridor, three of the world's 'biodiversity hotspots', are all located in South Africa.

Co-existence of various ethnic groups and biodiversity regions serve as drivers for the use of these plants for medicinal purposes (van Wyk *et al*., 2009). Reports show that approximately 4,000 species are significant in an ethnobotanical respect and 3,000 plants already serve a medicinally useful purpose (van Wyk *et al*., 2009; Street and Prinsloo, 2013). A limited number of medicinal plants have reached the commercial market. *Aspalathus linearis* and *Agathosma betulina*, commonly referred to as rooibos and Buchu, respectively, are two medicinally relevant plants recognized internationally and are a testament to the commercial benefits associated with well-understood plant species (Street and Prinsloo, 2013).

Recent research has expanded our scientific knowledge regarding the use of plants to treat microbial infections. Several *Comretum* species, which are used to treat colds, fevers and urinary tract infections (Mokoka *et al*., 2010; Mapfunde *et al*., 2016), have been shown to have significant antifungal properties against *Microsporum canis* and *Sporothrix schenckii* (Masoko *et al*., 2007). *Hypoxis hemerocallidea*, commonly known as the African potato, has been shown to possess antibacterial, antifungal and antiviral activity against a wide range of pathogenic strains that are generally related to acute infections (Buwa and Van Staden, 2006; Steenkamp *et al*., 2006). Similarly, extracts from the *Kigelia africana*, the African sausage tree, have been shownto inhibit the growth of both *Candida albicans* and *Staphylococcus aureus* (Binutu *et al*., 1996; Owolabi et *al.*, 2007).

The use of medicinal plants as an alternative therapeutic has been explored in a number of studies, with reports highlighting the potential to extract potent, yet safe, antifungal metabolites (Craig and Newman, 2001; Ugoh and Bejide, 2013). One main driver of this growing interest in medicinal plant use is derived from the curiosity of researchers to scientifically substantiate the traditional practice of using natural remedies, with the purpose of determining whether actual pharmacological effects are observed (Samy and Ignacimuthu, 2000; Masika *et al*., 2005). Consequently, plants that exhibit anticryptococcal activity may potentially serve as the first steps towards developing therapeutics that are more efficient and less toxic.

2.2 Aim and objectives

This study aimed to assess the antifungal properties of extracted plant bioactives from indigenous medicinal plants, against pathogenic *Cryptococcus* species.

The following objectives were identified:

1. Extraction of plant bioactives from the selected indigenous medicinal plants usingethanol, methanol, and hexane as extraction solvents.
2. Assessing the antifungal activity of crude extracts using disc diffusion assays.
3. Evaluating the antifungal efficacy of each crude extract by determining the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC).
4. Assessing the potential of crude plant extracts to inhibit ergosterol biosynthesis.

2.3 Methodology

2.3.1 Test organisms and culture conditions

Yeast strains used in this research study were obtained from the culture collection of Dr. Angela Botes, Mycology Laboratory, School of Molecular and Cell Biology, Faculty of Science, University of the Witwatersrand. *Cryptococcus deneoformans* CBS 132, *C. gattii* CBS 10575, *C. neoformans* ATCC H99, and *C. tetragattii* MRC 8871 were maintained through periodic transfer on Sabouraud dextrose agar (SDA, pH 5.6) plates supplemented with 200 mg/l chloramphenicol. *Cryptococcus deneoformans* CBS 132 and *C. neoformans* ATCC H99 strains were incubated at 37 °C, while *C. gattii* CBS 10575 and *C. tetragattii* MRC 8871 were incubated at 30 °C. All yeast strains were incubated for a period of 48 h and were thereafter stored at 4 °C. Yeast strains for experimental procedures were inoculated into Yeast Nitrogen Base (YNB,pH 5.5) broth (1.7 g/l YNB; 20 g/l glucose; 1 l distilled water) and incubated at 37 °C for 48 h on a rotary wheel (Stuart™ SB2 fixed speed rotator, United Kingdom). The cell cultures were then adjusted to an OD_{600} = 0.1 using fresh YNB broth.

2.3.2 Antifungal assays

2.3.2.1 Plant bioactive extraction

The indigenous plant species used in this study include the following: *Agathosma betulina* (Buchu), *Hypoxis hemerocallidea* (African potato) and *Kigelia africana* (Sausage tree). *Curcuma longa* (turmeric) was included as a control as it is already known to possess antifungal properties (Esfandiari and Hashemi, 2019). All plant material was purchased from Springbok Pharmacy (https://www.springbokpharmacy.co.za/). Plant bioactives were extracted according topreviously described methods (Masoko *et al*., 2007; Mokoka *et al*., 2010). Briefly, dried plant material was crushed into a fine powder using a pestle and mortar. Ethanol, methanol and hexaneextracts were prepared by mixing 4 g of the ground plant material with 40 ml (10 ml per gram) 95% (v/v) ethanol, methanol and/or hexane in a 50 ml falcon tube. The sample was vortexed for five minutes and left to stand at room temperature for one hour. The supernatant was filtered through Whatman No.1 filter paper into a pre-weighed falcon tube. The filtrate was air-dried, and the remaining pellet was re-suspended in acetone and stored at 4 °C.

2.3.2.2 Disc diffusion assays

The antifungal activity of the ethanol, methanol and hexane extracts were evaluated against *Cryptococcus deneoformans* CBS 132, *C. gattii* CBS 10575, *C. neoformans* ATCC H99, and *C. tetragattii* MRC 8871 through the use of the disc diffusion assay protocol as previously described (Bauer et al., 1966). This method was selected to efficiently determine whether plant species demonstrated antifungal activity against the *Cryptococcus* species involved in the study. Sterile paper discs were impregnated with ethanol, methanol and hexane extracts of each indigenous plant species at a range of concentrations (50, 100, 200, 400 800 and 1600 mg). Acetone impregnated discs served as a negative control. The prescribed antifungal amphotericin B served as a positive control (0.0005, 0.001, 0.002, 0.004, 0.008 and 0.016 mg). Impregnated discs were placed on pre- inoculated YNB agar plates prepared by inoculating *Cryptococcus* on YNB media and grown at 37 °C for 48 h.

Sterile cotton swabs were used to evenly streak inoculum on the plates. Five repetitions of the streaking action were performed with a 90° rotation of the plate each time (Jorgensen and

Ferraro, 1998). Sterile forceps were used to equally position the disc on the YNB agar. *Cryptococcus deneoformans* and *C. neoformans* strains were incubated at 35 °C, while *C. gattii* and *C. tetragattii* were incubated at 30 °C for 48 h. Visible zones of inhibition (ZOI) were measured to the nearest millimetre.

2.3.2.3 Minimum inhibitory concentration (MIC)

The MIC has been described as the lowest concentration of a solution, chemical or extract required to inhibit the growth of a species (Espinel-Ingroff *et al*., 2005).

RPMI 1640 was supplemented with 10% (w/v) glucose and 0.165M 3-(N-morpholino) propanesulfonic acid (MOPS) then adjusted to pH 7.0. The final volume was made up to 50 ml using distilled water. The final mixture was then filter sterilized and stored at 4 °C. Fresh cultures of each cryptococcal species were prepared by sub-culturing on SDA (pH 5.6) at 30 °C for 48 h. Five 1 mm diameter colonies were picked and resuspended in 5 ml 0.145 M saline solution and vortexed for 15 seconds. Yeast Stock Solutions (YSS) were then prepared by adjusting the cell suspension OD_{600} to 0.1 (0.5 McFarland standard) using fresh saline solution. The final yeast inoculum was prepared for the 48-well plates by diluting the YSS at a 1:100 ratio to obtain the Yeast Working Solution (YWS), followed by diluting the YWS at a 1:20 ratio. A total of 360 µl of the final yeast dilution (YWS) was added to the relevant well. Plant extract dilutions were prepared by serially diluting stock plant extracts (20 mg/ml). A total of 40 µl was added to the relevant wells yielding final concentrations ranging from 0.15625 to 10 mg/ml. Positive control wells using 40 µl RPMI 1640 and 360 µl final yeast inoculum was created. A negative control well (blank) using 360 µl RPMI 1640 and 40 µl plant extract was also created. The 48-well plates were incubated at 35 °C for 72 h. After the incubation period, wells were spectrophotometrically measured at 600 nm using a VICTOR Nivo Multimode plate reader (Perkin Elmer, Massachusetts, United States).

2.3.3.4 Minimum fungicidal concentration (MFC)

Minimum fungicidal concentration is defined as the lowest concentration at which either no growth occurred or fewer than three colonies were recorded (Espinel-Ingroff *et al*., 2002).

Suspensions prepared for the MIC analysis were used to complete this assay. A total of 10 µl from each well was aliquoted onto SDA (pH 5.6) and equally spread on agar plates using the hockey stick method. The plates were incubated at 30 °C for 24 to 48 h. After the incubation period, the number of colonies was counted.

2.3.4 Ergosterol assay

The impact of ethanolic *K. africana* extract on cell ergosterol content was evaluated. Ergosterol is a fungal sterol located in the cell membrane (Solanko *et al*., 2018). Due to the specificity of ergosterol and its requirement for fungal growth, it is a suitable target for many antifungals including amphotericin B, clotrimazole, fluconazole, itraconazole and miconazole (Alcazar-Fuoli and Mellado, 2013). Starter cultures were prepared by inoculating cryptococcal cells into 20 ml 2 x minimal media (MM, 15 mM glucose; 10 mM magnesium sulfate; 29.4 mM monopotassium phosphate; 13 mMglycine; 0.003 mM thiamine hydrochloride). Cultures were incubated on a platform orbital shaker (120 rpm) (LABCON® SPO-MP15 shaker, South Africa) at 37 °C for 48 h. After incubation,2 ml of starter culture was inoculated into 20 ml fresh 2x MM supplemented with 2.5 or 5 mg/ml ethanolic *K. africana* extracts, based on the calculated MFC for each species. A negative control set supplemented with acetone and a positive control set supplemented with 0.01 µg/ml amphotericin B was also prepared. All sets were incubated for 24 h at 37 °C on a platform orbital shaker at 120 rpm. After incubation, samples were transferred into pre-weighed falcon tubes and centrifuged (11 350 x g for 5 min) at 4 °C. Samples were washed with 20 ml distilled water and re-centrifuged under the same conditions. The net weight of each pellet was determined. Pellets were resuspended in 1.5 ml 25% (w/v) ethanolic potassium hydroxide and transferred to sterile glass test tubes. Glass test tubes were vortexed for 1 min and subsequently incubated in a water bath for one h at 85 °C. Tubes were removed from thewater bath and allowed to cool to room temperature. Once samples were cool, 0.5 ml distilled water and 1.5 ml n-heptane were added to each glass test tube. Samples were vortexed for three minutes and the aqueous layer was transferred to a sterile 1.5 ml Eppendorf tube. Finally, samples were diluted

(10x) in 100% ethanol in quartz cuvettes and the absorbance was read at 230 and 281 nm (S-22 UV/Visible Spectrophotometer, Germany).

2.3.5 Statistical analysis

All experimental procedures were performed in triplicate. Statistical values were further presented as their means and the standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, California, https://www.graphpad.com) to run unpaired t-test analyses and determine statistical significance. A p-value < 0.05 was considered significant.

2.4 Results and discussion

2.4.1 Disc diffusion assays

Disc diffusion assays for each plant extract were conducted in order to identify which plant species produce bioactives that are antifungal in nature.

2.4.1.1 *Hypoxis hemerocallidea*

No ZOI was observed when using any of the extracts from *H. hemerocallidea* against *C. deneoformans*, *C. neoformans* (Table 2.1) and *C. tetragattii* (Table 2.2). Minimal (0.5 – 1 cm), inconsistentantifungal activity was observed against *C. gattii* using the methanolic extracts (Table 2.2); however, the ethanolic and hexane extracts from *H. hemerocallidea* yielded no ZOI against *C. gattii*.

The utilization of *H. hemerocallidea* as a medicinal plant is well-documented in literature, with findings demonstrating a wide range of uses such as treating diabetes, hypertension and asthma (Ojewale, 2006; Boukes *et al*., 2008; Drewes *et al*., 2008). Research by Steenkamp and colleagues (2006) highlighted the ability of methanolic *H. hemerocallidea* extracts (at a final concentration of 100 μg/ml) to completely inhibit the growth of *Escherichia coli*, demonstrating effective antibacterial activity. However, findings obtained from this study do not correlate withprevious literature stating the potential antifungal activity of *H. hemerocallidea.*

Table 2.1: Antifungal activity of three plant extracts against *Cryptococcus neoformans* ATCC H99 and *C. deneoformans* CBS 132.

		Mean zones of inhibition in mm (± SEM)					
Fungal Strain	**Solvent: Plant extract**	**50 mg**	**100 mg**	**200 mg**	**400 mg**	**800 mg**	**1600 mg**
***Cryptococcus neoformans* ATCC H99**	E:A	0	0	0	0	0	0
	E:C	0	0	0	6 ± 0	8 ± 0	10 ± 0
	E:H	0	0	0	0	0	0
	E:K	0	0.5 ± 0.5	4 ± 2.0	10 ± 2.0	14 ± 2.0	18 ± 2.0
	M:A	0	0	0	2 ± 2	3 ± 3	6 ± 2
	M:C	0	0	0	6 ± 0	11 ± 1.0	11.5 ± 1.5
	M:H	0	0	0	0	0	0
	M:K	0	0	0	0	1.5 ± 0.5	4.5 ± 0.5
	H:A	0	0	0	0.5 ± 0.5	1.75 ± 0.25	4 ± 0
	H:C	0	0	0.5 ± 0.5	4 ± 0	6 ± 0	12 ± 0
	H:H	0	0	0	0	0	0
	H:K	0	0	1 ± 0	4 ± 0	11 ± 5.0	17 ± 1.0
***Cryptococcus deneoformans* CBS 132**	E:A	0	0	7 ± 1.0	12 ± 0	15 ± 1.0	16.5 ± 1.5
	E:C	0	0	0	1 ± 1.0	7 ± 1.0	11 ± 3.0
	E:H	0	0	0	0	0	0
	E:K	0	0	0	0	6 ± 0	11 ± 1.0
	M:A	0	0	2 ± 2.0	8 ± 0	11 ±1.0	13 ± 1.0
	M:C	0	3 ± 3.0	8 ± 2.0	10 ± 2.0	10.5 ± 1.5	12 ± 2.0
	M:H	0	0	0	0	0	0
	M:K	0	1 ± 1.0	5 ± 1.0	9 ± 1.0	12 ± 0	13.5 ± 0.5
	H:A	0	3 ± 1.0	8 ± 0	8.5 ± 0.5	11 ± 1.0	12 ± 0
	H:C	0	0	2 ± 0	4 ± 0	6 ± 0	8 ± 0
	H:H	0	0	0	0	0	0
	H:K	4 ± 0	8 ± 0	11 ± 1.0	12.5 ± 0.5	15 ± 1.0	18 ± 2.0

E: Ethanol solvent; M: Methanol solvent; H: Hexane solvent; A: *Agathosma betulina* extract; C: *Curcuma longa* extract; H: *Hypoxis hemerocallidea* extract*;* K: *Kigelia africana* extract.

± values denote the standard deviation of three replicates (n = 3)

Table 2.2: Antifungal activity of three plant extracts against *Cryptococcus gattii* CBS 10755and *C. tetragatti* MRC 8871.

Fungal Strain	Solvent: Plant extract	Mean zones of inhibition in mm (± SEM) 50 mg	100 mg	200 mg	400 mg	800 mg	1600 mg
***Cryptococcus gattii* CBS 10575**	E:C	0	0	0	7 ± 1.0	11 ± 1.0	16 ± 2.0
	E:H	0	0	0	0	0	0
	E:K	0	0	0	1 ± 1.0	3 ± 1.0	6 ± 0
	M:A	0	0	0	0	0	0
	M:C	0	0	2 ± 0	5 ± 1.0	8 ± 0	13 ± 1.0
	M:H	0	0	1 ± 1.0	0	1 ± 1.0	4 ± 0
	M:K	0	0	0	0	0	0
	H:A	0	0	0	0	2 ± 0	4 ± 0
	H:C	0	0	2 ± 0	6 ± 4.0	8 ± 4.0	9 ± 1.0
	H:H	0	0	0	0	0	0
	H:K	0	0	2 ± 0	5 ± 1.0	8 ± 2.0	11 ± 3.0
	E:A	0	0	0	0	2 ± 2.0	2 ± 2.0
***Cryptococcus tetragatii* MRC 8871**	E:C	0	0	0	5 ± 1.0	9 ± 1.0	14 ± 2.0
	E:H	0	0	0	0	0	0
	E:K	0	0	0	0	2 ± 2.0	3 ± 3.0
	M:A	0	0	0	0	0	0
	M:C	0	0	1 ± 1.0	7 ± 1.0	7 ± 3.0	13 ± 5.0
	M:H	0	0	0	0	0	0
	M:K	0	0	0	0	0	0
	H:A	0	0	0	2 ± 2.0	5 ± 1.0	10 ± 4.0
	H:C	0	0	0	7 ± 1.0	12 ± 2.0	10 ± 0
	H:H	0	0	0	0	0	0
	H:K	0	0	0	0	4 ± 0.0	10 ± 0
	E:C	0	0	0	7 ± 1.0	11 ± 1.0	16 ± 2.0

E: Ethanol solvent; M: Methanol solvent; H: Hexane solvent; A: *Agathosma betulina* extract; C: *Curcuma longa* extract; H: *Hypoxis hemerocallidea* extract*;* K: *Kigelia africana* extract. ± values denote the standard deviation of three replicates (n = 3)

A study conducted by Katerere and Eloff (2008) investigated the chemical and biological differences between aerial parts and tubers of *H. hemerocallidea*. Their findings highlighted that leaves obtained from *H. hemerocallidea* displayed a different chemical profile to the tubers and as a consequence, it would not be possible to substitute one section of the plant for another as the same inhibitory effects would not be observed (Katerere and Eloff, 2008). In relation to the current study, the possibility that a section of *H. hemerocallidea* that does not contain the appropriate bioactive components to induce inhibition of cryptococcal growth cannot be excluded.

2.4.1.2 *Agathosma betulina*

Ethanolic *A. betulina* extracts produced no ZOI against *C. neoformans* (Table 2.1) and *C. gattii* (Table 2.2). *Cryptococcus tetragattii* was mildly susceptible to the ethanolic extracts, with small ZOI observed at concentrations of 800 and 1600 mg (Table 2.2). The *Cryptococcus* species most susceptible to the ethanolic extracts of *A. betulina* was *C. deneoformans*. Large ZOI ranging from 7 to 16.5 mm in diameter between concentrations of 200 mg and 1600 mg (Figure 2.1)were observed. Methanolic-extracts were ineffective against *C. gattii* and *C. tetragattii* **(**Table 2.2), however, small ZOI were visible against *C. neoformans* and *C. deneoformans* (Table 2.1). Hexane-extracted bioactives induced ZOI in all four *Cryptococcus* species, with *C. deneoformans* being the most susceptible (Table 2.1) and *C. gattii* the least susceptible (Table 2.2).

The benefits of consuming/utilizing *A. betulina* products are already exploited for commercial gain. Touted for its antibacterial properties (Geetha *et al*., 2012), our study determined that both ethanolic and methanolic extracts of *A. betulina* possess limited antifungal activity against the select cryptococcal pathogens. However, bioactives extracted using hexane were able to inhibit the growth of all four pathogenic species to some degree. This is in line with research that has identified the aliphatic hydrocarbon, limonene, in the essential oils extracted from *A. betulina* as having antifungal activity (Jain and Agrawal, 2002; Yazdanparast and Barton, 2006; Fajinmi *et al*., 2018). Indeed, this volatile monoterpene has been shown to prevent the growth of the dermatophytic fungi *Trichophyton rubrum* and the food spoilage agent *Zygosaccharomyces rouxii* (Matsuoka *et al*, 1990; Chai *et al*, 2009; Chee *et al*., 2009; Omran *et al*., 2011).

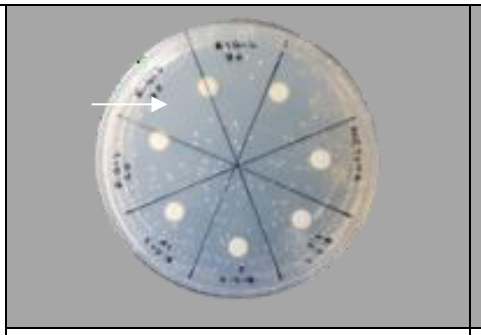
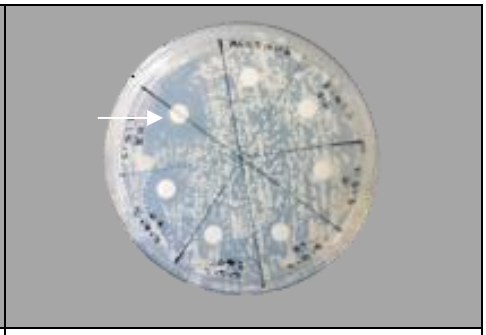
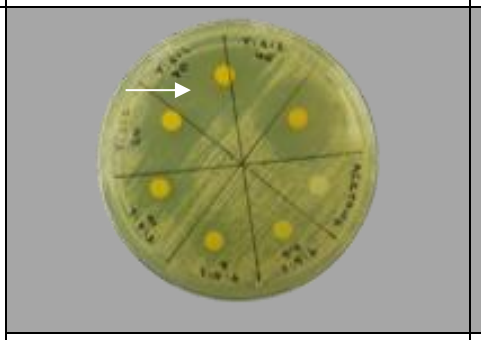
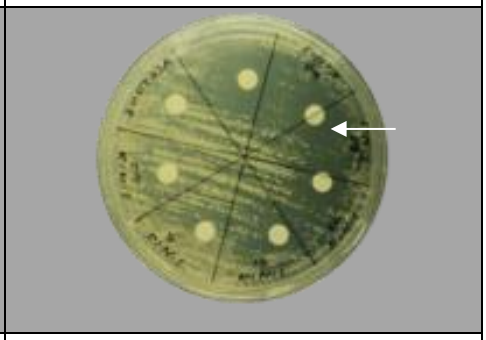
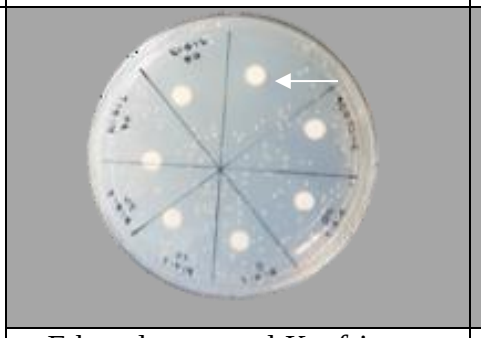
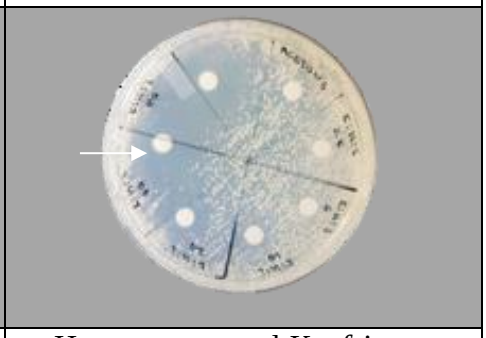
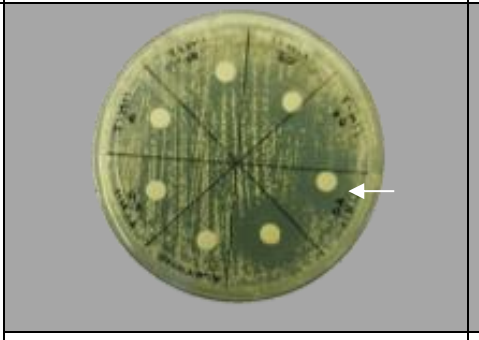
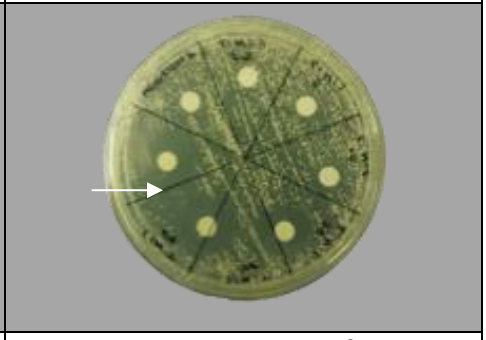
***C. deneoformans* CBS 132**		
	Ethanol-extracted *A. betulina*	Ethanol-extracted *K. africana*
***C. gattii* CBS 10575**		
	Ethanol-extracted *C. longa*	Hexane-extracted *K. africana*
***C. neoformans* ATCC H99**		
	Ethanol-extracted *K. africana*	Hexane-extracted *K. africana*
***C. tetragattii* MRC 8871**		
	Hexane-extracted *C. longa*	Hexane-extracted *K. africana*

Figure 2.1: Antifungal activity of *Agathosma betulina*, *Curcuma longa*, and *Kigelia africana*. Yeast Nitrogen Base (YNB, pH 5.5) plates showing zones of inhibition (ZOI) obtained when using plant extracts with concentrations ranging from 50 to 1600 mg against four pathogenic *Cryptococcus* species.

Further research has suggested that this is achieved by altering the cell membrane and the destruction of cellular proteins (Cai *et al*., 2019). It is likely that limonene and similar compounds may be present within the hexane *A. betulina* extracts that produced ZOI against the four *Cryptococcus* species tested, however further analysis into the identification of the bioactive compounds extracted should be performed.

2.4.1.3 *Kigelia africana*

The use of *K. africana* yielded results showing varying ranges of inhibition depending on the type of extraction solvent that was used. This was firstly seen in methanol-extracted *K. africana* samples where no ZOI were produced when used against *C. gattii* and *C. tetragatti* (Table 2.2). Small ZOI at 4.5 (± 0.5) mm were produced when methanol-extracted *K. africana* was used at a concentration of 1600 mg against *C. neoformans*. In contrast, hexane-extracted *K. africana* extracts at 1600 mg induced the largest ZOI in *C. deneoformans* at 18 (± 2.0) mm (Table 2.1), while the remaining three cryptococcal strains yielded ZOI ranging from 10 (± 0.0) to 17 (± 1.0) mm. Ethanol-extracted *K. africana* samples were most effective at inducing ZOI when used against *C. neoformans* and *C. deneoformans*. This result was observed in the measurement of *C. neoformans* being the only strain to record mean ZOI values of 0.5 (± 0.5) mm at 100 mg when ethanol-extracted *K. africana* was used. Moreover, at 1600 mg the ethanol-extracted *K. africana* samples induced the largest mean ZOI at 18 (± 2.0) mm (Table 2.2). It was also noted that the ethanol-extracted *K. africana* samples induced smaller ZOI against *C. gattii* and *C. tetragatti* (Table 2.2).

The use of ethanolic *K. africana* extracts in this study indicated that *C. neoformans* and *C. deneoformans* (Table 2.1) were more susceptible to the inhibitory effects of the plant bioactives when comparedto *C. gattii* and *C. tetragattii* (Table 2.2). Two separate studies have previously identifiedanticryptococcal activity against *C. neoformans*. Hamza and colleagues (2006) recorded inhibition through the use of fruit methanolic extracts, while Jain and Belsare (2009) found that chloroform extracts of *K. africana* stem bark inhibited *C. neoformans*, *Candia tropicalis* and *Mircosporum furfure*. Moreover, as of 2016, literature has acknowledged that approximately 145 phytoconstituents were previously isolated from varying sections of *K. africana*, with the main class of compounds being identified as iridoids, naphthoquinones and flavonoids (Bello *et al.*, 2016). Therefore, data recorded in the current study are in agreement with literature as ethanolic

K. africana extracts inhibited the growth of all four cryptococcal species. Further analyses are required to determine which specific bioactive compounds are involved in the growth inhibition.

2.4.1.4 *Curcuma longa*

Curcuma longa was the most successful of the four plant species that successfully inhibited the growth of all four *Cryptococcus* species, regardless of the extraction solvent that was used (Table 2.1 and 2.2).

The inclusion of *C. longa* in this study was based on previous literature indicating the existence of antifungal activity in this plant species. (Aggarwal *et al.*, 2007; Fidelis *et al*., 2019). This antifungal activity was highlighted in research conducted by Moghadamtousi and colleagues (2014) that reported antifungal activity against pathogenic yeast species such as *C. neoformans*. Their work also detailed how the potency of *C. longa* was lower when compared to antifungal treatments that were already on the market. These results are in line with previous studies and confirm that *C. longa* possesses potent antifungal properties.

Overall, *C. deneoformans* and *C. neoformans* proved to be more susceptible to the plant extracts when compared to their sister species *C. gattii* and *C. tetragatti*. This is in line with previous research noting higher resistance of the latter two species with regards to prescribed antifungals such as amphotericin B and fluconazole (Bernal-Martinez *et al*., 2010; Varma and Kwon-Chung, 2010; Harris *et al*., 2011; Gast *et al*., 2013).

Disc diffusion assays performed on *C. gattii* and *C. tetragatti* revealed that these fungal strains were more resistant as reduced ZOI measurements were recorded. The resistance recorded in these two strains could be attributed to factors related to the plant extract where insufficient dosages were tested and were unable to induce inhibition of fungal growth. Alternatively, active metabolites with inhibitory characteristics may not have been extracted (Katerere and Eloff, 2008).

2.4.2 MIC and MFC determination

Based on the results obtained in the disc diffusion assays, *H. hemerocallidea* was excluded from the remaining experimental procedures performed in this study as minimal to no antifungal activity was recorded for the extracted plant bioactives against all four *Cryptococcus* species.

Data generated while determining the MIC for each plant extract were inconclusive and inconsistent and are therefore not included in this discussion. This result may be due to the use of acetone that reduces the resolution of the spectrum or the pigmentation of the plant extracts, which could have interfered with the accuracy of the absorbance reading (Rocha *et al*., 2018).

The MFC assay revealed that at the maximum concentration of 10 mg/ml (Table 2.3), *A. betulina* plant extractions recorded no fungicidal activity against each *Cryptococcus* species as lawns grew on SDA plates after incubation at 30 °C for 48 h. The implication of this result suggests that in order for *A. betulina* to be utilized as a plant with active fungicidal characteristics, higher dosages may have to be administered to produce a desirable outcome. In comparison, ethanolic *K. africana* proved to be the most effective plant extract, yielding MFC values ranging from ~3.75 – 7 mg/ml (Table 2.3). *Cryptococcus gattii* appeared to be the most sensitive of the four cryptococcal species. Finally, the known antifungal *C. longa* produced, on average, the lowest MFC values across all the different extracts (Table 2.3); however, they still proved to be far less effective at producing ZOI when compared to amphotericin B measurements.

Table 2.3: Minimum fungicidal concentrations (MFC, mg/ml) of crude ethanolic, methanolic and hexane plant extracts against fourpathogenic *Cryptococcus* species.

Fungal species	**Plant species**	*Agathosma betulina*			*Kigelia africana*			*Curcuma longa*			AmB
	Solvent	Eth	Met	Hex	Eth	Met	Hex	Eth	Met	Hex	
Cryptococcus deneoformans CBS 132		> 10	> 10	> 10	~7.5	> 10	> 10	~3.75	~7.5	> 10	0.0025
C. gattii CBS 10575		> 10	> 10	> 10	~3.75	> 10	~7.5	5	~3.75	~3.75	0.0025
C. neoformans ATCC H99		> 10	> 10	> 10	~7.5	> 10	> 10	5	~7.5	> 10	0.000625
C. tetragatti MRC 8871		> 10	> 10	> 10	~7.5	> 10	> 10	~3.75	5	~3.75	0.0025

Eth: Ethanol solvent; Met: Methanol solvent; Hex: Hexane solvent; AmB: Amphotericin B (control).

2.4.3 Ergosterol assay

The total ergosterol content was determined for cells treated with ethanolic *K. africana* extract (2.5 or 5 mg/ml) and amphotericin B (0.01 µg/ml). Data obtained for the ergosterol content in *C. deneoformans* were inconclusive (data not shown). Exposure to the *K. africana* extractappeared to decrease in the total ergosterol content in *C. gattii* and *C. neoformans*, however, statistical analyses determined that the reduction was not significant. Interestingly, an increase in the total ergosterol content was observed in *C. tetragattii* (Figure 2.2). Samie and co-workers (2019) reported a similar phenomenon when they screened aerial tissue extracts from *Pelargonium sidoides* for antifungal activity against *C. neoformans*. They speculate that the rise in ergosterol content could be due to the stress imposed on the cells by the plant extract, howeverfurther research is required.

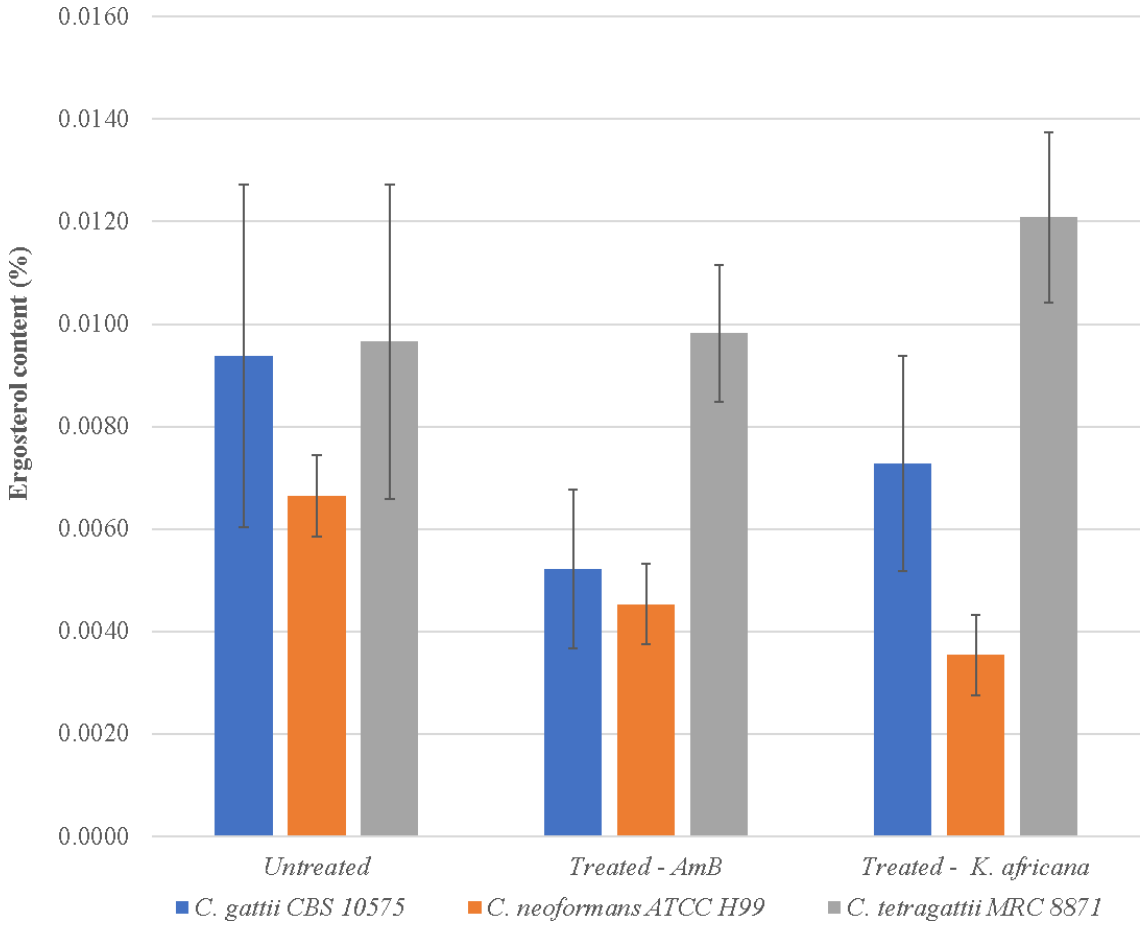


Figure 2.2: Mean ergosterol content (%) of untreated (acetone) cells and cells treated with either ethanolic *Kigelia africana* (2.5 or 5 mg/ml) extract or amphotericin B (Amb; 0.01 µg/ml). Error bars denote the standard error for three replicates (n = 3).

2.5 Conclusions and future work

The antifungal activity of several indigenous medicinal plants species was evaluated against pathogenic *Cryptococcus* species. Disc diffusion assay revealed that various extracts from *H. hemerocallidea* were ineffective against all four *Cryptococcus* species. The lack of antifungal activity in several plant extractsmay be attributed to a number of factors. Firstly, the selected medicinal plants may lack any bioactives that are effective against *Cryptococcus* species. Secondly, different parts of the plants often have different chemical profiles and are used for various purposes, therefore, this study may have utilised parts of the plant that did not contain the antifungal agents (Davey and Warrell, 2000; Street and Prinsloo, 2013). Finally, effective antifungal bioactives may have been overlooked as the solvents used may have had polarity ranges that were not specific for bioactives present in the plant species used (Hamza *et al*., 2006; Mokoka *et al*., 2010).

Analysis of the ergosterol assay suggests that ethanolic *K. africana* extracts reduced ergosterol content in *C. gattii* and *C. neoformans.* Although significant ergosterol inhibition was not recorded in this study, the finding that *K. africana* induced a reduction in these cryptococcal strains may serve as a foundation for future studies to explore the ergosterol inhibition in other medicinally significant plant species. In addition, research focused on identifying the exact metabolites responsible for antifungal activity are required to support further use of ethanolic *K. africana* as an alternative antifungal therapeutic.

CHAPTER 3: Antivirulence activity of *Kigelia africana* against pathogenic *Cryptococcus* species

3.1 Introduction

Pathogenic fungi continuously have to overcome a numerous range of host defence mechanisms in order to successfully establish an infection (Marcos *et al*., 2016). Amongst the defence mechanisms associated with humans, the most note-worthy systems would be the innate and adaptive immune responses (Romani, 2011). The innate immune response system has an important role in a number of processes which include the modulation of host-fungi interactions, protection of the host against fungal diseases through direct elimination of fungi, influencing fungal commensalism by modulating the impact that microbial communities possess, and the polarization of the adaptive immune response (Borriello *et al*., 2020). The pathogen elimination process can either be executed through phagocytic processes that target fungi residing within intracellular regions or by the secretion of microbicidal compounds that target non-digestible fungal components. Furthermore, the process of phagocytosis finally leads to the build-up of phagocytic cells at the interaction site between fungal and host cells. This interaction leads to the engulfment and degradation of these fungal cells within maturing phagosomes (Becker *et al*., 2015; Erwig and Gow, 2016). In addition to the early recognition of fungal pathogens byimmune cells, the innate immune system further serves the purpose of amplifying the immune response by triggering the recruitment of cells that function in the adaptive immune system, such as antigen-presenting cells, T lymphocytes and B lymphocytes (Lord and Vyas, 2019; Rich and Chaplin, 2019).

Fungal species have evolved their own range of strategies in response to these host defence mechanisms to improve their chances of survival (Romani, 2011). A major contributorto the survival of pathogenic fungi during infection are virulence factors (Kozel, 1995; Prates *et al*., 2013; Almeida *et al*., 2015). The central idea that virulence factors are components of a pathogen that can cause damage to the host is widely accepted. (Kozel, 1995; Pirofski and Casadevall, 2015). Cryptococcal pathogenesis is identified as a multi-factorial process as the likelihood that one specific virulence factor would be responsible for pathogen survival and dissemination are low (Kozel, 1995). To identify viable targets that inhibit successful fungal growth, it is necessary to consider the key factors involved in infection.

During host infection, reactive oxygen species are produced by macrophages and neutrophils in order to eliminate fungal pathogens that become internalized (Marcos *et al*., 2016). Pathogens may use enzymatic or non-enzymatic strategies to maintain redox homeostasis within hostphagocytic cells in order to overcome oxidative stress and repair damage to promote survival. (Chai *et al*., 2009; Marcos *et al*., 2016). *Cryptococcus neoformans* employs a variety of methods to carry out these enzymatic approaches, including antioxidant systems that employ superoxide dismutases, catalases, and peroxiredoxins, which are critical for cryptococcal cell survival in the oxidative environment of macrophages and neutrophils (Gerik *et al*., 2008). Non-enzymatic strategies against reactive oxygen species have been reported and occur in the form of metabolites that act in the detoxification process. The production of the pigment melanin bythe laccase enzyme is an example of a non-enzymatic defense strategy (Langfelder *et al*., 2003; Sharma *et al.,* 2018). Melanin reacts with reactive oxygen species to function as a sink for unpaired electrons that possess the potential to harm/damage the yeast (Langfelder *et al*., 2003). Interestingly, research by Sabiiti and colleagues (2014) identified that when fungal strains experienced high rates of phagocytosis by macrophages and reduced growth within cells *in vitro,*high levels of laccase activity were recorded, and strains exhibited resistance to antifungal therapeutics.

The enzyme urease has been linked with disease progression. Urease is an enzyme that is required for brain invasion during the late phase of infection as it promotes the sequestration of cryptococcal cells at microcapillary beds of the blood-brain barrier (Cox *et al*., 2000; Olszewski *et al*., 2004; Morrow and Fraser, 2013). Moreover, studies have proposed that the extracellular enzymatic degrading properties of urease that produce toxic ammonia from urea may impair the functioning of endothelial cells leading to increased membrane permeability (Taylor-Robinson *etal*., 1997; Morrow and Fraser, 2013).

Thus, targeting virulence factors that are important for successful infection and disease by cryptococcal species has been identified as a viable alternative therapeutic approach (Iyer *et al*, 2021). The targeting of virulence factors also provides a number of advantages, including reducing the rate of antifungal resistance due to weaker selective pressures being imposed, widening the spectrum of microbial targets and increasing the choice and availability of antifungal treatments (Kronstad *et al*., 2011; Zaragoza, 2019; Zaragoza, 2019).

3.2 Aim and objectives

This study aimed to assess the anti-virulence properties of ethanol-extracted *K. africana* against pathogenic *Cryptococcus* species.

The specific objectives of this study included the following:

1. Assess the impact of ethanol-extracted *K. africana* on laccase activity
2. Assess the impact of ethanol-extracted *K. africana* on urease activity

3.3 Methodology

Ethanolic *K. africana* extracts were prepared as previously described (Chapter 2). Air-dried extracts were resuspended in acetone.

3.3.1 Isolate preparation

Starter cultures were prepared by inoculation of 5 ml 2x minimal media (MM, 15 mM glucose; 10 mM magnesium sulfate; 29.4 mM monopotassium phosphate; 13 mM glycine; 0.003 mM thiamine hydrochloride) with 72 h old cultures previously grown on Sabouraud dextrose agar (SDA, pH 5.6). After incubation at 37 ºC for 72 h on a rotary wheel (Stuart™ SB2 fixed speed rotator, United Kingdom), cells were harvested via centrifugation (11 350 x g for 5 minutes) and washed twice using distilled water. Suspensions were counted using a standard haemocytometer and light microscope. The total number of cells were counted through the use ofthe following formula:

$$\frac{Average\ cell\ count}{16} \times dilution\ factor \times (4 \times 10^6)$$

Experimental cultures, positive control and negative control cultures were prepared in test tubes that contained 2x MM. Experimental cultures involved the inoculation of harvested cryptococcal cells (1 x 10^6 cells/ml) into 5 ml 2x MM, supplemented with ethanol-extracted *K. africana* plant extract at a sub-inhibitory concentration as determined in Chapter 2. Positive control cultures were prepared by inoculating cryptococcal cells (1 x 10^6 cells/ml) in 5 ml 2 x MM, supplemented with the same volume of acetone. Negative control cultures were prepared using 5 ml 2 x MM supplemented with ethanol-extracted *K. africana* extract. All three culture sets were incubated at 37 °C for 72 h on a rotary wheel.

3.3.2 Laccase assay

The protocol was adapted from Samie and co-workers (2019). Prepared cells (section 3.3.1) were harvested via centrifugation (11 350 x g for 5 minutes) and washed twice using distilled water. The harvested cells were then counted using a hemocytometer and inoculated into distilled water, supplemented with 5 mM L-3,4-dihydroxyphenylalanine (L-DOPA), to a final cell concentration of 5 x 10^6 cells/ml. Due to the light sensitivity of L-DOPA, tubes used were wrapped in tinfoil. All cultures were subsequently incubated at 37 °C on a rotary wheel and absorbance readings (S-22 UV/Visible Spectrophotometer, Germany) at a wavelength of 490 nm were taken every six hours for a period of 24 h.

3.3.3 Urease assay

The protocol was adapted from Samie and co-workers (2019). Prepared cells (section 3.3.1) were harvested via centrifugation (11 350 x g for 5 minutes) and washed twice using distilled water. The harvested cells were then counted using a hemocytometer and inoculated into distilled water, supplemented with 0.66 mM urea and 0.012 g/l phenol red indicator, to a final cell concentration of 5 x 10^6 cells/ml. All cultures were subsequently incubated at 37 °C on a rotary wheel and absorbance readings (S-22 UV/Visible Spectrophotometer, Germany) at a wavelength of 570 nm were taken every six hours for a period of 24 h.

3.3.4 Statistical analysis

All experimental procedures were performed in triplicate. Statistical values were further presented as their means and the standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, California, https://www.graphpad.com) to run unpaired t-test analyses and determine statistical significance.A *p*-value of < 0.05 was considered significant.

3.4 Results and discussion

3.4.1 Laccase assay

Laccase is a crucial enzyme involved in the biosynthesis of melanin, which contributes to fungal virulence (Nosanchuk and Casadevall, 2003; Zhu and Williamson, 2004). The contribution of melanin to virulence has previously been documented in the regulation of immune responses by cryptococcal species (Wang *et al*., 1995; Vecchiarelli and Monari, 2012). Research has demonstrated that melanized cells successfully downregulated the production of cytokines such as tumor-necrosis factor alpha (TNF-α) and reactive oxygen species, serving as confirmation of immune regulatory properties of melanin (Huffnagle *et al*., 1995; Tajima *et al*., 2019).

Melanized cryptococcal cells have melanin polymers deposited within the inner layer of the cell wall that impart brown-to-black pigmentation to cells as time passes (Nosanchuk and Casadevall, 2003). In addition to localization of melanin in the cell wall, work by Nosanchuk and Casadevall (2006) determined that melanin also contributes to the mechanical strength of the cell and increases resistance to enzymatic degradation from host immune system components. Eisenman and colleagues (2011) have previously recognized that melanin producing *Cryptococcus* species require an environment with exogenous catecholamines present in order to produce melanin. This finding was important to consider as most other melanin-producing fungi utilize endogenous precursors during melanin biosynthesis (Eisenman *et al*., 2011). Consequently, this study used L-DOPA to promote the synthesis of melanin as L-DOPA is oxidized to dopaquinone and is catalyzed by laccase (Williamson, 1994).

The laccase activity of each *Cryptococcus* species treated with ethanolic *K. africana* extracts was shown to be significantly inhibited. This result was observed in both the colour changes (Figure 3.1) and spectrophotometric absorbances (Figure 3.2) that were recorded. Visually, treated cultures were recorded as clear solutions after incubation for 24 h in the absence of any plantextract. In contrast, untreated cultures produced varying intensities of dark-coloured solutions in the same period demonstrating high rates of laccase activity and melanin production.

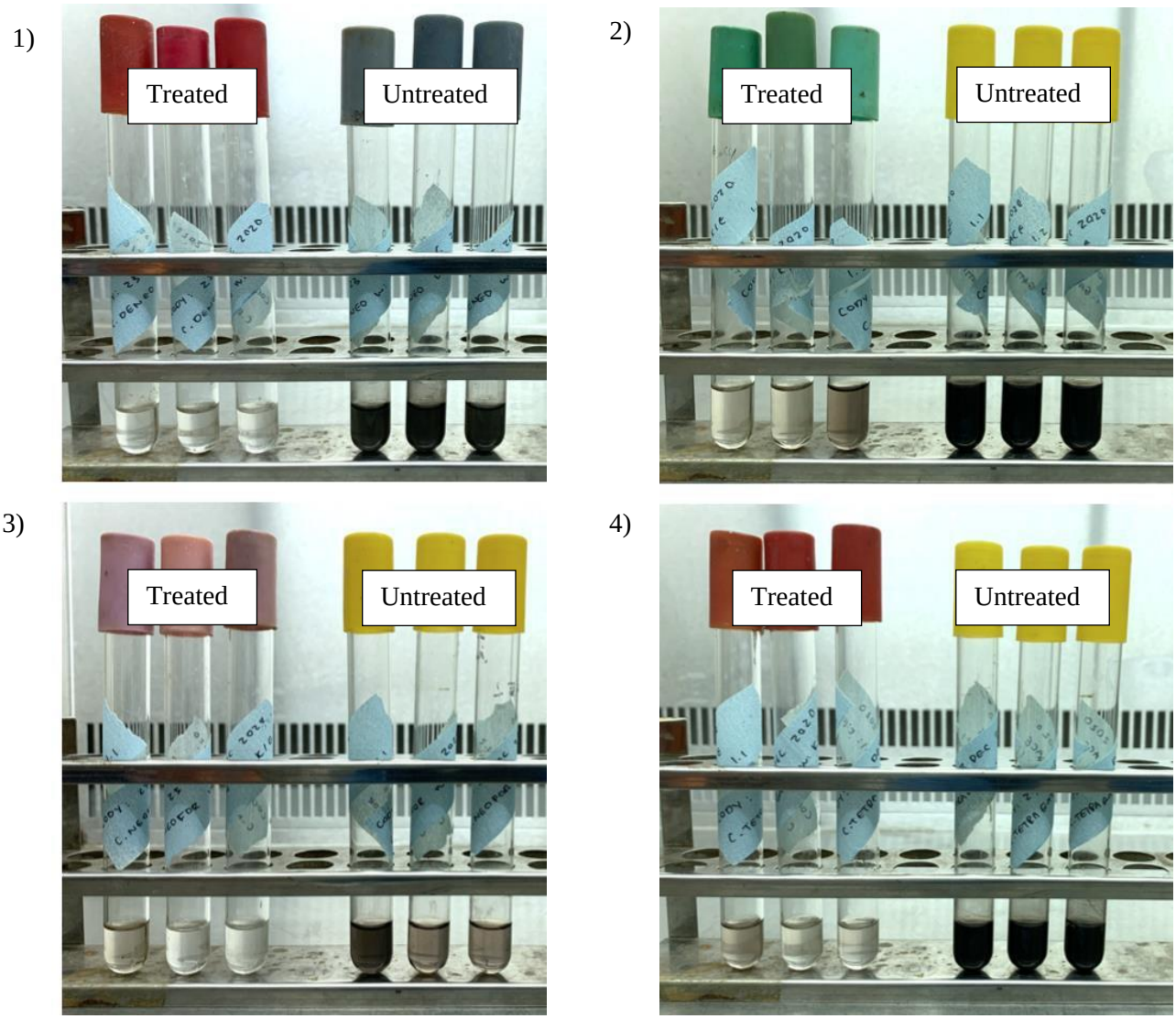


Figure 3.1: Laccase activity (melanin production) after 24 h incubation period at 37 °C. Tests were performed in triplicate showing treated cultures (left three test tubes) and untreated cultures (right three test tubes). 1) *Cryptococcus deneoformans* CBS 132; 2) *C. gattii* CBS 10575; 3) *C. neoformans* ATCC H99; 4) *C. tetragattii* MRC 8871.

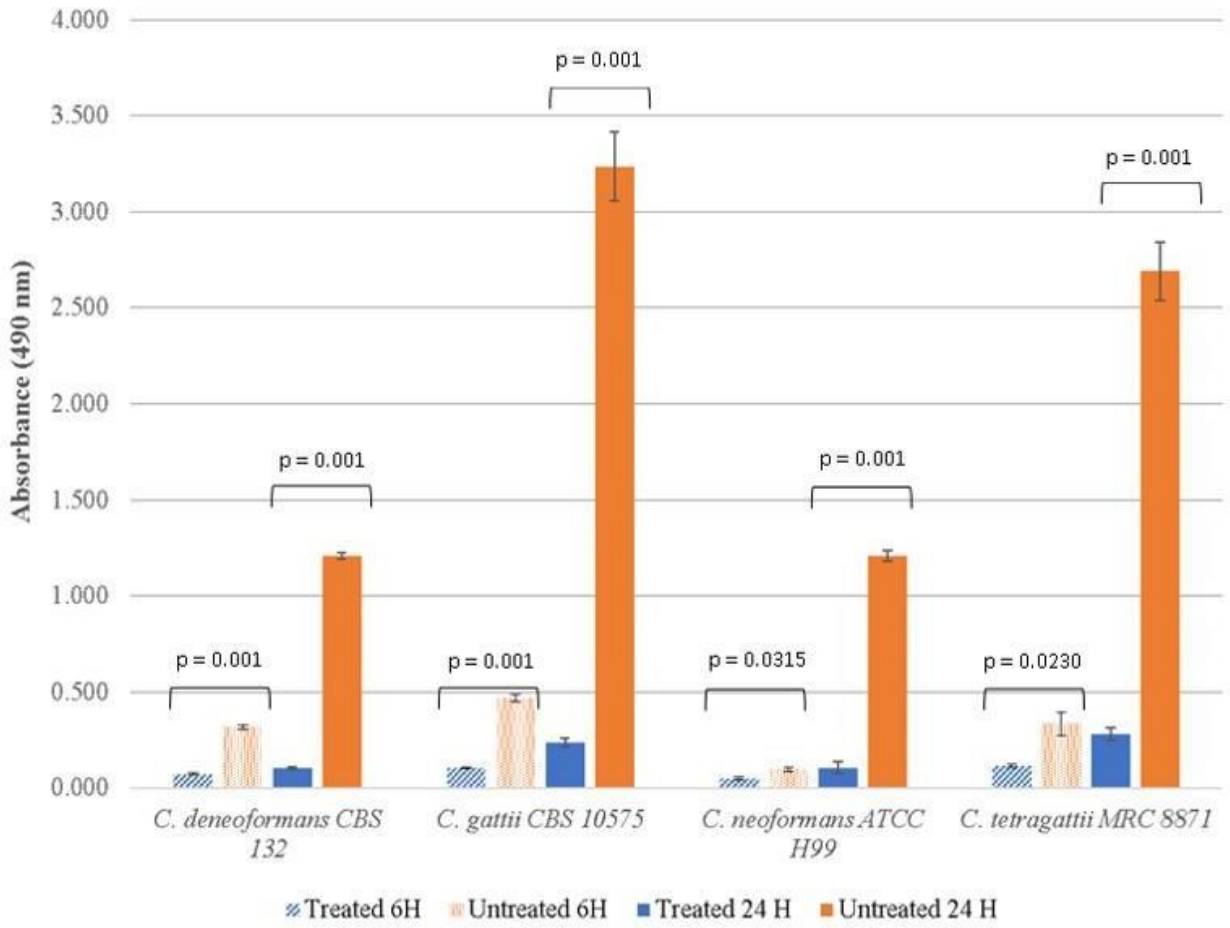


Figure 3.2: Mean absorbance (OD = 490 nm) indicative of laccase activity of four pathogenic cryptococcal species that were cultured in the presence (treated) and absence (untreated) of ethanolic *Kigelia africana* extract (2.5 or 5 mg/ml) for 24 h. Error bars denote standard error for three replicates (n = 3).

Spectrophotometric analysis revealed that ethanolic extracts significantly inhibited laccase activity in all four cryptococcal species (Figure 3.2). Upon analyzing the overall mean absorbance values for the laccase assay, it was noted that after 24 h, the untreated *C. gattii* and *C. tetragattii* yielded the highest mean absorbance for laccase activity at 3.235 (± 0.1796) and 2.691 (± 0.1529), respectively. Ethanolic *K. africana* significantly inhibited laccase activity of *C. neoformans* (*p*-value = 0.001) after 24 h. The same trend was observed for *C. deneoformans* after 24 h, where significant (*p*-value = 0.001) laccase inhibition wasrecorded. The most striking result was the effective laccase inhibition recorded for the interaction between *C. gattii* and ethanolic *K. africana*. After the 24 h incubation period (Figure 3.2), mean absorbance values measured a

highly significant reduction (*p*-value = 0.001) in laccase activity in *C. gattii* from 3.235 (± 0.1796) in the untreated cultures to 0.235 (±0.0218) in the samples treated with ethanol-extracted *K. africana*.

The implication of plant extracts showing favourable, effective laccase activity inhibition against each *Cryptococcus* species is that melanin production may be reduced (Williamson *et al*., 1997; Zhu and Williamson, 2004; Brown *et al*., 2012). Using a mouse model, research by Kwon-Chung and co-workers (1982) demonstrated that mice infected with a UV-generated melanin- deficient cryptococcal mutant showed increased rates of survival when compared to mice infected with the wild type. Similarly, Wang and co-workers (1996) determined that in vivo melanin deficient cells were more susceptible to phagocytosis and opsonization in comparison to melanized cells.

3.4.2 Urease assay

Urease is a nickel-dependent enzyme found in a wide variety of organisms (Morrow and Fraser, 2013). The enzyme catalyzes the hydrolysis of urea into carbon dioxide and ammonia (Callahan *et al*., 2005). Prompted by the unknown interaction between *C. neoformans* andmacrophages, Fu and co-workers (2018) studied the effect of urease on macrophages using wild- type strains isolated from a parental *Cryptococcus neoformans* H99 strain, a urease-mutant strainisolated from a *C. neoformans* URE1 deletion strain and a urease-competent strain isolated from a URE1 complemented strain. Their findings demonstrated that when transported inside macrophages, urease-competent *C. neoformans* strains initiated the promotion of non-lytic exocytosis and reduced the rate of intracellular replication, thereby suggesting the ability of urease enzymes to regulate virulence during infection.

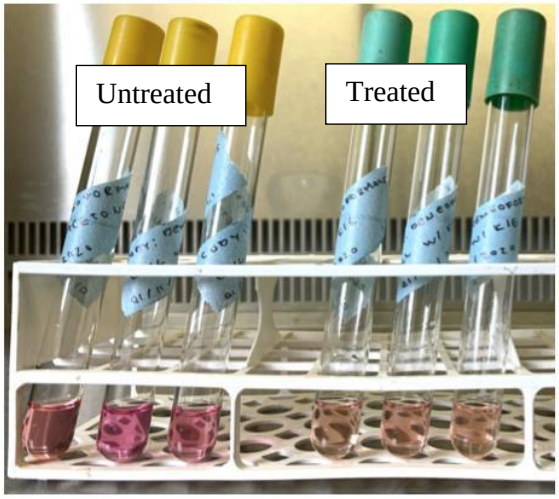
Our research revealed that ethanolic *K. africana* extracts inhibited urease activity in all four *Cryptococcus* species. Colour changes were documented after 24 h by the addition of the phenol red indicator (0.012 g/l). A medium supplemented with phenol red indicator will display colour shifts to yellow when the pH is lower than 7.0 and a shift to bright pink when the pH is within the range of 8.2 or greater (Liu *et al*., 2013). Untreated *Cryptococcus* species were able to catalyse the conversion of the available urea to ammonia and carbamate.

This conversion created an alkaline environment, turning the phenol red indicator bright pink (Figure 3.3). In contrast, cultures treated with ethanolic *K. africana* extracts demonstrated reduced urease activity as urea was not catalysed during the 24 h incubation period leading to the production of anacidic environment and yellow colour.

The reduction in urease activity was confirmed further by recording the absorbance values of each sample at 570 nm every six hours for 24 h (Figure 3.4). Findings revealed that *C. neoformans* had the highest urease activity evidenced by absorbance measurements ranging between 0.475 (± 0.041) and 0.700 (± 0.036). Additionally, significant differences between the treated and untreated samples at six (*p*-value = 0.0184) and 24 h (*p*-value = 0.017) incubationintervals were recorded for *C neoformans*. A predominant portion of clinical *C. neoformans* isolates produce large amounts of urease (Singh *et al*., 2013). Consequently, detection of urease activity has served as an avenue for rapid identification of *C. neoformans* during infection (Cox*et al*., 2000; Singh *et al*., 2013). Therefore, the finding that *C. neoformans* had the highest urease activity corresponds well with previous literature.

Results indicated that the *C. deneoformans* urease was the most resistant to the inhibitory effects of ethanolic *K. africana* extracts throughout the 24 h incubation period. Statistical analyses of the mean absorbance values determined that no significant difference was observed between the treated and untreated cells after the six (*p*-value = 0.3832) and 24 h (*p*-value = 0.0931) incubation periods (Table 3.4). In contrast, an important finding was the notable difference between the untreated and treated mean absorbance values recorded after six (*p*-value = 0.0043) and 24 h (*p*-value = 0.0056) incubation periods for *C. tetragattii*. Based upon this distinction in the difference measured for the assay performed on *C. tetragattii*, ethanolic *K. africana* plant extracts were most effective at inhibiting urease activity in this species compared to the remaining three cryptococcal species.

1)



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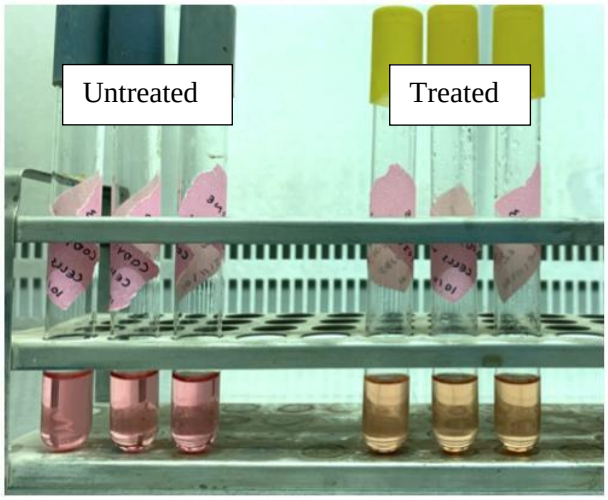

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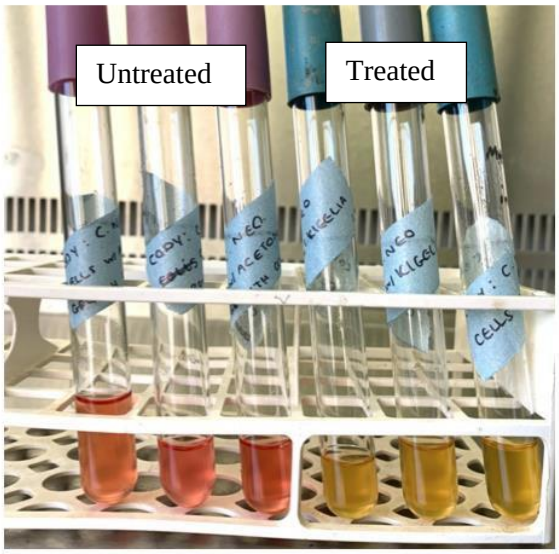

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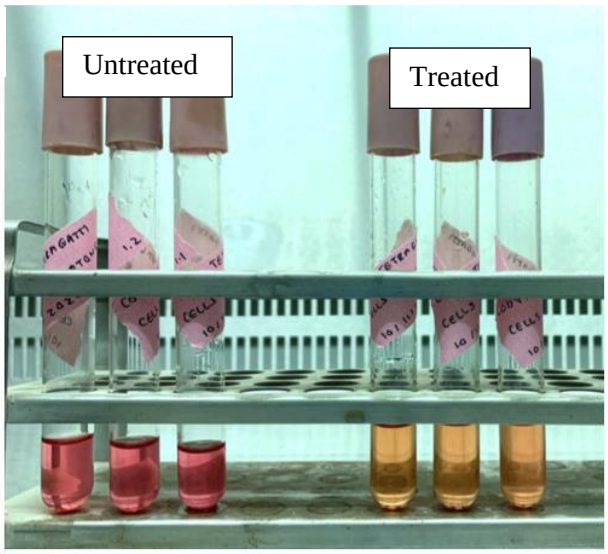

Figure 3.3: Urease activity after 24 h incubation period at 37 °C. Tests were performed in triplicate showing treated cultures (left three test tubes) and untreated cultures (right three test tubes). 1) *Cryptococcus deneoformans* CBS 132; 2) *C. gattii* CBS 10575; 3) *C. neoformans* ATCC H99; 4) *C. tetragattii* MRC 8871.

Studies have highlighted that urease-mediated ammonia has the capability to neutralize any acidic environment that fungal pathogens may be found within (Eaton *et al*., 1991; Singh *et al*., 2013). This trait is favoured as the ability to tolerate the severe pH of phagolysosomes is achieved. Thus, during infection, host defence mechanisms become more susceptible to cryptococcal infections as the conventional immune response are unable to clear/eliminate these pathogenic cells.

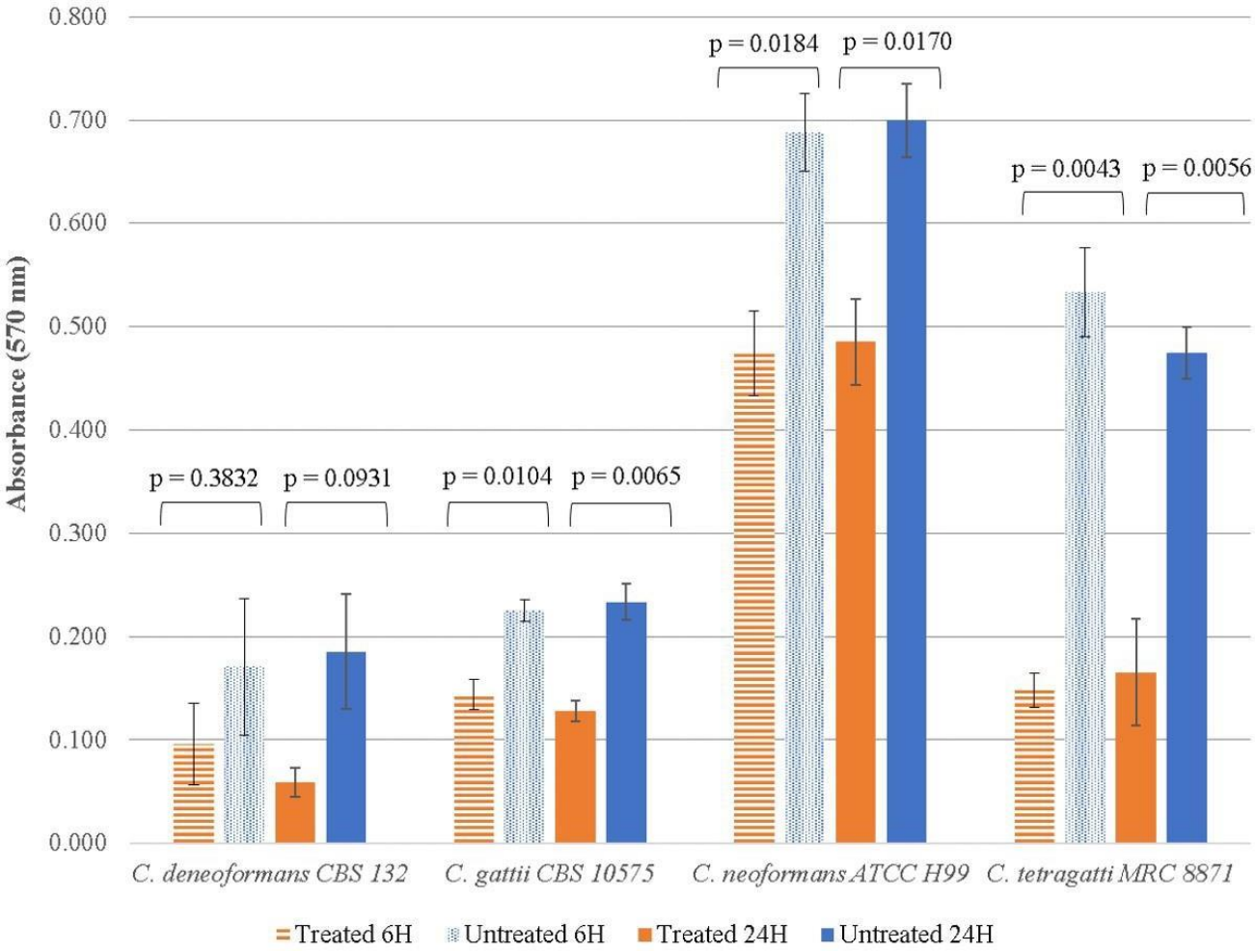

Figure 3.4: Mean absorbance (OD = 570 nm) indicative of urease activity of four pathogenic cryptococcal species that were cultured in the presence (treated) and absence (untreated) of ethanolic *Kigelia africana* extract (2.5 or 5 mg/ml) for 24 h. Error bars denote standard errorfor three replicates (n = 3).

The mechanism of enzyme inhibition by the *K. africana* extract could not be deduced from these assays. Regulation of the laccase enzyme is in response to environmental signals, including nutrient starvation, temperature fluctuations and oxidative stress (Zhu *et al*., 2004; Missal *et al*., 2005). Recent research determined that the pyrimidine analogues flucytosine, 5-fluorouracil and carmofur were able to inhibit melanin production in *C. neoformans* (Zimbres *et al*., 2019); however, they noted that this was not due to gene transcription inhibition of the *LAC* gene or direct binding of the compounds to the laccase enzyme. Similarly, expression of the *URE1* gene, which codes for the cryptococcal urease (*Ure1*), is dependent on the type of nitrogen available and the

current level of enzyme activity (Singh *et al*.,2013). Activation of the urease apoenzyme requires three accessory proteins, Ure4, Ure6, and Ure7, and successful functioning of the enzyme requires an active calcium transporter (Pmc1) (Squizani *et al*., 2017).

Several studies have identified various mechanisms of action where plant bioactive compounds are concerned. The alcoholic extracts of Ceylon leadwort, Indian sarsaparilla, Thumbai and Tridax daisy have been shown to specifically disrupt bacterial cell membrane potential, causing leakage of the cellular contents (Saritha *et al*., 2015). Similarly, the monoterpenoid phenols carvacrol and thymol, commonly extracted from oregano and thyme, have been shown to bind to and disrupt the bacterial cell membrane, while also significantly altering the bacterium's internal pH (*Lambert et al*., 2001). More recently, Freitas and co-workers (2019) determined that the flavonoid luteolin, extracted from certain *Syzygium* sp, can bind directly to the α-amylase catalytic site. Inhibition of this enzyme has been linked to decreased risks of developing diabetes mellitus and obesity. Interestingly, in 2004, Yi and co-workers reported that luteolin, exacted from the Chinese medicinal herb *Veronica linariifolia* Pall. ex Link, binds avidly to the surface spike protein of SARS-CoV. This interaction effectively interferes with the viral cell fusion process and entry into the host cell. These studies highlight the complex and varied molecular and physical mechanisms employed by plant bioactive compounds. Future studies will have to determine if the *K. africana* bioactives exploit one or several routes to inhibit laccase and urease activity.

3.5 Conclusions and future work

This study investigated the potential of ethanol extracted *K. africana* compounds to be utilized as an alternative treatment against *Cryptococcus* species by targeting known virulence factors. Considerable insight into the antivirulence activity of *K. africana* has been obtained in this study as the ability to significantly inhibit laccase activity in *C. gattii* was identified. In addition, significant laccase inhibition in the remaining *Cryptococcus* strains was also recorded. Following the laccase assay, urease activity of *Cryptococcus* species in the presence of *K. africana* plant extracts was also investigated. Results indicate that *C. tetragattii* was most susceptible to the urease inhibitory effects of ethanolic *K. africana* extract as the most significantreduction in activity was recorded against this strain. These results point to the promising application of ethanolic *K. africana* as a novel alternative therapeutic by targeting virulence factors associated with *Cryptococcus* species.

Further experimental investigations are required in order to identify specific metabolites within *K. africana* that are responsible for the laccase and urease enzyme inhibitory effects observed. Research protocols focused on integrating nuclear magnetic resonance spectroscopy (NMR) may serve as a viable route to understand and identify plant metabolites contributing to antivirulence. Furthermore, using NMR as a first-pass screen is beneficial due to the ease with which samples can be prepared for analysis and the high sample throughput associated with this technique (Ward *et al*., 2007; Leiss *et al*., 2011).

CHAPTER 4: Summary

The findings in this study add to the increasing body of research to understand how medicinal plants contribute to the treatment of diseases initiated by pathogenic fungal species. Taking this into account, plant bioactives were extracted from four medicinal plant species, namely *Agathosma betulina* (Buchu), *Curcuma longa* (Turmeric), *Hypoxis hemerocallidea* (African potato) and *Kigelia africana* (Sausage tree), using three solvents (methanol, ethanol and hexane) with a range of relative polarities. Extracted bioactives were used to conduct disc diffusion assays against four *Cryptococcus* species (*C. deneoformans* CBS 132; *C. gattii* CBS 10575; *C. neoformans* ATCC H99 and *C. tetragattii* MRC 8817) to investigate the antifungal activity of the plant species involved. Findings highlighted that *H. hemerocallidea* extracts were ineffective against all four *Cryptococcus* species and wastherefore excluded from further analyses. Coupled with the disc diffusion assays, minimal fungicidal concentration (MFC) assays determined that ethanolic *K. africana* extracts displayed the highest degree of fungicidal activity against *C. gattii* (~3.75 mg/ml) and were also effective in against the remaining species. Based on these findings, ethanolic *K. africana* extract was used to determine if the bioactives impacted the content of the popular antifungal target ergosterol. Findings from the ergosterol assay showed that ethanolic *K. africana* extracts reducedthe ergosterol content of *C. gattii* and *C. neoformans*, however, these results were not statistically significant.

Lastly, the anti-virulent activity of the ethanolic *K. africana* extract against pathogenic *Cryptococcus* species was assessed. The well-known virulence factors laccase and urease were selected as suitable targets for these evaluations. Generated data indicated that laccase and urease activity in all four *Cryptococcus* species was significantly inhibited, however the mechanism of this inhibition is unknown and more studies focusing on understanding this mechanism are required.

The implication of the results obtained in this study reveals that the indigenous medicinal plant species *A. betulina* and *K. africana* possess varying degrees of anti-cryptococcal activity. Similarly, ethanolic *K. africana* extract displayed anti-virulence activity in being able to inhibit the activity of two well-known cryptococcal virulence factors. These findings suggest that *K. africana* may be a relevant source of metabolites that may contribute to the antifungal treatment of cryptococcal-associated diseases. Furthermore, the anti-virulence activity recorded

for ethanolic *K. africana* in this study may contribute to the acquisition of metabolites that target a wider range of virulence factors, leading to reduced pathogenicity in cryptococcal strains.

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