UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Antifungal Activity of *Kigelia africana* Phytochemicals against *Cryptococcus neoformans*

By

Elsie Kanhanda

(2430022)

Dissertation

Submitted in fulfilment of the requirements for the degree

Master of Science

In

Molecular and Cell Biology

In the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

Supervisor: Dr A. Botes

June 2023

DECLARATION

I, Elsie Kanhanda (2430022), am a student registered for the degree of Master of Science (by dissertation).

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ABSTRACT

Cryptococcus neoformans is an opportunistic fungal pathogen which frequently causes cryptococcosis in immunocompromised individuals all over the world. Each year, approximately one million cases of cryptococcosis are reported worldwide, resulting in increased mortality. Cryptococcosis can be treated with a limited number of antifungal drugs, with amphotericin B and fluconazole being the preferred choices. The main challenge faced in the current treatment strategy for cryptococcosis is the development of antifungal resistance by *C. neoformans*. A different approach is therefore necessary to identify and develop novel antifungal drugs and targets. The traditional approach for antifungal drug development has been to target the growth of the pathogen but a potentially effective alternative would be to target the virulence factors. Virulence factors are the elements of a pathogen that can cause damage in the host and lead to disease progression. They are not deemed essential for cell survival and as such are exposed to less evolutionary pressures. The most recognized virulence factors of *C. neoformans* include the enzyme urease, synthesis of melanin, thermotolerance, and the production of a polysaccharide capsule. In a recent study, bioactive molecules were used to inhibit cryptococcal urease activity.

In **Chapter 1**, literature based on the classification, life cycle, geographic distribution as well as pathogenicity and virulence factors of *C. neoformans* is reviewed. This is followed by reviewing literature on the current treatment strategies for cryptococcosis, antifungal drug resistance mechanisms of *C. neoformans* and the role of plant extracts as prospects in antifungal therapy.

In **Chapter 2** differentially extracted *Kigelia africana* crude extracts were screened for their antifungal activity against *C. neoformans*. Ethanolic extracted crude extracts were selected as the best performing bioactives. A sub-lethal concentration of the crude extracts was established and used for the treatment of *C. neoformans* in the enzyme and biofilm assays. Laccase and urease activity were inhibited. Biofilm formation was inhibited, and pre-formed biofilms were disrupted. Metabolic activity of cryptococcal biofilms was significantly reduced.

The effect of treating *C. neoformans* with *K. africana* crude extracts on the expression of virulence associated genes was evaluated in **Chapter 3**. The expression of *COX1*, *LAC1*, and *URE1* was upregulated, whilst the expression of *CAP10* was downregulated.

The findings of this study reveal evidence that *K. africana* crude extracts indeed have antifungal and antivirulence effects against *C. neoformans* and can therefore be used in the development of novel antifungal drugs which can be used to treat cryptococcal infections.

RESEARCH OUTPUTS

Conference Outputs - Poster Presentations

1. Kanhanda, E., Botes, A. 2022. Antifungal Activity of *Kigelia africana*. World Antimicrobial Awareness Week (WAAW) Research Symposium 2022, 21 – 22 November 2022.

2. Kanhanda, E., Botes, A. 2022. Antifungal Activity of *Kigelia africana*. Molecular Biosciences Research Thrust (MBRT), 1 December 2022.

DEDICATION

I dedicate this work to;

My father Jonathan Kanhanda

And

My mother Lizzy Kanhanda

You are my strongest support system.

ACKNOWLEDGEMENTS

I thank God for blessing me with the grace to complete this endeavour.

I would like to express my deepest gratitude to my supervisor, Dr Botes, for the unwavering guidance and support throughout my research journey. Your insights, encouragement, and constructive feedback have been valuable in shaping the direction of my research and refining my ideas.

I extend my sincere appreciation to Dr Sizani as well as my colleagues from Botes Lab. Thank you for all the assistance.

To Alyssa Paul, thank you so much for being more than just a lab colleague. I appreciate all the support you gave me from the beginning till the end.

I'm forever indebted to my family for all the support you gave me. Thank you for believing in me even when I doubted myself.

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α: alpha

β: beta

ºC: Degrees Celsius

Δ: delta

<: less than

≤: less than or equal

µg: microgram

µl: microliter

µM: micromolar

+/-: plus minus

%: percent

®: registered trademark

™: trademark

5-FC: flucytosine

ACT1: β-actin gene

AIDS: acquired immunodeficiency syndrome

AmB: amphotericin B

BBB: blood brain barrier

CAP10: polysaccharide capsule associated gene

cDNA: complementary deoxyribonucleic acid

CNS: central nervous system

COX1: cytochrome c oxidase subunit 1 gene

CSF: cerebrospinal fluid

CV: crystal violet

ECM: extracellular matrix

FLC: fluconazole

GAPDH: glyeraldehyde-3-phosphate dehydrogenase

gDNA: genomic deoxyribonucleic acid

GXM: glucuronoxylomannan

HIV: human immunodeficiency virus

HSF: heat shock transcription factor

HSP: heat shock protein

IL-1: interleukin-1

ITS: internal transcribed spacer

LAC1: laccase gene

L-DOPA: L-3,4-dihydroxyphenylalanine

MAPK: mitogen activated protein kinase

MM: minimal media

PBS: phosphate buffered saline

RNA: ribonucleic acid

RT-qPCR: reverse transcription-quantitative polymerase chain reaction

TNF: tumour necrosis factor

UPR: unfolded protein response

URE1: urease gene

UV: ultraviolet

XTT: 2,3-Bis- (2-Methoxy-4-Nitro-5-Sulfophenyl) -2H-Tetrazolium-5-Carboxanilide

YPD: yeast extract peptone dextrose

CHAPTER ONE: LITERATURE REVIEW

1.1 *CRYPTOCOCCUS NEOFORMANS*

Cryptococcus neoformans is a pathogenic fungus which causes cryptococcosis in immunocompromised patients all over the world (Bermas and Geddes MacAlister, 2020). This yeast pathogen belongs to the phylum Basidiomycota, class Tremellomycetes, and family Cryptococcaceae (Srikanta *et al*., 2014). It was initially described as a human pathogen when it was extracted from a bone infection (Busse, 1894). The same yeast was later extracted from fermenting peach juice and was named *Saccharomyces neoformans* because of its exceptional colony form (Sanfelice, 1894). Eventually, the yeast was reclassified as a distinct species within the genus *Cryptococcus* and renamed *Cryptococcus neoformans* because it lacked the ability to form ascospores, which is a significant feature for *Saccharomyces* (Barnett, 2010). *Cryptococcus neoformans* has been further classified based on a combination of genotypic and phenotypic characteristics; *C. neoformans* (VNI and VNII), *C. deneoformans* (VNIV), as well as *C. neoformans* and *C. deneoformans* hybrid (VNIII) (Hagen *et al*., 2015).

Most of the cryptococcal infections result from *C. neoformans* and *Cryptococcus gatti* although over fifty *Cryptococcus* species exist (Maziarz and Perfect, 2016). Generally, *C. neoformans* typically infects individuals with weakened immune systems whereas *C. gattii* often infects immunocompetent individuals as well as immunocompromised patients (Mitchell *et al*., 1995; Chen *et al*., 2014). *Cryptococcus neoformans* strains are responsible for nearly 95% of cryptococcal infections and the rest (approximately 5%) of the infections result from *C. gatti* (Hagen *et al*., 2015). *Cryptococcus neoformans* is found all over the world, whilst *C. deneoformans* is mostly present in the European continent, *C. gattii* has traditionally been located only in tropical and subtropical areas, for instance Australia, Brazil, California, central Africa, southern Hawaii and Southeast Asia (Harris *et al*., 2011). Of late, *C. gattii* has been found in temperate climates such as Vancouver Island, the region of the United States that encompasses the Pacific Northwest, and portions of Europe, implying an ecological shift probably linked to global climate changes (Kidd *et al*., 2004).

Under a microscope, cells appear as budding, encapsulated, spherical yeast cells (Pal *et al*., 2014). In culture, *C. neoformans* grows quickly, producing cream-coloured colonies within 48 to 72 hours (Refai *et al*., 2014). Being able to thrive at 37ºC is a unique trait that differentiates *C. neoformans* from other *Cryptococcus* species (Refai *et al*., 2014). *Cryptococcus neoformans* can also be identified by formation of the polysaccharide capsule

and synthesis of melanin (Zhao *et al*., 2019). Urease activity is also an indication of the presence of *C. neoformans* (Coelho *et al*., 2014).

Cryptococcus neoformans has no specific habitat but is rather found in different types of environmental niches including bat guano, dairy products, Eucalyptus trees, pigeon droppings, soil, wood and vegetables (Godinho *et al.,* 2017; Movahed *et al*., 2014). It can survive in pigeon excreta for over twenty years (Pal *et al*., 2014). This fungal pathogen is known to infect humans, birds and animals such as baboons, cats, cattle, dogs, horses, pigs and sheep (Ior *et al*., 2015).

The life cycle of *C. neoformans* can either be asexual or sexual (Maziarz and Perfect, 2016). In the sexual cycle, haploid cells of α and a mating types (MATa and MATα) undergo meiosis, which results in the formation of clamp connections, basidia, and basidiospores (Lin *et al*., 2005). When there are limited nutrients in the environment, MATα and MATa cells often secrete pheromones that trigger cell-cell fusions (Maziarz and Perfect, 2016). The MATα strains epitomise the majority of clinical and environmental isolates (Lin *et al*., 2005). The asexual cycle includes reproduction by mitosis whereby haploid *C. neoformans* MATa strains undergo a developmental transition that includes filamentation and sporulation also referred to as fruiting (Lin *et al*., 2005). Fruiting is also triggered by desiccation, nutrient limitations, and pheromones (Lin *et al*., 2005). Mating between two strains of the same type (MATα–MATα) also occurs and has been proven to form the infectious spores that account for infections in humans (Maziarz and Perfect, 2016).

1.2 CRYPTOCOCCOSIS

Cryptococcal infections lead to almost a million cases of cryptococcosis worldwide, resulting in almost 625 000 deaths annually (Godinho *et al*., 2017; Cogliati, 2012). It is reported that cryptococcosis leads to almost 180,000 deaths in sub-Saharan Africa alone (Zaragoza, 2019). Cryptococcosis is an infectious disease caused by the yeasts from the genus *Cryptococcus*, with a wide range of clinical manifestations (Maziarz and Perfect, 2016).

Clinical manifestations of cryptococcosis can be in the form of pulmonary (asymptomatic colonization of the airways, life-threatening pneumonia, and pulmonary nodules) or the central nervous system (CNS) (altered mentation, cranial neuropathies, fever, headache, lethargy, memory loss, and signs of meningeal irritation) infections (Maziarz and Perfect,

2016). Cryptococcosis also presents as cutaneous, ocular, and prostate infections (Arechavala *et al.*, 2018). Laboratory diagnosis of cryptococcosis is achieved through extraction of *C. neoformans* from clinical specimen or by staining the body fluids using India ink (Sionov *et al*, 2010).

Ever since the introduction of antiretroviral treatment, the occurrence of cryptococcosis in developed countries has significantly decreased (Cogliati, 2012). Several studies have shown that early diagnosis and prompt treatment of cryptococcosis usually result in better outcomes and reduced mortality rates (Srikanta *et al*., 2014). However, it remains a challenge in areas where there is limited access to antiretroviral therapy, such as developing countries especially those within sub-Saharan Africa (Maziarz and Perfect, 2016; Zaragoza, 2019).

1.3 PATHOGENESIS AND VIRULENCE FACTORS

Humans are infected when they inhale desiccated yeast cells or basidiospores from environmental sources (Godinho *et al*., 2017). *Cryptococcus neoformans* can typically enter the host lungs and fail to cause any disease in an immunocompetent host (Gaylord *et al*., 2020). This is because the lungs have a distinguished immune response (Martin and Frevert, 2005). Their alveoli are covered with macrophages that are responsible for the phagocytosis and removal of such pathogens (Martin and Frevert, 2005). Lymphocytes and other myeloid cells are also functional in the lungs, as well as the surfactant which has proteins that are antimicrobial in nature (Martin and Frevert, 2005). To survive this complex environment, *C. neoformans* would have to; adjust to the host environment, develop mechanisms of immune evasion, and produce virulence factors (Esher *et al*., 2018). Most of the cryptococcal cells are therefore cleared by the immune system, although a few can remain dormant in the lungs (Gaylord *et al*., 2020).

When host immunity is compromised, however, new infections or the dormant form of *C. neoformans* can be revived and disseminated to the CNS (Srikanta, *et al*., 2014). This revival and dissemination is achieved through the production of virulence factors (Zaragoza 2019). These are prominent classes of gene products that assist *C. neoformans* to persist in the host via adaptation to specific environmental conditions as well as evasion and interruption of the host-defence mechanisms (Malachowski *et al*., 2021). Some of the virulence factors of *C. neoformans* include the formation of a polysaccharide capsule,

production of melanin, secretion of extra-cellular enzymes such as phospholipase and urease, and the development of tolerance to high temperatures (Iyer *et al*., 2021).

1.4 THERMOTOLERANCE

It is vital for the yeast pathogen to be tolerant towards high temperatures so that it can survive mammalian body temperatures and subsequently complete the task of pathogenesis (Casadevall, 2012). Previous studies on cryptococcal thermotolerance determined that there are some adjustments in gene expression when the yeast is exposed to mammalian body temperatures (Kraus *et al.*, 2004). According to Yang and co-workers (2017), the HSP90 (heat shock protein 90) is a key regulator of thermotolerance in *C. neoformans*. It is a crucial element in the folding and stabilization of proteins, and its overexpression has been shown to increase thermotolerance in *C. neoformans* (Chatterjee and Tatu, 2017). The heat shock transcription factor (HSF) is also crucial for the transcriptional regulation of thermotolerance genes in *C. neoformans* (Yang *et al.*, 2017). The HSF gene is upregulated in response to heat stress, and its activation leads to the transcription of HSP90 (Yang *et al.*, 2017). The unfolded protein response (UPR) pathway, which is important in the regulation of endoplasmic reticulum homeostasis of *C. neoformans* when exposed to environmental changes, is also involved in thermotolerance (Cheon *et al.*, 2014; Yang *et al.*, 2017). Upon exposure to high temperatures, such as those found in the human body during an infection, there is an increase in the number of misfolded proteins in the endoplasmic reticulum of *C. neoformans* (Cheon *et al.*, 2014). The UPR pathway helps the fungus to respond to this stress by upregulating the production of chaperone proteins that help to refold the misfolded proteins (Cheon *et al.*, 2014). Studies have shown that mutants of *C. neoformans* that are defective in the UPR pathway have reduced thermotolerance and are more susceptible to high temperatures (Cheon *et al*., 2011). This suggests that the UPR pathway plays a critical part in permitting the fungus to thrive in the host's body during an infection (Cheon *et al*., 2011).

1.5 POLYSACCHARIDE CAPSULE

The polysaccharide capsule of *C. neoformans* is a crucial virulence factor that makes up around 25% of its overall virulence, according to a multivariate linear regression analysis that assessed the impact of various virulence factors (Casadevall *et al*., 2019). Consequently,

mutants that lack a polysaccharide capsule are non-virulent (Casadevall *et al*., 2019). The major components that make up the polysaccharide capsule are glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal), as well as other minor elements namely, mannoproteins and chitin-like structures (Heiss *et al*., 2009; Rodrigues *et al*., 2007). The capsule aids *C. neoformans* to evade phagocytosis which happens in the early stages of lung infection and protects it from desiccation and oxidative stress (Mansour and Levitz, 2002; Bermas and Geddes-McAlister, 2020). Most of the epitopes, for example the mannoprotein MP88, that bind to macrophage receptors reside on the cell wall and tend to be concealed from phagocytic cells by the polysaccharide capsule (Aksenov *et al*., 1973). According to Zaragoza (2019), the secreted capsular polysaccharides also affect the host immune response in several ways (Zaragoza, 2019). Glucuronoxylomannan, the most abundant polysaccharide in the capsule, blocks neutrophil migration in various ways (Godinho *et al*., 2017). It prevents leukocytes from exiting from the blood vessels as it exudes chemo-attracting properties and reduces expression of chemokine receptors (Dong and Murphy, 1993). Also, it prevents leukocytes from binding to the endothelium through induction of L-selectin and E-cadherin shedding from neutrophils (Dong and Murphy, 1996). Glucuronoxylomannan and GalGXM also induce apoptosis of T cells by activating caspases 8 and 9 (Monari *et al*., 2008).

O'Meara and Alspaugh (2012) state that the regulation of the *C. neoformans* polysaccharide capsule is influenced by a variety of factors including low iron content in the host, host carbon dioxide levels, ambient pH, as well as low glucose and nitrogen levels in the host. Iron is essential for the growth and survival of *C. neoformans*, and the fungus has evolved several mechanisms to acquire iron from the host (Huang *et al*., 2002). These mechanisms include the production of siderophores, which are small molecules that bind to iron and facilitate its uptake by the fungus (Huang *et al*., 2002). However, during infection, the availability of iron in the host is often limited due to the host's innate immune response, which restricts the availability of iron to invading pathogens (Jung and Kronstad, 2008). When confronted with low iron conditions, *C. neoformans* increases the expression of genes responsible for the production of the polysaccharide capsule, aiding the fungus evade the host's immune system (Jung and Kronstad, 2008). This increased capsule production is thought to be a survival mechanism for *C. neoformans* under low iron conditions.

1.6 MELANIN PRODUCTION

Laccase is a phenoloxidase found in *C. neoformans* (Williamson, 1994). It is encoded by the gene *LAC1* (Salas *et al*., 1996). Laccase catalyzes the synthesis of melanin, which is considered a virulence factor, through the oxidation of phenolic substances such as L-3,4-dihydroxyphenylalanine (DOPA) (Salas *et al*., 1996). Melanin is a negatively charged, hydrophobic pigment of high molecular weight which is normally black or brown (Casadevall, Rasa, and Nosanchuk, 2000). Melanin has several properties that enable *C. neoformans* to be resistant to the host immune response and enhance survival in the environment (Nosanchuk & Casadevall, 2003). According to Hill (1992), the ability of melanin to absorb a broad range of the electromagnetic spectrum confers resistance to ultraviolet (UV) light and prevents photo-induced damage. When exposed to UV light, melanised *C. neoformans* cells are found to be less susceptible than non-melanised cells (Wang and Casadevall, 1994). Melanin production in *C. neoformans* contributes to antifungal drug resistance as melanin can physically bind to drugs, reduce drug uptake by the fungal cell, and protect the cell against oxidative stress (Wang and Casadevall, 1994). Pigmented *C. neoformans* cells tend to be resistant to the antifungal drug amphotericin B (Wang and Casadevall, 1994). Melanin has been shown to modulate the host immune response by inhibiting the production of pro-inflammatory cytokines, such as tumour necrosis factor (TNF) and interleukin-1 (IL-1) (Williamson, 1997). These cytokines have an important part in initiating the immune response by activating immune cells and inducing the production of other pro-inflammatory cytokines (Huffnagle *et al*., 1995). Inhibition of TNF and IL-1 production by melanin may therefore lead to a dampening of the immune response and a decrease in inflammation (Huffnagle *et al*, 1995). This means that melanin may help *C. neoformans* establish infections without triggering a strong immune response (Huffnagle *et al*, 1995). Melanin protects the yeast cells from oxidants that are produced by host effector cells and confers resistance to murine macrophages (Jacobson and Tinnell, 1993). According to a study by Wang and co-workers (1995), melanin-producing strains of *C. neoformans* are more virulent than their equivalent albino mutant strains. This indicates that melanin production is not necessary for the yeast's growth but crucial during pathogenesis (Wang and Casadevall, 1994). Disruption of the *C. neoformans* phenoloxidase pathway could be an effective strategy in drug discovery (Wang *et al*., 1995).

1.7 UREASE ACTIVITY

Urease is an extracellular enzyme that is vital for cryptococcal virulence (Godinho *et al*., 2017). Cox and co-workers (2000) state that it is rare for urease-negative *C. neoformans* strains to cause disease and as a matter of fact, rapid detection of urease activity is a common way of determining the presence of *C. neoformans* in clinical isolates. The main function of urease is to catalyse the hydrolysis of urea into ammonia and carbon dioxide (Cox *et al*., 2000). The reaction often gives rise to an increase in pH under physiological conditions even though *C. neoformans* prefers acidic environments (Coelho *et al.*, 2014; Cox *et al.*, 2000). The ammonia produced by urease can help to neutralize acidic environments, such as those found in phagolysosomes within immune cells, and this can help *C. neoformans* to avoid being killed by acid-based defenses (Coelho *et al.*, 2014). When ammonia is produced in the immediate vicinity of endothelial cells, it may lead to an augmented cryptococcal adherence to the endothelium either by mechanical effects such as changing the microvessel shape or by an induction of adhesion molecules on the surface of endothelial cells (Chen *et al*., 2003). The potential effects of ammonia include the possibility of inducing endothelial cell toxicity and impacting astrocytes, which could then lead to the weakening of the blood-brain barrier's integrity by causing the opening of endothelial cell junctions (Barluzzi *et al*., 2007). Indeed, urease activity is essential for *C. neoformans* to cross the blood brain barrier during pathogenesis (Osterholzer *et al*., 2009). According to Osterholzer and co-workers (2009), cryptococcal urease encourages the build-up of immature dendritic cells (DCs) within the lung. Dendritic cells are important immune cells that help to stimulate T cells and initiate an appropriate immune response to infection (Templeton *et al*., 2018). However, when DCs are immature, they are less effective at activating T cells and can actually promote a non-protective T2 immune response (Templeton *et al*., 2018). Developing urease inhibitors and vaccines may thus help treat or prevent cryptococcal infections (Cox *et al*., 2000).

1.8 CRYPTOCOCCAL BIOFILMS

Biofilms are highly structured microbial communities that are surface adhered and attached to each other, bound in a self-made protective extracellular matrix (ECM) (Ramage *et al*., 2011). Formation of biofilms is a representation of the most usual growth mode of microorganisms in nature (Martinez and Casadevall, 2015). During the formation of cryptococcal biofilms, a significant quantity of capsular polysaccharide is released, which

results in the development of an intricate three-dimensional framework, the ECM, which encases the cell population, providing them with maximum protection and mechanical stability (Martinez and Casadevall, 2005). The cells in a biofilm differ from their planktonic counterpart cells in that the biofilm enables protection from their surroundings, recalcitrance to chemical and physical stress, metabolic co-operation, and a community-based regulation of gene expression (Ramage *et al*., 2011).

The formation of biofilms is linked with persistent infections as it enhances resistance to the host's immune system and antimicrobial treatment (Ramage *et al*., 2011). The treatment of cryptococcosis is currently not optimal as the infection is challenging to eliminate using antifungal medications (Sanglard *et al*, 2003). Antifungal resistance in biofilms can reduce the efficacy of standard antifungal treatments, resulting in treatment failure and a rise in morbidity and mortality (Ramage *et al*., 2011). Numerous mechanisms have been proposed to explain the biofilm's resistance to antimicrobial agents, such as reduced drug penetration, increased drug efflux, and changes in drug targets (Fox *et al*., 2015). The main factor contributing to antifungal resistance in biofilms is reduced drug penetration (Bonhomme and d'Enfert, 2013). The ECM of the biofilm serves as a physical obstacle that restricts the access of antifungal drugs to the underlying cells, thus reducing the efficacy of the drugs (Bonhomme and d'Enfert, 2013). Another factor which contributes to antifungal resistance in biofilms is increased drug efflux (Jabra-Rizk *et al*., 2004). Biofilm cells have been shown to have increased efflux pump activity, which can reduce the intracellular concentration of antifungal drugs and decrease their efficacy (Jabra-Rizk *et al*., 2004). Changes in drug targets also contribute to antifungal resistance in biofilms (Mathé and Van Dijck, 2013). For example, the expression of drug targets may change in response to the oxidative stress associated with life in the biofilm, leading to reduced drug efficacy (Mathé and Van Dijck, 2013). Cryptococcal biofilms have proven to be quite resistant to amphotericin B (AmB) and caspofungin, and totally recalcitrant to fluconazole and voriconazole (Martinez and Casadevall, 2017). Use of very high concentrations of AmB and caspofungin for treatment results in significantly reduced metabolism of *C. neoformans* cells in biofilms (Martinez and Casadevall, 2017). However, these concentrations are so high they are above attainable levels *in vivo* after systemic administration (Martinez and Casadevall, 2017). The influence of antifungal resistance in biofilms on treatment outcomes is significant (Fox *et al*., 2015). This highlights the need for more effective strategies to combat antifungal resistance in biofilms,

including developing new antifungal drugs, new combination therapies, and improved treatment regimens (Fox *et al*., 2015).

1.9 TREATMENT

Cryptococcosis can be treated with a range of antifungal drugs that belong to three classes namely, azoles, flucytosine, and polyenes (Spickler 2013). These classes are often used in combination to effectively treat, or prevent, disseminated infections (Bermas and Geddes-McAlister, 2020).

The polyene amphotericin B (AmB) is the most effective and fastest acting antifungal against *C. neoformans* because of its strong fungicidal effects (Bermas and Geddes-McAlister, 2020). It is the primary treatment of cryptococcosis even though it can cause significant neuronal toxicity (Iyer *et al*., 2021). Administration of AmB requires the patient be hospitalised in order to manage the toxic side effects of AmB that include anaemia, electrolyte abnormalities, hypomagnesaemia and nephrotoxicity (Bermas and Geddes-McAlister, 2020). Lipid-based AmB is safer and more efficient compared to the conventional AmB; however, it is expensive and inadequately registered in low-resource countries, which makes it difficult to access (Bermas and Geddes-McAlister, 2020).

Fluconazole (FLC) falls under the class of azole drugs, is greatly used in the first phase of treatment and in the maintenance phase to prevent relapses (Godinho *et al*., 2017). It is favoured over other azole drugs because it can penetrate the CNS, has bioavailability of approximately 100%, tolerates gastric pH, and has been extensively studied within clinical settings (Godinho *et al*., 2017). Fluconazole is less effective when compared to AmB because it takes longer to clear infections from the cerebrospinal fluid (CSF) and has inferior clinical outcomes in comparison with AmB-based treatments (Bermas and Geddes-McAlister). The most recurrent adverse effects of FLC include gastrointestinal problems, headaches and skin rashes (Bermas and Geddes-McAlister, 2020).

Flucytosine (5-FC) is a synthetic antifungal drug that was first used to treat cryptococcosis in 1968 (Bermas and Geddes-McAlister, 2020). Flucytosine has adverse side effects, particularly hepatotoxicity and myelotoxicity, probably caused by toxic 5-FU plasma concentrations (Vermes *et al*., 2000). Currently, the use of 5-FC is most effective when combined with AmB (Bermas and Geddes-McAlister, 2020).

Treating cryptococcal meningitis in HIV patients is often in three stages; induction, consolidation and maintenance (Spadari *et al*., 2020). The primary therapy of cryptococcal meningitis relies on the state of the patients. The first-line induction treatment includes administration of AmB at a dose of 0.7 to 1.0mg per kilogram per day plus 5-FC at 100mg per kilogram per day for a (Perfect *et al*., 2010). For consolidation and maintenance, FLC is administered at a dose of 400mg per day for at least eight weeks and at 200mg per day for six to twelve months (Spadari *et al*., 2020). The dosage recommended for patients with pulmonary cryptococcosis and other nonmeningeal cryptococcosis forms is 400mg/day of FLC for six months to one year (Perfect *et al*., 2010). Where FLC is not available or contraindicated, 200mg of voriconazole twice a day, 200mg/day of itraconazole, and 400mg of posaconazole twice a day can be used as alternatives.

1.10 ANTIFUNGAL RESISTANCE MECHANISMS

Antifungal resistance is the condition where a fungal pathogen continues to survive and reproduce after being subjected to antifungal treatment (Robbins *et al*., 2017). The development of antifungal resistance is caused by the extensive use of antifungal products in medicine and agriculture, endorsing the evolution of recalcitrant strains, and the occurrence of novel fungal species (Bermas and Geddes-McAlister, 2020). Resistance is classified as either primary or secondary (Hanson *et al*., 2007). In primary resistance, microorganisms that have never been exposed to a particular antifungal become resistant to it (Hanson *et al*., 2009). Primary resistance is said to be rare in *C. neoformans*, when it does occur, it is associated with 5-FC and sometimes FLC (Hanson *et al*., 2009; Bermas and Geddes-McAlister 2020). Secondary resistance occurs because of previous exposure to a particular drug (Hanson *et al*., 2009). This form of antifungal resistance is often observed in *C. neoformans* with regards to the azole FLC (Hanson *et al*., 2009).

Cryptococcus neoformans develops resistance to antifungal drugs by mutating the drug target gene thereby reducing drug binding and efficacy of the drug (Robbins *et al*., 2017). Azoles, such as FLC, target the ergosterol biosynthetic enzyme lanosterol 14-alpha demethylase that is encoded by the *ERG11* gene. Azoles block the manufacture of ergosterol and induce build-up of the toxic sterol, 14-α-methyl-3,6-diol, that is produced by delta (7)-sterol 5(6)-desaturase (Erg3). This toxic sterol applies severe membrane tension on the cell. Studies suggest that resistance to azole is connected with mutations in *ERG11* (Kanafani and Perfect,

2007). An analysis of *ERG11* was conducted on five progressive *C. neoformans* isolates collected from a patient that had repetitive cryptococcosis (Bermas and Geddes-McAlister, 2020). The first four *C. neoformans* isolates were sensitive to FLC (with minimum inhibitory concentration (MIC) values of 1 to 2μg/mL). The last isolate developed resistance to fluconazole (with a MIC value of 32μg/mL) (Bermas and Geddes-McAlister, 2020). Analysis showed that there was a point mutation in the *ERG11* gene, which caused an amino acid substitution of glycine for serine, ultimately increasing the pathogens resistance to FLC.

In a population-based research of *C. neoformans* in the Gauteng province, South Africa, FLC susceptibility showed no significant change between 2002–2003 and 2007–2008 (Govender *et al*., 2011). Only 0.6% of incident isolates from 2002–2003 had a FLC MIC of ≥16 μg/ml (considered resistant) (Govender *et al*., 2011). A study that was carried out in Uganda showed that FLC MICs in 2010–2014 significantly increased compared to a previous study in 1998–1999 (Smith *et al*., 2015). The MIC_{50} and MIC_{90} values were 8μg/ml and 32μg/ml respectively in 2010–2014 whilst in in 1998–1999, an MIC_{50} of 4μg/ml and an MIC_{90} of 8μg/ml were recorded (Smith *et al*., 2015). Therefore, an increase in FLC resistance by *C. neoformans* in Uganda was observed within a decade (Smith *et al*., 2015). At the moment, decreased proportions of FLC recalcitrance are recorded in various states of Europe and North America as a result of increased access to anti-retroviral treatments and reduction in cases of cryptococcal meningitis (Bermas *et al*., 2020). Nevertheless, rates of resistance are ranging from at least 30% to 50% in countries across Africa and Asia, with the maximum percentages being recorded in South Africa (Bongomin *et al*., 2018).

Polyenes, such as AmB, bind directly to ergosterol in the fungal membrane (Kanafani and Perfect, 2007). This causes the formation of porin passages that result in the loss of transmembrane potential and compromised cellular function (Kanafani and Perfect, 2007). Antifungal resistance to polyenes is once again achieved by genetic mutations in the ergosterol biosynthesis pathway (Robbins *et al*., 2017). Specifically, mutations in the genes *ERG11*, *ERG3*, and *ERG6* have been associated with reduced susceptibility to polyene antifungals such as amphotericin B in *C. neoformans* (Robbins *et al*., 2017). *ERG11* encodes lanosterol 14α-demethylase, which is a key enzyme involved in the ergosterol biosynthesis pathway (Robbins *et al*., 2017). Mutations in *ERG11* can lead to reduced binding of polyenes to the cell membrane, resulting in decreased susceptibility to these drugs (Robbins *et al*., 2017). *ERG3* encodes sterol C5,6 desaturase, which catalyzes the conversion of dihydrolanosterol to ergosterol. Mutations in *ERG3* have been associated with a decrease in

ergosterol levels and an increase in the production of biosynthetic precursors like fecosterol and lichesterol, which can confer resistance to polyene antifungals (Sanglard *et al*., 2003). ERG6 encodes Δ (24)-sterol C-methyltransferase, which is involved in the synthesis of ergosterol from zymosterol. Mutations in *ERG6* can also lead to reduced ergosterol levels and an increase in the production of alternative sterols, which can contribute to polyene resistance. This results in reduced ergosterol levels and induces the replacement of ergosterol with biosynthetic precursors like fecosterol, lichesterol, and lansosterol (Bermas and Geddes-McAlister, 2020). Interestingly, resistance to AmB is also associated with an increase in catalase activity, which reduces susceptibility to oxidative damage (Kanafani and Perfect, 2007).

1.11 DEVELOPMENT OF NOVEL TREATMENTS

Due to the unique biology of the yeast pathogen, it is increasingly cumbersome to treat cryptococcal infections (Gaylord *et al*., 2020). One of the challenges faced in developing novel antifungals for treating *C. neoformans* is that, like other fungi, it lacks viable targets (Aaron *et al*., 2020). Since both fungi and mammalian cells are eukaryotes, most targets that are found in fungi are also present in human cells (Aaron *et al*., 2020). These shared targets increase the likelihood of host toxicity (Aaron et al., 2020; Campoy and Adrio, 2017). The traditional approach for antifungal drug development has been to target the growth of fungi, but a potentially effective alternative is to target pathogenesis mechanisms such as the polysaccharide capsule or melanin production (Aaron *et al*., 2020).

Plants have always been rich medicinal sources in various parts of Africa (Arkhipov *et al*., 2014). Therapeutic plants have been frequently reported in traditional systems of medicine for the treatment of both human and animal fungal infections and are considered to be a valuable source for the innovation of novel antifungal therapies (Mishra *et al*., 2020). Recent research shows that plants are rich in many bioactive secondary metabolites such as alkaloids, flavonoids, phenolics, saponins, and terpenoids; which display antifungal properties (Mishra *et al*., 2020). Arkhipov and co-workers (2014) carried out research to examine the ability of *K. africana* fruit extracts to prevent the growth cancerous and microbial cells. The outcome of the study validated the traditional African medicinal usage of *K. africana* in treating a wide range of diseases (Arkhipov *et al*., 2014). Samie and co-workers (2019) tested the antifungal activity of *Perlagonium sidoides* against *C. neoformans* (Samie *et al*., 2019). Their results

revealed that the plant extracts were fungicidal in nature (Samie *et al*., 2019). The plant extracts also presented antivirulence potential by significantly constraining laccase and urease activity while also decreasing the size of the polysaccharide capsule (Samie *et al*., 2019). These studies show evidence that medicinal plants can be regarded as a prospective source for antifungal drugs and can be used for multicomponent inhibition and virulence attenuation for future treatment schemes (Mishra *et al*., 2020; Samie *et al*., 2019).

1.11.1 *KIGELIA AFRICANA*

Kigelia africana (family Bigoniaceae) is one of the most recognized medicinal plants in Africa because of its pan tropical distribution (Arkhipov *et al*., 2014). *Kigelia africana* is usually referred to as sausage tree because of the shape of its fruit (Arkhipov *et al*., 2014). Traditional African healers use preparations from different parts of the tree to treat constipation, dysentery, gonorrhoea, rheumatism, ulcers, wounds, and most gynaecological complications among others (Bello *et al*., 2016). This wide range of medicinal use suggests that *K. africana* could be a source of important crude extracts that can be used as alternatives to current medicines (Arkhipov *et al*., 2014). Approximately 149 compounds have been isolated from *K. africana* which belong to coumarins, flavonoids, iridoids, limonoids, naphthoquinones, phenolics, steroids, terpenes, and terpenoids category, and are known to have a wide range of biological activity (Singh *et al*., 2018). Several experiments provide meaningful justification of their use (Bello *et al*., 2016; Arkhipov *et al*., 2014).

A study by Arkhipov and co-workers (2014), explored the medicinal properties of *K. africana* fruit extracts. The researchers focused on evaluating the antibacterial, antifungal, antigiardial, and anticancer activities of different extracts derived from the fruit of *K. africana* (). They prepared different extracts using solvents of varying polarity and then tested these extracts against a range of microorganisms, including bacteria, fungi, and the parasite *Giardia*, as well as cancer cell lines (Arkhipov *et al*., 2014). The fruit extracts of *K. africana* demonstrated significant antibacterial effects against various bacterial strains. The extracts showed particular efficacy against the Gram-positive bacteria *Bacillus cereus, Staphylococcus aureus* and *Staphylococcus epidermidis,* which are known to cause several infections and are often resistant to antibiotics. The extracts exhibited potent antifungal properties, effectively inhibiting the growth of *Aspergillus niger*, *Candida albicans* and *Saccharomyces cerevisiae*. This suggests the potential of *K. africana* extracts in the treatment

of fungal infections. The study also found that the fruit extracts displayed notable antigiardial activity. *Giardia* is a protozoan parasite that can cause gastrointestinal infections, and the findings suggest that *K. africana* extracts may be useful in combating this parasite. Lastly, researchers evaluated the cytotoxic effects of *K. africana* extracts on cancer cell lines (Arkhipov *et al*., 2014). The results indicated that the extracts have promising anticancer properties, inhibiting the growth of cancer cells.

A study by Semwal and co-workers (2014) investigated the antimicrobial activity of fruit extracts and purified fractions from *K. africana*. The authors tested the extracts and fractions against four pathogenic bacteria; *Escherichia coli*, *Enterococcus faecalis*, *Moraxella catarrhalis*, and *S. aureus*, and two yeasts; *Candida albicans* and *C. glabrata* using the microdilution method. They also isolated and identified the active compounds using various chromatographic and spectroscopic techniques. The fruit extracts and fractions showed significant antimicrobial activity against all the tested microorganisms, with minimum inhibitory concentrations (MICs) ranging from 0.039 to 0.625mg/ml. The most effective extract was the ethyl acetate extract, which had the lowest MICs against all the bacteria and yeast strains, followed by the methanol and the hexane extract. The active compounds isolated from the ethyl acetate fraction were identified as verminoside, a glycoside of iridoid lactone, and kigelinone, a naphthoquinone derivative. Verminoside and kigelinone showed potent antimicrobial activity against all the tested microorganisms, with MICs ranging from 0.004 to 0.156 mg/ml. The authors concluded that synergistic interactions of chemical constituents could be responsible for the antimicrobial activity of *K. africana* fruits.

1.12 PROBLEM STATEMENT

The emergence of fungal infections caused by *C. neoformans* poses a significant threat to public health, especially in immunocompromised individuals (Cogliati, 2012). Existing antifungal drugs are often associated with limited efficacy, toxicity, and the development of drug resistance (Bermas and Geddes-McAlister, 2020; Robbins *et al*., 2017). Recently, there has been a growing interest in exploring alternative sources for novel antifungal agents. *Kigelia africana*, a medicinal plant known for its diverse pharmacological properties, has shown promising antimicrobial activity against various pathogens (Bello *et al*., 2016). However, the potential of *K. africana* as an antifungal agent against *C. neoformans* and its ability to inhibit virulence factors remain largely unexplored.

Therefore, the objective of this study is to evaluate the effects of *K. africana* crude extracts on the growth and virulence of *C. neoformans*. The study aims to assess the effectiveness of differentially extracted *K. africana* crude extracts using disc diffusion assays, determine the lethal and sub-lethal concentrations of *K. africana* crude extracts using a minimum inhibitory concentration (MIC) protocol, and assess their impact on the key virulence factors of *C. neoformans*, such as biofilm formation, melanin production, and urease activity. Additionally, the study will explore the possible effects of *K. africana* crude extracts on the gene expression of virulence-associated genes (laccase, cytochrome oxidase, urease, and the polysaccharide capsule associated gene *CAP10*).

By elucidating the potential of *K. africana* as a natural antifungal agent against *C. neoformans*, this research may contribute to the development of alternative treatment options for cryptococcosis and help combat the challenges associated with conventional antifungal therapy.

1.13 AIM AND OBJECTIVES

The aim of the study was to evaluate the effects of *Kigelia africana* crude extracts on the growth and virulence of *Cryptococcus neoformans* cells.

1.13.1 SPECIFIC OBJECTIVES

1. Assessing the differentially extracted *K. africana* crude extracts using disc diffusion assay.
2. Determining the lethal and sub-lethal concentrations of *K. africana* crude extracts using a minimum inhibitory concentration (MIC) protocol.
3. Analyzing the effects of *K. africana* extracts on biofilm formation, enzyme, and metabolic activity.
4. Quantifying the expression levels of select virulence genes using reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis.

CHAPTER TWO: ANTIFUNGAL AND ANTIVIRULENCE PROPERTIES OF *KIGELIA AFRICANA* CRUDE EXTRACTS AGAINST *CRYPTOCOCCUS NEOFORMANS*

2.1 INTRODUCTION

Cryptococcus neoformans is a fungus that is a common cause of opportunistic infections in immunocompromised individuals, such as those with human immunodeficiency virus (HIV)/ acquired immunodeficiency syndrome (AIDS) or cancer (Desalermos *et al*., 2014). It is one of the main causal pathogens for cryptococcosis, which infects the lungs and the central nervous system (Cheon *et al*., 2014). Approximately, one million cases of cryptococcosis are recorded worldwide, resulting in at least 625000 deaths annually (Godinho *et al*., 2017). Some of the virulence factors that contribute to the pathogenicity of *C. neoformans* include: the polysaccharide capsule, melanin production, urease activity, and biofilm formation (Zaragoza, 2019).

The polysaccharide capsule of *C. neoformans* is a crucial virulence factor that makes up around 25% of its overall virulence, according to a multivariate linear regression analysis that assessed the impact of various virulence factors (Casadevall *et al*., 2019). Consequently, mutants that lack a polysaccharide capsule are non-virulent (Casadevall *et al*., 2019). The major components that make up the polysaccharide capsule are glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal), as well as other minor elements namely, mannoproteins and chitin-like structures (Heiss *et al*., 2009; Rodrigues *et al*., 2007). The capsule aids *C. neoformans* to evade phagocytosis which happens in the early stages of lung infection and protects it from desiccation and oxidative stress (Mansour and Levitz, 2002; Bermas and Geddes-McAlister, 2020).

Laccase is a phenoloxidase found in *C. neoformans* which is encoded by the *LAC1* gene (Williamson, 1994). Laccase catalyzes the synthesis of melanin, which is considered a virulence factor, through the oxidation of phenolic substances such as L-3,4-dihydroxyphenylalanine (DOPA) (Salas *et al*., 1996). Melanin is a negatively charged, hydrophobic pigment of high molecular weight which is normally black or brown (Casadevall *et al*., 2000). It enhances the yeast's survival in the environment, increases resistance to host defences, and is involved in the dissemination from the lungs to the brain (Nosanchuk and Casadevall, 2003).

Urease is an extracellular enzyme that is vital for cryptococcal virulence (Godinho *et al*., 2017). This enzyme's main function is catalysing the hydrolysis of urea into ammonia and carbon dioxide (Cox *et al*., 2000). The ammonia produced by urease can help to neutralize acidic environments, such as those found in phagolysosomes within immune cells, and this

can help *C. neoformans* to avoid being killed by acid-based defenses (Coelho *et al.,* 2014). When ammonia is produced in the immediate vicinity of endothelial cells, it may lead to an augmented cryptococcal adherence to the endothelium either by mechanical effects such as changing the microvessel shape or by an induction of adhesion molecules on the surface of endothelial cells (Chen *et al*., 2003). The potential effects of ammonia include the possibility of inducing endothelial cell toxicity and impacting astrocytes, which could then lead to the weakening of the blood brain barrier (BBB)'s integrity by causing the opening of endothelial cell junctions (Osterholzer *et al*., 2009). Indeed, urease activity is essential for *C. neoformans* to cross the BBB during pathogenesis (Osterholzer *et al*., 2009).

Biofilms are highly structured microbial communities that are surface adhered and attached to each other, bound in a self-made protective extracellular matrix (ECM) (Ramage *et al*., 2011). The most abundant polysaccharide in the capsule, GXM, is involved in the formation of biofilms (Martinez and Casadevall, 2006). The organisms in a biofilm are different from their planktonic counterparts in that the biofilm enables protection from their surroundings, recalcitrance to chemical and physical stress, metabolic co-operation, and a community-based regulation of gene expression (Ramage *et al*., 2011). Antifungal resistance in biofilms can reduce the efficacy of standard antifungal treatments, resulting in treatment failure and a rise in morbidity and mortality (Ramage *et al*., 2011).

According to Mishra and co-workers (2016), some previous studies have shown evidence that medicinal plants can be regarded as a prospective source for antifungal drugs and can be used for multicomponent inhibition and virulence attenuation for future treatment schemes. For example, the plant extracts of *Perlagonium sidoides* presented antivirulence potential by significantly constraining laccase and urease activity while also decreasing the size of the polysaccharide capsule (Samie *et al*., 2019). *Kigelia africana* (family Bigoniaceae) is one of the most recognized medicinal plants in Africa because of its pan tropical distribution, and several studies have concluded that it is a potential source of important crude extracts that can be used as alternatives (Arkhipov *et al*., 2014). The main goal of the current study is to explore antifungal and virulence attenuation properties of *K. africana* crude extracts against *C. neoformans*.

2.2 AIM AND OBJECTIVES

The study aimed to evaluate the effects of *Kigelia africana* crude extracts on the growth and select virulence factors of *Cryptococcus neoformans*.

2.2.1 SPECIFIC OBJECTIVES

1. Assessing the antifungal activity of differentially extracted *K. africana* crude extracts using disc diffusion assay.
2. Determining the minimum inhibitory concentration (MIC) of *K. africana* crude extracts.
3. Analysing the effects of *K. africana* crude extracts on virulence factors, specifically the activity of laccase and urease.
4. Analysing the effects of *K. africana* extracts on biofilm formation and cell viability using a crystal violet (CV) and a 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay.

2.3 MATERIALS AND METHODS

2.3.1 STRAINS AND CULTURE CONDITIONS

The *Cryptococcus neoformans* type strain, H99 used throughout this study was accessed from Dr Angela Botes' culture collection at the University of the Witwatersrand, Johannesburg, South Africa. *Cryptococcus neoformans* was maintained by periodic transfer to Sabouraud Dextrose Agar (SDA; pH 5) agar supplemented with 200mg/l chloramphenicol.

2.3.2 PLANT BIOACTIVE EXTRACTION

Plant bioactives were extracted from the medicinal plant *Kigelia africana* (African sausage tree) as described by Breda and co-workers (2016), with modifications. A total of 2g crushed *K. africana* dried fruit powder was mixed with 20ml of the extraction solvent, either ethanol (95% v/v) or distilled water in 50ml Falcon tubes. The samples were vortexed for 3-5min and subjected to centrifugation at 1372xg for 10min. The supernatant was then filtered through Whatman No. 1 filter paper into pre-weighed vials. Filtrates were air-dried and resuspended in acetone (for the crude extracts extracted using ethanol) or distilled water (for the crude

extracts extracted using distilled water) and stored at 4°C. The final concentration of the crude extracts was worked out using the formula: $\frac{extract\ weight}{acetone\ volume}$

2.3.3 DISC DIFFUSION ASSAY

The Kirby-Bauer susceptibility test was carried out following the protocol described by Hudzicki (2009) with some modifications. Five distinct colonies were transferred to 5ml physiological saline (0.85% w/v) using a sterile loop and vortexed for 3-5min to achieve the turbidity of the 0.5 McFarland standard. The sample was collected by dipping a sterile swab into the solution. Any excess fluid was removed. The swab was then used to streak the surface of a Mueller-Hinton agar (MHA) plate evenly. Streaking was repeated at least six times, with a 90º rotation of the plate each time. Filter paper discs were inoculated with standardized concentrations (250µg/ml) of amphotericin B (positive control), 0.85% (w/v) physiological saline (made up of 8.5g NaCl, 1l distilled water) (negative control), acetone (vehicle control), distilled water (vehicle control), 120µg/µl ethanolic extract (test), and 120µg/µl aqueous extract (test) and allowed to dry briefly. These discs were then transferred onto six distinct zones of the MHA plate pre-inoculated with *C. neoformans* and incubated at 37ºC for 48hr. The modification to incubate at 37ºC for 48 hours instead of 35ºC for 24 hours was based on empirical evidence suggesting that *C. neoformans* exhibits more reliable growth at the higher temperature and longer incubation period (Refai *et al., 2014)*. The modification aimed to enhance the accuracy and sensitivity of the assay by allowing more time for the organism to grow and form visible zones of inhibition around the discs. The assay was replicated three times and the diameter of each zone of inhibition was measured using a ruler and recorded in millimetres.

2.3.4 MINIMUM INHIBITORY CONCENTRATIONS (MIC)

Minimum inhibitory concentration (MIC), defined as the lowest concentration of antifungal showing no visible growth, was established to identify the sub-lethal dose for *K. africana* crude extracts, using a colourimetric assay (Singh *et al*, 2018; Samie *et al*., 2019). Yeast cells from overnight yeast extract peptone dextrose (YPD) (made up of 5g yeast extract, 10g peptone, and 10g glucose, dissolved in 500ml distilled water) culture were harvested by

centrifugation (5000xg for 8min) and washed twice with phosphate buffered saline (PBS) (made up of 4g NaCl, 0.1g KCl, 0.77g Na_2HPO_4, and 0.12g KH_2PO_4, dissolved in 500ml distilled water) then suspended in minimal media (MM) (30mM glucose, 20mM magnesium sulphate, 58.8mM potassium dihydrogen phosphate, 26mM glycine, 330mM 3-(N-morpholino) propanesulfonic acid and 6µM thiamine hydrochloride) at a concentration of $2x10^6$cells/ml. Subsequently, equal volumes of the diluted cell suspension and the *K. africana* crude extracts (1.25, 2.5, 5, 10, 15 and 20µg/µl) were added to 96 well plates yielding an inoculum concentration of $1x10^6$cells/ml. The test also included adding diluted cell suspensions to serially diluted amphotericin B (0.5, 1, 2, 4, 8, 16 and 32µg/ml) to different cells, to a final inoculum concentration of $1x10^6$cells/ml. The positive control wells had the diluted cell suspensions and acetone, and the negative control wells contained no cells. The 96 well plates were sealed and incubated at 37ºC on an orbital shaker (150rpm) for 24hr. A total of 150µl of each cell suspension was mixed with 50µl p-iodonitrotetrazolium chloride (INT; 0.2mg/ml) in new wells. Plates were sealed and incubated at 37ºC for 24hr. Subsequently, colour changes were recorded; pink wells indicated live cells while clear wells are indicative of cell death. Absorbance readings were taken at 600nm wavelength using a BOECO S-22 UV/Vis spectrophotometer.

2.3.5 ENZYME ASSAYS

2.3.5.1 PREPARATION OF YEAST INOCULUM

Enzyme assays were carried using the method outlined by Samie and co-workers (2019) with some modifications. Yeast cultures were prepared in test tubes by inoculating a single yeast colony into 5ml YPD broth followed by for 24hr incubation at 37ºC on a Stuart SB2 tube orbital rotator. Cells were harvested by centrifugation at 5000xg for 8min and washed twice with PBS. The cells were then re-suspended in distilled water before transfer to test medium at the specified cell concentrations.

2.3.5.2 LACCASE ASSAY

Cells were inoculated into 5ml of MM supplemented with 5ml of 40µg/µl of ethanol extracted *K. africana* crude extracts to an inoculum concentration of $1x10^6$cells/ml and 20µg/µl *K. africana* crude extracts. The untreated cells were inoculated into 5ml of MM supplemented with 5ml of acetone to an inoculum concentration of $1x10^6$cells/ml. For the negative control, no cells were added to the 5ml of MM supplemented with 5ml of 40µg/µl *K. africana* extract. All the samples were incubated at 37ºC on a Stuart SB2 tube orbital rotator for 48hr. Cells were then harvested by centrifugation at 5000xg for 8min and washed twice with PBS. The cells were then inoculated into 5ml sterile distilled water supplemented with 5mM L-3,4-dihydroxyphenylalanine (L-DOPA) to a concentration of $5x10^6$cells/ml. The test tubes were wrapped in tin foil and incubated at 37ºC on a Stuart SB2 tube orbital rotator for 48hr. Absorbance readings were taken after 5, 10, 30min, 1, 6 and 24hr of incubation at 490nm wavelength using a BOECO S-22 UV/Vis spectrophotometer.

2.3.5.3 UREASE ASSAY

Cells were inoculated into 5ml of MM supplemented with 5ml of 40µg/µl of ethanol extracted *K. africana* crude extracts to a cell concentration of $1x10^6$cells/ml and 20µg/µl *K. africana* crude extracts. The untreated cells were inoculated into 5ml of minimal media supplemented with 5ml of acetone to a final concentration of $1x10^6$cells/ml. For the media control, no cells were added to the 5ml of MM supplemented with 5ml of 40µg/µl *K. africana* extract. All the samples were incubated at 37ºC on a Stuart SB2 tube orbital rotator, for 48hr. Cells were then harvested by centrifugation at 5000xg for 8min and washed twice with PBS. The cells were then inoculated into 5ml sterile distilled water supplemented with 0.66mM urea and 0.012g/l phenol red indicator to a concentration of $5x10^6$cells/ml. The samples were incubated at 37ºC on a Stuart SB2 tube orbital rotator. Absorbance readings were taken after 5, 10, 30min, 1, 6 and 24hr of incubation at 570nm wavelength using a BOECO S-22 UV/Vis spectrophotometer.

2.3.6 BIOFILM ASSAYS

2.3.6.1 BIOFILM FORMATION

The biofilm assays were carried out following the protocol outlined by Kumari and co-workers (2017) with some modifications. Isolates were grown on SDA for 72hr at 37˚C. One colony was picked and further sub-cultured in 5ml MM. Cells were harvested by centrifugation (5000xg for 8min) and washed twice with PBS. The cell concentration was adjusted to $1x10^7$cells/ml in fresh MM using a haemocytometer. A total of 100µl cell suspension was transferred into each well of a flat bottomed 96-well plate. The media control wells had 100µl of MM with either acetone or *K. africana* crude extracts. For the untreated cells, 100µl MM was inoculated with $1x10^7$cells/ml of the cells only. The test wells contained 100µl of MM plus $1x10^7$cells/ml inoculum and 20µg/µl *K. africana* crude extracts. The other test wells contained 100µl of minimal media plus cells and 4µg/ml amphotericin B. The plate was sealed with parafilm and incubated at 37˚C for 48hr at 75rpm, whereas the protocol by Kumari and co-workers (2017) states 30˚C for 24hr at 150rpm. By extending the incubation time, increasing the temperature, and reducing the agitation speed, it facilitated the formation of more robust and mature biofilms, allowing for a better assessment of their responses to the treatment. Wells were aspirated to remove unattached cells and then washed with 150µl of PBS. The wells were drained by pipetting PBS out and then inverting plates on a paper towel to remove residual PBS. Then 100µl of fresh MM was added to wells. The plate was incubated at 37˚C for 72hr with shaking at 75rpm to allow for biofilm formation.

2.3.6.2 CRYSTAL VIOLET ASSAY

Following biofilm formation, the wells were aspirated to remove the unattached cells. The wells were washed with 200µl of PBS. The plate was then dried for 20min at 37˚C in the biosafety cabinet. A total of 110µl 0.4% (w/v) aqueous crystal violet solution was added to each well then incubated for 45min at 37^{o}C. The wells were then washed with 200µl Milli-Q water three times. The wells were destained with 100µl of 95% (v/v) ethanol for 45min at room temperature. A total of 100µl ethanol was transferred to new wells and absorbance was measured using a VICTOR Nivo Multimode plate reader at 595nm.

2.3.6.3 THE 2,3-BIS- (2-METHOXY-4-NITRO-5-SULFOPHENYL)-2H-TETRAZOLIUM-5-CARBOXANILIDE (XTT) ASSAY

The CyQUANT™ XTT cell viability assay kit (Thermo Fisher Scientific) was used following the protocol outlined by the manufacturer. The XTT reagent was thawed in a 37^{o}C water bath, then vortexed. The Electron Coupling Reagent was thawed at room temperature and vortexed. The XTT reagent and the Electron Coupling reagent were mixed in the ratio 6:1, respectively, to produce the XTT working solution. After biofilm formation, wells were aspirated and washed with 200µl of PBS. The plate was left to dry for 20min in the biosafety cabinet. For all the wells containing cells (treated and untreated), as well as the media control (without cells), 100µl of MM was added. Then 70µl of the XTT working solution was added to each well. The plate was incubated at 37^{o}C for 4hr with shaking at 75rpm. Absorbance was read at 450nm using a VICTOR Nivo Multimode plate reader.

2.4 RESULTS AND DISCUSSION

2.4.1 DISC DIFFUSION ASSAY

The disc diffusion method works by placing a disc infused with antimicrobial agents on agar that has been pre-inoculated with the test microbe (Hudzicki, 2009). As the disc absorbs moisture, the antimicrobial agents diffuse outwards in a radial pattern through the agar medium, generating a concentration gradient of antimicrobial agent (Tendencia, 2004). At the periphery of the disc, the concentration of antimicrobial agents is high and decreases progressively as the distance from the disc increases (Tendencia, 2004). Eventually, the concentration becomes too low to inhibit the growth of the microbe, which can then proliferate without any hindrance (Hudzicki, 2009). After incubation, if the antimicrobial agent inhibits the growth of the microbe, a transparent zone or halo appears around the disc (Tendencia, 2004).

Disc diffusion assays for differentially extracted crude extracts were conducted to identify which solvent extracted crude extracts would be most effective against *C. neoformans*. Crude extracts extracted using ethanol as the extraction solvent are referred to as ethanolic *K. africana* crude extracts (120µg/µl) and were resuspended in acetone after air-drying. Those extracted using distilled water as the extraction solvent are referred to as aqueous

K. africana crude extracts (120µg/µl) and were resuspended in distilled water after air-drying. In addition, a positive control (amphotericin B, 0.25µg/µl) was included in the experiment.

The results of the assay (Figure 2.1) showed that the ethanolic crude extracts produced a clear zone of inhibition (ZOI), with an average of 16mm (Table 2.1), while aqueous crude extracts had no effect on the growth of *C. neoformans* as there was no ZOI observed (Figure 2.1). The positive control, amphotericin B (AmB), produced a significantly larger ZOI than the ethanolic crude extracts (Figure 2.1) with an average of 29.67mm (Table 2.1), indicating its potent antifungal activity. As anticipated, all of the negative controls had no ZOI.

Figure 2.1: Antifungal activity of differentially extracted *Kigelia africana* crude extracts (120µg/µl). Acetone, AMB – amphotericin B (250µg/ml), ETH extract – *K. africana* ethanolic crude extracts (120µg/µl), dH_2O – distilled water, aq extract – *K. africana* aqueous crude extracts (120µg/µl), Phys Saline – physiological saline.

Table 2.1: Zones of inhibition resulting from antifungal activity against *Cryptococcus neoformans.*

Antifungal agent	Mean Zone of Inhibition (mm)
Acetone	0 (+/- 0)
Amphotericin B (250µg/ml)	29.67 (+/- 1.69)
Distilled water	0 (+/-0)
Kigelia africana aqueous extract (120µg/µl)	0 (+/-0)
Kigelia africana ethanolic extract (120µg/µl)	16 (+/-1)
Physiological saline	0 (+/-0)

The fact that ethanolic crude extracts produced a ZOI suggests that they possess antifungal properties against *C. neoformans* and the absence of a ZOI for aqueous crude extracts implies that they may not have any significant antifungal activity towards *C. neoformans (*Bonev *et al*., 2008). This discrepancy between the aqueous and ethanolic crude extracts' antifungal properties suggests that the active compounds in the plant material extracted using water may have been different or less concentrated compared to those extracted using ethanol (Agyare *et al*., 2013). The antifungal traits displayed by ethanolic crude extracts are consistent with previous studies that have reported the antifungal activity of *K. africana* crude extracts. In a study conducted by Arkhipov and co-workers (2014), extracts obtained from the fruit of *K. africana* were found to have broad antifungal activity, inhibiting the growth of 75% of the tested fungal species, including a strain of *Candida albicans*. Agyare and co-workers (2013) also noted that crude extracts extracted using methanol and ethanol were more effective than those extracted using water. The larger ZOI of AmB is expected as it is a potent and clinically proven antifungal agent.

Acetone and distilled water were used as vehicle controls in the assay, as the crude extracts were dissolved in these solvents. Acetone has been reported to have antimicrobial activity, for example, a study by Dyrda and co-workers (2019) found that acetone has a deleterious effect on the growth and viability of some fungi. The study found that the growth of *C. albicans* and *Aspergillus niger* was inhibited by acetone concentrations greater than

20% (v/v) (Dyrda *et al*., 2019). Including acetone as a vehicle control was crucial to account for any potential effects that the solvent might have had on *C. neoformans*. Water is regarded as a neutral solvent that does not have any inherent antimicrobial activity (Sommer *et al*., 2017). Including water as a vehicle control also ensured that any observed effects were due to the crude extract and not the solvent itself. Therefore, the vehicle controls provided confidence in the assay's validity, as they showed no inhibitory effect on the growth of *C. neoformans*. Based on these results, the *K. africana* ethanolic crude extracts were selected for use in treating cells in all the assays performed in this study.

2.4.2 MINIMUM INHIBITORY CONCENTRATION (MIC)

The minimum inhibitory concentration is the lowest concentration of a particular antimicrobial agent required to inhibit the growth of a microorganism (Kumari, 2017). The basis of the assay is to expose a known quantity of the microorganism to a range of concentrations of the antimicrobial agent, and then observe the lowest concentration that prevents visible growth of the organism after a specified incubation period (Andrews, 2001). The MIC can be used to determine the susceptibility of a microorganism to different antimicrobial agents, and it is a key tool in guiding the selection of appropriate antimicrobials for the treatment of infectious diseases (Arkhipov *et al*., 2014). This study used a colorimetric microdilution method with p-iodonitrotetrazolium (INT) to determine the MICs of AmB and *K. africana* crude extracts against *C. neoformans* H99. To establish the MIC value from the colorimetric microdilution technique, the lowest concentration of the antimicrobial agent that completely inhibited the visible growth of the fungi in the microtitre plate was observed (Andrews, 2001). This was achieved by visually examining the wells and determining the concentration at which there was no visible growth, as indicated by the lack of colour change from colourless to pink or red in the presence of INT (Andrews, 2001). The INT is a redox indicator which is colourless in its oxidized form but turns pink or red in the presence of actively metabolizing cells (Podczasy and Wei, 1988).

The MICs for AmB and *K. africana* crude extracts could not be established as the results were inconclusive. All the treated wells had growth in them, and this was shown by the colour change to pink. We determined the IC50 (0.004µg/µl) and IC80 (0.016µg/µl) of AmB as well as the IC80 (15µg/µl) and IC90 (20µg/µl) of *K. africana* crude extracts using the

equation; % inhibition = $\frac{\text{untreated cells absorbance} - \text{treated cells absorbance}}{\text{untreated cells absorbance}}$ X 100. The IC50 for *K. africana* crude extracts is at a concentration lower than the range we tested. We took 20µg/µl (IC90) *K. africana* crude extracts as the sub-lethal concentration and used it for treating *C. neoformans* cells in all the experiments carried out in this study.

A study by Arkhipov and coworkers tested the susceptibility of some fungi to *K. africana* crude extracts and found the MIC values ranging from 0.8412 to 2.768µg/µl. Another study by Agyare and co-workers (2013) investigated the MIC of *K. africana* extracts against various fungi and bacteria. The results showed that the MIC of the *K. africana* extracts against the tested fungi ranged from 2.5µg/µl to 5µg/µl. The extracts showed effective activity against *C. albicans*, with an MIC of 2.5µg/µl. The study concluded that *K. africana* extracts have potential as a natural antifungal agent. Our results do not necessarily correlate with those found in both studies as our MIC could not be established. However, the variety in the results obtained from these studies confirms the antifungal activity of *K. africana* crude extracts can vary depending on the particular species being tested and specific test conditions.

2.4.3 LACCASE ACTIVITY

As previously mentioned, the enzyme laccase is important in the virulence of *C. neoformans* (Bermas and Geddes-McAlister, 2020). Laccase catalyzes the synthesis of melanin, a crucial virulence factor which allows the organism to survive in the host by evading the immune system (Eisenman *et al*., 2007). Melanin protects the cryptococcal cell from high temperatures, oxidative stress, and ultraviolet (UV) radiation (Bermas and Geddes-McAlister, 2020). According to Hill (1992), the ability of melanin to absorb a wide range of the electromagnetic spectrum provides protection against ultraviolet (UV) radiation and inhibits harm caused by photo-induced damage. When exposed to UV light, melanised *C. neoformans* cells are found to be less susceptible than non-melanised cells (Wang and Casadevall, 1994). According to a study by Wang and co-workers (1995), melanin-producing strains of *C. neoformans* are more virulent than their equivalent albino mutant strains.

The occurrence of melanin production in untreated cells, indicated by a change in colour to dark brown and black in the test tubes indicates laccase activity (Figure 2.2A). The slight brown colour observed in the reaction mixtures of the test tubes shown in Figure 2.2B after

24 hours indicates a reduction in laccase activity subsequent to treatment with *K. africana* crude extracts. The results obtained are consistent with the findings of Samie and co-workers (2019) who used a colorimetric assay to measure the activity of the cryptococcal laccase in the presence of different concentrations of *Pelargonium sidoides* extracts (Samie *et al*., 2019). The results showed that the *P. sidoides* extracts had a dose-dependent inhibitory effect on laccase activity in *C. neoformans* (Samie *et al*., 2019). At the highest concentration tested (1mg/ml), the extract inhibited laccase activity by 72.8% compared to the control group (Samie *et al*., 2019). The researchers also found that the extract reduced the production of melanin in *C. neoformans*, which is consistent with the inhibition of laccase activity (Samie *et al*., 2019).

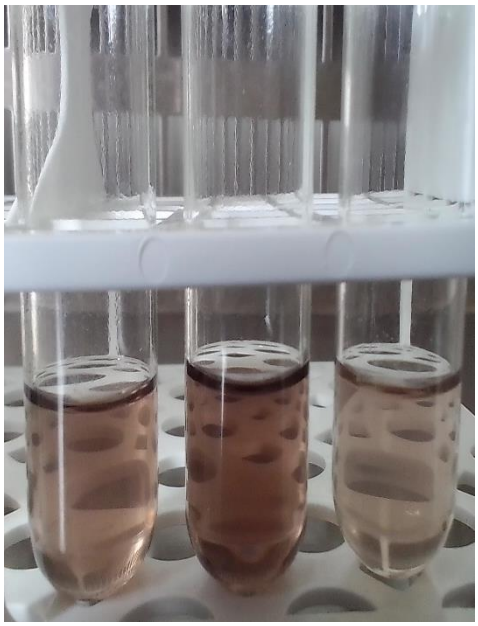
A.  B. 

Figure 2.2: Laccase assay results for *Cryptococcus neoformans* cells untreated (A) and treated (B) with *Kigelia africana* (20µg/µl) crude extracts after 24hr.

In our study, there was a progressive increase in laccase activity with increase in incubation time (Figure 2.3). For the untreated cells, there was large increase in enzyme activity at the 6hr point. This progressive increase is likely caused by an increase in the number of cells, during the exponential phase of cell growth, hence producing more enzyme to catalyse melanin production. The highest laccase activity was observed at the 24hr time point. The laccase activity in treated cells was significantly reduced throughout the incubation period compared to the laccase activity in untreated cells ($p \leq 0.05$). Therefore, *K. africana* crude

extracts are capable of inhibiting laccase activity in *C. neoformans* cells and could be used to target virulence factors in treating cryptococcal infections. These results show evidence that medicinal plant extracts can be considered as a potential future source for antifungal drug development and can be used for multicomponent inhibition and virulence attenuation for future treatment schemes (Mishra *et al*., 2020; Samie *et al*., 2019).

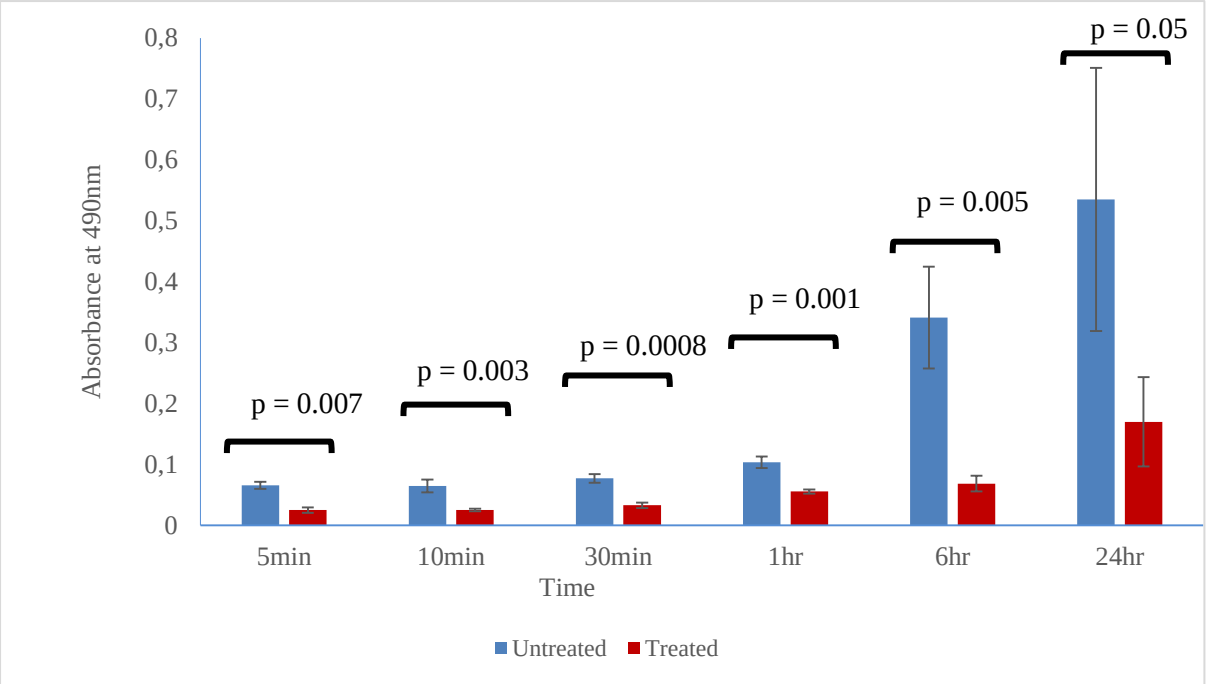


Figure 2.3: Laccase assay results for *Cryptococcus neoformans* cells treated (red) and untreated (blue) with *Kigelia africana* (20µg/µl) crude extracts at different time intervals. Bars denote standard error of three replicates.

2.4.4 UREASE ASSAY

Cryptococcus neoformans produces high levels of urease to help the yeast survive and grow in the host by neutralizing acidic environments (Singh *et al*., 2013). Urease is also essential for crossing the BBB during pathogenesis (Godinho *et al*., 2017). The BBB normally maintains a tight junction between endothelial cells that form the blood vessels in the brain, creating a physical barrier that prevents large molecules and pathogens from crossing into the brain (Cox *et al*., 2000). However, the increased pH due to urease activity can disrupt these tight junctions, making it easier for *C. neoformans* to cross the BBB and invade the brain

(Cox *et al*., 2000). The urease assay measures the production of ammonia and is used to determine urease activity in *C. neoformans* (Cox *et al*., 2000).

Urease activity was inhibited by the plant extracts and the colour change to light orange (Figure 2.4A) is an indication that the cells could not catalyse the conversion of urea into ammonia, carbon dioxide and water. This shows that the *K. africana* crude extracts can inhibit urease activity or production. In untreated cells, urease successfully converted urea into ammonia resulting in the pink colour change (Figure 2.4B). Similarly, the study by Samie and co-workers (2019) also used a colorimetric assay to monitor urease activity in the presence of different concentrations of *P. sidoides* extracts. The results showed that the *P. sidoides* extracts had a dose-dependent inhibitory effect on urease activity in *C. neoformans* (Samie *et al*., 2019). At the highest concentration tested (1mg/ml), the extract inhibited urease activity by 85.9% compared to the control group (Samie *et al*., 2019).

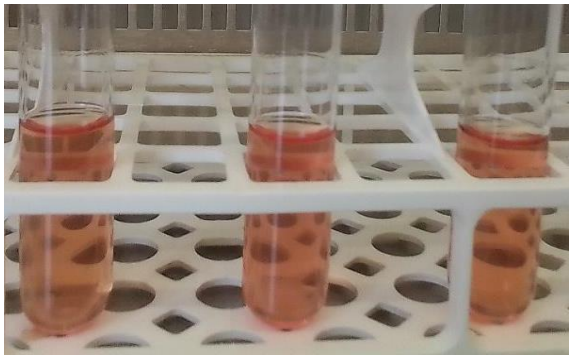
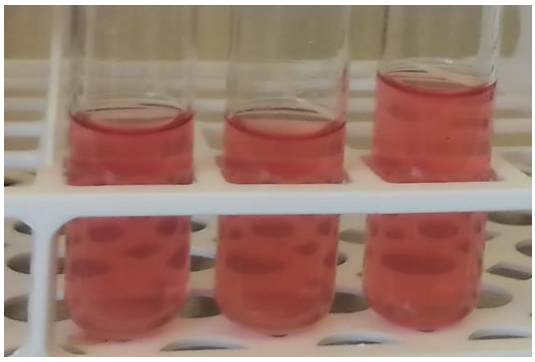
A.  B. 

Figure 2.4: Urease assay results for *Cryptococcus neoformans* cells treated (A) with *Kigelia africana* crude extracts (20µg/µl) and untreated (B) after 24hr.

Results denote that there was progressive increase in urease activity (breakdown of urea) in in all samples (treated and untreated) (Figure 2.5). However, the absorbance values show that urease activity was significantly higher in untreated cells compared to treated cells ($p < 0.05$) up to the 6hr incubation period. The highest enzyme activity was noted at the 6hr time point for the untreated cells, whilst it was noted at 24hr in treated cells. The decrease in enzyme activity at 24hr in the untreated cells could be as a result of substrate depletion (decreased amount of urea) and hence the reaction was now approaching the plateau phase. The results confirm that *K. africana* crude extracts are capable of inhibiting urease activity in *C. neoformans* cells and can be used to target virulence factors in treating cryptococcal infections.

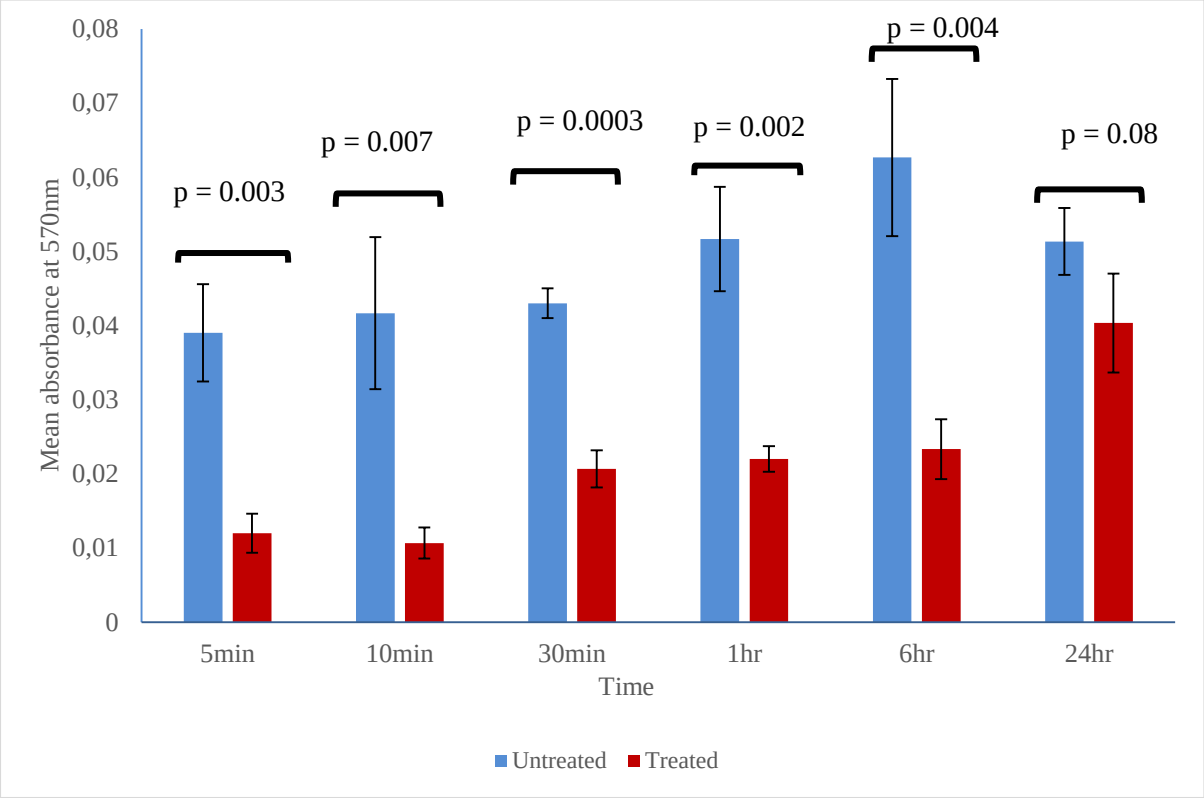


Figure 2.5: The graphical representation of the urease assay results of the treated (with 20µg/µl *Kigelia africana* crude extracts) and untreated reactions at different time intervals. Bars denote standard error of three replicates.

2.4.5 BIOFILM FORMATION

According to Ramage and co-workers (2011), it is crucial to comprehend the role of cryptococcal biofilms in antifungal resistance and pathogenesis for the development of new therapies and strategies to combat *C. neoformans* infections. As mentioned earlier, biofilms are highly structured microbial communities that are surface adhered and attached to each other, bound in a self-made defensive extracellular medium (Ramage *et al*., 2011). The importance of cryptococcal biofilms in antifungal resistance lies in their ability to protect the fungal cells from the host immune system and from antifungal drugs (Martinez and Casadevall, 2015). The matrix of the biofilm acts as a physical barrier that prevents drugs from penetrating and killing the fungal cells within (Martinez and Casadevall, 2015). Biofilm formation by *C. neoformans* has been observed on medical devices such as catheters, which

often lead to persistent infections that are difficult to treat (Ramage *et al*., 2011). Cells in biofilms also cannot be engulfed by phagocytic cells because the extracellular matrix (ECM) of the biofilms is thick and dense, making it difficult for phagocytes to penetrate and reach the individual cells within the biofilms (Aslanyan *et al*., 2017). The presence of biofilms can therefore complicate the treatment of cryptococcal infections, as they can confer higher antimicrobial resistance and increased difficulty in immune clearance compared to their planktonic counterparts (Jabra-Rizk *et al*., 2004). In our study, the biofilms formed by *C. neoformans* cells were stained with crystal violet then decolourized using 95% (v/v) ethanol. Absorbance of the decolourization agent reflects the number of cells in the biofilms formed.

2.4.6 PRE-BIOFILM FORMATION

Cryptococcus neoformans cells were treated with *K. africana* crude extracts (5, 10 and 20µg/µl) before biofilm formation, and the absorbance readings were taken after biofilm formation. Biofilm formation percentages were calculated based on the absorbance readings and were plotted (Figure 2.6). The results show that treatment with *K. africana* crude extracts inhibited biofilm formation as evidenced by lower biofilm formation in treated biofilms than in untreated biofilms (Figure 2.6). The highest concentration of crude extracts (20µg/µl) resulted in the lowest biofilm formation (17.3%), indicating the greatest inhibition of biofilm formation. The lowest concentration of crude extracts caused the highest biofilm formation among treated biofilms (52.5%). The results from the crystal violet assay of cells treated with crude extracts before biofilm formation suggest that the crude extracts have the ability to inhibit biofilm formation in a dose dependent manner.

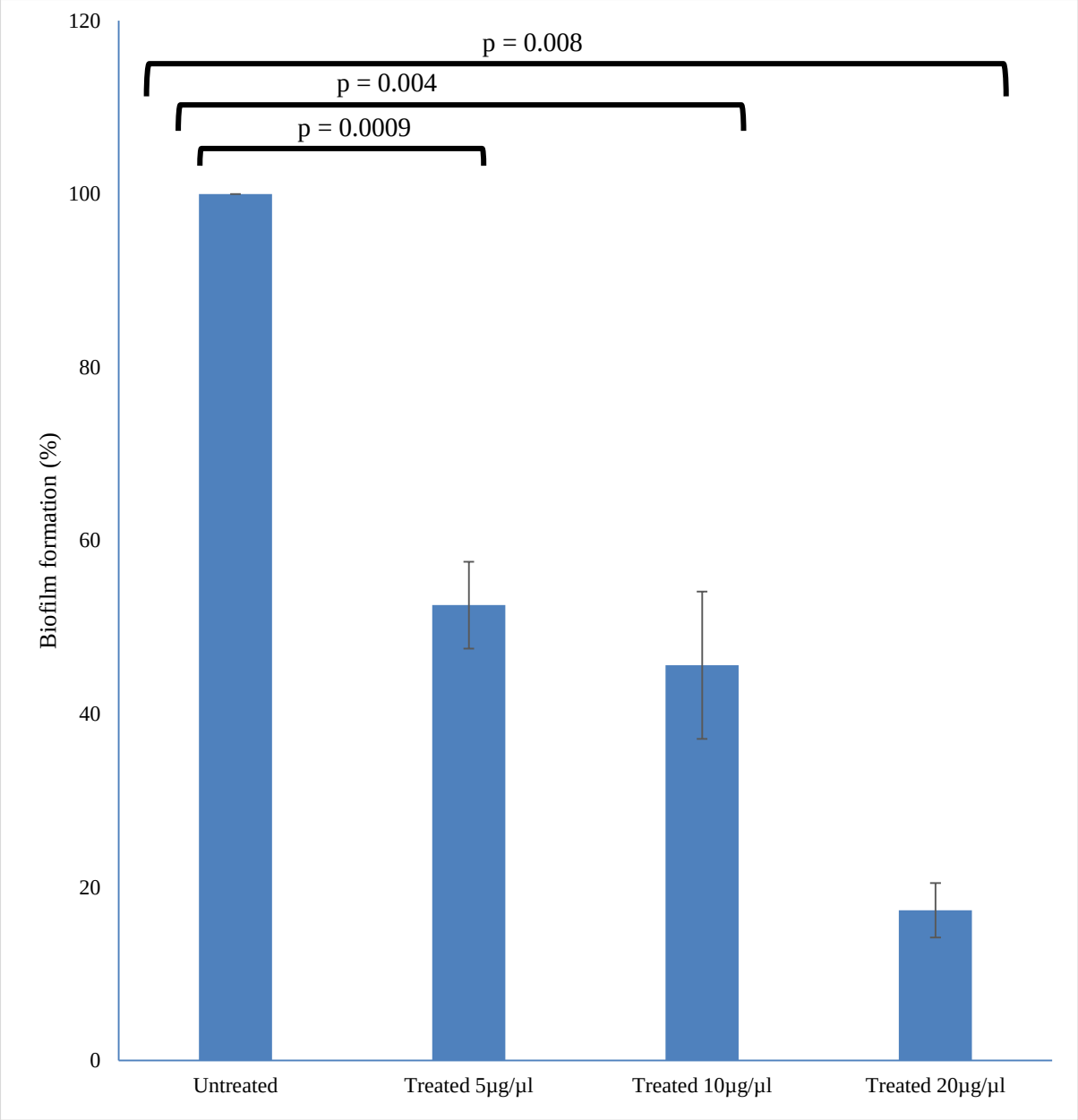


Figure 2.6: The crystal violet assay results of treated (with 5, 10 and 20µg/µl *Kigelia africana* crude extracts) and untreated reactions before biofilm formation. Bars denote standard error of three replicates.

2.4.7 POST BIOFILM FORMATION

Similarly, the ability of the crude extracts to disrupt already formed biofilms was evaluated. The cells were first allowed to form biofilms, and then treated with 5, 10 and 20µg/µl of *K. africana* crude extracts. The results show that treatment with *K. africana* crude extracts disrupted pre-formed biofilms as shown by lower biofilm formation in treated biofilms than

in untreated biofilms (Figure 2.7). The treated wells had less dense biofilms, with the highest concentration of crude extracts (20µg/µl) resulting in the lowest biofilm formation (10.3%), suggesting the greatest disruption of biofilms. The lowest concentration of crude extracts caused the highest biofilm formation among treated biofilms (71.8%). The results from the crystal violet assay of cells treated with crude extracts after biofilm formation suggest that the crude extracts have the ability to disrupt biofilms in a dose dependent manner, and as such have the potential to be used as active ingredients in the formulation of novel antifungal drugs for the treatment of cryptococcal infections as they can disrupt the pre-formed cryptococcal biofilms.

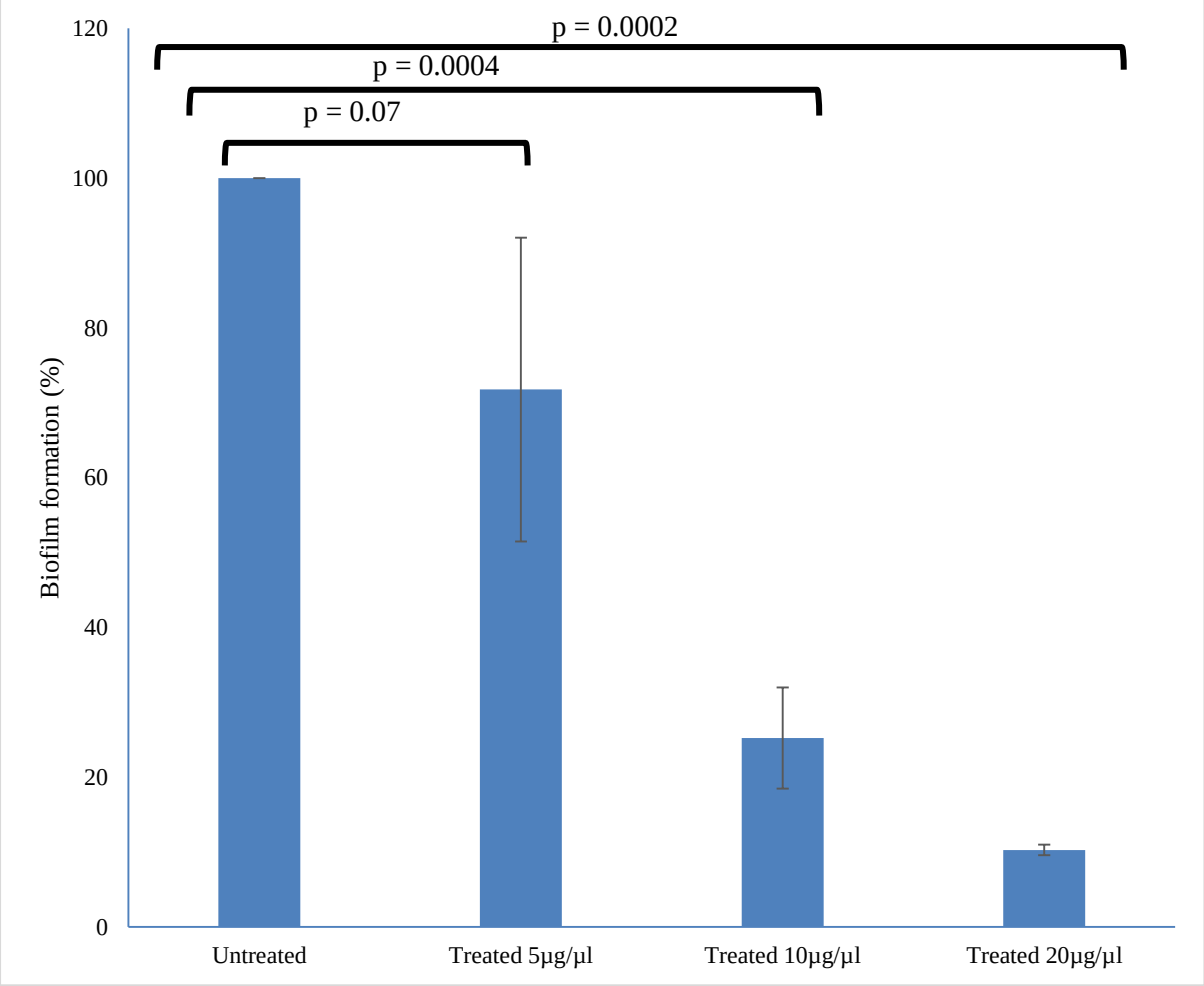


Figure 2.7: The crystal violet assay results of treated (with 5, 10 and 20µg/µl *Kigelia africana* crude extracts) and untreated reactions after biofilm formation. Bars denote standard error of three replicates.

2.4.8 THE 2,3-BIS- (2-METHOXY-4-NITRO-5-SULFOPHENYL)-2H-TETRAZOLIUM-5-CARBOXANILIDE (XTT) ASSAY

The 2,3-Bis- (2-Methoxy-4-Nitro-5-Sulfophenyl) -2H-Tetrazolium-5-Carboxanilide (XTT) assay is a method used to evaluate the viability and metabolic activity of biofilms (Kumari *et al*., 2017). Metabolically active yeast cells use their mitochondrial dehydrogenases to convert the XTT salt into the XTT formazan product (Martinez and Casadevall, 2006). This results in a colour change to dark orange (Martinez and Casadevall, 2006). The intensity of the colour change is proportional to the quantity of metabolically viable cells and is assessed using a microtiter plate reader (Martinez and Casadevall, 2006). The XTT assay offers a reliable method to quantify both cell viability and metabolic activity, making it a valuable tool for evaluating the efficacy of antifungal agents and treatments against cryptococcal biofilms (Martinez *et al*., 2010).

2.4.9 PRE-BIOFILM FORMATION

Cells were cultured and treated with *K. africana* crude extracts before biofilm formation and the absorbance readings were taken after biofilm formation. Metabolic activity percentages were calculated based on the absorbance readings and were plotted (Figure 2.8). The biofilms treated with crude extracts had lower metabolic activity than the untreated biofilms. The highest concentration of crude extracts (20µg/µl) resulted in the lowest metabolic activity (34.4%), indicating the lowest number of viable cells in the biofilm. The lowest concentration of crude extracts resulted in the highest metabolic activity among treated biofilms (71.2%). These results suggest that *K. africana* crude extracts can reduce metabolic activity in biofilms in a dose dependent manner, and therefore have the potential to be used as active ingredients in the formulation of novel antifungal drugs for the treatment of cryptococcal infections as they can inhibit the formation of cryptococcal biofilms.

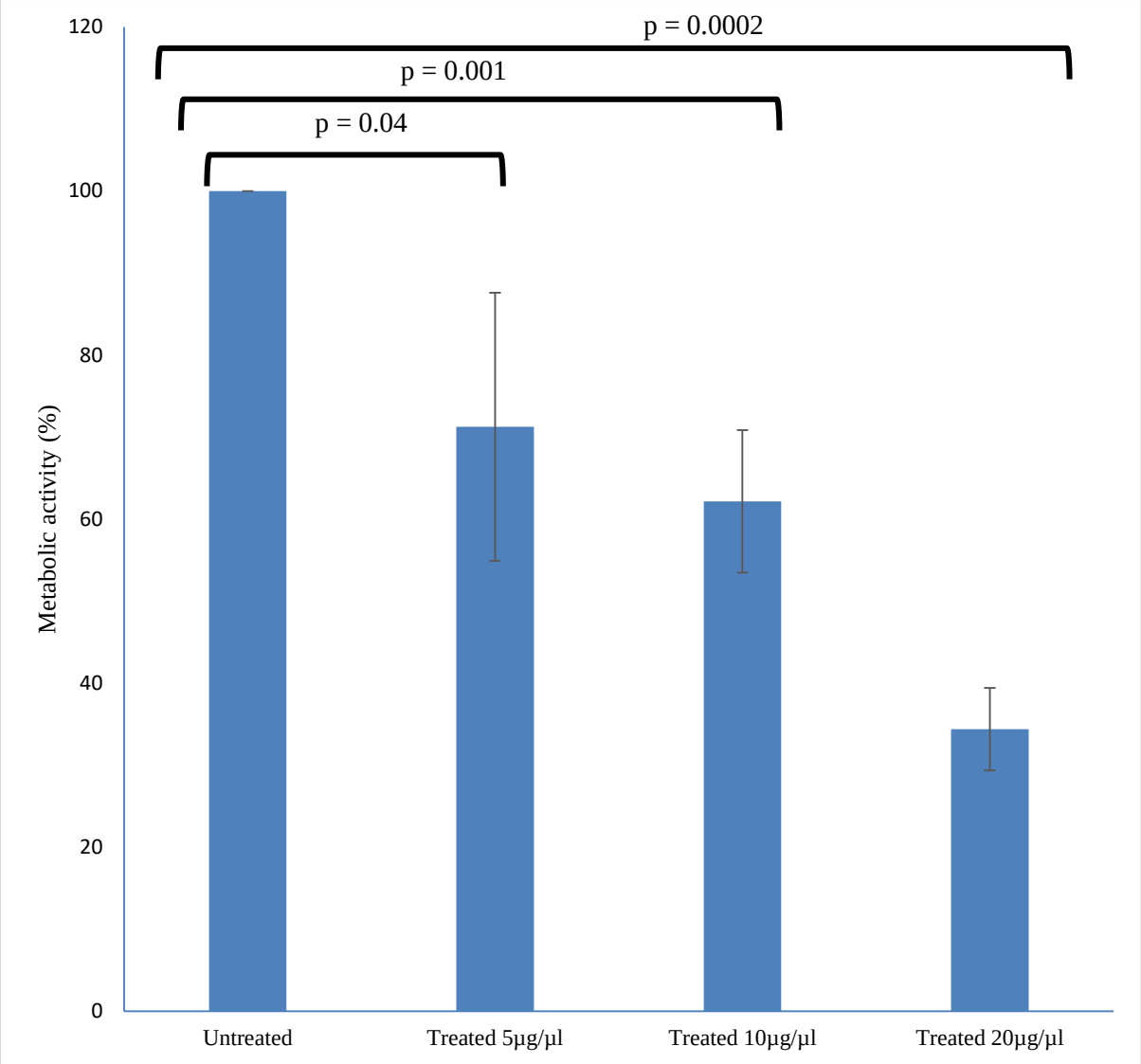


Figure 2.8: 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay results of treated (with 5, 10 and 20µg/µl *Kigelia africana* crude extracts) and untreated reactions before biofilm formation. Bars denote standard error of three replicates.

2.4.10 POST BIOFILM FORMATION

In the second XTT assay, *C. neoformans* cells were allowed to form biofilms before treatment with *K. africana* crude extracts and the absorbance readings were taken after biofilm treatment. Metabolic activity percentages were calculated based on the absorbance readings and were plotted (Figure 2.9). Treated biofilms had low metabolic activity (Figure 2.9). The biofilms treated with the highest concentration of crude extracts (20µg/µl) had the lowest metabolic activity (47.3%) suggesting that disruption of pre-formed biofilms by

K. africana crude extracts resulted in a lower number of viable cells. Treatment with the lowest concentration of *K. africana* crude extracts resulted in the highest metabolic activity among the treated biofilms (85.2%) These results suggest that *K. africana* crude extracts can reduce metabolic activity and the number of viable cells in biofilms in a dose dependent manner.

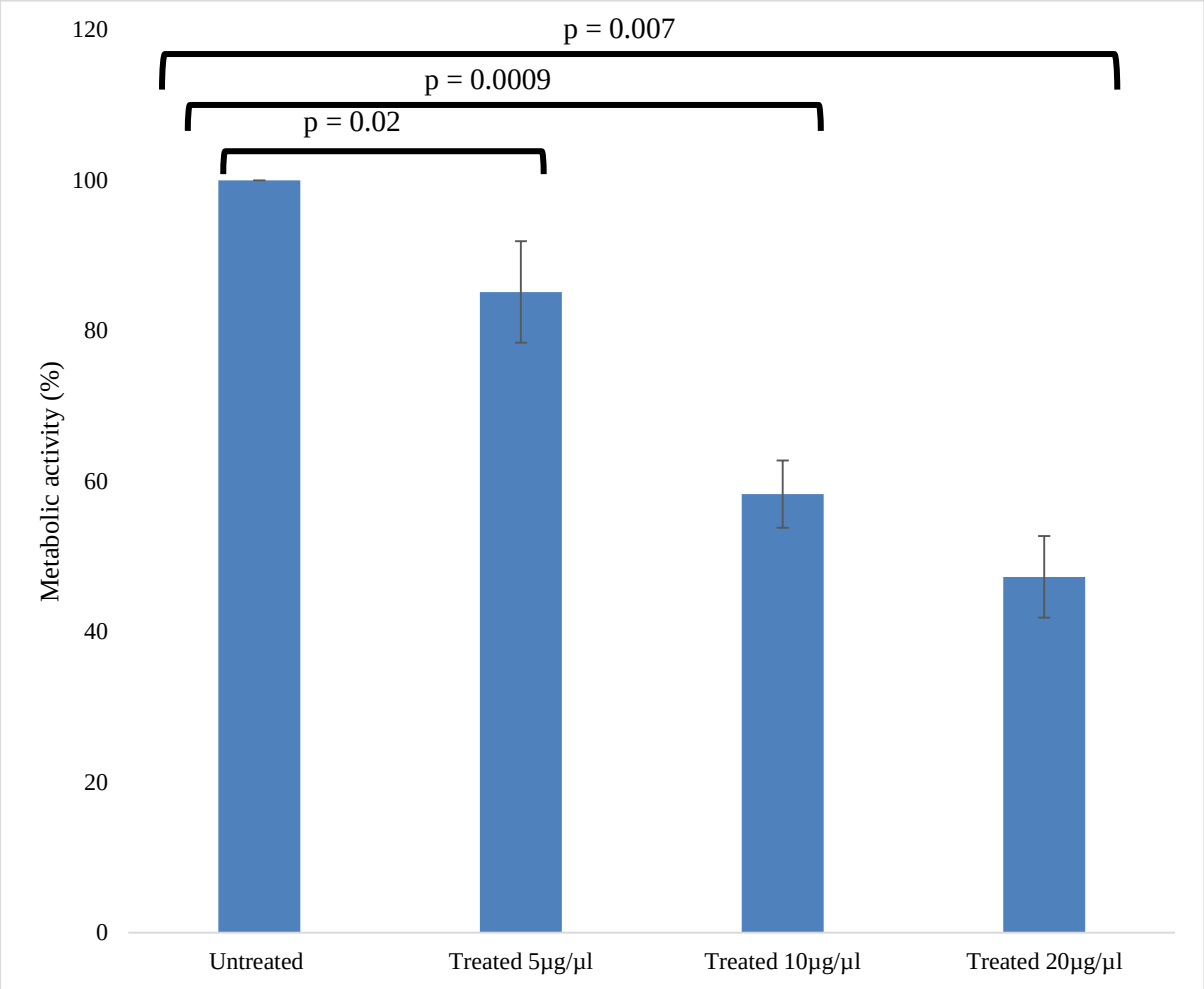


Figure 2.9: 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay results of treated (with 5, 10, and 20µg/µl *Kigelia africana* crude extracts) and untreated reactions after biofilm formation. Bars denote standard error of three replicates.

In a study by Kumari and co-workers (2017) the XTT assay was used to evaluate the metabolic activity of *C. neoformans* and *Cryptococcus laurentii* biofilms in the presence of essential oil components. The results showed that the metabolic activity of both *C. neoformans* and *C. laurentii* cells decreased in the presence of the essential oil components (Kumari *et al*., 2017). This decrease in metabolic activity was dose dependent, meaning that

higher concentrations of essential oil components resulted in greater inhibition of metabolic activity (Kumari *et al*., 2017). This is the same trend observed in our XTT assay results (Figure 2.8 and 2.9), where the metabolic activity of *C. neoformans* cells decreased with an increase in concentration of *K. africana* crude extracts.

2.5 CONCLUSIONS

Kigelia africana crude extracts extracted using ethanol are more effective antifungal agents than *K. africana* crude extracts extracted using distilled water, and this was proven by our disc diffusion assay results. Antifungal activity of *K. africana* crude extracts against *C. neoformans* was also confirmed by the colourimetric broth microdilution assay, as the crude extracts inhibited growth of cells in a dose dependent mode. Virulence attenuation properties of *K. africana* crude extracts were confirmed by results of the laccase and urease assays where both enzymes' activities were inhibited in treated cells. The results from the CV and XTT assays showed that *K. africana* crude extracts can inhibit biofilm formation, disrupt already formed biofilms, and reduce the number of viable cells in biofilms. Conclusively, the study has shown that *K. africana* crude extracts are indeed effective against *C. neoformans* and can therefore be used in the development of novel antifungal drugs which can be used to treat cryptococcal infections. Identification of the particular bioactive compounds responsible for these antifungal and antivirulence activities could result in the development of more effective antifungal agents.

CHAPTER THREE: DIFFERENTIAL EXPRESSION OF VIRULENCE ASSOCIATED GENES FOR *CRYPTOCOCCUS NEOFORMANS* TREATED WITH *KIGELIA AFRICANA* CRUDE EXTRACTS

3.1 INTRODUCTION

The fungus *Cryptococcus neoformans* is a disease-causing agent known to cause cryptococcosis, particularly in individuals with compromised immune systems (Desalermos *et al.*, 2014). The virulence of *C. neoformans* is attributed to many factors, such as the production of laccase, urease, cytochrome c oxidase subunit 1 and a polysaccharide capsule (Zaragoza, 2019).

Laccases are multicopper oxidases that catalyse the oxidation of a variety of phenolic and non-phenolic compounds, using molecular oxygen as the electron acceptor (Zhu and Williamson, 2004). Laccases occur in various organisms, such as fungi, plants, and bacteria (Casadevall *et al.*, 2000). In *C. neoformans*, laccase has a crucial part in the synthesis of melanin which is vital in the virulence of this opportunistic fungal pathogen (Coelho and Casadevall, 2014). According to Cordero and co-workers (2020), expression of the laccase gene is regulated in response to relevant biological stresses such as low nutrient conditions and increase in temperature. *LAC1* expression is regulated by conserved elements of the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) and high-osmolarity glycerol (HOG) response signalling pathways (Cordero *et al., 2020*). Under starvation conditions, the cAMP/PKA pathway is linked to localization and stimulation of *LAC1* expression, whereas the HOG pathway suppresses the expression of *LAC1* (Cordero *et al., 2020*).

Urease is an enzyme that catalyses the hydrolysis of urea into carbon dioxide and ammonia, which can help the fungus to survive in the host environment by neutralizing the acidity of the host's immune response (Godinho *et al.*, 2017). The expression of the urease gene in *C. neoformans* is known to be regulated by several factors, including availability of nutrients and temperature (Toplis *et al.*, 2020). The expression of the urease gene is upregulated in nutrient-limited conditions and at a human physiological temperature of 37°C, suggesting that urease may play a role in nutrient acquisition and survival of *C. neoformans* when exposed to mammalian body temperatures (Toplis *et al.*, 2020; Kraus *et al.*, 2004). Urease gene expression is also upregulated in *C. neoformans* when it is grown *in vitro* under conditions that mimic the environment of the central nervous system (CNS) (Olszewski *et al.*, 2004). Upregulation of the urease gene expression by *C. neoformans* promotes microvascular sequestration *in vivo*, which enhances CNS invasion (Olszewski *et al.*, 2004).

Cytochrome c oxidase subunit 1 (*COX1*) is a crucial enzyme in the process of oxidative phosphorylation, which is essential for the production of energy in *C. neoformans* (Akhter *et al*., 2003; Toffaletti *et al.,* 2003). It has been hypothesized that overexpression of *COX1* may be linked to an increase in the production of energy, which is crucial for the survival of *C. neoformans* in harsh surroundings (Toffaletti *et al.,* 2003).

The polysaccharide capsule is a critical virulence factor of *C. neoformans* (Zaragoza, 2019). It is composed primarily of glucuronoxylomannan (GXM), a complex polysaccharide that is synthesized and secreted by the organism (Robertsons *et al*., 2014). The polysaccharide capsule of *C. neoformans* has various effects on host cells, which include hindering phagocytosis, impeding recruitment of inflammatory cells, elevating co-stimulatory molecules, suppressing the delayed-type-hypersensitivity response, and decreasing antibody production during fungal infection (Lupo *et* al., 2008). Expression of the polysaccharide capsule associated genes is critical for the proper synthesis and assembly of the polysaccharide capsule and is regulated in response to various stress conditions, such as limited nutrients (Okabayashi *et al*., 2005).

Samie and co-workers (2019) established that plant bioactive compounds have the potential to attenuate virulence in *C. neoformans* and can hence be regarded as prospective sources for producing novel antifungal drugs. In another study, the use of a naturally occurring compound (propolis) to treat *C. neoformans* resulted in the downregulation of laccase, urease, and polysaccharide capsule associated gene expression (Thammasit *et al*., 2018). *Kigelia africana* is one of the most recognized medicinal plants in Africa, and several studies have concluded that it is a potential source of important crude extracts that can be used as alternatives (Arkhipov *et al*., 2014). The main goal of our study is to evaluate the influence of *K. africana* crude extracts on the expression of select virulence associated genes of *C. neoformans*.

3.2 AIM AND OBJECTIVES

The aim of this study is to evaluate the expression levels of select virulence associated genes in the presence of *Kigelia africana* crude extracts.

3.2.1 SPECIFIC OBJECTIVES

1. Extracting ribonucleic acid (RNA) from *C. neoformans* untreated and treated with *K. africana* crude extracts.
2. Identifying genes that are upregulated or downregulated in response to treatment with the crude extracts.

3.3 MATERIALS AND METHODS

3.3.1 STRAINS AND CULTURE CONDITIONS

The *Cryptococcus neoformans* strain and culture conditions used in this study are the same described in section 2.3.1.

3.3.2 PLANT BIOACTIVE EXTRACTION

Plant bioactives were extracted from the medicinal plant *Kigelia africana* (African sausage tree) as described in section 2.3.2.

3.3.3 TREATMENT OF *CRYPTOCOCCUS NEOFORMANS*

Cryptococcus neoformans was grown on Sabouraud Dextrose Agar (SDA) for 72hr at 37^{o}C. One colony was picked using a sterile loop and added to a test tube containing 5ml of minimal medial (MM) (30mM glucose, 20mM magnesium sulphate, 58.8mM potassium dihydrogen phosphate, 26mM glycine, 330mM 3-(N-morpholino) propanesulfonic acid and 6µM thiamine hydrochloride). The test tubes were incubated at 37^{o}C on a Stuart SB2 tube orbital rotator for 48hr. The cells were harvested by centrifugation (5000xg for 8min) then washed twice with phosphate buffered saline (PBS) (137mM sodium chloride, 2.7mM

potassium chloride, 10mM disodium hydrogen phosphate, and 1.8mM potassium dihydrogen phosphate). The cells were then inoculated into test tubes containing 5ml of MM supplemented with 40µg/µl *K. africana* crude extracts to make a final concentration of $1x10^6$cells/ml *C. neoformans* and 20µg/µl *K. africana* crude extracts. Untreated test tubes had cells inoculated into 5ml of MM supplemented with acetone to a final cell concentration of $1x10^6$cells/ml. After inoculation, the untreated and treated (with 20µg/µl *K. africana* crude extracts) cells were incubated at 37^{o}C on a Stuart SB2 tube orbital rotator for 1hr. A total of 1ml of the treated and untreated cells was transferred into different 1.5ml Eppendorf tubes and were immediately flash-frozen in liquid nitrogen and stored at -80^{o}C.

3.3.4 RIBONUCLEIC ACID (RNA) EXTRACTION

Frozen cells were thawed and centrifuged at 5000xg for 8min, and the supernatant was discarded. A total of 1ml TRIzol® reagent was added to the cells in a tube and mixed thoroughly. The cells suspended in TRIzol® reagent were transferred to bead tubes and subjected to bead beating using the Disruptor Genie® for 3min at high speed. The mixture was incubated at room temperature for 5min. A total of 300µl of chloroform was added to the mixture, vortexed for 15s and incubated at room temperature for 5min. This was centrifuged at 12000xg for 15min at 4^{o}C. The upper aqueous and organic phase was extracted and transferred to a new tube. An equal volume of chloroform was added, and the mixture was vortexed briefly, then centrifuged at 12000xg for 15min. The aqueous phase was transferred to a new tube and an equal volume of 100% (v/v) isopropanol was added to precipitate the RNA. This was incubated at room temperature for 45min and centrifuged at 12000xg for 15min at 4^{o}C. The pellet was washed with 100µl of 75% (v/v) ethanol and centrifuged at 12000xg for 5min. The pellet was air-dried for 10min and resuspended in 50µl diethyl pyrocarbonate (DEPC) water, then passed through genomic deoxyribonucleic acid (gDNA) removal columns from the Monarch® Total RNA Miniprep kit (New England Biolabs). RNA concentrations were determined using the Nanodrop spectrophotometer.

3.3.5 INTERNAL TRANSCRIBED SPACER POLYMERASE CHAIN REACTION (ITS PCR)

The internal transcribed spacer (ITS) region was amplified by polymerase chain reaction (PCR) using the Taq 5X Master Mix from New England Biolabs following the manufacturer's instructions, on a Bio-Rad T100 Thermocycler, to check for the presence of gDNA in the RNA samples (extracted from cells untreated and treated with 20µg/µl *K. africana* crude extracts). A positive control (gDNA sample) was included. The ITS primers used were ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2R (TGTGTTCTTCATCGATG) (Gardes and Bruns, 1993). The following incubation steps were followed; initial denaturation at 95°C for 30s, denaturation at 95°C for 30s (30 cycles), annealing at 55°C for 30s (30 cycles), extension at 68°C for 1min (30 cycles), and final extension at 68°C for 5min. The PCR products were visualized on a 1% (w/v) agarose gel supplemented with 10µg/µl ethidium bromide under ultraviolet (UV) light.

3.3.6 FIRST STRAND COMPLEMENTARY DNA (cDNA) SYNTHESIS

The LunaScript® RT SuperMix Kit (New England Biolabs) was used to form complementary DNA (cDNA) from the extracted RNA samples by reverse transcription, following the manufacturer's instructions. For each 20µl reaction, 4µl of LunaScript® RT SuperMix was mixed with RNA (1µg final concentration) and nuclease-free water (to 20µl) and added to PCR tubes. These were incubated in a BioRad T100 thermal cycler with the following incubation steps; primer annealing at 25^{o}C for 2min, cDNA synthesis at 55^{o}C for 10min, and heat inactivation at 95^{o}C for 1min. The resulting cDNA concentrations were determined using the Nanodrop spectrophotometer. The cDNA samples were stored at -20^{o}C.

3.3.7 PRIMERS

The study aimed to investigate the expression levels of four target genes (cytochrome oxidase c subunit 1 gene - *COX1,* polysaccharide capsule associated gene - *CAP10,* laccase gene *LAC1,* and urease gene - *URE1*) in *C. neoformans* using reverse transcription quantitative polymerase chain reaction (RT-qPCR). The selection of the urease, polysaccharide capsule associated, laccase, and cytochrome c oxidase1 genes as targets in the RT-qPCR experiment

was based on the fact that these genes have been well-documented as key virulence factors in *C. neoformans* (Zaragoza, 2019: Godinho *et al*., 2017), and our study aimed to evaluate the effects of *K. africana* crude extracts on the virulence of *C. neoformans*. Urease, for example, has been shown to play a role in fungal survival and proliferation within host tissues, while capsule formation is known to contribute to immune evasion and persistence within the host (Singh *et al*., 2013; Coelho *et al*., 2014). Laccase has been linked to the ability of *C. neoformans* to grow in iron-limited environments, which is important for fungal survival in the host (Wang and Casadevall, 1994). Cytochrome c oxidase1 is an enzyme involved in oxidative phosphorylation, which is essential for energy generation in cells (Toffaletti *et al*., 2003). Two commonly used housekeeping genes, β-actin – *ACT1* and glyeraldehyde-3-phosphate dehydrogenase gene - *GAPDH*, were used as internal controls for the experiment. Housekeeping genes are defined as genes that are constitutively expressed in all tissues and under all conditions and are necessary for basic cellular functions (Thellin *et al*., 1999). These genes encode for proteins that are involved in essential cellular processes, such as metabolism, DNA replication and repair, and protein synthesis (Thellin *et al*., 1999). Housekeeping genes are commonly used as internal standards in gene expression studies to normalize the expression levels of target genes, because it is assumed that their expression is constant across different samples and experimental conditions (Dheda *et al*., 2018). Primers for the housekeeping and target genes were obtained from the literature (Table 3.1).

Table 3.1: Gene-specific primers used for reverse transcription quantitative polymerase chain reaction.

Gene	Primer name	Primer Sequence (5' – 3')	Reference
ACT1	ACT1F ACT1R	CCTTCTACGTCTCTATCCAG TTTCAAGCTGAGAAGACTGG	Salas *et al*., 1996
CAP10	CAP10F CAP10R	GGATGCAGAGTTGAGGAAGA TATCCGTTGTAGGTCATGGC	Okabayashi *et al*., 2005
COX1	COX1F COX1R	CTGGTATGACACTACACAAGATGCCTC ACCAGCTAGAACTGGGATACATAGGA	Alanio *et al*., 2011
GAPDH	GAPDHF GAPDHR	TGAGAAGGACCCTGCCAACA ACTCCGGCTTGTAGGCATCAA	Xue *et al*., 2010
LAC1	LAC1F LAC1R	AGAAGGGAAGGAAGGTGATG TATACCTCACAACCGCCAAT	Alanio *et al*., 2011
URE1	URE1F URE1R	CGATTCTGGCACAAATGCTAT AGAGCCCTGTCAATGACT	Alanio *et al*., 2011

3.3.8 GRADIENT POLYMERASE CHAIN REACTION

The optimal melting temperature (Tm) for all the primers was determined using gradient PCR on cDNA. A total of 5µl Taq (5x) Master Mix (NEB) was mixed with 0.2µM forward primer (ACT1F, CAP10F, COX1F, GAPDHF, LAC1F, or URE1F), 0.2µM reverse primer (ACT1R, CAP10R, COX1R, GAPDHR, LAC1R, or URE1R), 3.6ng/µl cDNA and 18µl nuclease-free water in PCR tubes. Gradient PCR was run on a BioRad T100 thermal cycler under the following incubation conditions; initial denaturation at 95^{o}C for 30s, denaturation at 95^{o}C for

30s (30 cycles), primer annealing at 62, 60, 58, and 56°C for 30s (30 cycles), extension at 68°C for 45s (30 cycles), and final extension at 68°C for 5min. The PCR amplicons were visualized on a 3% (w/v) agarose gel supplemented with 10µg/µl ethidium bromide under ultraviolet UV light.

3.3.9 QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

The Luna® Universal qPCR Master Mix was used to amplify the cDNA samples, abiding with the manufacturer's instructions. A total of 10µl Luna® Universal qPCR Master Mix was mixed with 0.2µM forward primer (ACT1F, CAP10F, COX1F, GAPDHF, LAC1F, or URE1F), 0.2µM reverse primer (ACT1R, CAP10R, COX1R, GAPDHR, LAC1R, or URE1R), cDNA (<100ng final concentration) and nuclease-free water (up to 20µl) and added to BioRad 8-tube strips sealed using the BioRad Optical Flat 8-cap strips. The qPCR reaction was run on a BioRad CFX 96 machine with the following incubation steps; initial denaturation at 95°C for 1min, denaturation at 95°C for 15s (40 cycles), annealing and extension at 56°C for 30s, with plate read (40 cycles) and melt curve from 55-95°C (1 cycle). The resulting gene expression data was analyzed using CFX Maestro (BioRad).

3.4 RESULTS AND DISCUSSION

3.4.1 RNA QUANTIFICATION

Quantification of RNA is an essential step in the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) workflow (Bustin *et al*., 2009). RT-qPCR is a highly sensitive technique used to quantify gene expression levels (Fleige and Pfaffl, 2006). It involves converting RNA to cDNA using reverse transcription, followed by amplification of the cDNA using PCR (Fleige and Pfaffl, 2006). Therefore, the accuracy and reliability of RT-qPCR results depend on the quality and quantity of the RNA used as input (Bustin *et al*., 2009). RNA quantification provides important information about the concentration and purity of RNA samples (Bustin *et al*., 2009). Accurate quantification of RNA ensures that the same amount of RNA is used for reverse transcription, which helps to ensure that differences in the gene expression levels observed are due to biological differences and not technical variations caused by using different amounts of RNA (Fleige and Pfaffl, 2006). In this study, RNA

concentrations were determined using the Nanodrop spectrophotometer (Table 3.2). The results confirm that high concentrations of RNA were successfully extracted.

Table 3.2: Concentrations of the ribonucleic acid extracted from *Cryptococcus neoformans* cells treated with 20µg/µl *Kigelia africana* crude extracts and untreated.

Sample	Concentration (ng/µl)	260/280 Ratio
Untreated 1	333.5	1.78
Untreated 2	325.7	1.77
Untreated 3	125.2	1.58
Treated 1	691.9	1.56
Treated 2	454.7	1.94
Treated 3	90.9	1.55

3.4.2 INTERNAL TRANSCRIBED SPACER POLYMERASE CHAIN REACTION (ITS PCR)

According to Hashemipetroudi and co-workers (2018), contamination of RNA samples with gDNA can lead to overestimation of the amount of target mRNA in gene expression analysis. In this study, the ITS region was amplified via PCR to detect potential gDNA contamination (Figure 3.1).

On the agarose gel, a band of approximately 650 base pairs (bp) was observed in the gDNA positive control, indicating successful amplification of the ITS region (Figure 3.1). No PCR products were detected in the RNA samples or the NTCs, indicating the absence of gDNA contamination in the RNA samples, which is crucial for accurate downstream applications such as RT-qPCR (Hashemipetroudi *et al*., 2018). The absence of a PCR product in the NTCs also indicates that there was no contamination from other sources, such as the reagents or the laboratory environment (Bustin and Nolan, 2004). These results provide confidence in the

quality of the RNA samples used in this experiment and support the use of these samples in downstream applications.

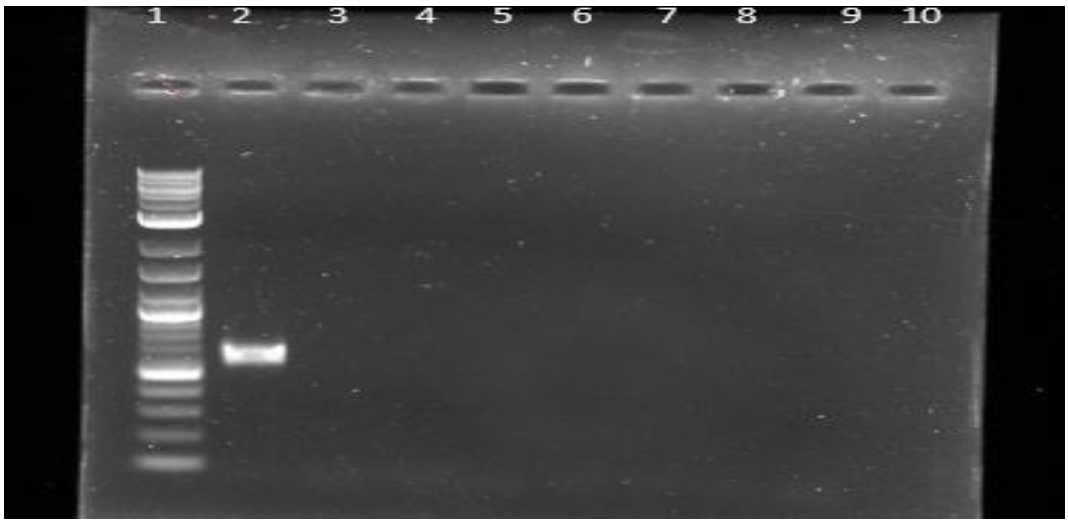


Figure 3.1: Agarose gel electrophoresis results of the internal transcribed spacer polymerase chain reaction (ITS PCR) products. Lane 1 – New England BioLabs 1kb plus deoxyribonucleic acid (DNA) ladder, lane 2 – genomic DNA (positive control) lane 3 to lane 5 – untreated ribonucleic acid (RNA) sample, lane 6 to lane 8 – treated RNA sample, lane 9 – no template control (NTC).

3.4.3 cDNA QUANTIFICATION

Quantification of cDNA is another important step in the RT-qPCR workflow and should be performed prior to PCR amplification to ensure the accuracy and reliability of the results (Bustin *et al*., 2009). It allows for normalization of RT-qPCR results to account for variations in the amount of input cDNA (Bustin *et al*., 2009). Normalization helps to ensure that differences in gene expression levels between samples are not due to differences in the amount of cDNA used but instead due to biological differences (Bustin *et al*., 2009).

Furthermore, cDNA quantification can help to identify potential sources of error such as variations in the efficiency of reverse transcription, variations in the quality of RNA samples, or variations in the efficiency of PCR amplification (Bustin and Nolan, 2004). Identifying these sources of error can help to optimize the RT-qPCR workflow and improve the accuracy

and reliability of the results (Bustin and Nolan, 2004). The LunaScript® RT SuperMix Kit New England Biolabs was used to synthesize cDNA from the extracted RNA samples by reverse transcription, the resulting cDNA concentrations were determined using the Nanodrop spectrophotometer (Table 3.3).

Table 3.3: Concentrations of complementary deoxyribonucleic acid (cDNA) synthesized from the ribonucleic acid (RNA) samples extracted from *Cryptococcus neoformans* cells untreated and treated with 20µg/µl *Kigelia africana* extract.

Sample	cDNA concentration (ng/µl)	260/280
Untreated 1	1321.5	1.75
Untreated 2	743.8	1.79
Untreated 3	828.7	1.82
Treated 1	900.8	1.79
Treated 2	716.9	1.85
Treated 3	1253.5	1.78

3.4.4 GRADIENT POLYMERASE CHAIN REACTION

Gradient PCR is a technique used to optimize PCR conditions, such as annealing temperature, magnesium chloride ($MgCl_2$) concentration, and primer concentration (Robertson and Walsh-Weller, 1998). It involves running the PCR reaction in a thermal cycler with a gradient function, where each column of the reaction plate contains a different temperature or concentration (Ishii and Fukui, 2001). In this study, gradient PCR was used to optimize the annealing temperature for the primers for genes of interest (*COX1, CAP10,*

LAC1, and *URE1*) and the housekeeping genes (*ACT1* and *GAPDH*). The results showed all of the primers had the same optimum annealing temperature.

3.4.5 REVERSE TRANSCRIPTION QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR)

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a powerful tool for studying changes in gene expression (Taylor *et al*., 2010). In this study, RT-qPCR was used to measure the expression levels of laccase, cytochrome c oxidase, urease, and the polysaccharide capsule associated gene (*CAP10*) in *C. neoformans* treated with *K. africana* crude extracts. RT-qPCR is particularly useful because it allows for the precise quantification of RNA transcripts in a sample, even at low levels. It also has a high degree of sensitivity, specificity, and reproducibility, making it an ideal method for detecting changes in gene expression (Taylor *et al*., 2010).

The results of the RT-qPCR indicate that treatment of *C. neoformans* cells with *K. africana* crude extracts led to the upregulation of laccase (1.08-fold), cytochrome c oxidase 1 (2.35 fold), and urease (1.47 fold), as well as the downregulation of the polysaccharide capsule associated gene (*CAP10*) (-4.41 fold) (Figure 3.2). The observed changes in gene expression suggest that *K. africana* crude extracts may have the potential to interfere with *C. neoformans* virulence by altering the expression of key genes involved in fungal survival and proliferation within the host.

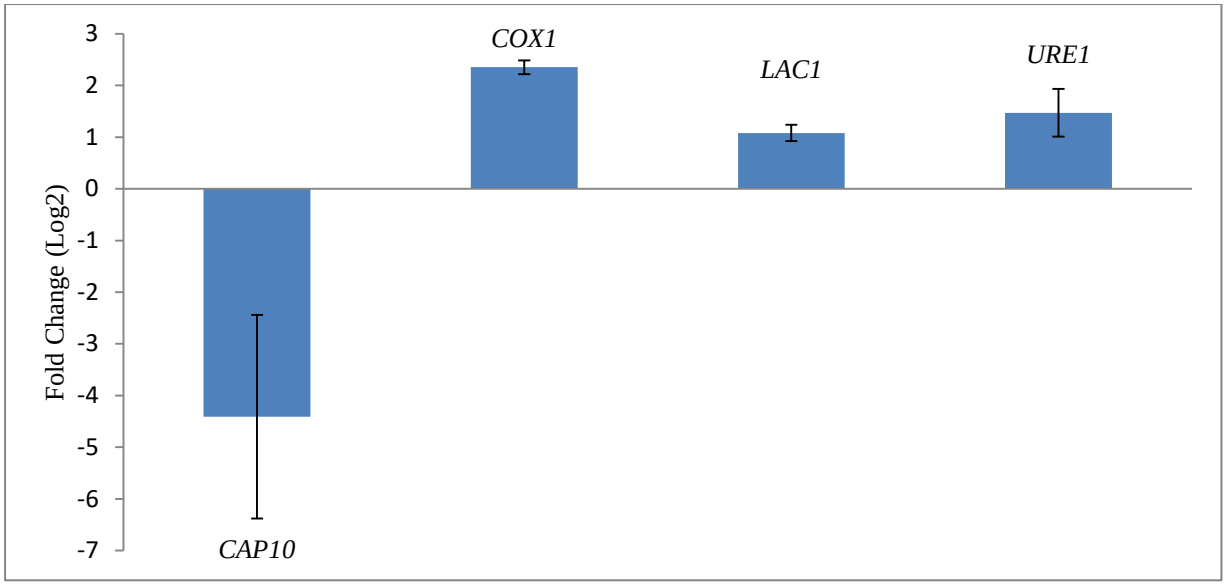


Figure 3.2: Fold changes of target genes (polysaccharide capsule associated gene (*CAP10*), cytochrome c oxidase subunit 1 (*COX1*), laccase (*LAC1*), and urease (*URE1*)), in response to treatment with 20µg/µl *Kigelia africana* crude extracts, normalized against the housekeeping genes β-actin (*ACT1*) and glyeraldehyde-3-phosphate dehydrogenase (*GAPDH*) using CFX Maestro (BioRad). Bars denote standard error of three replicates.

Laccase is known to be important in the melanization of *C. neoformans* cell walls, which can help the fungus evade host immune responses (Williamson, 1997). Melanin has several properties that enable *C. neoformans* to be resistant to the host immune response and enhance survival in the environment (Nosanchuk & Casadevall, 2003). These include; the ability to interfere with phagocytosis by inhibiting the binding of complement proteins C3b and C4b to the surface of the fungal cell (Nosanchuk & Casadevall, 2003); having antioxidant properties, which enable it to scavenge free radicals and other reactive oxygen species that are produced by the host immune system as part of its defence against infection (Williamson, 1997); and the ability of melanin to absorb a wide range of the electromagnetic spectrum provides protection against ultraviolet (UV) radiation and inhibits harm caused by photo-induced damage (Hill, 1992). A study by Missall and co-workers (2005) investigated the role of the thiol-specific antioxidant Tsa1 in regulating the expression of two laccase genes, *LAC1* and *LAC2*, in *C. neoformans.* The authors found that in the absence of Tsa1, the expression of *LAC1* was significantly upregulated in response to oxidative and copper stress, while the expression of *LAC2* was not affected (Missall *et al*., 2005). This suggests that *LAC1* plays a specific role in the response to oxidative and copper stress, while *LAC2* may have other

functions (Missall *et al*., 2005). The upregulation of *LAC1* expression in response to oxidative and copper stress suggests that it plays a role in the detoxification of reactive oxygen species by synthesising melanin, which provides protection against oxidative stress (Missall *et al*., 2005). The upregulation of *LAC1* expression in response to *C. neoformans* treatment with *K. africana* crude extracts, in our study, suggests that the crude extracts can induce oxidative stress in *C. neoformans*.

Similarly, urease is also important for cryptococcal survival in the host as it plays a key role in nitrogen metabolism and pH regulation (Godinho *et al*., 2017). The blood brain barrier (BBB) normally maintains a tight junction between endothelial cells that form the blood vessels in the brain, creating a physical barrier that prevents large molecules and pathogens from crossing into the brain (Cox *et al*., 2000). However, the increased pH caused by the activity of urease can disrupt these tight junctions, enabling *C. neoformans* to cross the BBB and invade the brain (Cox *et al*., 2000). A study by Yu and co-workers (2020) found that the expression of the urease gene in *C. neoformans* varied in response to different biologically relevant stresses. For example, the expression of the urease gene was upregulated in response to low pH, which is a stress condition commonly encountered by the fungus in the host environment (Yu *et al*., 2020). Similarly, in the present study the urease gene expression was upregulated in response to treatment with *K. africana* crude extracts which has antifungal properties against *C. neoformans* and therefore, a biologically relevant stress.

The upregulation of the laccase and urease genes as shown in the RT-qPCR results (Figure 3.2) did not correspond to an increase in laccase and urease enzyme activities as determined previously (Chapter 2). There are various possible reasons for this discrepancy, including post-transcriptional modification (alternative splicing, RNA stabilization by polyadenylation, or 5’ capping, which could affect the amount and quality of the laccase and urease proteins produced) and/or the presence of enzyme inhibitors (Fan *et al.,* 2005). Also, the *K. africana* crude extracts used in the study may also have inhibitory effects on the laccase and urease enzymes directly, which could explain the observed reduction in activity in the assays despite the upregulation of gene expression.

Cytochrome c oxidase subunit 1 is a mitochondrial enzyme involved in oxidative metabolism and energy production, which is essential for *C. neoformans* to survive in the host environment (McDade *et al*., 2002). Toffaletti and co-workers (2003) suggest that the upregulation of *COX1* expression is linked to a stress response for *C. neoformans* induced by

harsh conditions in its surrounding. This means that when the yeast is under stress, it responds by increasing the expression of *COX1*, possibly to help produce more energy for survival (Toffaletti *et al*., 2003). In the current study, *COX1* expression was upregulated in response to treatment of *C. neoformans* with *K. africana* crude extracts, which suggests the crude extracts may have induced a stress response in the yeast through activation of signalling pathways such as the mitogen activated protein kinase (MAPK) pathway, which has been shown to regulate *COX1* (Brown *et al*., 2007). The upregulation of *COX1* could also mean that the crude extracts may contain compounds that act as activators of *COX1* gene expression. These compounds could directly interact with the regulatory elements of the *COX1*, such as the promoter region, and stimulate its transcription (Hood *et al*., 2009). It is also possible that the *K. africana* crude extracts may have altered the cellular metabolism of *C. neoformans* in a way that indirectly stimulates *COX1* expression. For example, plant phenolic compounds can induce oxidative stress in the cells leading to an increase in demand for cytochrome c oxidase subunit 1 activity and thus upregulation of *COX1* expression to meet this demand (Shalaby and Horwitz, 2015; Akhter *et al*., 2003).

The study by Kim and co-workers (2014) demonstrated that the fungicidal activity of *o*-vanillin (a substance that is derived from the main constituent of vanilla bean extract) is associated with the generation of oxidative stress, leading to mitochondrial dysfunction, reduced ATP production, elevated reactive oxygen species (ROS) generation, and eventually, cell death in *C. neoformans*. These findings correlate with the upregulation of *COX1* expression in response to treatment of *C. neoformans* with *K. africana* crude extracts, which may have caused oxidative stress in the cells. It is possible that the oxidative stress in *C. neoformans* treated with *K. africana* crude extracts also caused mitochondrial dysfunction, resulting in lower metabolic activity as depicted by the XTT assay results (Chapter 2). The XTT assay measures metabolic activity in cells by assessing the ability of viable cells to convert the tetrazolium salt XTT into a coloured formazan product, which is proportional to the quantity of viable cells present (Martinez and Casadevall, 2006).

The downregulation of the polysaccharide capsule associated gene (*CAP10*) in cells treated with *K. africana* crude extracts is significant because the polysaccharide capsule is a major virulence factor in *C. neoformans* (Godinho *et al*., 2017). The polysaccharide capsule aids *C. neoformans* to evade phagocytosis which happens in the early stages of lung infection and protects it from desiccation and oxidative stress (Mansour and Levitz, 2002; Bermas and Geddes-McAlister, 2020). Hence the downregulation of the polysaccharide capsule may

enhance the susceptibility of *C. neoformans* to host immune defenses, making it easier to eliminate by the host (Zaragoza, 2019). A study by Thammasit and co-workers (2018) investigated the potential of propolis (a naturally occurring substance produced by honeybees) on primary virulence factors of *C. neoformans* and its influence on the expression of virulence associated genes. They found that the expression of the polysaccharide capsule associated genes was downregulated in *C. neoformans* treated with ethanol extract propolis (EEP) (Thammasit *et al*., 2018); and this correlates with our results.

The downregulation of *CAP10* in the RT-qPCR results corresponds with the inhibition and eradication of biofilms (Chapter 2). The polysaccharide capsule of *C. neoformans* contributes to the formation and maintenance of biofilms, by providing a surface for cells to adhere to and by producing extracellular polysaccharides that contribute to the matrix. (Martinez and Casadevall, 2015). Biofilm-based crystal violet assays were used in this study to assess the ability of *K. africana* crude extracts to prevent biofilm formation and to disrupt biofilms in *C. neoformans* cells. The decrease in biofilm formation, as indicated by low absorbance readings, could be attributed to the downregulation of *CAP10*. Without the protective barrier provided by the capsule, the fungal cells may be more susceptible to mechanical and chemical stress, which could lead to decreased viability and biofilm formation (Martinez and Casadevall, 2006). The downregulation of *CAP10*, prevention of biofilm formation, and disruption of biofilms associated with treating *C. neoformans* cells with *K. africana* crude extracts could mean that the crude extracts have the potential to be used in treatment of cryptococcal infections (Arkhipov *et al*., 2014)

3.5 CONCLUSIONS

The results of this study demonstrate that treatment of *C. neoformans* with *K. africana* crude extracts affects the regulation of laccase, cytochrome oxidase, urease, and the polysaccharide capsule associated gene expression. Upregulation of the laccase, cytochrome oxidase, and urease gene expression in response to treatment of *C. neoformans* with *K. africana* crude extracts confirms that the crude extracts created unfavourable conditions for the proliferation of *C. neoformans* hence this virulence associated genes were overexpressed to enable survival of the yeast in the harsh environment. Downregulation of the polysaccharide capsule associated gene expression could lead to reduced production of the polysaccharide capsule, which in turn could make *C. neoformans* more susceptible to host immune defences or

antifungal drugs. Therefore, *K. africana* crude extracts have the potential to be utilized in the development of novel antifungal drugs which can be used to treat cryptococcal infections.

CHAPTER FOUR: SUMMARY

4.1 SUMMARY

In this study, the effectiveness of *Kigelia africana* crude extracts as potential antifungal agents was tested against *Cryptococcus neoformans* H99 using a variety of assays. The crude extracts were extracted from crushed plant material using ethanol and distilled water as extraction solvents. The disc diffusion assay results proved that *K. africana* crude extracts extracted using ethanol are more effective antifungal agents than *K. africana* crude extracts extracted using distilled water. Antifungal activity of *K. africana* crude extracts against *C. neoformans* was also confirmed by the colourimetric broth microdilution assay, as the crude extracts inhibited growth of cells in a dose dependent mode. Virulence attenuation properties of *K. africana* crude extracts were confirmed by results of the laccase and urease assays where both enzymes' activities were inhibited in the cells that were treated with *K. africana* crude extracts. The results from the CV and XTT assays showed that *K. africana* crude extracts can inhibit biofilm formation, disrupt already formed biofilms, and reduce the number of viable cells as well as metabolic activity in biofilms.

In addition to these assays, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine the effects of *K. africana* crude extracts on the expression of virulence-associated genes (*CAP10, COX1, LAC1,* and *URE1*) in *C. neoformans*. The RT-qPCR results showed that the expression of COX*1*, *LAC1*, and *URE1* was upregulated, which confirms that the crude extracts created unfavourable conditions (such as oxidative stress) for the proliferation of *C. neoformans* hence these virulence associated genes were overexpressed to enable survival of the yeast in the harsh environment. Downregulation of the polysaccharide *CAP10* expression reveals that *K. africana* crude extracts are capable of suppressing the expression *CAP10* in *C. neoformans*. This could lead to reduced production of the polysaccharide capsule, which in turn could make *C. neoformans* more susceptible to host immune defences as well as antifungal drugs.

Overall, this study provides evidence that *K. africana* crude extracts are indeed effective against *C. neoformans* and can therefore be used in the development of novel antifungal drugs which can be used to treat cryptococcal infections. *Kigelia africana* crude extracts could also be included in antifungal therapies where they can be used to increase susceptibility of *C. neoformans* to synthetic antifungal drugs.

4.2 LIMITATIONS

The study focused solely on the efficacy of *K. africana* crude extracts against *C. neoformans*. It did not consider other *C. neoformans* strains or fungal species, hence the generalizability of the findings could be limited to the specific organism tested. The study primarily relied on *in vitro* assays to evaluate the antifungal activity of the crude extracts. While these assays provide initial evidence, they do not necessarily reflect the complexities of an *in vivo* environment. Further studies involving animal models or clinical trials would be necessary to assess the efficacy and safety of these extracts in a more realistic setting.

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