

Title: *Galenia africana* extract as an alternative to typical azoles against
Candida species

By

Charnice Rene' Mouton

Student number: 3239975



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Supervisor: Professor C Africa

Co-supervisor: Professor J Klaasen

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Key words

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Abstract

Candidiasis is a common infection caused by *Candida* species, often associated with immune compromised patients. Due to the prevalence of candidal resistance to typical azole treatments, alternative forms of therapies should be investigated. The use of medicinal plants as an alternative form of therapy has become a topic of interest in the scientific community. *Galenia africana* has shown a lot of potential as an antimicrobial plant extract. In this study, the antifungal activity of the ethanolic and ethyl acetate extraction of *Galenia africana* was investigated against fluconazole-resistant and susceptible *Candida* species. High-performance liquid chromatography and nuclear magnetic resonance were employed to separate, isolate and purify the active compounds of *Galenia africana* followed by evaluating the antifungal activity using broth microdilution, synergistic and time kill assays. Three compounds were isolated from *Galenia africana*, identified as (*S*)-5,7-dihydroxy flavone (1), (*E*)-2',4'-dihydroxychalcone (2), and (*S*)-2',5,7,-trihydroxyflavanone (3). The results showed significant antifungal activity below 500µg/ml which confirms that *Galenia africana* can be used as a potential antifungal against fluconazole resistant strains of *Candida*.

Declaration

I declare that '*Galenia africana* extract as an alternative treatment to typical azoles against *Candida* species' is my own work and has not been submitted before for any degree or examination. All the sources I have used has been indicated and acknowledged by complete references.

Charnice Rene' Mouton

Signed:



Date:1.12.22.....

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List of Abbreviations

Abc1p- ATP-binding cassette transporter

AIDS – Acquired Immunodeficiency Syndrome

ATCC – American Type Culture Collection

ATP- Adenosine triphosphate

AUC- Area Under the Curve

CdCDR1- *Candida dubliniensis* *Candida* Drug Resistance gene 1

CDR1- *Candida* Drug Resistance gene 1

CDR2- *Candida* Drug Resistance gene 2

CFU- Colony Forming Units

CLSI – Clinical Laboratory Standards Institute

cm- Centimetres

DMSO- Dimethyl sulfoxide

ERG11-Sterol 14 α -demethylase

EtOAc- Ethyl Acetate

EtOH- Ethanol

FCZ- Fluconazole

FIC index- Fractional Inhibitory Concentration index

g – Grams

h- Hour/hours

H₂O- Water

H₂SO₄- Vannilin reagent stain

HIV – Human Immunodeficiency Virus

IC50- Fifty percent Inhibitory Concentration

IFD- Invasive Fungal Disease

INT- p-iodonitrotetrazolium chloride

KBDD- Kirby Bauer Disk Diffusion

Kg- Kilograms

Kgbw- Kilograms per body weight

L-Litre

MC- Micro-colonies

MDR- Multiple Drug Resistance

MDR1- Multidrug Resistance Protein 1

MeOH- Methanol

MF- Major Facilitator transporter

MIC – Minimum Inhibitory Concentration

mL – Milliliter

mm – Millimeter

NCPF – National Collection of Pathogenic Fungi

NMR- Nuclear Magnetic Resonance Spectroscopy

NPV- Negative Predictive Value

OIs -Opportunistic Infections

PPV- Positive Predictive Value

R- Resistant

ROC- Receiver Operating Characteristic Curve

Rpm- Revolutions per minute

RPMI- Roswell Park Memorial Institute medium

S- Susceptible

SDA- Sabouraud Dextrose Agar

S-DD- Susceptible-Dose Dependant

SPSS – Statistical Product and Service Solutions

TLC- Thin-Layer Chromatography

UPC2- Sterol uptake control protein 2

UV254/360- Ultra violet light at 254nm and 360nm

v/v – Volume to volume

WHO- World Health Organisation

YNBG – Yeast Nitrogen Base Agar

µg - Microgram

μl – Microliter

μM - Micrometer

$^{\circ}\text{C}$ – Degrees centigrade

59-MHC- 9-methoxyhydnocarpin

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Chapter I: Introduction and Literature Review

Introduction

The genus *Candida* belongs to the family *Saccharomycetaceae*, known to inhabit the normal flora of the oral cavity, vagina and digestive tract, causing disease when the hosts defense is compromised (Seagle *et al*, 2021). As a pleomorphic fungus, it is able to alternate between a benign yeast form to invasive pseudohyphae/hyphae (Chow *et al*, 2021).

The prevalence of fungal infections caused by *Candida* species has increased dramatically over the last decade (Koehler *et al*, 2019; Daneshnia *et al*, 2023) and their ability to form drug- resistant biofilms plays a big role in their infectious development (Kaur and Nobile, 2024). Some species of this genus is known to cause several unwanted infections, particularly in patients who are immune compromised or exposed to prolonged antifungal therapy (Fischer *et al*, 2022). More than 17 different species of *Candida* have been reported as opportunistic human pathogens (Baniodeh *et al*, 2022) and are known to cause both superficial and systemic infections (Macias-Paz *et al.*, 2022). A global study completed by the Global Action Fund for Fungal Infections (GAFFI), revealed an estimated mortality rate of 40%, classifying candidemia as the fifth largest life-threatening fungal species (Chibabhai, 2022).

Saranya *et al* (2014) reported *Candida albicans* as one of the seven most common *Candida* species causing 90% of invasive infections. The ability of *Candida* species to alternate between yeast and hyphal forms (Mayer *et al*, 2013), together with their inherent virulence factors enhances their pathogenicity which makes it difficult to treat as antifungal resistance has become more common (Sardi *et al*, 2013). *Candida* species ability to alter forms together with their inherent virulence factors alter the regulation of multidrug efflux transporters and the sterol composition which in turn influences the cell structure, preventing the transport of antifungals into the cell (Arendrup and Patterson, 2017; Sanglard and Odds, 2002). With the development of resistance to common azole treatments, the interest in natural medicaments such as plant extracts and oils continues to grow (Holetz *et al*, 2002).

In recent studies, medicinal plants have shown significant activity against bacterial, viral and fungal infections (Akram *et al*, 2018; Manandhar *et al*, 2019; Akwongo *et al*, 2024) with the

use of natural products and their essential oils in antimicrobial drug testing procedures (Yuan *et al*, 2016). Most oils are composed of volatile compounds within plants which are responsible for their antioxidant and antimicrobial properties (Marcos-Arias, 2011). According to Al-Judaibi and Al-Yousef (2014), medicinal plants have a high antimicrobial effect due to the presence of secondary metabolic compounds. These compounds are reduced from the plants' metabolism and include several flavonoids, alkaloids, tannins, phenols and terpenes (Al-Judaibi and Al-Yousef, 2014). Plant flavonoids are phenolic compounds, known for their defensive traits against insects, fungi and viruses (Ferdes, 2018). Flavonoids play an important role in the growth, development and immune responses of plants, allowing them to act as immune stimulators. Many plants enhance biological immune responses through the complement activation of innate immunity, rapid growth of lymphocytes and the stimulation of macrophages (Rawat and Anand, 2012). Synthetic drugs act as immune stimulators in the same way and are often prescribed in the treatment of several diseases and infections, but most of these drugs are associated with side effects and complications. This can be detrimental when used over a long period of time (Karimi *et al*, 2015). On the other hand, natural medicine has been used for decades, treating infections through the enhancement of the body's immunity with fewer side effects (Nisar *et al*, 2017). According to Mahlo *et al* (2016), medicinal plants should be screened to identify whether they can be used as alternative treatments to synthetic drug agents. This can be achieved through the identification of the main source of activity in a plant species which lies in the isolation of their active compounds, fractions and metabolites (Fabricant and Farnsworth, 2001; R'ios and Recio, 2005). Therefore, a keen interest in the discovery of active plant compounds is imperative to investigate alternative and natural ways to treat diseases and infections.

South Africa is home to a diverse range of plant species, amounting to about 30 000 in total (Van Wyk *et al*, 1997). Around 80% of South Africans make use of plants and traditional medicine to meet their primary health care needs (Aremu *et al*, 2021). *Galenia africana* is a Cape-endemic species indigenous to the Namaqualand region of South Africa (Van Wyk, 2008). It is a woody perennial shrub with pale stems and hairless leaves, only growing 0.5-1 m high (Le Roux *et al*, 1994). The indigenous people have used this plant to treat an array of ailments including toothache, eye infections, asthma, inflammation as well as venereal diseases (Watt and Breyer-Brandwijk., 1962). Past studies have shown that *G. africana* can be used as an antifungal treatment (Pool *et al*, 2009; Vries *et al.*, 2005), antibacterial agent (Ticha *et al.*, 2014) and even in the treatment of *Mycobacterium smegmatis* and

Mycobacterium tuberculosis causing tuberculosis (Mativandlela *et al.*, 2009). Phytochemical studies have identified a range of bioactive constituents in *G. africana* – especially flavonoids and chalcones. For example, (S)-5,7-dihydroxyflavanone (pinocembrin) and (E)-2',4-dihydroxychalcone have been isolated from this plant. These types of compounds are known to exhibit antimicrobial activities and could act via membrane disruption or efflux pump inhibition (Heredia *et al.*, 2022).

In this study, *Galenia africana* was investigated as a potential antifungal against several species of *Candida* with a view to developing an alternative to azoles for the treatment of *Candida*. The susceptibility, antimicrobial activity and dose-dependent timing of *G. africana* against nine type strains of *Candida* was tested through the broth microdilution, synergy and time kill assays.

1.1 Candida

Most *Candida* species are commensals of the human body (Vázquez-González *et al*, 2013), allowing them to be more prevalent as opportunistic pathogens due to the patient being immunocompromised (Keighley *et al*, 2019). The ability of *Candida* spp to alternate between a yeast and pseudohyphatic form together with the underlying disease affects the pathogenicity in these patients (Guarner and Brandt, 2011). The hyphal (pathogenic) form is coupled with tissue damage and epithelial cell invasion, enabling deeper penetration into host tissues and facilitating immune evasion and persistent infection. This genus contains heterogeneous anamorphic yeasts and comprises about 196- 200 species (Eggimann *et al*, 2003), the most reported species being *Candida albicans*, which is specific to humans (Brandt and Lockhart, 2012).

To date, more than 150 species of *Candida* have been identified, of which about 15 are most commonly isolated from patients with opportunistic infections and classified as infectious. These species include *Candida albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae*, *C. dubliniensis*, *C. pelliculosa*, *C. kefyr*, *C. lipolytica*, *C. famata*, *C. inconspicua*, *C. rugosa*, and *C. norvegensis* (Pfaller and Diekema, 2007). Globally, invasive *Candida* infections affect an estimated number of 1–2 million people per year, causing over one million deaths. South Africa bears a heavy burden: it was estimated that ~5,421 cases of candidemia occur annually in the country, driven by HIV/TB syndemics and limited resources (Schwartz *et al*, 2019). Historically *C. albicans* caused ~95% of *Candida* infections, but in recent decades non-albicans species (e.g. *C. glabrata*, *C. parapsilosis*, *C. tropicalis*) have risen markedly (Keighley *et al*, 2019).

1.2 Clinical manifestations of candidiasis in Africa

Common manifestations of candidiasis include thrush, atrophic stomatitis, mucocutaneous candidiasis, and vulvovaginitis, which in most cases are self-limiting, especially if the patient is not immunocompromised. With these manifestations, basic hygiene and treatment of the infection will ensure eradication.

Opportunistic infections (OIs) particularly those associated with HIV/AIDS, remain one of the main causes of illness and mortality in patients particularly from sub-Saharan Africa (Oladele *et al*, 2020). This is mainly due to the lack of resources as a result of poverty and

poor surveillance of HIV progression which often results in opportunistic fungal infections (Firacative *et al*, 2020). These factors can contribute to the increase in disease progression as well as compromising of the patients' quality of life (Bongomin *et al*, 2022).

Despite the development of antifungal prophylaxis therapies, the development of invasive infections caused by *Candida* species continues to increase leading to mortality in patients (McCort and Tsai, 2023). According to the study completed by Schwartz *et al*, 2019, it has been estimated that 5421 cases of candidemia are reported annually as a result of HIV, TB and limited resources available (poverty). *C. albicans* continues to be the leading cause of candida associated infections with an estimated 95% infection rate but, in the past decade the cases of non-albicans species such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. auris* have risen (Govender *et al*, 2021). These findings as well as studies by (Oladele *et al*, 2020), (Govender *et al*, 2021) and (Tsay *et al.*, 2020) prove that the geographical region of HIV can contribute to the rate of illness and mortality in patients. This is mainly due to the lack of resources and treatment failure of candidiasis as a result of resistant species (Dangarembizi *et al*, 2022).

1.3. The use of azoles in the treatment of *Candida* infections

Patients that receive long-term fluconazole therapy are prone to develop resistance to treatment which can be noted by the decreased *in vitro* susceptibility of *C. albicans* to fluconazole (Whaley *et al*, 2017). *C. albicans* possesses classic virulent mechanisms namely biofilm formation, hyphal morphogenesis and the secretion of hydrolytic enzymes, which can lead to their pathogenicity (Jabeen *et al.*, 2023). However, recent research indicates that the presence of these virulent traits is not directly linked to antifungal resistance. In fact, antifungal resistance varies between different species and strains, with some non-albicans species developing resistance independent of the virulence traits mentioned (Bohner *et al*, 2022). In a study done by Song *et al* (2015), the minimum inhibitory concentrations (MIC) of several antifungals were tested against *Candida* species isolated from patients with oral candidiasis. They found that the MIC range for fluconazole against *C. albicans* was 0.064 to 64 µg/ml which confirms that it is susceptible when compared to the CLSI standard.

Murphy and Bicanic (2021) confirmed that antifungal resistance is increasing in clinically significant *Candida* species. While *C. albicans* remains the most prevalent pathogen in

invasive candidiasis, emerging non-*albicans* species such as *C. glabrata* and *C. auris* exhibit increasingly high levels of azole resistance and frequent multidrug resistance. In a retrospective study completed in the Galveston–Houston region (2016–2024), *C. albicans* isolates remained largely susceptible to echinocandins. However, fluconazole resistance is increasing among both *C. albicans* and non-*albicans* species (Nguyen and Ren, 2025).

1.4 Resistance to typical azoles by the *Candida* species

1.4.1 Emerging /acquired resistance

Several studies (Abrantes *et al*, 2014; Leon *et al*, 2002; Makhado *et al*, 2014; Saranya *et al*, 2014; Yapar, 2014) have shown that the number of *Candida* species that develop resistance over time is increasing. A lot of insight has been gained into the investigation of non-*albicans* species due to the development of resistance in species such as, *Candida parapsilosis*, and *C. glabrata* and *C. tropicalis* (Whaley *et al*, 2017). Epidemiological studies have shown that *C. parapsilosis* and *C. tropicalis* are the most common non-*albicans* species in Brazil and in comparison, *C. glabrata* and *C. krusei* are less likely to cause candidiasis (Nucci *et al*, 2010).

In a study completed by Huang *et al* (2000), *Candida parapsilosis* was recognised as the most common pathogen associated with candidaemia in neonatal intensive care units. *Candida parapsilosis* is commonly isolated from patients with end-stage renal disease resulting in the infection of peritoneal dialysis catheters which could lead to *Candida* associated peritonitis (Ramage *et al*, 2006). During 1997-1999, a study completed in 71 medical centres from the United States, Canada, Latin America, and Europe showed an increase in candidaemia related infections. Overall, the highest causative agents of these yeast infections were *Candida albicans* (55%), *C. glabrata* and *C. parapsilosis* (15%), *C. tropicalis* (9%) and other *Candida* species (6%) (Oxman *et al*, 2010). Data captured from the SENTRY Antimicrobial Surveillance Program during 2008-2009 show *C. parapsilosis* as the second most common infectious agent in Latin America (Nucci *et al*, 2013; Pfaller *et al*, 2011).

Recent data from European, Latin and North American neonatal intensive care units (NICUs) confirm that candidemia in infants is predominately caused by *C. parapsilosis*. Predisposing disease such as prematurity, central venous catheters (CVCs), total parenteral nutrition (TPN) and prior echinocandin exposure contribute to invasive infections (Lona-Reyes *et al.*, 2022; Maria *et al.*, 2023; Wang *et al.*, 2023).

The exposure of fluconazole in patients with bloodstream infections could be seen as an independent risk factor as a result of emerging fluconazole resistant *Candida* species (Garnacho-Montero *et al*, 2010). Recent studies have shown that the resistance of *C. parapsilosis* is associated with the single-nucleotide polymorphism (SNP) over expression of the CDR1 and ERG11 genes which could compromise the antifungal activity of fluconazole (Zhang *et al*, 2015; Souza *et al*, 2015). Additional studies have confirmed that the molecular resistance of *C. parapsilosis* to fluconazole not only involves the over expression but also the missense mutations of ERG11 as well as the over expression of efflux pumps CDR1 and MDR1 (Berkow *et al*, 2015; Grossman *et al*, 2015).

C. glabrata is known to develop resistance when exposed to typical azole treatments which correlates with it showing the highest incidence of resistance among clinical *Candida* isolates (Oxman *et al*, 2010). Several studies have identified clinical multidrug-resistant *C. glabrata* isolates (Whaley *et al*, 2017) which were also resistant to echinocandin (Pham *et al.*, 2014). The presence of activating mutations in Pdr1, the zinc cluster transcription factor and the increased expression of ERG11 has been linked to the mode of resistance (Arendrup and Patterson, 2017; Whaley *et al*, 2017). *C. glabrata* can also cause candidemia particularly in patients suffering from hematologic malignancy and diabetes.

A high mortality rate has also been associated with *C. tropicalis* particularly in the elderly. These infections are often associated with malignancies, such as patients suffering from acute myeloid leukemia (Whaley *et al*, 2017). There has been a lot of contradicting information published about whether *C. tropicalis* is susceptible or has developed resistance to antimicrobials over time. According to Choi *et al* (2016), fluconazole susceptibility is determined by the variability in sterol 14 α -demethylase (ERG11) gene expression and less fluconazole-susceptible groups while non-fluconazole susceptible groups would show a higher level of variability in ERG11 gene expression. In a study completed by Whaley *et al* (2017), the sterol uptake control protein 2 (UPC2) gene, a gene responsible for encoding the transcriptional regulator of the ERG11 gene, showed no difference in its expression between fluconazole resistant or control isolates. This suggests that the overexpression of ERG11 gene has no significance in the presence of fluconazole resistant isolates.

In a study completed by Jin *et al* (2018) azole resistance in *C. tropicalis* was shown to be linked to the Multidrug Resistance Protein 1 (MDR1) and *Candida* Drug Resistance gene 1

(CDR1) genes, which are responsible for the over expression of the efflux pumps, Mdr1p and Cdr1p. The decreased expression of *C. tropicalis* MDR1 and a gene (with a high homology to *C. albicans* CDR1) was seen in isolates with reduced susceptibility to fluconazole. In fluconazole resistant isolates there was an increased expression of MDR1 and CDR1 (Jin *et al*, 2018). In conclusion, the overexpression of MDR1 as well as Y132F and S154F (the amino acid substitutions) in Erg11p both play a role in the development of fluconazole resistance in *C. tropicalis* clinical isolates.

1.4.2 Innate resistance

There are several innate mechanisms characteristic to the genus *Candida* that can result in the development of resistance to typical antifungal treatments such as, the up regulation of ERG11, alterations in ERG11p and the alteration of sterol composition (Arendrup and Patterson, 2017; Berkow and Lockhart, 2017; Whaley *et al*, 2017). The intracellular accumulation of antifungals can sometimes be compromised and has been associated with the up regulation of multidrug efflux transporters (Pfaller and Diekema 2012). These transporters include *Candida* Drug Resistance gene 1 (CDR1), *Candida* Drug Resistance gene 2 (CDR2), *Candida dubliniensis* *Candida* Drug Resistance gene 1 (CdCDR1), Major Facilitator transporter (MF) and Multidrug Resistance Protein 1 (MDR1). The development of resistance in *Candida* species is often as a result of different synergistic combinations of molecular mechanisms. This refers to the changes in their cell structure, efflux pumps, chromosomal changes as well as complications with the antifungal target of the cell (Sanglard and Odds, 2002).

Candida species can readily adhere to medical devices and biomaterials through the formation of biofilms, which play a key role in promoting microbial persistence and growth (Kumar *et al.*, 2021). A critical clinical concern with these biofilms is their enhanced resistance to commonly used antifungal agents, particularly azoles and echinocandins (Pierce *et al.*, 2021). In vivo, effective eradication of *Candida* biofilms often necessitates the removal of the infected device or substrate, which is frequently unfeasible in immunocompromised patients due to clinical risks (Perlin *et al.*, 2023). The observed resistance has been linked to multiple factors, including limited drug penetration into the biofilm, reduced metabolic activity of embedded cells, nutrient limitation, upregulation of resistance-associated genes, and the presence of ‘persister’ cells that can withstand antifungal assault (Silva *et al.*, 2022).

Therefore, it is important that we understand the innate mechanisms of *Candida* species so that the development of resistance can also be understood.

1.5 The need for alternative treatments to typical azoles

The continuous use of antifungals in the treatment of *Candida* species has led to the emergence of azole resistance. This typically occurs as the result of the cross-resistance to many unrelated drugs referred to as multiple drug resistance (MDR). This condition is a serious complication which limits the treatment available to the patient as only a few effective treatments can be used (White *et al*, 2002).

The main concern in the treatment of resistant species is the fact that most clinics in disadvantaged areas do not have the resources to conduct antimicrobial assays. These local clinics are unable to provide the patient with the most effective treatment needed to prevent complications due to opportunistic infections. As an outcome, most patients are given typical azole treatments which only results in further colonization of the organism and no eradication (Africa and Abrantes, 2017).

In order to prevent the development of candidal resistance there needs to be an understanding of the factors that contribute to this, namely, limitations in clinical diagnosis and evaluation, the lack of proper diagnostic equipment, as well as the lack in understanding the mechanisms behind fungal resistance (the genetic mutations and over expression of genes). Therefore, it is imperative, particularly in poverty-stricken areas, that cost-effective microbial assays be implemented to accurately identify and diagnose patients. In doing this, the gap in primary health care between the under-privileged and privileged will be narrowed as alternative treatments can be developed and be made widely available. A recommended alternative to the conventional treatment of *Candida* includes the use of traditional medicines such as medicinal plants.

1.6 Medicinal plants as antimicrobial agents

The use of herbal extracts has been utilized in traditional medicine for hundreds of years and is still incorporated in many cultures as antimicrobial agents. According to Okoye *et al* (2014), about 80% of people across the world (mostly in Africa) still depend on the use of herbal medicine to treat various diseases. About 25% of medical drugs in developing

countries make use of the medicinal properties in plants which are made up of herbs and their derivatives (Azaizeh *et al*, 2000).

Various studies have revealed that most herbalists use almost every segment of herbal plants to treat various ailments. They believe that plants possess active ingredients that serve as antimicrobial agents against opportunistic infections (Mativandlela, 2009; Parekh and Chanda, 2007; Okpekon *et al.*, 2004). Plant extracts and oils are not only widely available but are also cost-effective in treating an infection or preventing disease (Al-Judaibi and Al-Yousef, 2014). In some cultures, particularly in the rural and under privileged areas in South Africa, plant extracts and oils are used as a primary source to treat a variety of ailments and conditions before consulting a doctor (Duarte *et al*, 2005). Most African people typically living in rural areas do not have access to primary health care so the use of medicinal plants is quite common. Not only are medicinal plants easily accessible and affordable, but it has also been part of their culture for centuries (Okigbo *et al*, 2009).

The antimicrobial potential of medicinal plant extracts represents a promising response to the pressing issue of antimicrobial resistance. These plants contain diverse bioactive compounds—such as flavonoids, alkaloids, saponins, and phenolics—that act through mechanisms like membrane disruption, enzyme inhibition, and oxidative stress induction. Additionally, many extracts act synergistically with conventional antibiotics, enhancing their efficacy and lowering resistance risk. However, the field currently faces key challenges: variability in extraction methods, lack of standardized antimicrobial assays, and a scarcity of pharmacokinetic and clinical data. Future research must address these gaps by optimizing extraction protocols, developing robust testing frameworks, and conducting rigorous *in vivo* and clinical studies to translate phytochemistry into safe, standardized antimicrobial therapies (Vaou *et al.*, 2021).

1.7 The use of plant extracts against *Candida*

Rajkowska *et al* (2017) investigated the effect of essential oils as potential antifungal agents and found that tea tree, thyme, peppermint, clove and peppermint oil showed a high antifungal activity to several *Candida* strains. The MICs ranged from 0.03–8.0% v/v (Rajkowska *et al*, 2017). Another study done by Zbikowska *et al* (2017) examined the antimicrobial and antiradical activity of five different species of the Genus *Bergenia*. Overall,

the most effective antimicrobial substance found in these leaves was hydroquinone. Hydroquinone is formed by the degradation of arbutin, a glycoside that can be extracted from many medicinal plants. Despite its toxicity, hydroquinone can be used at low concentrations to prevent the growth of microbes in the human body (Zbikowska *et al*, 2017). Studies investigating whether plants and their extracts have antimicrobial, antifungal and antibacterial effects on the *Candida* species (Helmy and Bakr, 2014; McGaw *et al*, 2008; Santos *et al*, 2018) showed that most plants have antimicrobial properties and can even work in synergy with other compounds (Nascimento *et al*, 2000; Rukayadi *et al*, 2009).

1.8 Synergism in medicinal plants

A study completed by Stermitz *et al* in 2000, revealed that the use of cationic berberine alkaloids could be used to create a multiple drug resistant inhibitor. Berberine is a quaternary ammonium salt produced by *Berberis fremontii*, a species of barberry plants. Berberine produces a substance known as 59-methoxyhydrnocarpin (59-MHC). On its own, 59-MHC had no antimicrobial activity against the MDRs present in *Staphylococcus aureus* but when used in synergy with berberine and other NorA substrates it was seen to have an inhibitory effect. Hence, a substance/compound could be ineffective on its own to prevent microbial growth but if used in synergy with a synthetic drug it could have an inhibitory effect (Stermitz *et al*, 2000).

In a study done by Kumar *et al* (2015) the synergistic effect of α - and β -asarones isolated from *Acorus calamus* was investigated in combination with fluconazole, clotrimazole and amphotericin B drugs against several pathogenic *Candida* species. They found that at lower concentrations of asarones and drugs, there was anti-candidal activity present (fractional inhibitory index <0.5) and the synergistic activity of these combinations also inhibited the biofilm formation by *Candida* species (Kumar *et al*, 2015).

The synergistic activity of fluconazole and two compounds, namely isoquinolone analogue 15 and phthalazinone analogue 24 against several resistant isolates of *C. albicans* was confirmed by Mood *et al* (2017). In this study, the checkerboard assay was completed in which the fractional inhibitory concentration indices with fluconazole was less than 0.17 for compound 15 and less than 0.12 for compound 24. These two compounds also presented strong antifungal activity with isavuconazole (a relatively new azole drug) at a lower

concentration when compared to fluconazole. This confirms that isoquinolone analogue 15 and phthalazinone analogue 24 not only demonstrates good synergistic activity with fluconazole, but also serves to increase the efficacy of isavuconazole (Mood *et al*, 2017).

1.9 *Galenia africana* as a potential antimicrobial agent

Galenia africana is an aromatic woody shrub that belongs to the family *Aizoaceae*. It is often referred to as ‘kraalbos’, ‘perdebos’ but mostly ‘geelbos’ due to the change in colour of the plant over time (Van Wyk B-E *et al*, 1997). *G. africana* is indigenous to the Northern Cape, Namaqualand and Karoo where the indigenous people make use of this plant to treat various ailments and infections including toothache, skin diseases, inflammation and venereal diseases (Watt and Breyer-Brandwijk, 1962).

Investigations of the toxicological effects of this plant have shown that many forage animals indigenous to the area of the plant, developed ascites, bloating of the stomach and liver damage typically during adverse drought conditions (Van der Lugt *et al*, 1992). Despite the above-mentioned adverse effects on animals, *G. africana* has shown a lot of potential as an antimicrobial agent particularly in humans to treat common infections.

Research done by Vries *et al* (2005), showed antimicrobial activity of *G. africana* against the following species *Alternaria sp.*, *Fusarium sp.*, *Cylindrocorynion sp.*, *Phaeoemoniella chlamydospora*, *Eutypalata* and *Botrytis cinerea*. Researchers believe that these antimicrobial properties are as a result of the important compounds produced by the plant namely flavonoids and chalcones (Maiko, 2010; Vries *et al* 2005). Flavonoids can have an inhibitory effect on several lifestyle diseases including inflammation and allergies. Chalcones are open chain flavonoids with similar inhibitory effects as flavonoids and are also responsible for the antioxidant properties of higher green plants (Ticha *et al*, 2014). By investigating these compounds in *G. africana* researchers can begin to understand how and why this plant possesses its antimicrobial properties.

1.9.1 Organic compounds found in *Galenia africana*

Several studies have demonstrated that *Galenia africana* possesses a rich profile of flavonoid and chalcone compounds that contribute significantly to its antimicrobial activity. Ticha *et al.* (2014) successfully isolated eleven flavonoids from *G. africana*, including (E)-2',4'-

dihydroxychalcone, (S)-7-hydroxyflavanone, (E)-2',4'-dihydroxy-2,3-dihydrochalcone, (S)-5,7-dihydroxyflavanone, (S)-2',5,7-trihydroxyflavanone, and (S)-5,7-dihydroxy-2'-methoxyflavanone. Notably, (E)-2-hydroxy-3',6'-dimethoxychalcone exhibited potent antimycobacterial activity with a MIC₉₉ of 5 µM against *Mycobacterium tuberculosis* (H37Rv) strains (Ticha *et al.*, 2014). Earlier work by Vries *et al.* (2005) identified overlapping compounds including 5-hydroxy-7-methoxyflavanone, 5,7-dihydroxyflavanone, dihydroechinoidinin, and 2',4'-dihydroxychalcone, further reinforcing the hypothesis that the antimicrobial effects of *G. africana* are closely linked to its flavonoid constituents. Mativandlela *et al.* (2009) also reported the isolation of three known flavonoids and one novel chalcone, with (2S)-5,7,2'-trihydroxyflavanone and (E)-2',4'-dihydroxychalcone showing notable anti-tuberculosis activity. In addition to their intrinsic antimicrobial effects, certain compounds from *G. africana* demonstrated synergistic potential when combined with conventional antibiotics. For instance, (E)-3,2',4'-trihydroxy-3'-methoxychalcone and (2S)-5,7,2'-trihydroxyflavanone significantly enhanced the efficacy of isoniazid, with the latter reducing the minimum inhibitory concentration (MIC) by 16-fold and yielding a fractional inhibitory concentration (FIC) of 0.12, indicative of strong synergism (Mativandlela *et al.*, 2009). These findings collectively highlight the therapeutic potential of *G. africana* and underscore the importance of its flavonoid-rich phytochemistry in the development of novel antimicrobial strategies.

1.10 Summary and Statement of research problem

Candidiasis is one of the most common nosocomial infections acquired, where the crude mortality ranges from 20-61% depending on the geographical location (Kreusch and Karstaedt, 2013). With the increase in immune-compromising infections such as HIV/AIDS and malignant diseases, the risk of developing an opportunistic infection (such as a fungal infection) also increases. Amongst the leading fungal pathogens known to cause infections in immunocompromised patients is the genus *Candida*. *Candida* species can pose a threat to the innate immunity of the patient especially with the identification of resistant species (Yapar, 2014). Thus, it is a cause of great concern particularly in the sub-Saharan regions of Africa where the prevalence of HIV infection is the highest in the world.

Patients from developing countries will continue to suffer from opportunistic infections associated with HIV as a result of late diagnosis, poor adherence to treatment, drug resistance, poverty, poor nutrition and high exposure to infectious agents to name a few (Rubaihayo *et al*, 2016). Limited surveillance data on *Candida* species prevalence and antifungal resistance hampers effective control of candidiasis in HIV-infected individuals, potentially contributing to increased morbidity and mortality (Africa and Abrantes, 2017). In order to prevent the increase in morbidity and mortality rates, it is imperative that the use of natural medicaments and alternative therapies be investigated as a safer way to treat candidiasis in immune compromised patients.

The use of medicinal plants as a treatment aid has been known to exist since ancient times; even in this day and age medicinal plants are used as the basis for many synthetic medicaments (Nascimento *et al*, 2000; Avijgan *et al*, 2006). *Galenia africana* is an indigenous South African plant that is known to flourish in the Namaqualand and Karoo regions of the country (Van Wyk *et al*, 1997). The people that live there use this plant as an antiseptic to treat common ailments and infections (Ticha *et al*, 2014). Although the extracts of the plant showed antimicrobial activities (Mativandlela *et al*, 2008, Ticha *et al*, 2014, Vries *et al*, 2005), not a lot of research has been aimed at investigating the antifungal activity of *G. africana* compounds against *Candida* species. The expected impact of this research is to provide critical data on the antifungal efficacy of *G. africana* natural extract against drug resistant *candida* species. In addition, findings from this study will provide unique insights into the synergistic effects of *G. africana* natural extract and fluconazole. Another aspect of

this research is to find the exact point at which *Candida* growth is prevented, which is significant to identify the dose-dependent timing.

Research Questions

1. Can *G. africana* be developed as an alternative form of treatment against *Candida* strains resistant to typical azole treatments?
2. Does the type of solvent EtOH or EtOAc used during the extraction of *G. africana* have an effect on the susceptibility of the *Candida* species?

Aim

To investigate the application of *G. africana* extract as a possible antifungal agent for the treatment of fluconazole-resistant candidiasis

Objectives of the research

- To identify whether *G. africana* has potential antifungal activity against non-*albicans* species, preventing possible opportunistic infections.
- To investigate whether there is a difference in effect between the ethanolic fraction and the ethyl acetate fraction of *G. africana* on the growth of *Candida* species.

Chapter II: Materials and Methods

2.1 Chemical analysis of *Galenia africana*

2.1.1 Extraction of *G. africana*

Approximately 1 kg of *G. africana* was collected prior to use from a farm in Ceres (33.4007° S, 19.2950° E), Cape Winelands, Western Cape Province. The soft leaves were removed from the branches and cut up finely. The leaves were blended with 3 L of methanol and extracted overnight in a water bath at 60°C. The plant-methanol solution was filtrated from the plant debris and evaporated under pressure at 50°C using a rotary evaporator resulting in 12.3 g of the total plant extract. The total extract appeared thick, sluggish and dark green in colour. A second extraction was completed whereby the methanol collected from the concentration of the first batch of the plant was reused to ensure optimal usage of the plant material.

2.1.2 Preparation of column chromatography

Approximately 12.3 g of the extract was dissolved in a minimal amount of methanol. Small amounts of the dissolved extract were then combined with silica, and dried overnight. Cotton wool was placed at the bottom of the column using a sterile steel rod. This was to allow the mobile phase to flow through and prevent particle leakage. Approximately 30 cm of silica was added to the column, and it was compacted carefully to ensure that the silica was evenly spread and no air bubbles were present. The dried extract was then added and 5-8 cm of silica was placed above the extract, ensuring that the extract was not disturbed when the solvent system was added.

Table 1 outlines the solvent concentrations used during the chromatography process. Fractions of approximately 0.5 L each were collected into flasks and subsequently concentrated using a rotary evaporator. The number of compounds present in each major fraction was assessed using thin-layer chromatography (TLC). The dried fractions were then weighed, transferred into labeled glass vials, and stored for further analysis.

Table 1: The solvent system used for the first chromatography column on the crude extract of *Galenia africana*

Fractions	Hexane (%)	Ethyl acetate (%)	Volume (L)
1	100	-	0.5
2	100	-	0.5
3	95	5	0.5
4	95	5	0.5
5	95	5	0.5
6	95	5	0.5
7	90	10	0.5
8	90	10	0.5
9	90	10	0.5
10	90	10	0.5
11	75	5	0.5
12	75	5	0.5
13	75	5	0.5
14	75	5	0.5
15	60	40	0.5
16	60	40	0.5
17	60	40	0.5
18	60	40	0.5

2.1.3 Concentration of *Galenia africana*

Using the rotary evaporator, the filtered solution was placed into a water bath at 50°C which allowed the methanol to evaporate and condensate via a cooling system (10°C). The recovered methanol was collected in a glass flask and reused for a second round of extraction after it had been separated from the initial plant extract. At the end of this process, the extract was in its most concentrated form, with the removal of water and methanol.

2.1.4 Storage of extract fractions

Prior to the storage of these fractions, empty sterile vials were weighed and the fractions were transferred from the flasks into the vials. The vials were then placed underneath a fume hood to remove the excess solvent and the fractions were weighed. All vials were stored in the fridge at 4°C until further use.

2.1.5 Purification of *Galenia africana*

The total extract was purified using column silica gel chromatography, whereby the extract was exposed to different concentrations of solvents (Table 1). As the solvent system changes different fractions were separated and collected (Figure 1).

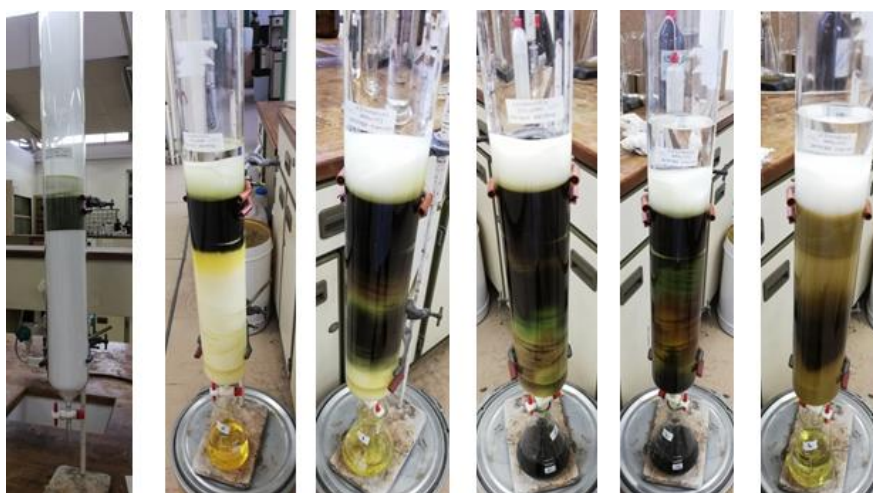


Figure 1: Column chromatography (7 x 30 cm, 500g silica gel) completed on *Galenia africana*.

Thin layer chromatography plates for the first column, where each compound (within the total extract) is depicted by different colours. The TLC plate was detected by UV254/360 and vanillin (H_2SO_4 reagent). Similar fractions were then combined, weighed and labelled.

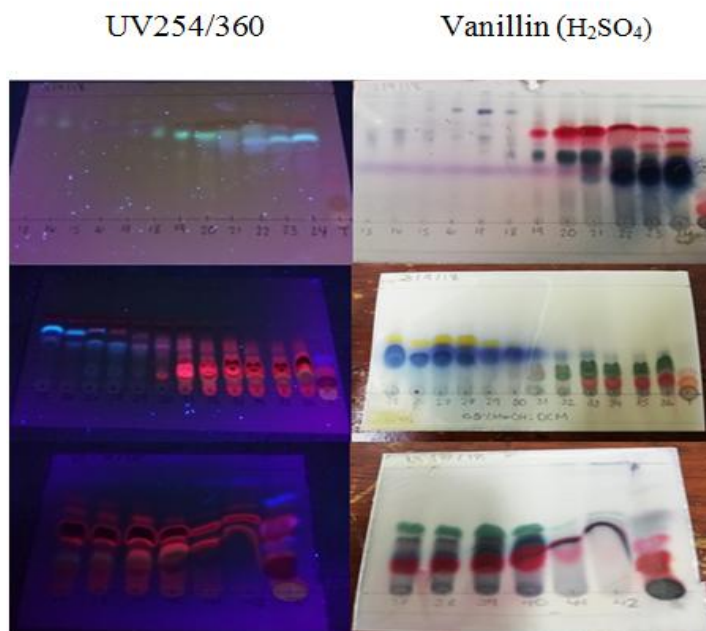


Figure 2: Thin layer chromatography plates for the first column, detected by UV254/360 and vanillin (H_2SO_4 reagent).

The 4th fraction (2.14 g) was selected as it contained all the compounds of interest and was separated using silica gel column chromatography into sub-fractions below.

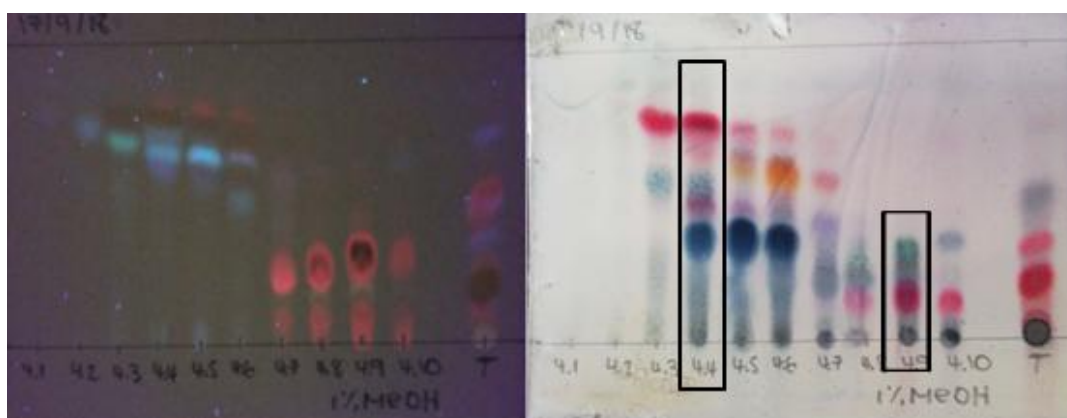


Figure 3: Sub-fractions of fraction 4 seen through ultra violet light at wavelengths of 254 nm and 360 nm (UV254/360) and vanillin (H_2SO_4 reagent).

The purpose of running sephadex columns was to purify compounds for nuclear magnetic resonance spectroscopy (NMR) analysis. The solvent system used for all sephadex columns was 100% ethanol. As shown in Figure 3, the sephadex column completed on fraction 4.4 resulted in the first NMR sample (1) with a weight of 10 mg. Fraction 4.9 was also separated using a sephadex column with the solvent system (95% MeOH in H₂O) which resulted in two more sub-fractions namely 4.9II and 4.9 III. Fractions 4.9 II and 4.9 III were selected and a sephadex column was completed resulting in another two pure compounds (2 and 3) with a weight of 9.5 mg and 8.7 mg, respectively. The fourth NMR sample (4) was separated from the total fraction 4 (138.8 mg) which was kept as a reference. From this reference another 4 fractions were collected (IV I, IV II, IV III and IV IV) and fraction IV II (88 mg) was separated by a sephadex column resulting in fraction IV II (1) and IV II (2). Another sephadex column was run on fraction IV II (2) with a weight of 34.5 mg which resulted in 9.6 mg of compound 10.5A

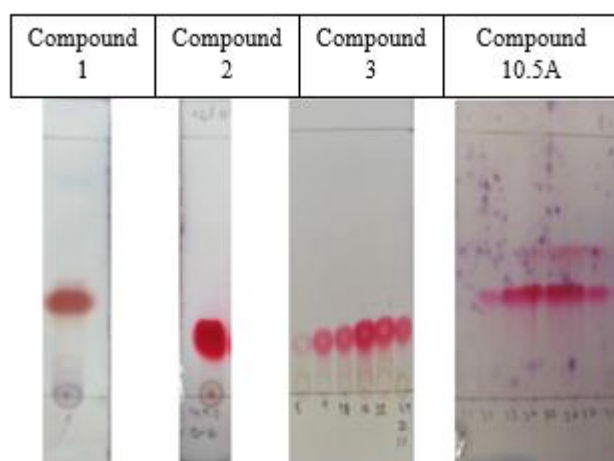


Figure 4: Thin layer chromatography plates sent for NMR analysis detected by vanillin (H₂SO₄ reagent).

2.1.6 Preparation of the total fraction in DMSO

The total fraction of *Galenia africana* was diluted in 1 mg/ml Dimethyl sulfoxide (Cat. no. D4540, Sigma Aldrich). The working solution was further diluted to a final concentration of 20% DMSO with the extract in RPMI which was used for the subsequent antimicrobial assays as described in the next chapter.

2.1.7 Identification of active compounds in *Galenia africana* using NMR Spectroscopy

The NMR spectra of 3 purified compounds were recorded at 25 °C in CDCl₃, using Bruker Avance 400 NMR spectrometer (1H at 400 MHz, 13C at 100 MHz) seen in Figure 5-10. The chemical structure of each compound was determined using the carbon-hydrogen framework for each compound. The chemical shifts of 1H (δ H) and 13C (δ C) in ppm were determined relative to solvent signal.

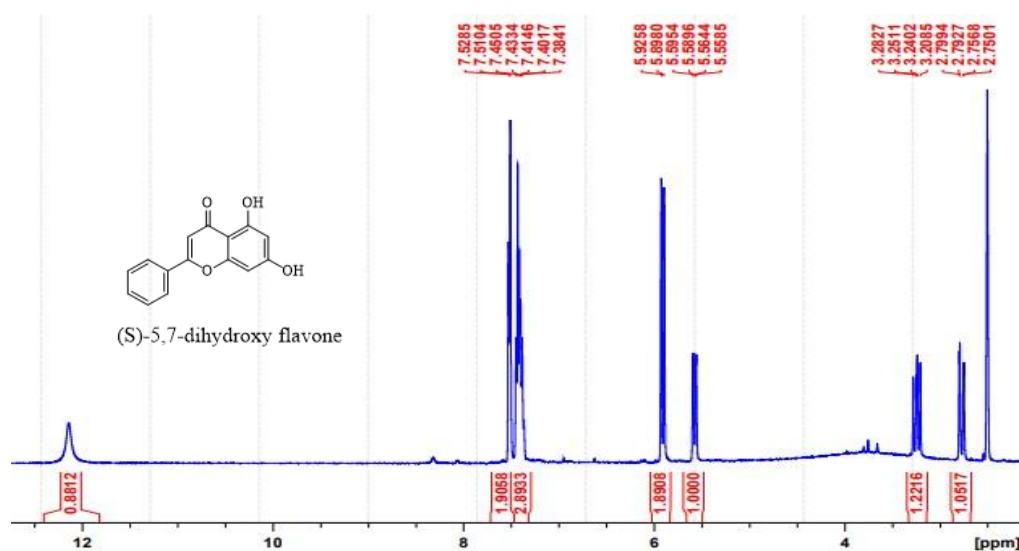


Figure 5: ¹H NMR spectrum of compound 1

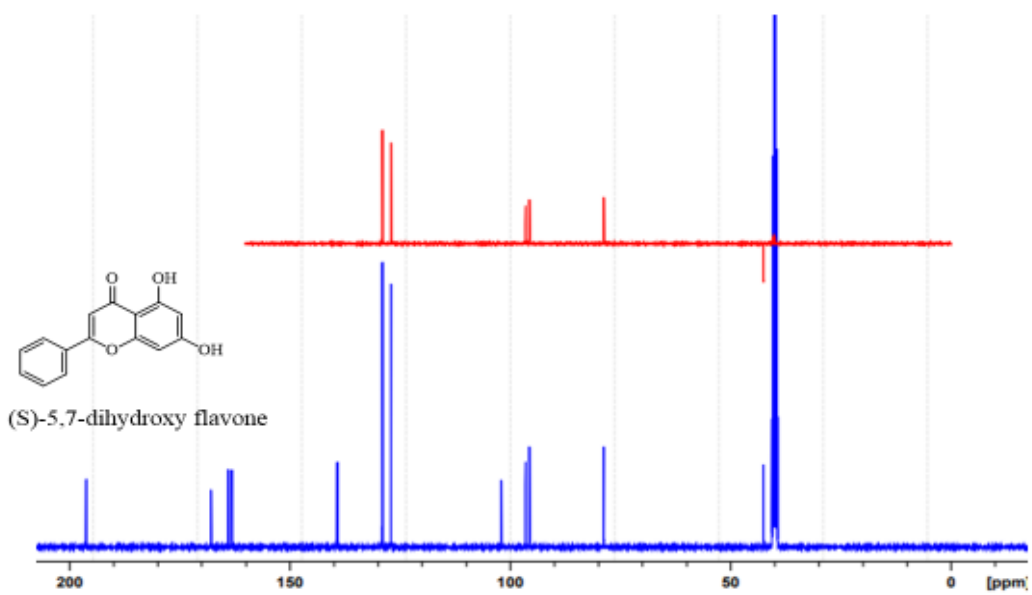


Figure 6: ^{13}C NMR spectrum of compound 1

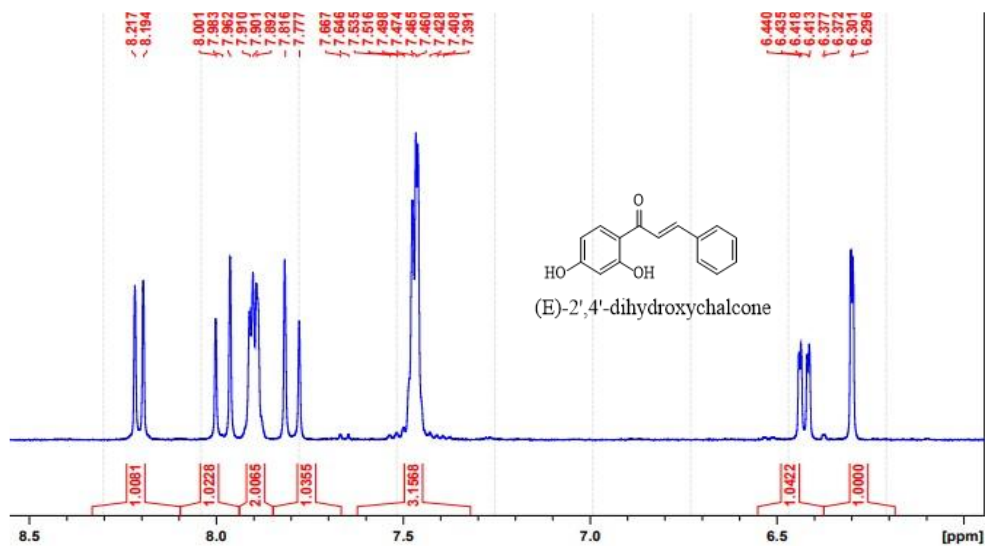


Figure 7: ^1H NMR spectrum of compound 2

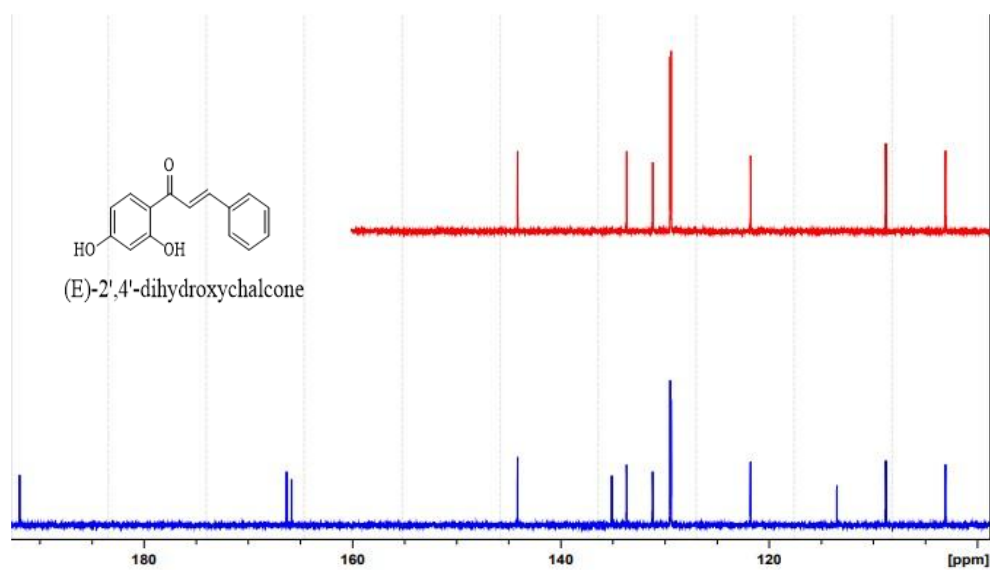


Figure 8: ^{13}C NMR spectrum of compound 2

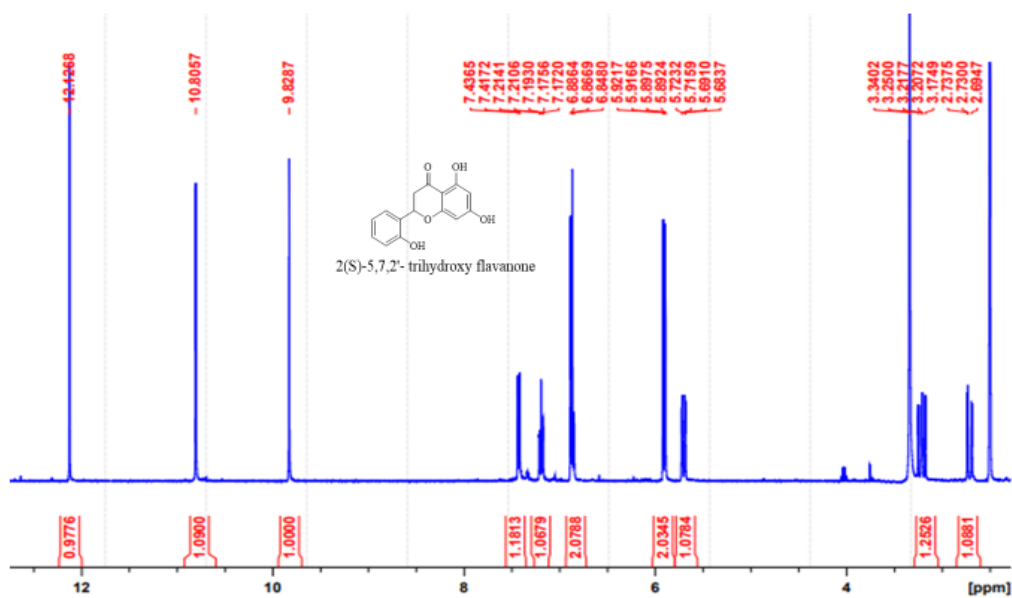


Figure 9: ^1H NMR spectrum of compound 3

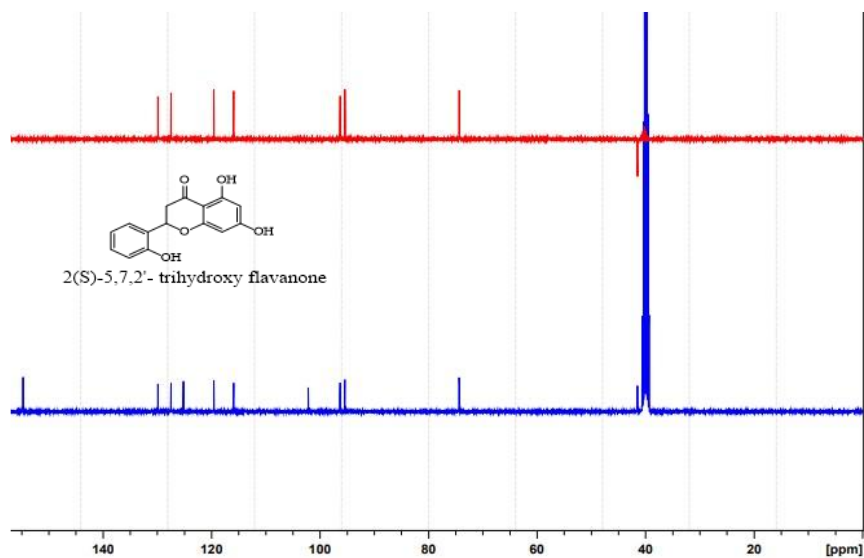


Figure 10: ^{13}C NMR spectrum of compound 3

2.2 Antimicrobial assays

2.2.1 Identification of *Candida* species used in this study

Candida species for this study were obtained from the microbial storage bank in the Medical Biosciences Department of the University of the Western Cape, Bellville, South Africa. Of the 330 isolates, 184 *Candida* were identified using ID API 32C (Cat. No. 20600, bioMérieux SA, France) and VITEK (Cat. No. V2020-SYS, Thermo Scientific, USA).

Confirmatory tests were completed on modified Fluka chromogenic *Candida* identification agar (Cat. no. 94382, Sigma-Aldrich, USA) with respective selective supplement (Cat. no. 68067, Sigma-Aldrich, USA), Oxoid chromogenic *Candida* agar (Cat. no. CM1002A, Oxoid, UK), with respective selective supplement (Cat. no. SR0231E, Oxoid, UK) of which 12 species were identified as *C. albicans* (69 isolates, 37.5%), 2.7% as *C. tropicalis* (5 isolates), 23.4% as *C. dubliniensis* (43 isolates), 10.9% as *C. glabrata* (20 isolates), 0.5% as *C. globosa* (1 isolate), 0.5% as *C. guilliermondii* (1 isolate), 2.7% as *C. kefyr* (5 isolates), 2.7% as *C. krusei* (5 isolates), 1.1% as *C. magnoliae* (2 isolates), 0.5% as *C. membranifaciens* (1 isolate), 1.1% as *C. parapsilosis* (2 isolates), 2.2% as *C. sake* (4 isolates) and 14.1% as *C. humicola* (26 isolates).

2.3 Media preparation

2.3.1 Sabouraud dextrose agar

Sabouraud 4% dextrose agar (Cat. no. 84088, Sigma Aldrich) was prepared by adding 65 g in 1 L distilled water and autoclaved prior to use.

2.3.2 Yeast Nitrogen Based Agar

Bacteriological agar was prepared by adding 1.3 g (Cat. no. A6686, Sigma Aldrich) per 100 ml distilled water and autoclaved. The yeast nitrogen base (10x concentration) was prepared separately by adding 6.7 g yeast nitrogen base (Cat. no. 239210, Difco) to 5 g dextrose (Cat. no. G8270, Sigma Aldrich) and diluted in distilled water. This nutrient solution was then filter sterilized using a 50 ml syringe and 0.2 µm filter (Cat. no. 25NS, MSI filters, USA) into a 50 ml sterile centrifuge tube and added to the sterile bacteriological agar before it solidified.

2.3.3 Roswell Park Memorial Institute (RPMI) medium

The RPMI-1640 medium was prepared by adding RPMI powder (10.43 g with L-glutamine) (Cat. no. R6504, Sigma Aldrich) to 800 ml distilled water. Approximately 18 g Glucose (Ref. no. G8270, Sigma Aldrich) and 34.53 g Mops (Ref. no. M1254, Sigma Aldrich) was added to the RPMI medium and the turbidity was adjusted with sodium hydroxide (Ref. no. S2770, Sigma Aldrich) using a pH meter (Crison GLP21, Spain) to 7-7.2 pH. Subsequently, the RPMI was filter sterilized using a 0.22 µm filter (Cat. no. 25NS, MSI filters, USA) and several aliquots were made in 50 ml sterile screw cap tubes.

2.4 Preparation of extract

The *G. africana* extract was prepared as ethanolic crystals (brown in colour) with a liquorice odour. The *G. africana* extract was made up as a 20% (200 mg/ml) stock solution in RPMI. This extract solution was dissolved by placing it in a spectrafuge (Cat. no. C5090725, Labnet International Inc. USA,) for 5 minutes at the 6.5 (x1000) rpm. Once this was completed the extract was filter-sterilized using a 0.45 µm filter. The concentration of the extract was determined by conducting a series of doubling dilutions in proportionate amounts. The primary objective of this technique was to estimate the concentration of the unknown sample by counting the number of colonies that grew on the agar plates from the serially diluted

samples. A standard formula was then applied to calculate the original concentration of the sample, $CFU/mL = \text{Number of Colonies} \times \text{Dilution Factor} / \text{Volume Plated (mL)}$ (Ben-David and Davidson, 2014).

2.5 Kirby-Bauer disk diffusion method

2.5.1. Impregnation of filter discs with the ethanolic fraction of *Galenia africana*

The *Galenia africana* ethanolic extract was diluted to a final volume of 500 μl in sterile eppendorf tubes. Eight serial dilutions were completed in RPMI with concentrations ranging from 100- 1.5625 mg/ml. Each concentration was prepared in triplicate. Sterile filter paper discs (Cat. no. SF2462, Lasec) were prepared by saturation with 40 μl of each extract concentration and left to dry aseptically overnight at 37 °C.

2.5.2. Resuscitation and seeding of *Candida* species

Candida species were resuscitated by inoculation into Sabouraud broth (Cat. no. S3306, Fluka Analytical) and incubated at 37°C for 24 h. Each strain was streaked aseptically onto Sabouraud dextrose agar (SDA) for single colony formation and incubated at 37 °C for 24 h. A single colony of each *Candida* species was isolated and diluted in 5 ml of sterile saline solution. Each test tube was placed in a nephelometer (SN. 437R06N124, Trek Diagnostics Systems Ltd) to measure its cell density and to standardize the cell cultures to the 0.5 McFarland standard at 3×10^8 CFU/mol (Ref. no. R20410, Remel, Kansas) (Barry *et al*, 1979). The cell suspension was diluted to 1:100 in RPMI media according to the Clinical and Laboratory Standards Institute standard reference procedure document M27/A2 (NCCLS, 2002).

2.5.3 Susceptibility testing

SDA plates were inoculated to form a lawn of *Candida* growth by aseptically swabbing the plate with the standardized cell suspension (0.5 McFarland standard at 3×10^8 CFU/mol). Each agar plate was divided into four equal quadrants and four impregnated disks were placed in the center of each quadrant. Eight concentrations ranging from 100 mg/ml- 1.5625 mg/ml were used. The plates were incubated at 37 °C for 24 h after which the zones of inhibition were measured. The negative control was a 50% ethanol impregnated filter paper disc (40 μl) and the positive control was the commercial 25 μg fluconazole disc (Ref. no.

CT1806B, Oxoid, UK). Post incubation, the zone of inhibition (a clear zone showing no growth surrounding the antimicrobial disk) was measured from the start to the end of the clear zone. The filter paper disk (9 mm) was subtracted from the total clear zone and classified as sensitive, intermediate or resistant based on the size of the zone (). A zone of inhibition that was smaller than the standard zone for a particular antifungal was interpreted as resistant to the antimicrobial (Barry *et al*, 1979).

The susceptibility of the nine type strains of *Candida* to FCZ was tested in a concentration range of 64 µg/ml-0.25 µg/ml. According to the Clinical and Laboratory Standards Institute M27A3 approved standard protocol (Clinical and Laboratory Standards Institute, 2008). The approved MIC breakpoint values indicate susceptibility at a concentration <8 µg/ml, susceptible-dose dependant (S-DD) at 16-32 µg/ml and resistance at ≥ 64µg/ml.

2.6. Broth Microdilution assay

A working suspension of each *Candida* species was prepared by first diluting the culture 1:50 in RPMI medium, followed by a further 1:20 dilution to achieve a final concentration of $1-5 \times 10^3$ CFU/mL. In a 96-well plate, 100 µL of RPMI broth was added to wells in columns 2 to 11. Then, 100 µL of *G. africana* extract at a concentration of 200 mg/mL (20%) was added to column 2. Once the *Candida* cell suspension was added, the starting concentration of the extract became 5 mg/mL (5%). A serial dilution of the extract was performed from column 2 through column 11 (wells A–E). Column 1 served as a control with undiluted extract at 20%. After adding 100 µL of the cell suspension to each experimental well, the final concentration of *G. africana* extract ranged from 10% to 0.00976% (i.e., 100 to 0.0976 mg/mL).

Controls: The growth controls comprised of 100 µl cells without the extract and 100 µl broth without extract respectively. The fluconazole control (Ref. no. F8929, Sigma Aldrich) was added at a final concentration of 32 µg/ml (100 µl of FCZ and 100 µl of cell suspension). The sterility controls used were 200 µl of the RPMI broth and a 200 µl saline control respectively.

The plate was sealed using a sealing film (Ref. no. 030017, Sigma Aldrich) and incubated at 37°C for 24 h. Post incubation, 40 µl of 95% p-iodonitrotetrazolium chloride (INT) (Ref. no. 10406, Sigma Aldrich) at a concentration of 0.2 mg/ml was added into each well and the plate was incubated for another 2 h. Antimicrobial activity was confirmed by removing 10 µl from clear wells and inoculating it onto SDA followed by incubation for 24 h at 37°C. The

lack of fungal growth after incubation confirmed the MIC. The MIC was interpreted as the lowest concentration at which there was no visible growth, as seen by the lack of colour change to pink.

2.7 Synergy assay

Each *Candida* isolate was grown on SDA plates for 24 h and the turbidity was adjusted to the 0.5 McFarland standard. A 1:10 fungal suspension in RPMI resulted in 1×10^6 to 5×10^6 CFU/ml. The first 3 columns (Row A-C) were used as MIC controls for the MIC combination of *G. africana* and FCZ (Column 1, A-C), $\frac{1}{2}$ MIC combination (Column 2, A-C,) and $\frac{1}{4}$ combination (Column 3, A-C).

In column 2-6 and 8-12 (F-H) a volume of 50 μ l RPMI was added. A volume of 100 μ l FCZ was added at 2x MIC for each *Candida* species (as determined by broth microdilution) in column 1 and column 7 (F-H) and a serial dilution (50 μ l) was completed from column 1-6 and 7-12 (F-H). The $\frac{1}{2}$ MIC and $\frac{1}{4}$ MIC of *G. africana* was added to a volume of 50 μ l to the wells in column 1-6 and 7-12 (F-H). The 1:10 cell suspension was then added to all wells at volume of 100 μ l. The plates were sealed using a sealing film and incubated at 37°C for 24 h and the MIC values for the compound alone, the FCZ alone and the combinations were observed. (Nageeb *et al*, 2015; Pankey *et al*, 2014). To maintain unbiased results, this assay was performed in triplicate for each *Candida* isolate. The cell suspension in RPMI without the compound was used as a positive control. The sterility controls used were 200 μ l RPMI and 200 μ l saline.

The use of the fractional inhibitory concentration (FIC) index was calculated as the FIC of drug A+ FIC of drug B which can be calculated by using the following formulas:

$$\text{FIC of drug A} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}}$$

$$\text{FIC of drug B} = \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

If the FIC index was ≤ 0.5 it was recorded as synergistic. A FIC index of >0.5 but ≤ 4 was interpreted as indifference and antagonism defined by a FIC index >4 (Nageeb *et al*, 2015; Pankey *et al*, 2014).

2.8 Time-kill assay

The purpose of the time kill assay is to identify the rate of inhibition or cell death by observing the growth pattern for antimicrobials. By doing this, the fungistatic or fungicidal activity can be determined and used to improve dose dependant timing (Burgess *et al*, 2000). The synergy was also determined by performing the time-kill curve for each isolate using the *G. africana* extract at $\frac{1}{2}$ MIC and $\frac{1}{4}$ MIC. The starting inoculum ranged from 1×10^5 to 5×10^5 CFU/ml as explained in the synergy methodology above. About 100 μ l of $\frac{1}{2}$ MIC of *G. africana* and 100 μ l of $\frac{1}{2}$ MIC of FCZ were combined, mixed on a vortex mixer and 100 μ l of this combination was dispensed into one well. This procedure was repeated for the $\frac{1}{4}$ MIC as well. All wells containing RPMI with $\frac{1}{2}$ MIC and $\frac{1}{4}$ MIC combinations were inoculated with 100 μ l of the *Candida* cells and incubated at 37°C for 24 h in a shaking incubator. At the following predetermined time points; 0, 3, 6, 9, 12 and 24 h, 40 μ l aliquots of each concentration was removed aseptically and streaked onto SDA plates (Nageeb *et al*, 2015; Pankey *et al*, 2014). These plates were then incubated for 24 h at 37°C and colony counts were performed.

Fungicidal activity was defined as = 3Log_{10} CFU/ml reduction in the colony count relative to the initial inoculums. Synergism was achieved with $\geq 2\text{Log}_{10}$ CFU/ml decrease by the combination compared to the most active agent. Antagonism was defined as a $\geq 2 \text{Log}_{10}$ CFU/ml increase by the combination compared to the most active agent (Nageeb *et al*, 2015; Pankey *et al*, 2014).

Chapter III: Results

3.1 Purification and identification of 3 active compounds in *Galenia africana*

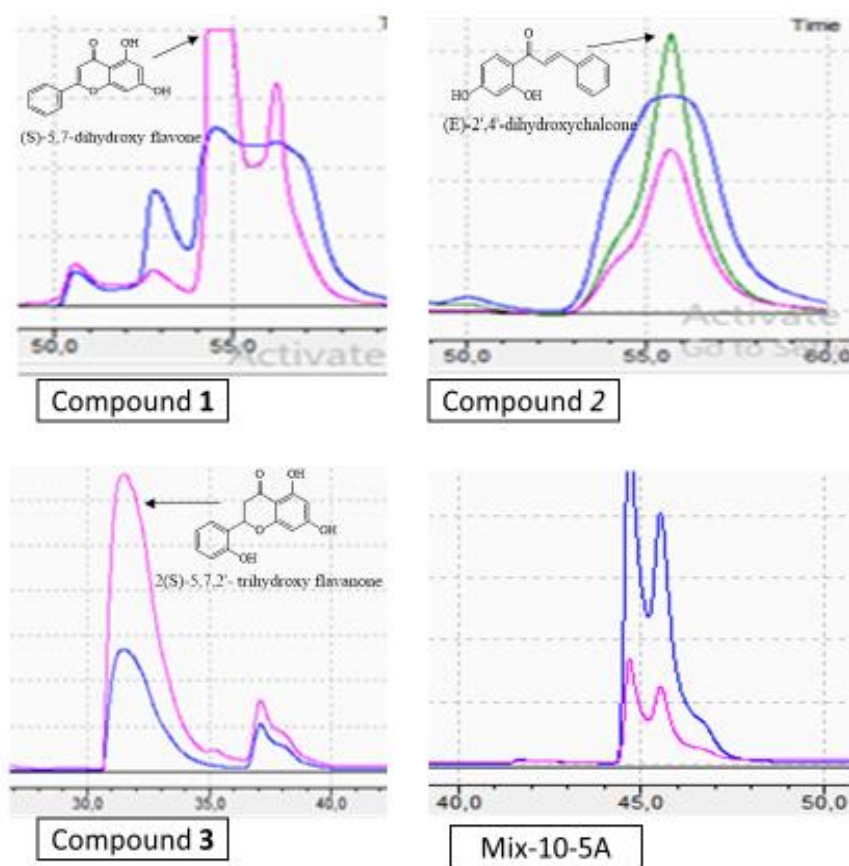


Figure 11: HPLC, isolation retention time at 35-37% DIW and 65-67% Methanol.

3.2 Chemical analysis of pure compounds in *Galenia africana*

The structure of compound 1 was confirmed by comparison of the obtained NMR data with those published in the literature to be (*S*)-5,7-dihydroxy flavone (Ticha *et al*, 2015), compound 2 was confirmed by comparison of the obtained NMR data with those published in the literature to be (*E*)-2',4'-dihydroxychalcone (2) (Mativandlela *et al*, 2009; Ticha *et al*, 2015) and the structure of compound 1 was determined to be 2(*S*)-5,7,2'- trihydroxy flavanone (3) (Mativandlela *et al*, 2009) and compound 4 as mix we can refer to as fraction (10-5A).

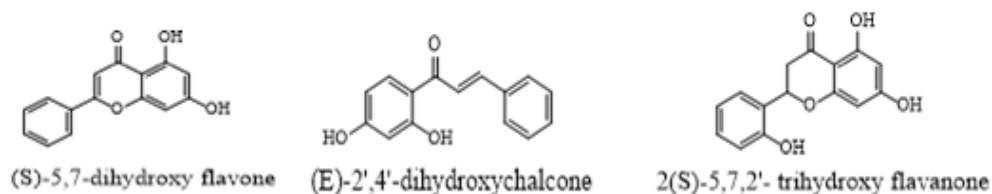


Figure 12: The chemical structures of the most important compounds isolated from *Galenia africana*.

Compound 1

Compound 1 was identified as (*S*)-5,7-dihydroxy flavone based on the analysis of the NMR data and comparison with literature. The ^1H NMR spectrum of 1 showed two meta coupled protons at H-8, δ 5.89 and H-6, δ 5.92 (s, each; $J=2.9$ Hz) and assigned to ring B; four protons at 5.58 (m, 7.45 Hz); 6.56 (m, 7.43 Hz); 7.19 (dd, 1.7, 7.5 Hz), and 7.42 (d, 7.5 Hz) assigned for protons 2', 6', 4' and 3', 5' of ring B; and two protons at 2.77 (dd, 17.0, 2.9 Hz), 3.24 (dd, 17.0, 12.9 Hz) and 5.58 (dd, 12.9, 2.9 Hz) assigned for protons H-3eq, H-3ax and H-2 of ring C. The carbon 13 and DEPT-135 analysis showed 15 carbons classified as 1 methylene (CH_2) at 42.6 (t, C-3); 7 methines (CH) at 78.78 (d, C-2); 95.6, 96.5 (d, each, C-6-8), 102.0 (d, C-3'), 126.8 (d, C-5'), 127.0 (d, C-6'), 129.0 (d, C-4'); 7 fully substituted carbons at 102. (s, C-10), 139.2 (s, C-1'), 126.8 (s, C-2'), 163.1/163.9 (S, each, C 9/5), 167.9 (s, C-7), and 196.2 s, C-4). The observed values from ^1H and ^{13}C NMR match with the

structure ((*S*)-5,7-dihydroxy flavone or pinocebrin. The same compound was reported previously from the same plant (Mativandlela *et al*, 2009).

Compound 2

Compound 2 was identified as (*E*)-2',4'-dihydroxychalcone based on the analysis of the NMR data and comparison with literature. The ¹H NMR spectrum of **2** showed two meta coupled protons at 5.89 and 5.91 (*d* each; *J*=2.1 Hz) and assigned to ring B; four protons at H-3', δ 6.29 (*d*, 2.4 Hz); H-5', δ 6.42 (*dd*, 8.9, 2.4 Hz); δ 7.91 (*m*, 15.0, 7.79 Hz), and δ 8.2 (*d*, 8.9 Hz) assigned for protons 3', 5' and 6' of ring A; and two protons at 7.79 (*dd*, 15.0 Hz, H- α), 8.0 (*d*, 15.0 Hz, H- β). The carbon 13 and DEPT-135 analysis showed 15 carbons classified as 103 (*d*, C-3'); 108.8 (C-5'); 113.5 (*s*, C-1'); 121.8 (*d*, C- α), 129.4 (*dd*, C-2/6'), 129.5 (*dd*, C-3/5); 131.2 (*d*, C-4), 133.7 (*d*, C-6'); 135.1 (*d*, C-1); 144.1 (*d*, C- β), 165.8 (C-2'); 166.3 (*s*, C-4') and 191.9 (C=O). The observed values from ¹H and ¹³C NMR match with the structure ((*E*)-2',4'-dihydroxychalcone. The same compound was reported previously from the same plant (Mativandlela *et al*, 2009; Ticha *et al*, 2014).

Compound 3

Compound 3 was identified as 2(*S*)-2',5,7,-trihydroxyflavanone based on the analysis of the NMR data and comparison with literature. The ¹H NMR spectrum of **3** showed two meta coupled protons at 5.89 and 5.91 (*d* each; *J*=2.1 Hz) and assigned to ring B; four protons at 6.86 (*t*, 7.5 Hz); 6.87 (*d*, 7.4 Hz); 7.19 (*td*, 1.7, 7.5 Hz), and 7.42 (*d*, 7.5 Hz) assigned for protons 3', 5', 4', and 6' of ring A; and two protons at 2.71 (*dd*, 17.2, 3.2 Hz), 3.21 (*dd*, 17.2, 13.1 Hz) and 5.71 (*dd*, 13.1, 3.2 Hz) assigned for protons H-3_{eq}, H-3_{ax} and H-2 of ring C. the carbon 13 and DEPT-135 analysis showed 15 carbons classified as 1 methylene (CH₂) at 41.6 (*t*, C-3); 7 methines (CH) at 74.4 (*d*, C-2); 95.4/96.4 (*d* each, C6/8), 116.0 (*d*, C-3'), 119.6 (*d*, C-5'), 127.5 (*d*, C-6'), 129.9 (*d*, C-4'); 7 fully substituted carbons at 102.2 (*s*, C-10), 125.2 (*s*, C-1'), 154.7 (*s*, C-2'), 163.6/163.9 (*S*, each, C 9/5), 167.1 (*s*, C-7), and 196.8 (*s*, C-4). The observed values from ¹H and ¹³C NMR match with the structure 2(*S*)-2',5,7,-trihydroxyflavanone. The same compound was reported previously from the same plant (Mativandlela *et al*, 2009; Ticha *et al*, 2014).

3.3 Fluconazole susceptibility testing

Figure 13 demonstrates the susceptibility and resistance to FCZ (25 μ g). Susceptibility is shown by *C. dublinensis* with a clear zone of inhibition of 27 mm surrounding the FCZ impregnated disk (Figure 9a), intermediate resistance is demonstrated by *C. luistanae* with the presence of micro-colonies in the susceptibility area (Figure 9b) and resistance is seen by *C. krusei* with complete growth around the FCZ impregnated disk (Figure 9c). FCZ susceptibility was completed on YNBA and incubated at 37°C for 24 h.

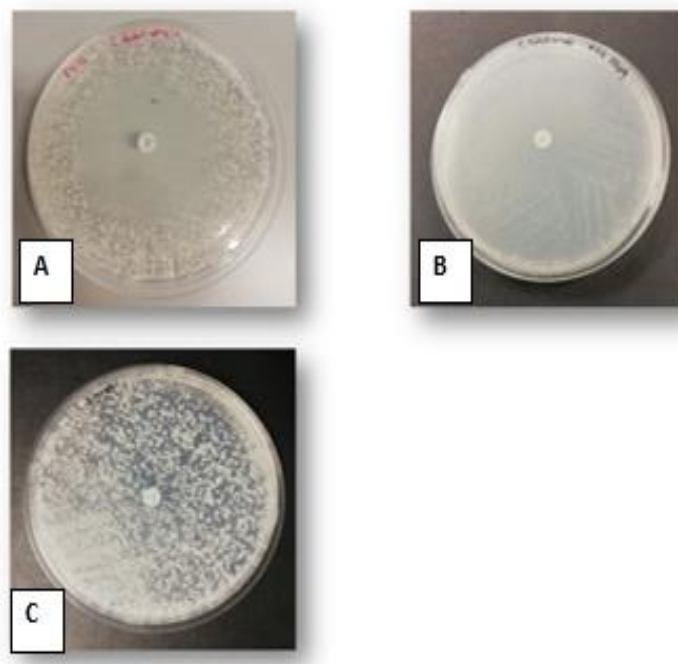


Figure 13: Fluconazole susceptibility completed on yeast nitrogen-based agar for *Candida dublinensis* (A), *Candida luistanae* (B) and *Candida krusei* (C).

Table 2 shows the susceptibility of FCZ (25 µg) on nine type strains of *Candida* using the disk diffusion method. From the table below, it is evident that five out of the nine *Candida* species were susceptible to FCZ at 25 µg and two species showed complete resistance. *Candida parapsilosis* ATCC 22019 presented no zone of inhibition with micro-colonies, which also indicates resistance.

Table 2: The susceptibility of fluconazole (25 µg) against nine type strains of *Candida* using the Kirby Bauer disk diffusion method on YNBA. N= 78.

<i>Candida</i> species	Average zone of inhibition (mm)
<i>C. albicans</i> ATCC 90028	21.67
<i>C. albicans</i> NCPF 3281	23
<i>C. dublinensis</i> NCPF 3949a	27
<i>C. glabrata</i> ATCC 26512	0
<i>C. kefyr</i> ATCC 4135	21
<i>C. krusei</i> ATCC 2159	0
<i>C. lusitanae</i> ATCC 34449	2 with MC
<i>C. parapsilosis</i> ATCC 22019	0 with MC
<i>C. tropicalis</i> ATCC 950	24.3

*0- indication of resistance (overgrowth of species on agar plate)

*MC- indication of micro-colonies present

3.4 Antifungal susceptibility testing

3.4.1 *Galenia africana* ethanolic extract

Figure 14 demonstrates the disk diffusion assay performed on SDA for a susceptible strain (*C. tropicalis*) and resistant strain (*C. lusitanae*) of *Candida*. The numbers observed represent the following concentrations: 1 (100 mg/ml); 2 (50 mg/ml); 3 (25 mg/ml) and 4 (12.5 mg/ml). No zones of inhibition are observed for both species. The purpose of this assay was to evaluate the susceptibility of *G. africana* at concentrations ranging from 100- 12.5 mg/ml. All nine type strains showed no zones of inhibition.

Figure 15 shows the negative controls used for the ethanolic fraction of *G. africana*. The growth of *C. keyfr* in the presence of the RPMI and the ethanol (50%) impregnated disk reveal that both controls had no effect on the growth of the organism. These controls were used for all nine type strains of *Candida*, completed in triplicate and no zone of inhibition was observed.

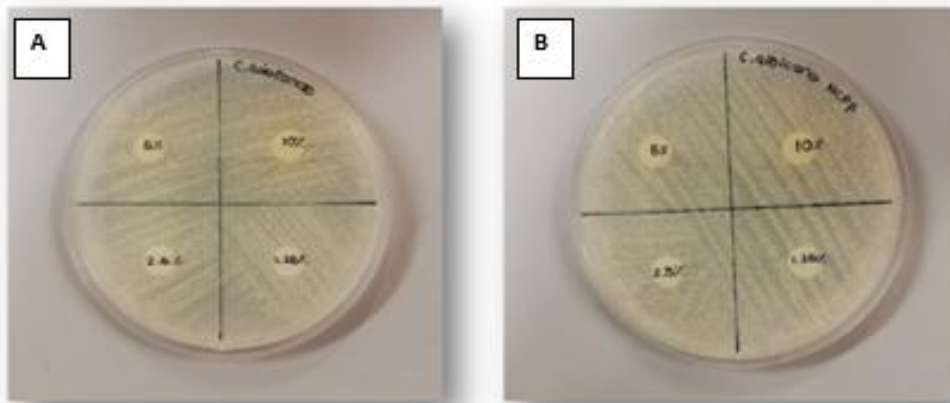


Figure 14: Susceptibility of the ethanolic extract of *Galenia africana* using the disk diffusion method for *Candida albicans* NCPF (A) and *Candida lusitanae* (B).

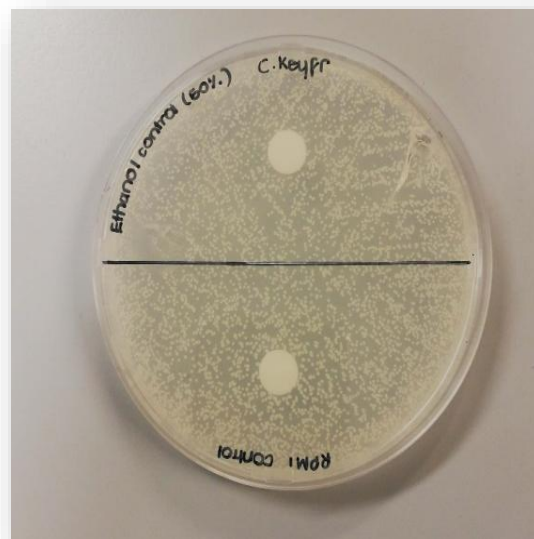


Figure 15: The effect of 50% ethanol and RPMI on the growth of *Candida keyfr*.

3.4.2 *Galenia africana* ethyl acetate extract

Figure 16 demonstrates the resistance of *Candida* species to the ethyl acetate extract of *Galenia africana* using the disk diffusion method. YNBA was used to evaluate the susceptibility of *Candida* species against *G. africana* at 4 concentrations ranging from 100-12.5 mg/ml. In figure 12a and 12b, it is evident that even at the highest concentration of the extract there was no zone of inhibition present for *C. keyfr* and *C. glabrata*. The percentages observed represent the following concentrations: 10% (100 mg/ml); 5% (50 mg/ml); 2.5% (25 mg/ml) and 1.25% (12.5 mg/ml). No zones of inhibition were observed for the nine type strains of *Candida*.

Figure 17 demonstrates the effect of the controls used in the susceptibility of *Candida keyfr* (a) and *Candida glabrata* (b). In figure 12a and 12b there is complete growth of both *Candida* species which indicates that Roswell Park Memorial Institute (RPMI) medium and dimethyl sulfoxide (DMSO) at 20% (v/v) had no effect on the susceptibility of the Candidal species. No zones of inhibition were observed in all *Candida* species.

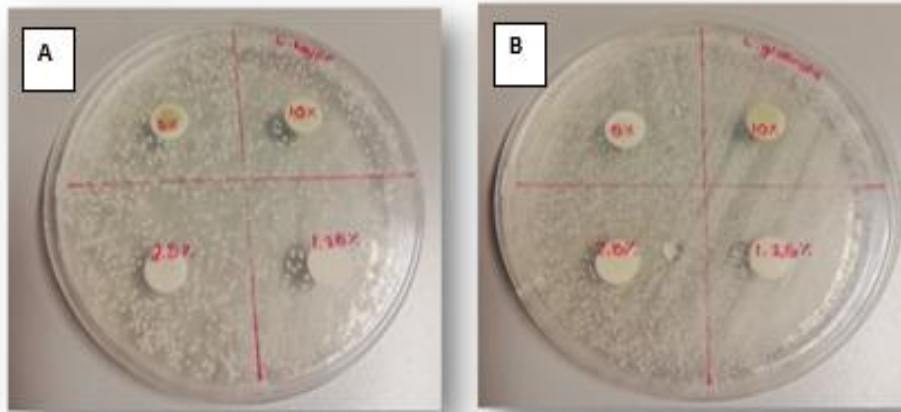


Figure 16: Susceptibility testing *Candida keyfr* (A) and *Candida glabrata* (B) using the ethyl acetate extract of *Galenia africana*

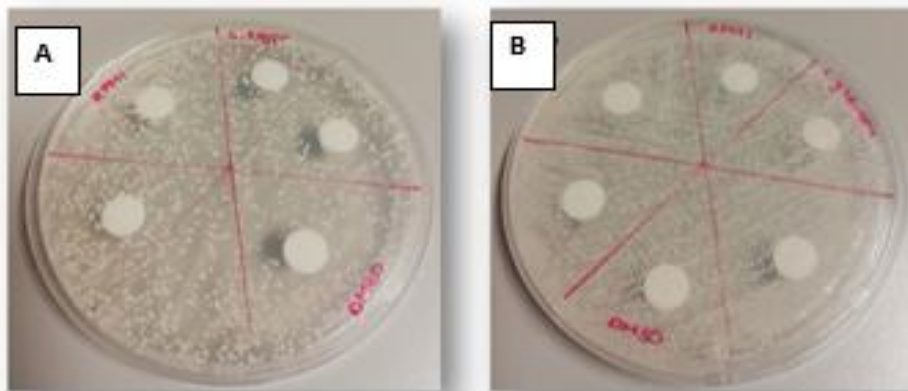


Figure 17: The effect of RPMI and DMSO on the growth of *Candida keyfr* (A) and *Candida glabrata* (B).

Figure 18 demonstrates the MIC of FCZ required to inhibit the growth of *C. tropicalis* (Row A-C) and *C. albicans* ATCC (Row F-H). *Candidal* species were exposed to a FCZ concentration range between 64-0.25 $\mu\text{g/ml}$ then incubated at 37°C for 24 h. Column 12 contains the control wells for both *C. tropicalis* and *C. albicans* ATCC which include; the growth control with 200 μl *Candidal* cells (12A and 12F), sterility control with 200 μl RPMI (12B and 12G) and distilled water (12C and 12H). The bright pink/red colour is seen clearly at the low concentration range, where the MIC of *C. tropicalis* was identified at 2 $\mu\text{g/ml}$ and the MIC of *C. albicans* ATCC was identified at 16 $\mu\text{g/ml}$. All experiments were completed in triplicate and repeated twice.

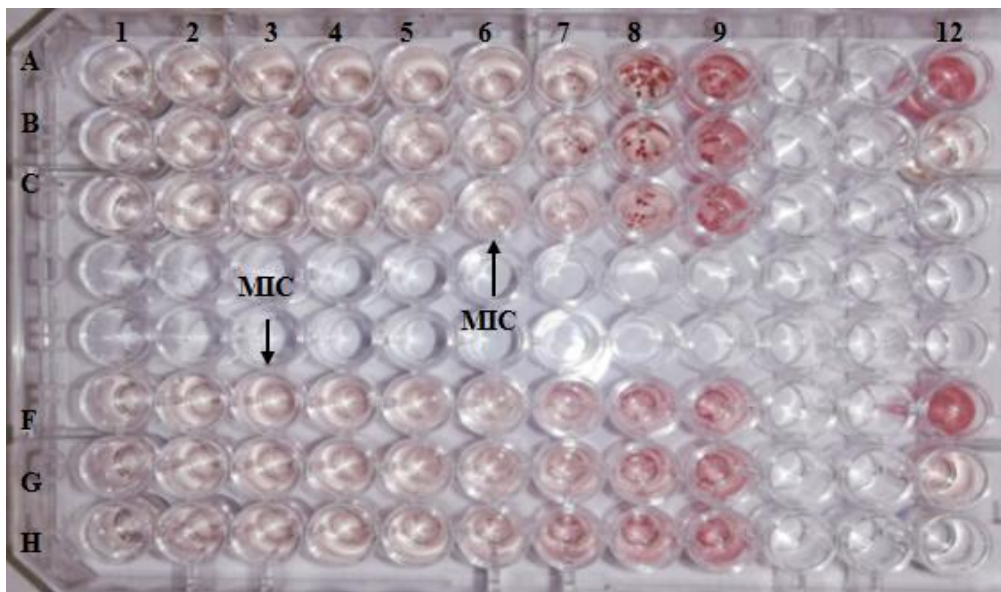


Figure 18: Screening of minimum inhibitory concentration of fluconazole using the broth microdilution method for *Candida tropicalis* (A-C) and *Candida albicans* ATCC (F-H).

Figure 19 demonstrates the MIC FCZ of *C. albicans* NCPF (Row A-C) and *C. dublinensis* (Row F-H). *Candidal* species were exposed to FCZ concentration range between 64-0.25 $\mu\text{g/ml}$ and incubated at 37°C for 24 h. Column 12 contains the control wells for both *C. albicans* NCPF and *C. dublinensis* which include; the growth control with 200 μl *Candidal* cells (12A and 12F), sterility control with 200 μl RPMI (12B and 12G) and distilled water (12C and 12H). To ensure accuracy, 40 μl from each experimental well was inoculated on SDA and the MIC was identified at the lowest concentration with no colony formation. Each assay was completed in triplicate and repeated twice.

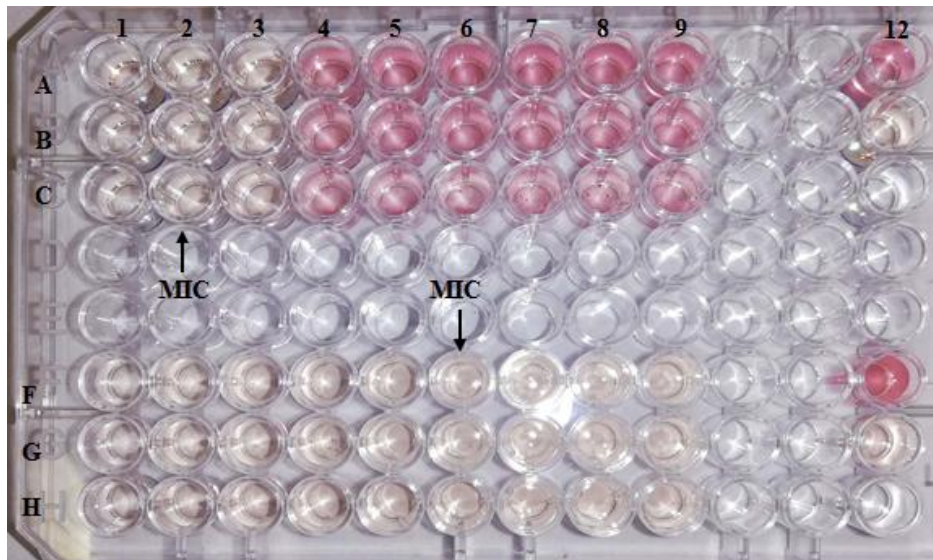


Figure 19: Screening of minimum inhibitory concentration of fluconazole using the broth microdilution method for *Candida albicans* NCPF (A-C) and *Candida dublinensis* (F-H).

Table 3 shows the susceptibility testing of FCZ using the BMD method. The starting concentration of FCZ was 64 µg/ml serially diluted in RPMI to 0.25 µg/ml. The MIC was observed after the INT reagent was added and confirmed after inoculation onto SDA.

Table 3: The minimum inhibitory concentration of fluconazole against 9 type strains of *Candida* using the microdilution method.

<i>Candida</i> species	Fluconazole MIC (µg/ml)
<i>C. albicans</i> ATCC 90028	16
<i>C. albicans</i> NCPF 3281	32
<i>C. dublinensis</i> NCPF 3949a	2
<i>C. glabrata</i> ATCC 26512	64
<i>C. keyfr</i> ATCC 4135	1
<i>C. krusei</i> ATCC 2159	64
<i>C. luistanae</i> ATCC 34449	1
<i>C. parapsilosis</i> ATCC 22019	2
<i>C. tropicalis</i> ATCC 950	1

Fluconazole MIC breakpoint values: susceptibility (S) at a concentration <8 µg/ml, susceptible-dose dependant (S-DD) at 16-32 µg/ml and resistance at ≥ 64 µg/ml (Clinical and Laboratory Standards Institute, 2008).

3.5 Antifungal screening of *Candida* species

3.5.1 Broth microdilution screening on ethanolic extract of *Galenia africana*

Figure 20 demonstrates the MIC of the ethanolic fraction of *G. africana*. In figure 20A, the formation of *Candidal* colonies at 100 mg/ml indicates resistance of *C. keyfr* to ethanolic fraction of *G. africana*. Figure 20B shows no colonies at the highest concentration of *G. africana* which indicates that the MIC is at 100 mg/ml for *C. albicans* NCPF. The control wells for both figure 20A and 20B are positive (12A), and the sterility control used, RMPI (12C) shows no colony formation indicating sterility. FCZ at the concentration of 32 µg/ml (12B) shows no growth for both fungal species which indicates susceptibility to FCZ at 32 µg/ml for both *Candida* species. Each experiment was completed in triplicate and confirmation of the MIC was completed by inoculation of 40 µl on SDA for 24 h at 37 °C. Growth of *Candida* species can be seen by the formation of cream-white colonies at the bottom of the 96-well plate. A resistance pattern can be seen by an increase in the fungal colonies as the concentration of *G. africana* decreases from wells 1-9.

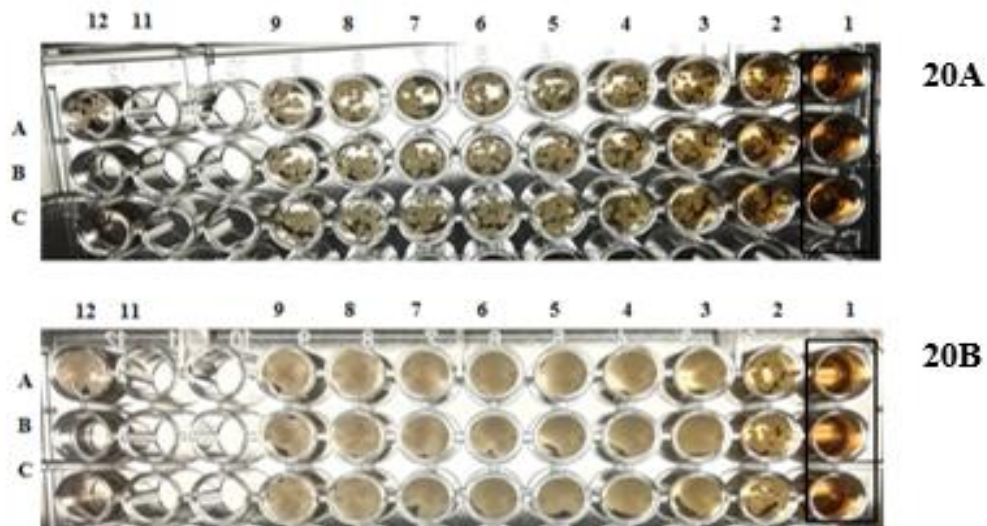


Figure 20: Broth microdilution testing the susceptibility of *Candida keyfr* and *Candida albicans* NCPF using the ethanolic extract of *Galenia africana*

Table 4 summarizes the results of the BMD assays completed in 96 well plates, with a starting concentration of 100 mg/ml of the ethanolic fraction of *Galenia africana*. Seven out of the nine *Candida* species showed a MIC of 100 mg/ml. The more susceptible *Candida* species, *C. albicans* ATCC 90028 and *C. tropicalis* ATCC 950 showed a lower MIC at 50 mg/ml of the ethanolic extract.

Table 4: The minimum inhibitory concentration (MIC) of the ethanolic extract of *Galenia africana* against 9 type strains of *Candida* using the microdilution method.

<i>Candida</i> species	MIC (mg/ml)
<i>C. albicans</i> ATCC 90028	50
<i>C. albicans</i> NCPF 3281	100
<i>C. dublinensis</i> NCPF 3949a	100
<i>C. glabrata</i> ATCC 26512	100
<i>C. keyfr</i> ATCC 4135	100
<i>C. krusei</i> ATCC 2159	100
<i>C. luistanae</i> ATCC 34449	100
<i>C. parapsilosis</i> ATCC 22019	100
<i>C. tropicalis</i> ATCC 950	50

3.5.2 Broth microdilution screening on the ethyl acetate extract of *Galenia africana*

Figure 21 demonstrates the antimicrobial activity of the EtOAc extraction of *G. africana* on *C. albicans* ATCC 90028 (Rows A-C) and *C. albicans* NCPF 3281 (Rows F-H). The EtOAc extraction of *G. africana* was serially diluted in RPMI (columns 2-9) after the stock was prepared (200 mg/ml). The following controls were used for *C. albicans* ATCC 90028 (Row A-C) and *C. albicans* NCPF 3281 (Row F-H): growth control containing 200 μ l *Candida* cells in RPMI (12A and 12F), sterility control- 200 μ l RPMI (Well 12B), positive control- 100 μ l FCZ in 100 μ l cells (Well 12C and 12G), negative control-DMSO at concentration of 20% in RPMI added to 100 μ l cells (Well 12D and 12H) and DMSO at concentration of 10% in RPMI added to 100 μ l cells (Well 12E and 11G). The colorimetric change from yellow to pink indicates the growth of cells, whereas no colour change indicates the MIC value. INT reagent (0.2mg/ml) was used as a confirmatory indication of cell growth.

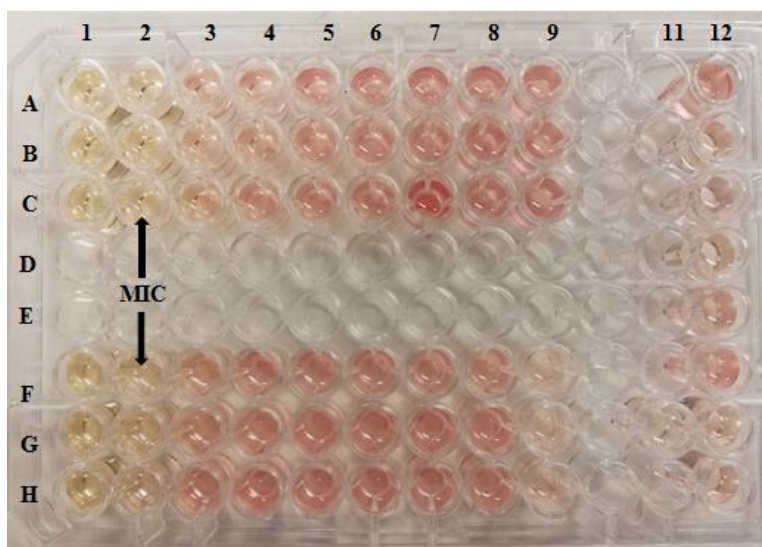


Figure 21: Broth microdilution assay for the ethyl acetate extract of *Galenia africana* showing the minimum inhibitory concentration of *Candida albicans* ATCC 90028 (A-C) and *Candida albicans* NCPF 3281 (F-H)

Figure 22 demonstrates the antimicrobial activity of the ethyl acetate fraction of *G. africana* on *C. glabrata* ATCC 26512 (Rows A-C) and *C. krusei* ATCC 2159 (Rows F-H). The controls used for *C. glabrata* ATCC 26512 and *C. krusei* ATCC 2159 were represented as follows: growth control containing 200 μ l *Candidal* cells in RPMI (Well 12A and 12F), RPMI alone (Well 12B), FCZ at 64 μ g/ml (Well 12C and 12G), DMSO at concentration of 20% in RPMI added to 100 μ l cells (Well 12D and 12H) and DMSO at concentration of 10% in RPMI added to 100 μ l cells (Well 12E and 11G).

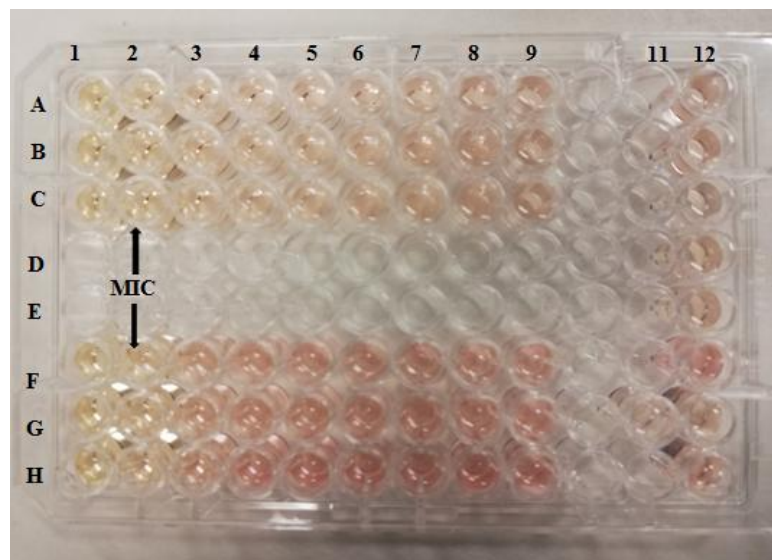


Figure 22: Broth microdilution assay for the ethyl acetate extract of *Galenia africana* showing the minimum inhibitory concentration of *Candida glabrata* (A-C) and *Candida krusei* (F-H).

Table 5: The minimum inhibitory concentration of the ethyl acetate extract of *Galenia africana* against nine type strains of *Candida* using the microdilution method.

<i>Candida</i> species	MIC (mg/ml)
<i>C. albicans</i> ATCC 90028	50
<i>C. albicans</i> NCPF 3281	50
<i>C. dublinensis</i> NCPF 3949a	50
<i>C. glabrata</i> ATCC 26512	50
<i>C. keyfr</i> ATCC 4135	50
<i>C. krusei</i> ATCC 2159	50
<i>C. luistanae</i> ATCC 34449	50
<i>C. parapsilosis</i> ATCC 22019	50
<i>C. tropicalis</i> ATCC 950	50

3. 6 Synergistic activity of *Galenia africana*

3.6.1 Ethanolic extract of *Galenia africana* in combination with fluconazole

Figure 23 demonstrates the synergistic interaction between *G. africana* and FCZ against a susceptible species of *Candida*, *C. albicans* ATCC. On the left, the superior view of the plate is seen next to the inferior view showing the fungal colonies at the base of the plate. Column 1 (Row A-C) shows the combination of MICs for extract extract (50 mg/ml) and FCZ (16 µg/ml). In column 2 (Row A-C) the combination of the ½ MIC and in column 3 (Row A-C) the combination for the ¼ MIC values of *G. africana* and FCZ. Column 1-6 (Rows F-H) shows the serial dilution of FCZ in dH₂O (16 – 0.5 µg/ml) in combination with ½ MIC of *G. africana* (25 mg/ml). Column 7-12 (Row F-H) demonstrates the serial dilution of FCZ (16 – 0.5 µg/ml) in combination with 1/4 MIC of *G. africana* (12.5 mg/ml). In column 12 (superior view), the following controls are used; the growth control in Row A, the negative control RMPI and sterility control dH₂O. Both the negative control and sterility control is clear, showing no contamination and the growth control was positive, by indication of the INT reagent.

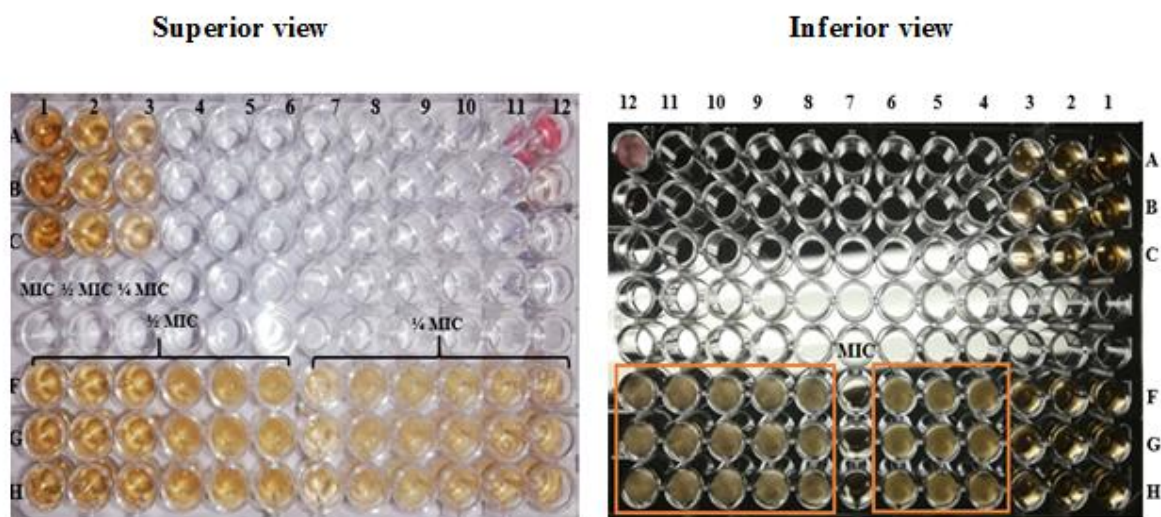


Figure 23: The synergistic activity of the ethanolic extract of *Galenia africana* (50 mg/ml) and fluconazole (16 µg/ml) against *Candida albicans* ATCC.

The synergistic activity of the ethanolic extract of *G. africana* and FCZ was tested against *C. krusei* (Figure 24). At the MIC combination for both *G. africana* and FCZ no biofilm formation and turbidity were seen and confirmation on SDA showed no growth at *G. africana* (100 mg/ml) and FCZ (64 µg/ml). In columns 2-6 (Row F-H) biofilm formation can clearly be seen as well as in columns 8-12 (Rows F-H) which is an indication of complete resistance of *C. krusei*. Columns 1 and 7 (Rows F-H) appear to be clear and no biofilm formation can be seen, this does not indicate a MIC range. The inferior view shows the presence of micro-colonies was seen at the ½ MIC (Column 2, Row A-C) as well as the ¼ MIC (Column 3, Row A-C) combinations. The presence of micro-colonies in column 1 and 7 (Row F-H) indicated by arrows, shows that even at ½ MIC and ¼ MIC of *G. africana* in combination with 64 µg/ml FCZ there was complete resistance shown by *C. krusei*. Fungal colony formation can be seen from columns 2-6 and 8-12 (Row F-H) noted by the orange outline. The MIC combination of *G. africana* (100 mg/ml) and FCZ(64 µg/ml) no growth was seen for *C. krusei*.

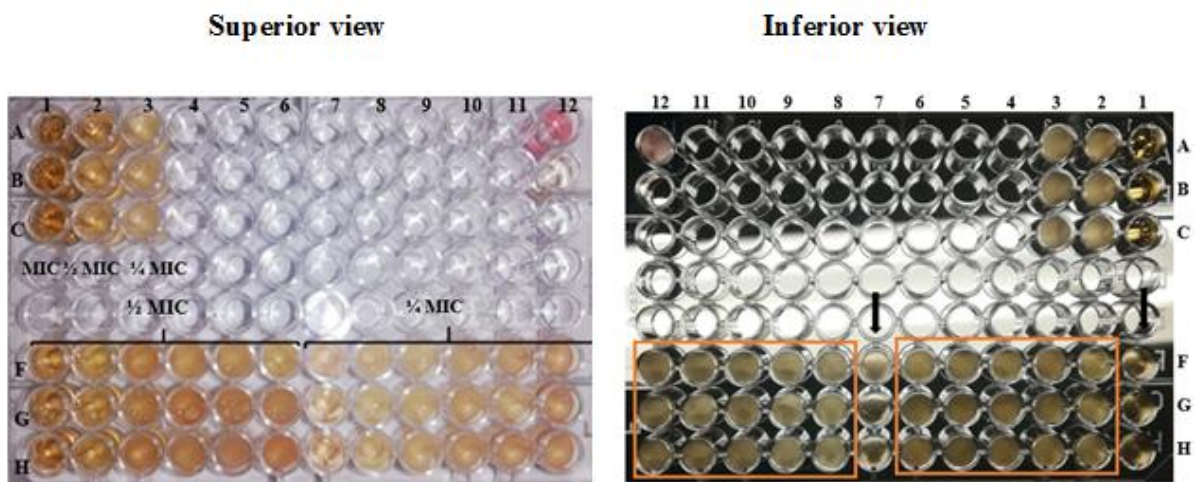


Figure 24: The synergistic interaction of ethanolic extract of *Galenia africana* (100 mg/ml) and fluconazole (64 µg/ml) against *Candida krusei*.

Table 6 demonstrates the BMD method used to test the synergy of the ethanolic fraction of *G. africana* and FCZ. The combination of the extract and antifungal was completed against five type strains of *Candida*, three susceptible to FCZ and two resistant species. In table 6, the MIC of the ethanolic extract alone (MIC E), the MIC for FCZ alone (MIC FCZ), the MIC for the combination of the ethanolic extract and fluconazole (MIC E + FCZ) is shown. *C. krusei* and *C. glabrata* showed no MIC (-) and growth was seen in all wells, indicating that all tested strains were resistant to the concentrations of the extract and antifungal.

Table 6: Synergistic activity of the ethanolic extract of *Galenia africana* and fluconazole against five type strains of *Candida*

<i>Candida</i> species	MIC (E) mg/ml	MIC (FCZ) µg/ml	MIC (E+FCZ) mg/ml (µg/ml)	FIC Index	Outcome
<i>C. albicans</i> ATCC 90028	50	16	12.5 (16)	1.25	Indifference
<i>C. albicans</i> NCPF 3281	100	32	25 (16)	0.75	Indifference
<i>C. tropicalis</i> ATCC 950	50	2	25 (4)	2.5	Indifference
<i>C. krusei</i> ATCC 2159	100	64	-	-	Resistant
<i>C. glabrata</i> ATCC 26512	100	64	-	-	Resistant

FIC index: ≤ 0.5~synergistic; >0.5 but ≤4~ indifference; >4~Antagonistic

3.6.2 Ethyl acetate extract of *Galenia africana* in combination with fluconazole

Figure 25 demonstrates the combination treatment between *G. africana* and FCZ against a S-DD species of *Candida*, *C. albicans* NCPF. In column 1 (Row A-C) the MIC of the ethyl acetate extract of *G. africana* (50 mg/ml) was tested, column 2 (Row A-C) the MIC for FCZ (32 µg/ml) can be observed with a colorimetric change to pink. Column 3 (Row A-C) shows the combination of the MICs for both *G. africana* (50 mg/ml) and FCZ (32 µg/ml). Column 4 (Row A-C) shows the combination of ½ MIC values and column 5 (Row A-C) shows the combinations for the ¼ MIC values. The colorimetric change of the INT reagent indicates growth at the MIC of FCZ alone, the ½ MIC and ¼ MIC combinations. Column 1-6 (Rows F-H) shows the serial dilution of FCZ in dH₂O (32- 1 µg/ml) in combination with ½ MIC of *G. africana* (25 mg/ml). In column 7-12 (Row F-H) the serial dilution of FCZ (32- 1 µg/ml) in combination with ¼ MIC of *G. africana* (12.5 mg/ml) is demonstrated. The MIC is identified with an orange outline in Column 7 (Rows F-H) at 12.5 mg/ml *G. africana* and 32 µg/ml FCZ. In column 12, the following controls are represented; the growth control in (12A), the negative control RMPI (12B) and sterility control dH₂O (12C). Both the negative control and sterility control is clear showing no contamination and the growth control was positive, by indication of the INT reagent.

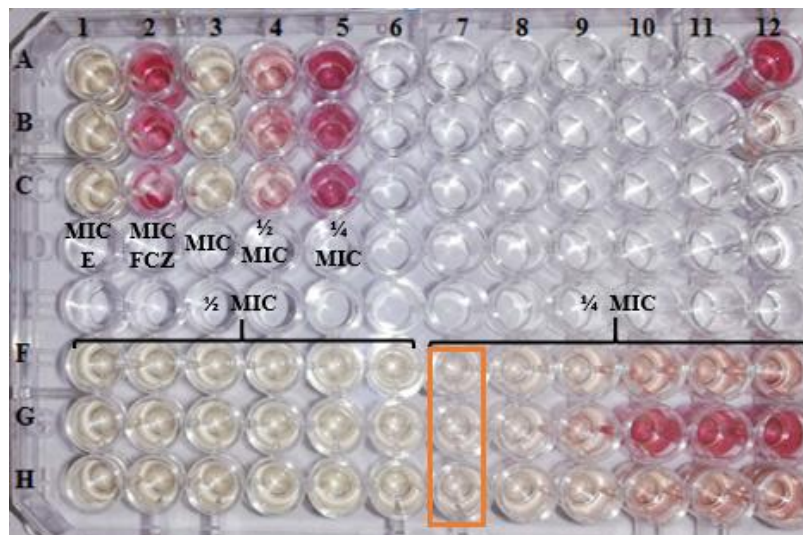


Figure 25: The synergistic interaction of ethyl acetate extract of *Galenia africana* and fluconazole against *Candida albicans* NCPF.

Figure 26 shows the synergistic interaction between *G. africana* and FCZ against a susceptible species of *Candida*, *C. tropicalis*. In column 1 (Row A-C) the MIC of the ethyl acetate extract of *G. africana* (50 mg/ml) is seen. In column 2 (Row A-C) the MIC for FCZ (2 µg/ml) is depicted and in column 3 (Row A-C) the combination of the MICs for both *G. africana* and FCZ. Column 4 (Row A-C) shows the combination of the ½ MIC values for *G. africana* (25 mg/ml) and FCZ (1 µg/ml) and column 5 (Row A-C) shows the combinations for the ¼ MIC values. In column 1-6 (Rows F-H), the serial dilution of FCZ in dH₂O (8- 0.25 µg/ml) in combination with ½ MIC of *G. africana* (25 mg/ml) can be seen. In column 7-12 (Row F-H) the serial dilution of FCZ (8- 0.25 µg/ml) in combination with ¼ MIC of *G. africana* (12.5 mg/ml) is demonstrated. In column 12, the controls are seen in column 12, growth control (12A), the negative control RMPI (12B) and sterility control dH₂O (12C). Both the negative control and sterility control is clear showing no contamination and the growth control was positive, by indication of the INT reagent (0.2mg/ml).

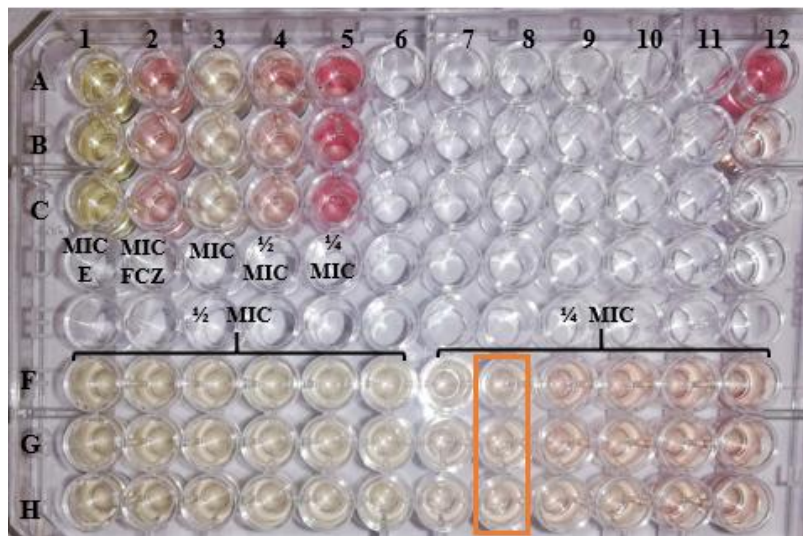


Figure 26: The synergistic interaction of ethyl acetate extract of *Galenia africana* and fluconazole against *Candida tropicalis*.

Table 7 demonstrates the broth microdilution method used to test the synergy of the ethyl acetate fraction of *G. africana* and FCZ. The combination of the extract and antifungal was completed against five type strains of *Candida*, three susceptible to FCZ and two resistant species.

Table 7: Synergistic activity of the ethyl acetate fraction of *Galenia africana* and fluconazole against five type strains of *Candida*.

<i>Candida</i> species	MIC (E) mg/ml	MIC (FCZ) µg/ml	MIC (E+FCZ) mg/ml (µg)	FIC Index	Outcome
<i>C. albicans</i> (ATCC 90028)	50	16	25 (8 µg)	1	Indifference
<i>C. albicans</i> (NCPF 3281)	50	32	12.5 (32)	1.25	Indifference
<i>C. tropicalis</i> (ATCC 950)	50	2	12.5 (4)	2.25	Indifference
<i>C. krusei</i> (ATCC 2159)	50	64	-	-	Resistant
<i>C. glabrata</i> (ATCC 26512)	50	64	-	-	Resistant

FIC index: ≤ 0.5~synergistic; >0.5 but ≤4~ indifference; >4~Antagonistic

C. krusei and *C. glabrata* showed no MIC (-) and fungal growth was seen in all wells therefore the outcome was resistant.

3.7 Time- Kill results

3.7.1 Time-Kill analysis of the ethanolic extract of *Galenia africana* in combination with fluconazole

The time-kill curve plotted against *C. albicans* (ATCC 90028) using the ethanolic extract of *G. africana*, showed that after 3 h a fungicidal effect was observed at both the MIC of *G. africana* and MIC combination with MIC FCZ (E+FCZ). At the MIC FCZ (16 µg/ml) a reduction in cell growth after 3 h incubation followed by a gradual increase in cells from 6 till 24 h was observed. The ½ and ¼ MIC combinations showed a 0.99 and 0.74 Log₁₀ decrease in cell growth after 3 h incubation, followed by a 0.6 and 0.56 Log₁₀ CFU/ml increase in cell growth. This suggests that there was no synergistic activity between *G. africana* and fluconazole at the ½ and ¼ MIC combinations. A plateau in cell growth is seen at the 9 h incubation point followed by steady increase in cell growth from 12 till 24 h.

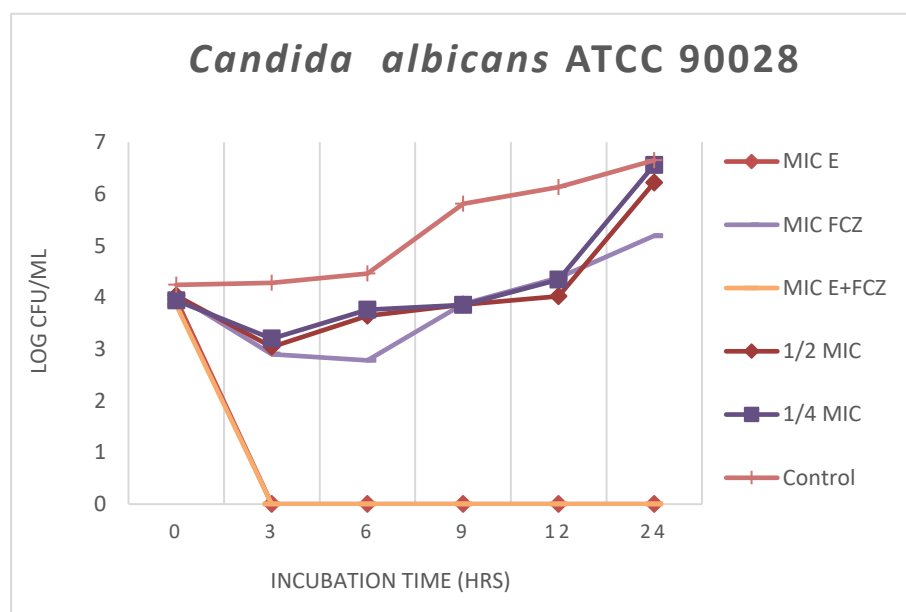


Figure 27: Time-kill curve for the combinations of the ethanolic extract of *Galenia africana* and fluconazole against *Candida albicans* ATCC over a period of 24 h.

The FCZ sensitive, *Candida tropicalis* (ATCC 950) showed a slight plateau in cells seen after 3 h for the MIC FCZ, ½ MIC, ¼ MIC EtOH extraction of *G. africana* and growth control. After 3 h, the fungistatic activity of MIC FCZ (2 µg/ml) was seen by a 0.81 Log₁₀ CFU/ml reduction in cells followed by a gradual increase in growth after 6 h reaching a plateau towards 24 h. Antagonism (> 3Log) was seen at the ½ and ¼ MIC combinations of the EtOH extract of *G. africana* and FCZ with a 3.25 and 3.35 Log₁₀ increase in fungal growth after 24 h (Rodriguez *et al*, 2010). At the MIC FCZ alone, *C. tropicalis* showed indifference with a 1.01 Log₁₀ increase in fungal growth after 24 h. This suggests that all three combinations reacted similarly and no synergistic activity was seen at ½ MIC and ¼ MIC concentrations.

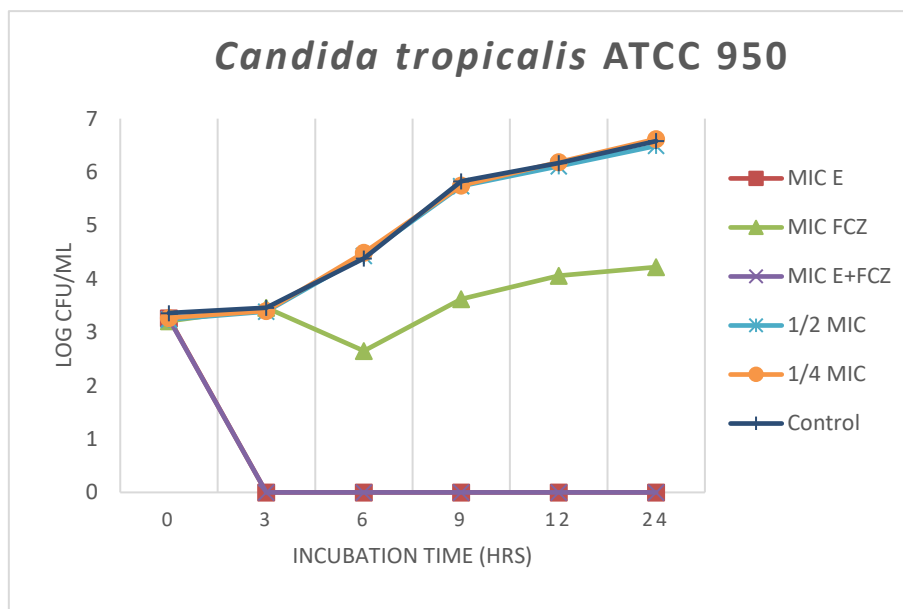


Figure 28: Time-kill curve for the combinations of the ethanolic extract of *Galenia africana* and fluconazole against *Candida tropicalis* (ATCC 950) over a period of 24 h.

The FCZ resistant, *Candida glabrata* (ATCC 26512) showed a 0.24 Log₁₀ reduction in cells seen by the MIC FCZ (64 µg/ml) at the 3 h incubation point, followed by a 1.23 Log₁₀ increase in cell growth at 6 h and a gradual increase in cell growth can be seen till 24 h. The EtOH ½ and ¼ MIC combinations of *G. africana* and FCZ showed a similar growth pattern seen by 0.32 and 0.52 Log₁₀ CFU/ml increase in cells at 3 h incubation point. The ½ and ¼ MIC combinations showed a 4.05 and 4.14 Log₁₀ CFU/ml increase in fungal growth after 24 h which according to Johnson *et al* (2004), is classified as an antagonism (≥3 Log increase in colony count). This confirms no synergistic activity for both the ethanolic ½ and ¼ MIC combinations with FCZ. The growth control shows a steady increase in cells from 0 h till 24 h. The EtOH and EtOAc extract of *G. africana* showed fungicidal activity against *C. glabrata* after 24 h at MIC and MIC + FCZ.

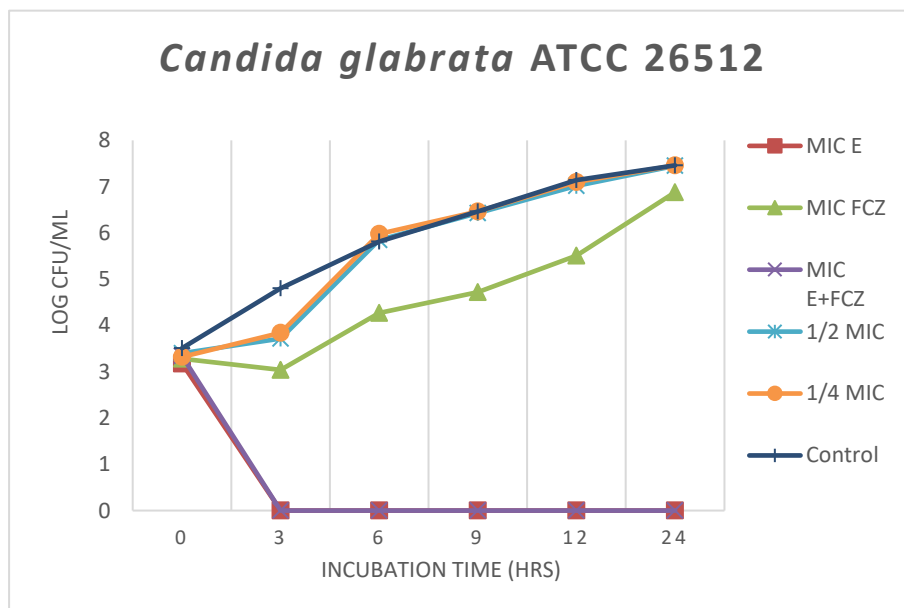


Figure 29: Time-kill curve for the combinations of the ethanolic extract of *Galenia africana* and fluconazole against *Candida glabrata* (ATCC 26512) over a period of 24 h.

The FCZ resistant *Candida krusei* (ATCC 2159) showed a 1.89 Log₁₀ CFU/ml increase in fungal growth after treatment at MIC FCZ (64 µg/ml) after 24 h which confirms the indifference in the result. The ½ and ¼ MIC of EtOH extraction of *G. africana* and FCZ showed a 1.31 Log₁₀ and 1.49 Log₁₀ CFU/ml decrease in cells at 3 h incubation. A plateau is seen from 9 till 12 h after which a peak in fungal growth by 1.75 Log₁₀ CFU/ml at 24 h is observed against the ½ MIC combination. The ¼ MIC combination showed a rapid increase by 1.49 Log₁₀ CFU/ml in fungal growth after 3 h till 9 h followed by a plateau in fungal growth till 12 h. A 0.75 Log₁₀ CFU/ml increase in fungal growth can be seen till 24 h. The ½ and ¼ MIC combinations of *G. africana* and FCZ (MIC E+FCZ) showed a 1.98 and 1.82 Log₁₀ CFU/ml increase in fungal growth after 24 h classifying as indifference with no synergistic effect.

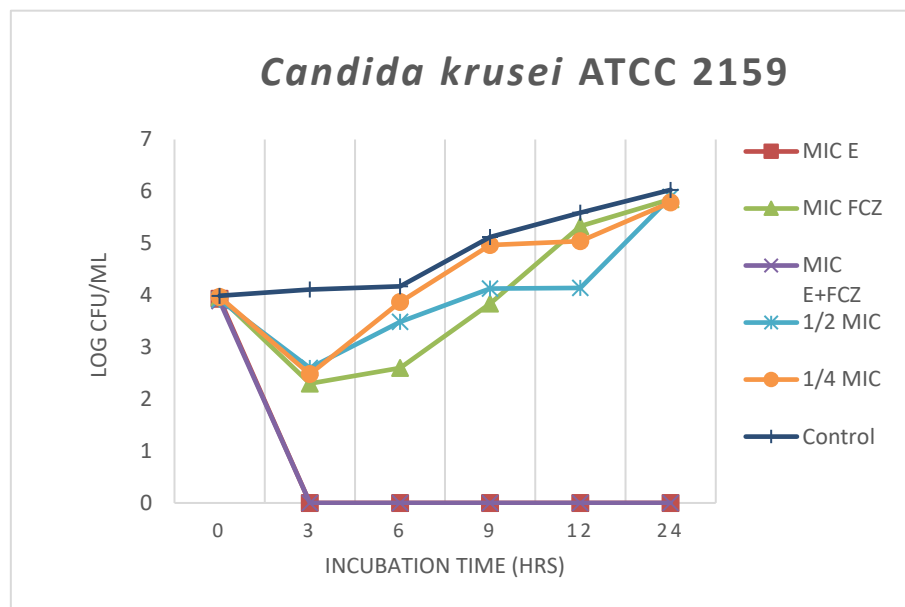


Figure 30: Time-kill curve for the combinations of the ethanolic extract of *Galenia africana* and fluconazole against *Candida krusei* (ATCC 2159) over a period of 24 h.

3.7.2 Time-Kill analysis of the ethyl acetate extract of *Galenia africana* in combination with fluconazole

Figure 27-30 shows the time-kill curves plotted using the ethyl acetate extract of *G. africana* against *C. albicans* (ATCC 90028), FCZ sensitive *Candida tropicalis* (ATCC 950), FCZ resistant *Candida glabrata* (ATCC 26512) and *Candida krusei* (ATCC 2159). The ethyl acetate extract of *G. africana* shows fungicidal activity against each *Candida* species at the MIC after 3 h incubation which was seen with the combination of the MIC for *G. africana* and FCZ (MIC E+FCZ) as well. After 3 h, the ½ MIC and ¼ MIC combinations shows a slight decrease in cells followed by a gradual increase till 24 h. The same can be seen at the MIC for FCZ.

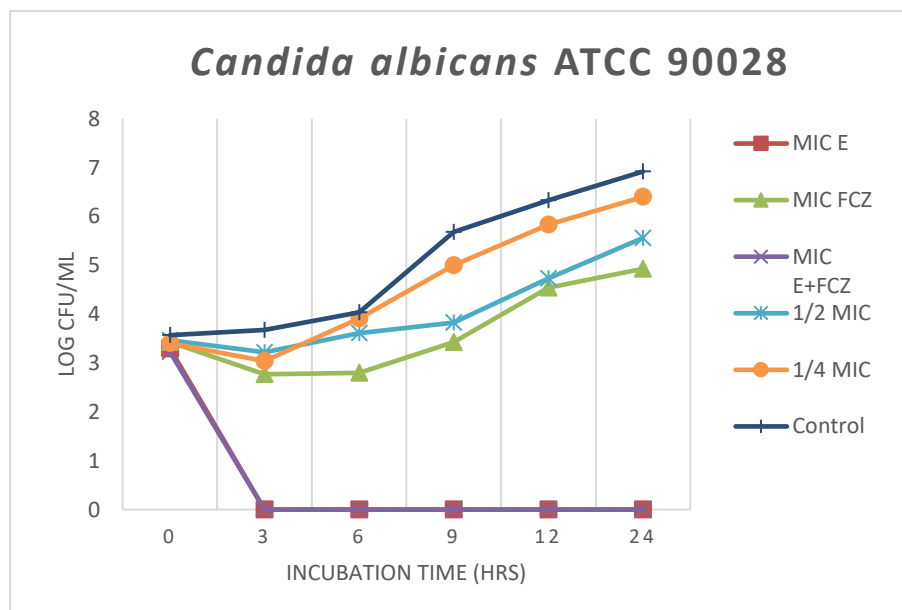


Figure 31: Time-kill curve for the combinations of the ethyl acetate extract of *Galenia africana* and fluconazole against *Candida albicans* (ATCC 90028) over a period of 24 h.

The time-kill curve plotted against *C. tropicalis* (ATCC 950) using the EtOAc extract of *G. africana* showed a 1.2 Log₁₀ reduction at 3 hr incubation for MIC FCZ (2 µg/ml) followed by a steady increase in cell growth towards 24 h. The growth control showed a steady increase in cells after the 0 h incubation point till 6 h. A gradual increase in cells is seen from 6 h till 12 h and the control reaches a plateau towards 24 h. At the ½ and ¼ MIC combinations of the EtOAc extract of *G. africana* and FCZ, a 1.74 and 1.97 Log₁₀ CFU/ml increase in *C. tropicalis* was seen after 24 h. This is classified as indifference (<2 Log increase in cell growth). The negative control, FCZ showed a 0.88 Log₁₀ increase in fungal growth. The growth control for the EtOAc extract showed a 1.82 Log₁₀ CFU/ml increase in cell growth. Analysis of the graph shows that after 3 h of incubation, the MIC *G. africana* (50 mg/ml) and the combination of *G. africana* and FCZ (MIC E+FCZ) there was a fungicidal effect of the EtOAc extract resulting in no growth of *C. tropicalis*. *C. albicans* ATCC and *C. tropicalis* showed a similar growth curve for the ½ and ¼ MIC combinations with the ethanolic extract of *G. africana* and FCZ. There was an initial decrease in cell growth followed by a gradual increase in cell growth after 24 h. The FCZ controls for these susceptible species showed a ≤1.5 Log₁₀ CFU/ml increase in cell growth after 24 h showing indifference.

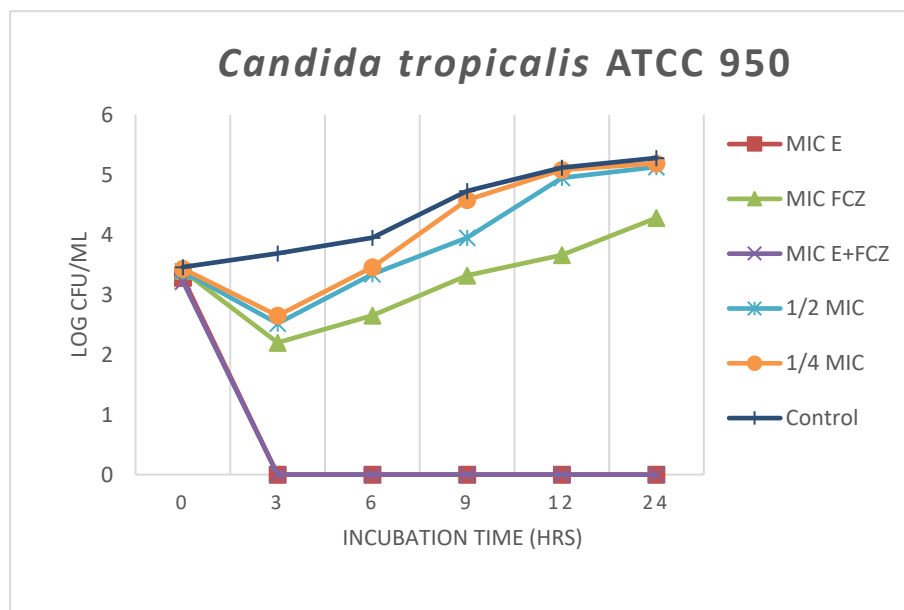


Figure 32: Time-kill curve for the combinations of the ethyl acetate extract of *Galenia africana* and fluconazole against *Candida tropicalis* (ATCC 950) over a period of 24 h.

The time-kill curve plotted against *C. glabrata* (ATCC 26512) using the EtOAc extract of *G. africana* showed a similar growth pattern for the MIC FCZ and ½ MIC combination. After 3 h, a 0.44 and 0.32 Log₁₀ CFU/ml decrease in cell growth was seen, followed by a steady increase of 1.14 and 1.17 Log₁₀ CFU/ml at 6 h. A peak in the cell growth by 0.96 and 0.95 Log₁₀ CFU/ml was seen from 12 till 24 h. The MIC FCZ for the EtOH and EtOAc extract of *G. africana* showed a 3.6 and 2.77 Log₁₀ CFU/ml increase of *C. glabrata*, which is the highest Log₁₀ CFU/ml difference when compared to the other *Candida* species. The ethyl acetate ¼ MIC combination with FCZ showed a 0.29 Log₁₀ CFU/ml decrease in cell growth at 3 h, followed by a 1.74 Log₁₀ CFU/ml peak in growth at 6 h. A gradual increase in cell growth was seen at 6 h till 24 h incubation. The EtOAc extract of *G. africana* showed antagonism at the ½ and ¼ MIC combinations with a 2.99 and 3.11 Log₁₀ CFU/ml increase in *C. glabrata* after 24 h. The growth control showed a steady increase in cell growth by 3.37 Log₁₀ CFU/ml from 0 till 24 h.

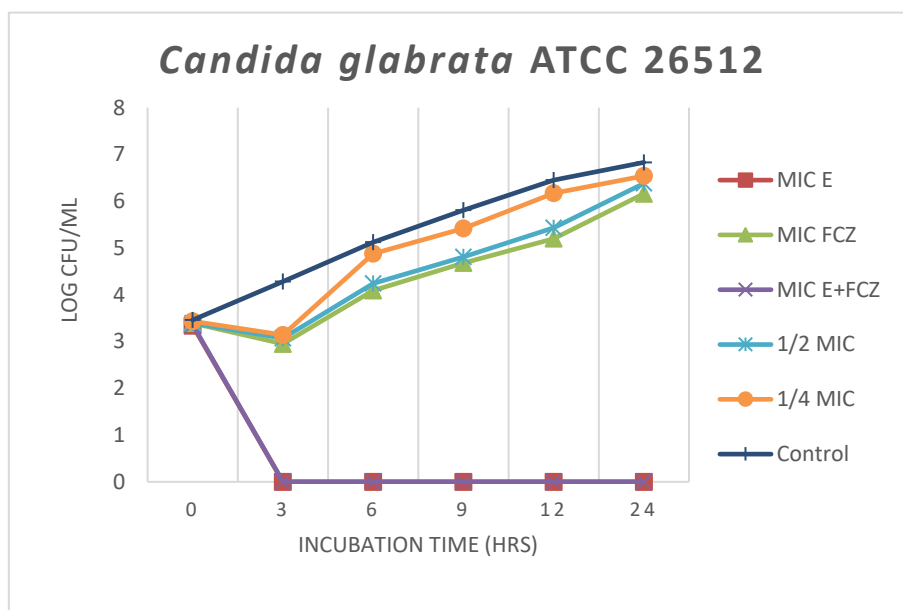


Figure 33: Time-kill curve for the combinations of the ethyl acetate extract of *Galenia africana* and fluconazole against *Candida glabrata* (ATCC 26512) over a period of 24 h.

The time kill curve plotted against *C. krusei* (ATCC 2159) using the EtOAc extract of *G. africana* showed that only 3 h was required to induce a fungicidal effect by *G. africana* at the MIC (50 mg/ml) and combination of MICs with FCZ (50 mg/ml and 64 µg/ml). The MIC FCZ (64 µg/ml) showed a 0.82 Log₁₀ decrease in cells after 3 h, followed by a 0.29 Log₁₀ increase in cells at 6 h. A peak in cell growth can be seen by a 0.89 Log₁₀ increase at 9 h followed by a slight plateau in cell growth at 12 h. After 24 h incubation, a 0.83 Log₁₀ increase in cells is seen with no fungicidal activity. The EtOAc extract showed a similar growth curve for the ½ and ¼ MIC combinations of *G. africana* and FCZ. After 3 h incubation, a 0.21 Log₁₀ decrease in cells was seen for both the ½ and ¼ MIC combinations followed by a 0.42 and 0.43 Log₁₀ increase in cells at 6 h. After 6 h incubation, a gradual increase in the cell growth was seen till 24 h. Indifference was seen at both the EtOAc ½ and ¼ MIC combinations with a 1.56 and 1.57 Log₁₀ CFU/ml increase in *C. glabrata* after 24 h with no synergistic activity. The growth control showed a 1.93 Log₁₀ gradual increase in cell growth from 0 h till 24 h. The FCZ and growth control for the EtOAc extract of *G. africana* showed a 1.4 and 1.93 Log₁₀ CFU/ml increase in cell growth after 24 h.

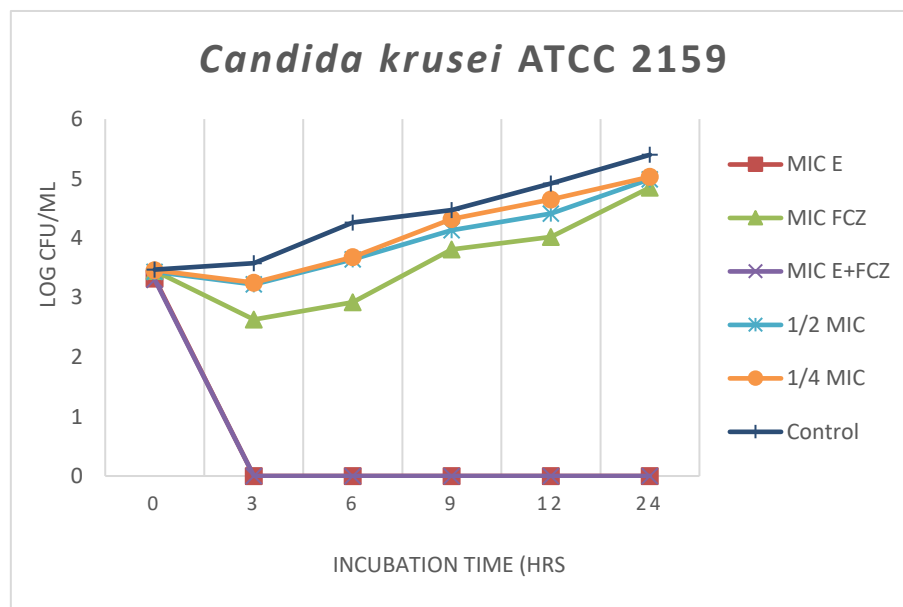


Figure 34: Time-kill curve for the combinations of the ethyl acetate extract of *Galenia africana* and fluconazole against *Candida krusei* (ATCC 2159) over a period of 24 h

3.8 Statistical analysis

A normality test was completed on the raw data (Appendix A) for the time-kill study of *C. albicans* ATCC, *C. glabrata*, *C. krusei* and *C. tropicalis*. The normality test revealed that the data was not normally distributed due to the fungicidal effect of both fractions of *G. africana*. The large discrepancy in the cell counts (CFU/ml) for the different treatment groups was observed therefore, the ANOVA (Analysis of Variance) test could not be used but instead the Kruskal Wallis rank test was suggested. The Kruskal Wallis test was completed for each *Candida* species to determine whether there was a difference between each treatment at the 3 h incubation. This time point was selected because at 3 h incubation, a fungicidal effect of both fractions of *G. africana* was seen at both the MIC of *G. africana* and the MIC combination with FCZ.

Appendix B showed the statistical analysis completed on the time-kill assays for both the ethanolic and ethyl acetate fractions of *Galenia africana*. The Kruskal Wallis rank test was used to determine the significant difference between the different treatments (Chan and Walmsley, 1997). The high CFU/ml difference between the fungicidal treatments of *G. africana* (at MIC alone and in combination MIC FCZ) in comparison with the other treatments for each *Candida* species is largely responsible for the high p-value and chi square value. From these results, it was seen that both the ethanolic and ethyl acetate fractions of *G. africana* showed a chi-square value of $X^2 (5) = 5.00$ for all *Candida* species. This high chi-square value suggests that the different combinations of treatments are independent. The p-value for both fractions was 0.4159 ($P > 0.05$) suggesting that there is no statistical significance between the different treatments (Khodavandi *et al*, 2010). Further statistical analysis was completed by comparing both the ethanolic and ethyl acetate fractions of the *Galenia africana* extract against each *Candida* species. The chi-square probability test revealed a value of $X^2 (11) = 11$ and a p-value of 0.4433 which indicates that there is no statistical difference between the two fractions of *G. africana* for all the *Candida* species.

3.9 MIC evaluation of pure compounds of *Galenia africana*

Figure 35-36 shows the serial dilution of pure compounds 1-3 and 10-5A, with a starting concentration of 500µg/ml (Column 1; A-C; F-H) after cell suspension was added. Each figure was taken after inoculation of INT reagent (0.2mg/ml) and read after 2 hours incubation. The controls used in this experiment can be seen in column 12, A-1:10 cell suspension in RPMI (positive control); B-MIC of FCZ (32µg/ml); C-DMSO (1:50 dilution); D-RPMI and E- Saline. MIC confirmation was completed on SDA and incubated for 24h (Appendix C).

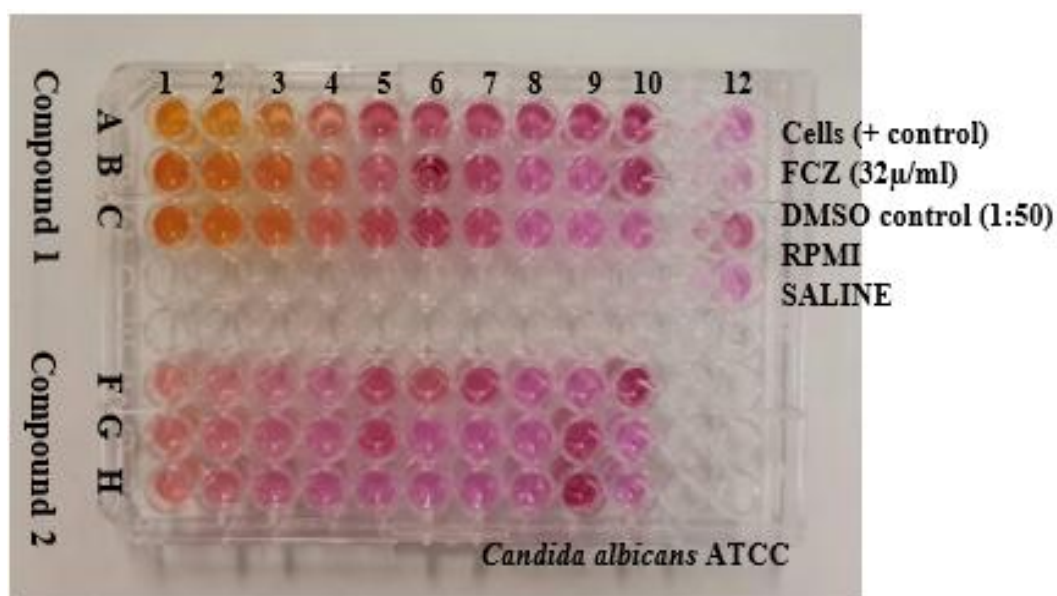


Figure 35: Broth microdilution method used to determine MIC of pure compounds 1 and 2 against *C. albicans* (ATCC 90028)

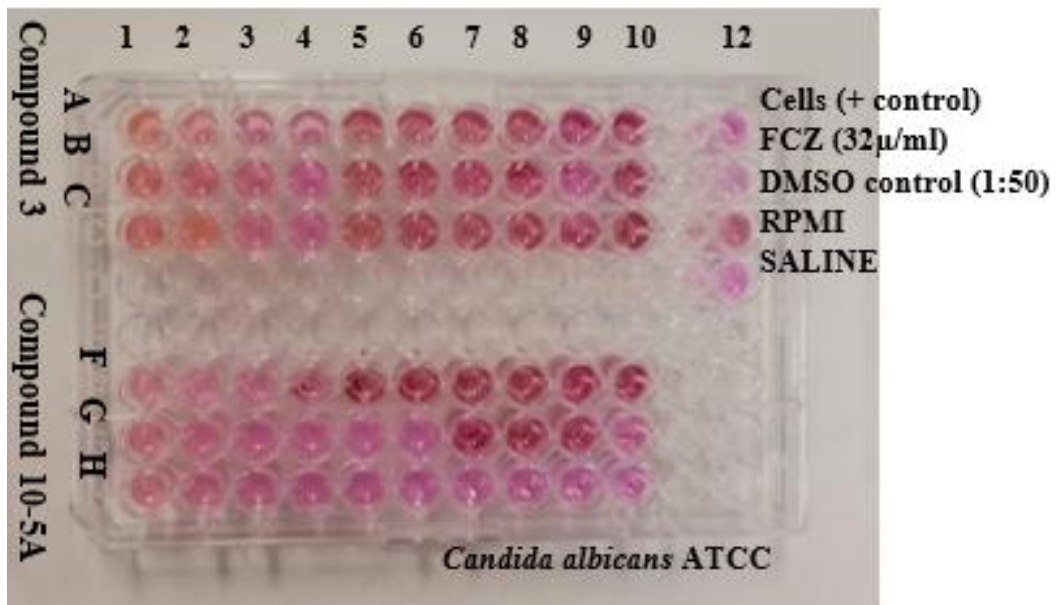


Figure 36: Broth microdilution method used to determine MIC of pure compounds 3 and 10-5A against *C. albicans* (ATCC 90028).

Figure 37-38 shows the serial dilution of pure compounds 1-3 and 10-5A, with a starting concentration of 500 μ g/ml (Column 1; A-C; F-H) after cell suspension was added. Each figure was taken after inoculation of INT reagent and read after 2 hours incubation. The controls used in this experiment can be seen in column 12, A-1:10 cell suspension in RPMI (positive control); B-MIC of FCZ (32 μ g/ml); C-DMSO (1:50 dilution); D-RMPI and E-Saline. MIC confirmation was completed on SDA and incubated for 24h (Appendix D).

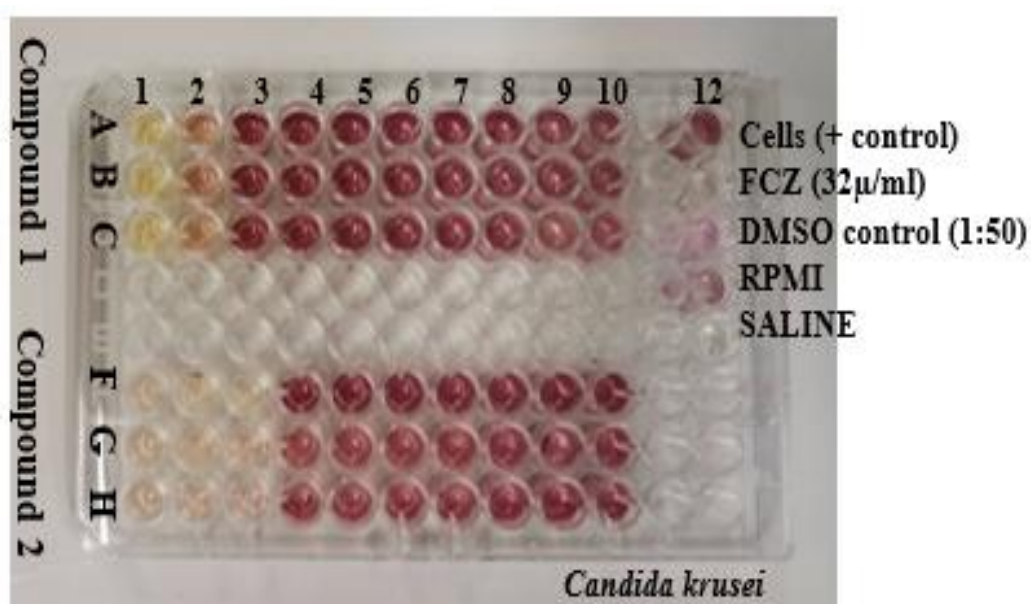


Figure 37: Broth microdilution method used to determine MIC of pure compounds 1 and 2 against *C. krusei* (ATCC 2159)



Figure 38: Broth microdilution method used to determine MIC of pure compounds 3 and 10-5A against *C. krusei* (ATCC 2159)

Table 7 shows the MIC range of 3 pure compounds and a mixture of two compounds (10-5A) isolated from *G. africana* against five species of *Candida*. The antifungal activity of pure compounds 1 and 2 ((*S*)-5,7-dihydroxy flavone (1) and (*E*)-2',4'-dihydroxychalcone (2)) show the lowest MIC range when compared to the overall antifungal effects of the compounds on the five species of *Candida*. All the isolated pure compounds show a MIC \leq 1 mg/ml which shows a significant antifungal activity against the five strains of *Candida* (Mapfumari *et al*, 2022). Pure compound (*S*)-2',5,7,-trihydroxyflavanone (3) and compound mixture (10-5A) shows a MIC range between 250-500 μ g/ml whereas the resistant strain, *C. krusei* showed the highest MIC at 1 mg/ml when treated with compound mixture (10-5A).

Table 7: The minimum inhibitory concentration of pure compounds (1-3) (*S*)-5,7-dihydroxy flavone (1), (*E*)-2',4'-dihydroxychalcone (2), and (*S*)-2',5,7,-trihydroxyflavanone (3) of *G. africana* against 5 species of *Candida*.

<i>Candida</i> Species	Minimum inhibitory concentration of 3 pure compounds and a mixture of compound 4 (10-5A) (μ g/ml)			
	1	2	3	4 (10-5A)
<i>C. albicans</i> (ATCC 90028)	250	62,5	125	500
<i>C. albicans</i> (NCPF 3281)	250	250	125	500
<i>C. krusei</i> (ATCC 2159)	500	62,5	125	1000
<i>C. dublinensis</i> (NCPF 3949a)	250	62,5	125	500
<i>C. tropicalis</i> (ATCC 950)	250	125	125	500

Chapter IV: Discussion

Nature provides our species with an array of plants, many that provide antimicrobial activity through the compounds they produce. For this reason, pharmaceutical companies continue to investigate the mechanism of action associated with natural medicine (Soliman *et al*, 2017). Plants contain diverse natural compounds, the most common group being polyphenols as it exhibits antimicrobial, anticancer and anti-inflammatory mechanisms (Soliman *et al*, 2017). A plants natural defense system involves the production of numerous secondary metabolites which include alkaloids, terpenoids, essential oils and polyphenols (Pang *et al*, 2021). Polyphenols inhibit cell growth by damaging the bacterial cell membrane, compromising the production of targeting the bacterial efflux pumps and the production of inhibiting enzymes (Bubonja-Šonje *et al*, 2020). The development of novel, inexpensive and alternative ways of treating resistant strains of *Candida* has become a well investigated area of research (Soliman *et al*, 2017). The aim of this study was to investigate *G. africana* as an alternative method to treat resistant *Candidal* associated infections. Therefore, the need to determine whether the type of solvents used during the process of fractionation affects the MIC determination and if *G. africana* has an antifungal activity against non-albicans species should be investigated.

4.1 Susceptibility of *Candida* species

KBDD assays were completed using both the EtOH fraction and EtOAc fraction of *Galenia africana* on nine type strains of *Candida*. The susceptibility was tested on SDA, MHA and YNBA. No zones of inhibition were seen for both fractions of *G. africana* with the disk diffusion assays. Due to the consistency of these results, the type of media used did not have a direct effect on the diffusion rate of the extract into the agar. A study completed by Jiang (2011) and Klančnik *et al* (2010) revealed that the disk diffusion assay is not the most reliable in identifying the antimicrobial activity of a natural plant extract. Both studies advised alternative methods of antimicrobial testing such as the agar dilution and broth microdilution method. According to Moreno *et al* (2006), the cause of a slow diffusion rate is dependent on the polarity of the natural substance used and no zone of inhibition does not necessarily indicate that the plant extract has no antimicrobial activity. In other words, the less polar the natural substance is, the slower the rate of diffusion will be.

The negative controls used for the EtOH fraction of *G. africana* were 50% ethanol in dH₂O and RPMI. There was complete growth of all nine type strains of *Candida* in the presence of

50% ethanol and RPMI disks which indicates that both ethanol and RPMI had no effect on the antimicrobial activity of ethanolic extract of *G. africana*. The negative controls for the ethyl acetate fraction of *G. africana* were DMSO and RPMI which showed no zone of inhibition for all nine *Candida* species. The positive control used for susceptibility testing was a 25 µg FCZ disk.

The overall susceptibility of FCZ to all nine type strains of *Candida* is summarized where *C. albicans* ATCC, *C. albicans* NCPF, *C. dublinensis*, *C. kefyr* and *C. tropicalis* showed susceptibility to FCZ. *C. lusitanae* and *C. parapsilosis* showed intermediate resistance with the presence of micro-colonies and *Candida glabrata* and *Candida krusei* showed complete resistance. Due to the unreliability of the KBDD method, the BMD method was completed to observe the MIC for both extractions of *G. africana* and to determine the FCZ breakpoints for the nine type strains of *Candida*.

4.2 Broth microdilution

4.2.1 Fluconazole susceptibility

The MIC of FCZ for each *Candida* species was tested in a concentration range of 64 -0.25 µg/ml. It is evident that the columns before the initial colour change was not the actual MIC, for this reason all wells before the colour change were inoculated onto SDA to confirm the exact MIC. The growth controls for all nine type strains of *Candida* were positive indicating that there were cells present in all the experimental wells. The sterility controls RPMI (used as the diluent for serial dilution) and distilled water (used as a diluent for FCZ) showed no growth which confirms that aseptic techniques were followed. The MIC for *C. tropicalis* was identified at 2 µg/ml of FCZ and the MIC for *C. albicans* ATCC was identified at 16 µg/ml. This confirms that *C. tropicalis* is susceptible and *C. albicans* ATCC is susceptible dose dependant (S-DD) (Clinical and Laboratory Standards Institute, 2008).

The visible colour change seen in the 96-well microtitre plate confirmed the MIC of *C. albicans* NCPF at 32 µg/ml of FCZ. *C. dublinensis* showed no visible colour change in any of the wells but the growth control showed a colour change to red. Due to the variability of the INT results, confirmation of the MIC was completed on SDA. From these results, we can determine that the INT reagent could either have been expired or the number of cells present

at that concentration was not able to reduce the INT reagent releasing the red dye. The MIC of *C. dublinensis* was confirmed at 2 µg/ml of FCZ.

The susceptibility of the nine type strains of *Candida* to FCZ was classified according to the CLSI FCZ breakpoint values (Clinical and Laboratory Standards Institute, 2008). These results revealed that, *C. tropicalis*, *C. dublinensis*, *C. kefyr*, *C. lusitanae* and *C. parapsilosis* were susceptible to FCZ as the MIC was less than 8 µg/ml. *C. albicans* ATCC and *C. albicans* NCPF was S-DD with a MIC between 16 and 32 µg/ml. Lastly, *C. krusei* and *C. glabrata* was classified as resistant to FCZ because the MIC was 64 µg/ml.

These findings highlight the growing need for alternative antifungal agents, particularly against fluconazole-resistant strains such as *C. glabrata* and *C. krusei*. Several studies have reported that medicinal plant extracts possess promising antifungal activity. For instance, Kumar *et al.* (2015) demonstrated that β-asarone from *Acorus calamus* significantly enhanced fluconazole efficacy against *Candida albicans*, with p-values < 0.01 indicating strong synergistic effects. Similarly, Mood *et al.* (2017) reported that isoquinolone derivatives showed statistically significant inhibition (p < 0.05) of fluconazole-resistant *C. albicans* strains. Moreover, Rajkowska *et al.* (2017) found that essential oils from *Thymus vulgaris* and *Syzygium aromaticum* had MICs significantly lower than fluconazole for various *Candida* strains (p < 0.05). These findings support the antifungal potential of natural compounds like chalcones and flavonoids, similar to those found in *Galenia africana*, especially in the treatment of resistant fungal infections. The integration of such plant-based compounds in antifungal therapy could offer a viable alternative or complement to azole treatment in clinical settings.

4.2.2 Ethanolic extract of *Galenia africana*

The antimicrobial activity of *G. africana* was further investigated with a confirmatory assay, the BMD method. *C. kefyr* showed complete resistance to *G. africana* with the presence of microcolonies at a concentration of 100 mg/ml. The MIC of *C. albicans* NCPF can be observed with a clear well at a concentration of 100 mg/ml of *G. africana*. All MICs was confirmed by INT reagent (0.2mg/ml). All growth control wells for the nine type strains of *Candida* were positive, and the sterility control used (RMPI), was clear indicating sterility. The FCZ control used for all type strains was 64 µg/ml. Of the nine type strains of *Candida*; seven type strains had a MIC of 100 mg/ml for the EtOH extract of *G. africana*. *C. albicans*

and *C. tropicalis* were more susceptible to the EtOH extraction of *G. africana* at a lower concentration of 50 mg/ml. This confirms that the polarity of the compounds has an effect on the diffusion rate of *G. africana* and that the BMD method was better at interpreting the overall antimicrobial activity of this extract.

The MIC of *G. africana* is quite high if compared to the recent literature for the breakpoints of natural extracts against fungal species. A recent study has been completed on the toxicity of *G. africana* which could change the interpretation of this. The starting concentration used in the BMD assay was 200 mg/ml which is higher than the standard concentrations used for fungal, antimicrobial and susceptibility testing. According to Van Vuuren (2008), if the MIC values of a plant extract is below 8.0 mg/ml it suggests that the extract has some antimicrobial activity but if the MIC is below 1.0 mg/ml it is a better indication of good antimicrobial activity. The toxicity of *G. africana* tested by Ng'uni *et al* (2018) revealed that the EtOH extract had no toxic *in vivo* effects on female and male Sprague-Dawley rats at concentrations of 300 or 2000 mg/kgbw. The rats were administered by oral gavage once and standard conditions were maintained until day 15 when necropsy was done. The overall toxicity of *G. africana* was assessed by observing the body weight of the rats post administration of the extract and through autopsy. Clinically, the rats were healthy and their behaviour was normal with no abnormalities in their internal organs after the autopsy was completed (Ng'uni *et al*, 2018). In conclusion, the EtOH extraction of *G. africana* has shown antifungal activity and even at such high concentrations of the extract it has proven to be non-toxic to mammalian cells.

4.2.3 Ethyl acetate extract of *Galenia africana*

The antimicrobial activity of the EtOAc extract of *G. africana* was tested using the BMD method. The MIC of susceptible species of *Candida*, *C. albicans* ATCC 90028 and *C. albicans* NCPF 3281 was observed by the lack of colour change by INT reagent as 50 mg/ml. The ability of the organism to reduce the INT reagent indicates that the ethyl acetate fraction of *G. africana* was no longer able to inhibit growth at 2.5% (25 mg/ml), leaving the cells viable for growth (Al-Bayati, 2008). The growth controls for all nine type strains of *Candida* were positive. RPMI alone was used as a sterility control which showed no growth with a lack of colour change. The FCZ control used was at a concentration of 64 µg/ml was negative for all type strains of *Candida* except *C. krusei* and *C. glabrata*. DMSO was used as a

negative control due to its low solubility in an aqueous solution. At the highest concentration of DMSO (10% v/v) in RPMI there was growth of each *Candida* species even though there was a lack of colour change, and this too was confirmed on SDA. Hence, DMSO does not affect the growth or viability of the fungal cells and the concentration of the extract is solely responsible for antimicrobial activity (Silva *et al*, 2017).

The EtOAc extract of *G. africana* was tested against more resistant species of *Candida*, *C. glabrata* ATCC 26512 and *C. krusei* ATCC 2159. The MIC was seen at 50 mg/ml for both *C. glabrata* and *C. krusei*. The growth controls for *C. glabrata* and *C. krusei* were positive, in the case of *C. glabrata* the INT reagent was not as red as with the results for *C. krusei*. The sterility control, RPMI was clear and the negative control, FCZ at 64 µg/ml showed a lack of colour change to pink. The positive control used was DMSO at a concentration of 10% in RPMI and 5% in RPMI. At both concentrations of DMSO, there was complete growth of *C. glabrata* and *C. krusei*.

A summary of BMD assay results using the EtOAc extraction of *G. africana* revealed a lower MIC of 50 mg/ml for all nine type strains of *Candida*. The comparison of the EtOH and EtOAc extract of *G. africana* revealed that the EtOAc extract had a lower MIC than the ethanolic extract. This result could be due to the different solvent systems used during the process of fractionation (Bacon *et al*, 2017; Dhanani *et al*, 2017; Sultana *et al*, 2009), suggesting that the use of EtOAc during fractionation could yield better antimicrobial results as certain active compounds would not be lost during this process (De Zoysa *et al*, 2019).

4.3 Synergistic interaction of *Galenia africana* and fluconazole

A lot of difficulty is seen in determining which method of synergy is most accurate because there is no standardized method to test synergistic activity (Nageeb *et al*, 2015). Various methods can be used to test synergy which are reproducible and interpreted accordingly (Pankey and Ashcraft, 2005). The synergistic effect of the MIC EtOH and EtOAc extract of *G. africana* alone and in combination with FCZ was tested against two susceptible (*C. albicans* ATCC and *C. tropicalis*), one S-DD (*C. albicans* NCPF) and two resistant strains of *Candida* (*C. glabrata* and *C. krusei*). The BMD method was used to identify the MIC of *G. africana* in combination with FCZ (E+FCZ) and classified according to the FIC index.

The five type strains showed no growth at the MIC of *G. africana* and the combination of MICs for *G. africana* and FCZ. This indicates that at the MIC of *G. africana* (EtOH and EtOAc extract) was solely responsible for the fungicidal activity against the *Candida* species. Even though no synergy was seen overall comparing the EtOH and EtOAc extract of *G. africana* in combination with FCZ, the MIC for the combination of EtOAc extract *G. africana* with FCZ showed a lower MIC range for the susceptible strains of *Candida* than the EtOH extract. Evaluation of the FIC indices testing synergism for the susceptible strains of *Candida*, showed an indifference in antifungal activity when exposed to EtOH and EtOAc during extraction of *G. africana* (Table 6 and 7). The resistant species, *C. krusei* and *C. glabrata* also showed resistance to both extract of *G. africana* and FCZ. No synergy was observed at $\frac{1}{2}$ and $\frac{1}{4}$ MIC combinations of EtOH and EtOAc extract of *G. africana* in combination with FCZ as the FIC indices was >0.5 . This suggests that the type of solvent (EtOH and EtOAc) used during the extraction process did not have a major effect on the potency of *G. africana* and that *G. africana* alone, possesses antifungal activity evident from studies completed by Heredia *et al*, 2022; Mativandlela *et al*, 2009; Ticha *et al*, 2014; Vries *et al*, 2005.

In a comparative antifungal study completed by Lestyningrum *et al* (2019), extracted EtOAc mint leaves showed a lower MIC in comparison to the EtOH extract mint leaves. Mint leaves contain alkaloids and saponin, which possess antimicrobial activity and facilitates cell death by reducing the sterol membrane of fungal cells (Sujana *et al*, 2013). The phenolic compounds will often occupy the EtOAc extraction, aiding in a high phenolic antimicrobial activity when compared to other solvent extractions (Yu *et al*, 2002).

4.4 Time-Kill analysis of the ethanolic and ethyl acetate extract of *Galenia africana* in combination with fluconazole

Time kill kinetic assays are used to determine the relationship of a drug/treatment on microbial activity over time. This can be interpreted as the fungistatic or fungicidal interaction of a microorganism in response to the antimicrobial agent (Balouiri *et al*, 2016). The time-kill assay is known as the golden standard to test synergy, this method yields more dynamic and extensive information about bacterial/fungal cell death, which is not provided by other methods testing synergism (Espinoza *et al*, 2020).

In the present study, time-kill curves were plotted against *C. albicans* (ATCC 90028), FCZ sensitive *C. tropicalis* (ATCC 950) and FCZ resistant, *C. glabrata* (ATCC 26512) and *Candida krusei* (ATCC 2159) to determine the synergy of the MIC EtOH and EtOAc extraction of *G. africana* alone and in combination with FCZ (Figures 23-30). The MIC values for the *Candida* spp were confirmed prior by synergism assay confirmation in 96-well plates (Figure 19-22). The current study revealed that only 3 h was required to kill 99.9% of *C. albicans* (ATCC 90028), *C. tropicalis* (ATCC 950) and *C. glabrata* (ATCC 26512) and *C. krusei* (ATCC 2159) at their respective MIC EtOH and EtOAc extraction of *G. africana* alone and in combination with FCZ. At $\frac{1}{2}$ and $\frac{1}{4}$ MIC combinations of EtOH and EtOAc extraction of *G. africana* in combination with FCZ (MIC E+FCZ), fungistatic activity was observed for all 4 type strains of *Candida* as the cell growth gradually increased after 6h. Similarly, the fungistatic activity of FCZ was seen after 6 h of exposure against both resistant and susceptible *Candida* spp, with an initial decrease in cell growth at 3h exposure followed by a gradual increase in growth at 6-24h (Lewis and Graybill, 2008). This suggests that the fungicidal activity is solely due to the antifungal activity of *G. africana* alone and not FCZ, as cell growth continued from 6hr-24h exposure.

In a comparative time kill study completed by John *et al* (2019), the use of K21 compound in combination with FCZ showed an inhibition of FCZ-resistant and susceptible *Candida* after only 2h of exposure. A significant synergistic effect was seen with the combination of K21 with FCZ. Only 2h of exposure was required to kill 99.9% of *C. albicans* (ATCC 90028), *C. albicans* (NCPF 3281), *C. glabrata* (ATCC 26512), *C. krusei* (ATCC 2159) and *C. dubliniensis* (NCPF 3949a) indicating that K21 compound has significant antifungal activity.

A comparative study, investigating *in vitro* susceptibility of *Acinetobacter baumannii* by (Sopirala *et al*, 2010) revealed that the Etest showed a better correlation in synergistic activity with the time kill assay than the checkerboard MTT assay. The limitations of the time-kill method are that it is time consuming, expensive and requires adequate expertise in a clinical laboratory setting (Eliopoulos and Eliopoulos, 1988). The standardization of synergistic techniques according to CLSI for routine antimicrobial testing is required as common azole treatments continue to develop drug-resistant mechanisms (Orhan *et al*, 2005). Future recommendations to optimize synergy testing by investigating the alternative methods of synergy such as Etest and checkerboard assay and correlation with time-kill results (Bidaud *et al*, 2021).

4.5 MIC of compound 1,2,3 and 10.5A

Similar studies completed by Ticha *et al* (2014) and Heredia *et al* (2022) identified the major phytochemical groups in *G. africana* extract, proven to exhibit antifungal activity. These compound groups include aliphatics, aliphatic triterpenoids, fatty acids, flavonoids, and phenolic compounds (Mativandlela *et al*, 2009; Ticha *et al*, 2014). In this study, the isolation of 4 pure compounds in *G. africana* was completed to optimize the MIC of FCZ susceptible and resistant *Candida* species (Table 7). The FCZ susceptible *C. albicans* (ATCC 90028), *C. tropicalis* (ATCC 950), *C. albicans* (NCPF 3281), *C. dublinensis* (NCPF 3949a) and FCZ resistant *C. krusei* (ATCC 2159) showed significant antimicrobial activity with MIC range < 1000µg/ml (Mapfumari *et al*, 2022).

The isolated compounds 2 ((E)-2',4'-dihydroxychalcone) and 3 (2(S)-2',5,7,-trihydroxyflavanone) showed the most significant antimicrobial activity with MIC ranges between 62.5-250 µg/ml. The MIC ranges obtained for compound 1 ((S)-5,7-dihydroxy flavone) and 6 (mixture of 2 compounds) show a higher MIC range between 250-1000 µg/ml, suggesting that these compounds are less potent than (E)-2',4'-dihydroxychalcone) and 2(S)-2',5,7,-trihydroxyflavanone. FCZ resistant *C. krusei* had the highest MIC for ((S)-5,7-dihydroxy flavone) and compound 6 (500 µg/ml and 1000 µg/ml), an indication of moderate antifungal activity (Shakhathreh *et al*, 2016). To maintain the same efficacy of killing in a resistant and a susceptible strain, it is advised to expose the resistant strain to a longer incubation time than to initially increase the concentration of the antifungal (Li *et al*, 2017). Despite the crude EtOH extract and total EtOAc extract of *G. africana* showing antifungal activity at high concentrations of *G. africana* (100mg/ml and 50mg/ml), the further isolation of pure compounds of *G. africana*, (S)-5,7-dihydroxy flavone, (E)-2',4'-dihydroxychalcone and (2(S)-2',5,7,-trihydroxyflavanone) showed a lower antifungal activity < 1000µg/ml. Similar studies show that the most active antifungal compounds within *G. africana* belong to the family of flavonoids and chalcones, (S)-5-hydroxy-7-methoxyflavanone, (2S)-5,7,2'-trihydroxyflavanone, (E)-2',4'-dihydroxychalcone and (E)-2',4'-dihydroxydihydrochalcone (Heredia *et al*, 2022; Mativandlela *et al*, 2009; Ticha *et al*, 2014; Vries *et al*, 2005).

The present study revealed that the isolated compounds of *G. africana* showed significant antifungal activity against FCZ susceptible and resistant *Candida* strains. The isolated compounds (S)-5,7-dihydroxy flavone (1), (E)-2',4'-dihydroxychalcone (2) and 2(S)-2',5,7,-

trihydroxyflavanone (3) of *G. africana* showed significant antifungal activity against FCZ resistant *Candida* species. This suggests that *G. africana* could potentially be used as an antifungal model to treat several FCZ resistant strains of *Candida* and that the use of different solvent systems had no drastic affect the overall fungicidal activity of *G. africana*. The further isolation, characterization and MIC optimization of active compounds within *G. africana* should be investigated as significant antifungal activity was seen below 1mg/ml (Ríos et al, 2005; Gibbons et al, 2004).

4.6 Summary and Conclusion

With the emergence of resistant non-*albicans* species, *C. krusei* and *C. glabrata* the application of common antifungals has become a new challenge (Yan *et al*, 2012). Therefore, a great need to develop novel and new alternative treatments to prevent widespread resistance of antimicrobials is a necessity (Appiah *et al*, 2017). Several studies were completed to determine the antifungal activity of both the ethanolic and ethyl acetate fractions of *G. africana*. Susceptibility testing was completed on nine type strains *Candida* which did not show any antifungal activity against all species for both the ethanolic and ethyl acetate fractions, for this reason the BMD method was completed as a confirmatory assay. All nine type strains were most sensitive to the ethyl acetate fraction of *G. africana* at 50 mg/ml. With the ethanolic fraction, seven of the nine type strains (*C. albicans* NCPF 3281, *C. dublinensis* NCPF 3949a, *C. glabrata* ATCC 26512, *C. keyfr* ATCC 4135, *C. krusei* ATCC 2159, *C. luistanae* ATCC 34449, *C. parapsilosis* ATCC 22019) had a MIC of 100 mg/ml. *Candida albicans* ATCC 90028 and *Candida tropicalis* ATCC 950 showed the most susceptibility against both fractions of *G. africana* at MICs of 50 mg/ml. *C. glabrata* and *C. krusei* proved to be very resistant, whereas *C. albicans* ATCC and *C. tropicalis* showed more sensitivity to *G. africana*.

In this study, the combination of *G. africana* and FCZ showed no synergistic interaction; mostly indifference was seen with the EtOH and EtOAc extractions of *G. africana* and FCZ. *C. albicans* ATCC, *C. albicans* NCPF and *C. tropicalis* classified as indifferent according to the FIC index (>0.5 but ≤ 4). *Candida krusei* and *C. glabrata* showed complete resistance to the combination of *G. africana* (ethanolic and ethyl acetate fraction) and FCZ as cell growth was confirmed by INT reagent at $\frac{1}{2}$ and $\frac{1}{4}$ MIC combinations. The time kill results revealed that only 3 h was required to kill 99.9% of *C. albicans* ATCC, *C. tropicalis*, *C. glabrata* and *C. krusei*. Even at the higher cell density (1:10) required to test synergy studies, *G. africana* showed fungicidal effect against both FCZ sensitive and resistant *Candida* spp. Both the EtOH and EtOAc extract of *G. africana* have shown significant fungicidal activity against the susceptible species of *Candida* at their MICs and combination of MICs with FCZ. This shows that the fungicidal activity is solely as a result of *G. africana* as no synergy was seen with FCZ at $\frac{1}{2}$ and $\frac{1}{4}$ MIC combinations. The isolation of pure compounds (*S*)-5,7-dihydroxy flavone, (*E*)-2',4'-dihydroxychalcone and 2(*S*)-2',5,7,-trihydroxyflavanone (compounds 1-3) showed significant antifungal activity < 1000 $\mu\text{g/ml}$ for *C. albicans* (ATCC 90028), *C. tropicalis* (ATCC 950), *C. albicans* (NCPF 3281) and *C. krusei* (ATCC 2159) (Ríos *et al*, 2005; Gibbons *et al*, 2004).

Traditional knowledge and literature indicate that, *G. africana* can be used to treat an array of health ailments (Watt and Breyer-Brandwijk., 1962) and a few studies have proven that it has antibacterial and antifungal properties (Vries *et al*, 2005; Ticha *et al*, 2014; Mativandlela *et al*, 2009; Mativandlela *et al*, 2008). In conclusion, this study shows that *G. africana* can be used to inhibit the growth of FCZ susceptible and resistant species of *Candida*. Even at higher concentrations of the extract, the toxicology study completed by Nguni *et al* (2018) on the EtOH extract of *G. africana* showed no form of toxicity against the rats even at 2x the concentration used in this study. Future recommendations for the optimization of this study would be to isolate more pure compounds within *G. africana* and to investigate the antifungal activity of pure compounds within *G. africana* in combination with nanoparticles. Another suggestion would be to use these isolated compounds and test its antifungal activity against more resistant strains of *Candida*.

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Appendix A

Time- kill assay testing the synergy of the ethanolic fraction of *G. africana* and fluconazole against *Candida albicans* ATCC

Ethanolic Fraction						
Time (hours)	MIC E	MIC FCZ	MIC E+FCZ	1/2 MIC	1/4 MIC	Control
0	9200	10900	7100	10800	8700	17400
3	0	800	0	1100	1600	18900
6	0	600	0	4400	5700	29100
9	0	7500	0	7200	7100	223000
12	0	23900	0	10500	21700	136000
24	0	158000	0	1730000	1670000	1890000

Time- kill assay testing the synergy of the ethyl acetate fraction of *G. africana* and fluconazole against *Candida albicans* ATCC

Ethyl Acetate Fraction						
Time (hours)	MIC E	MIC FCZ	MIC E+FCZ	1/2 MIC	1/4 MIC	Control
0	1990	2720	1700	2960	2580	940
3	0	590	0	2390	1100	200
6	0	630	0	6900	8100	1100
9	0	2700	0	4100	102000	480000
12	0	43000	0	9700	680000	2140000
24	0	35000	0	54000	950000	8400000

The fungicidal activity of the ethanolic fraction of *G. africana* against *Candida albicans* ATCC at 3 hours

EF_Treatment	Cell growth
MIC E	0
MIC FCZ	200
MIC E+FCZ	0
1/2 MIC	400
1/4 MIC	300
Control	13500

The fungicidal activity of the ethyl acetate fraction of *G. africana* against *Candida albicans* ATCC at 3 hours

EAF_Treatment	Cell growth
MIC E	0
MIC FCZ	430
MIC E+FCZ	0
1/2 MIC	1660
1/4 MIC	1780
Control	3800

Time- kill assay testing the synergy of the ethanolic fraction of *G. africana* and fluconazole against *Candida tropicalis*

Ethanolic Fraction						
Time (hours)	MIC E	MIC FCZ	MIC E+FCZ	1/2 MIC	1/4 MIC	Control
0	1800	1620	1780	1730	1880	2270
3	0	2910	0	2470	2520	2860
6	0	450	0	26800	31200	23900
9	0	4200	0	550000	560000	680000
12	0	11600	0	1280000	1540000	1480000
24	0	16500	0	3100000	4200000	3800000

Time- kill assay testing the synergy of the ethyl acetate fraction of *G. africana* and fluconazole against *Candida tropicalis*

Ethyl Acetate Fraction						
Time (hours)	MIC E	MIC FCZ	MIC E+FCZ	1/2 MIC	1/4 MIC	Control
0	18900	25400	16700	24800	26900	28600
3	0	160	0	3300	4500	4900
6	0	450	0	2200	2900	8900
9	0	2100	0	14800	38000	54000
12	0	4600	0	91000	121000	132000
24	0	18900	0	134000	256000	189000

The fungicidal activity of the ethanolic fraction of *G. africana* against *Candida tropicalis* at 3 hours

EF_Treatment	Cell growth
MIC E	0
MIC FCZ	2910
MIC E+FCZ	0
1/2 MIC	2470
1/4 MIC	2520
Control	2860

The fungicidal activity of the ethyl acetate fraction of *G. africana* against *Candida tropicalis* at 3 hours

EAF_Treatment	Cell growth
MIC E	0
MIC FCZ	160
MIC E+FCZ	0
1/2 MIC	3300
1/4 MIC	4500
Control	4900

Time- kill assay testing the synergy of the ethanolic fraction of *G. africana* and fluconazole against *Candida glabrata*

Ethanolic Fraction						
Time (hours)	MIC E	MIC FCZ	MIC E+FCZ	1/2 MIC	1/4 MIC	Control
0	1500	1900	2200	2500	2100	3200
3	0	1100	0	5300	6900	62000
6	0	18700	0	710000	960000	640000
9	0	53000	0	2680000	2870000	2900000
12	0	320000	0	10500000	12700000	13800000
24	0	1760000	0	28300000	28900000	28600000

Time- kill assay testing the synergy of the ethyl acetate fraction of *G. africana* and fluconazole against *Candida glabrata*

Ethyl Acetate Fraction						
Time (hours)	MIC E	MIC FCZ	MIC E+FCZ	1/2 MIC	1/4 MIC	Control
0	2210	2450	2300	2530	2680	2870
3	0	890	0	1260	1370	19000
6	0	12400	0	17300	76000	132000
9	0	48000	0	65000	260000	640000
12	0	157000	0	267000	1470000	2790000
24	0	1440000	0	2400000	3500000	6800000

The fungicidal activity of the ethanolic fraction of *G. africana* against *Candida glabrata* at 3 hours

EF_Treatment	Cell growth
MIC E	0
MIC FCZ	200
MIC E+FCZ	0
1/2 MIC	400
1/4 MIC	300
Control	13500

The fungicidal activity of the ethyl acetate fraction of *G. africana* against *Candida glabrata* at 3 hours

EAF_Treatment	Cell growth
MIC E	0
MIC FCZ	890
MIC E+FCZ	0
1/2 MIC	1260
1/4 MIC	1370
Control	19000

Time- kill assay testing the synergy of the ethanolic fraction of *G. africana* and fluconazole against *Candida krusei*

Ethanolic Fraction						
Time (hours)	MIC E	MIC FCZ	MIC E+FCZ	1/2 MIC	1/4 MIC	Control
0	8600	9200	7900	8300	9400	11200
3	0	200	0	400	300	13500
6	0	400	0	3100	7400	14900
9	0	6900	0	13600	94000	132000
12	0	216000	0	44000	109000	390000
24	0	710000	0	780000	620000	1080000

Time- kill assay testing the synergy of the ethyl acetate fraction of *G. africana* and fluconazole against *Candida krusei*

Ethyl Acetate Fraction						
Time (hours)	MIC E	MIC FCZ	MIC E+FCZ	1/2 MIC	1/4 MIC	Control
0	2110	2830	1990	2680	2960	2980
3	0	430	0	1660	1780	3800
6	0	840	0	4400	4800	18100
9	0	6500	0	13500	13600	29400
12	0	10500	0	26000	45000	84000
24	0	71000	0	98000	106000	253000

The fungicidal activity of the ethanolic fraction of *G. africana* against *Candida krusei* at 3 hours

EF_Treatment	Cell growth
MIC E	0
MIC FCZ	200
MIC E+FCZ	0
1/2 MIC	400
1/4 MIC	300
Control	13500

The fungicidal activity of the ethyl acetate fraction of *G. africana* against *Candida krusei* at 3 hours

EAF_Treatment	Cell growth
MIC E	0
MIC FCZ	430
MIC E+FCZ	0
1/2 MIC	1660
1/4 MIC	1780
Control	3800

Appendix B

Statistical analysis

The following tables show the statistical analysis of the time kill assay for each *Candida* species. Both the ethanolic and ethyl acetate fractions of *Galenia africana* was analysed using the Kruskal Wallis rank test. The chi-square probability test (X^2) and a p-value were used to determine whether there is a significant difference between the treatment groups or if these groups are dependant.

The Kruskal- Wallis rank test completed on *C. tropicalis* using the ethanolic fraction of *G.africana*

Kruskal-wallis equality-of-populations rank test

treatmen~01	Obs	Rank Sum
EFA_1/2MIC	1	4.00
EFA_1/4MIC	1	5.00
EFA_CONTROL	1	6.00
EFA_MICE	1	1.50
EFA_MICEFCZ	1	1.50
EFA_MICFCZ	1	3.00

chi-squared = 4.857 with 5 d.f.
probability = 0.4336

chi-squared with ties = 5.000 with 5 d.f.
probability = 0.4159

The Kruskal- Wallis rank test completed on *C. tropicalis* using the ethyl acetate fraction of *G.africana*

Kruskal-wallis equality-of-populations rank test

treatm~t	Obs	Rank Sum
1/2 MIC	1	4.00
1/4 MIC	1	6.00
Control	1	5.00
MICE	1	1.50
MICE+FCZ	1	1.50
MICFCZ	1	3.00

chi-squared = 4.857 with 5 d.f.
probability = 0.4336

chi-squared with ties = 5.000 with 5 d.f.
probability = 0.4159

The Kruskal Wallis test was completed by comparing both the ethanolic and ethyl acetate fractions of the *G. africana* extract against *C. tropicalis*.

Kruskal-wallis equality-of-populations rank test

treatmen~02	Obs	Rank Sum
1/2 MIC	1	10.00
1/4 MIC	1	12.00
Control	1	11.00
EFA_1/2MIC	1	6.00
EFA_1/4MIC	1	7.00
EFA_CONTROL	1	8.00
EFA_MICE	1	2.50
EFA_MICEFCZ	1	2.50
EFA_MICFCZ	1	5.00
MICE	1	2.50
MICE+FCZ	1	2.50
MICFCZ	1	9.00

chi-squared = 10.615 with 11 d.f.
probability = 0.4760

chi-squared with ties = 11.000 with 11 d.f.
probability = 0.4433

Kruskal- Wallis rank test completed on *C. albicans* using the ethanolic fraction of *G. africana*.

. kwallis EF_Cellgrowthca , by(Treatment_ca)
Kruskal-wallis equality-of-populations rank test

Treatme~a	Obs	Rank Sum
1/2 MIC	1	4.00
1/4 MIC	1	5.00
Control	1	6.00
MIC E	1	1.50
MIC E+FCZ	1	1.50
MIC FCZ	1	3.00

chi-squared = 4.857 with 5 d.f.
probability = 0.4336

chi-squared with ties = 5.000 with 5 d.f.
probability = 0.4159

Kruskal- Wallis rank test completed on *C. albicans* using the ethyl acetate fraction of *G. africana*.

```
. kwallis EAF_Cellgrowthca , by( Treatment_ca )
Kruskal-wallis equality-of-populations rank test
```

Treatme~a	Obs	Rank Sum
1/2 MIC	1	6.00
1/4 MIC	1	5.00
Control	1	3.00
MIC E	1	1.50
MIC E+FCZ	1	1.50
MIC FCZ	1	4.00

```
chi-squared = 4.857 with 5 d.f.
probability = 0.4336

chi-squared with ties = 5.000 with 5 d.f.
probability = 0.4159
```

The Kruskal Wallis test was completed by comparing both the ethanolic and ethyl acetate fractions of the *G. africana* extract against *C. albicans*.

```
. kwallis EF_Cellgrowthca_01 , by( Treatment_ca_01 )
Kruskal-wallis equality-of-populations rank test
```

Treatmen~01	Obs	Rank Sum
1/2 MIC	1	8.50
1/2 MICEAF	1	11.00
1/4 MIC	1	10.00
1/4 MICEAF	1	8.50
Control	1	12.00
ControlEAF	1	5.00
MIC E	1	2.50
MIC E+FCZ	1	2.50
MIC EAF	1	2.50
MIC EAF+FCZ	1	2.50
MIC FCZ	1	7.00
MIC FCZ EAF	1	6.00

```
chi-squared = 10.577 with 11 d.f.
probability = 0.4794

chi-squared with ties = 11.000 with 11 d.f.
probability = 0.4433
```

Kruskal- Wallis rank test completed on *C. glabrata* using the ethanolic fraction of *G. africana*.

. kwallis EF_Cellgrowthcg , by(Treatment_cg)

Kruskal-wallis equality-of-populations rank test

Treatme~g	Obs	Rank Sum
1/2 MIC	1	4.00
1/4 MIC	1	5.00
Control	1	6.00
MIC E	1	1.50
MIC E+FCZ	1	1.50
MIC FCZ	1	3.00

chi-squared = 4.857 with 5 d.f.
probability = 0.4336

chi-squared with ties = 5.000 with 5 d.f.
probability = 0.4159

Kruskal- Wallis rank test completed on *C. albicans* using the ethyl acetate fraction of *G. africana*.

. kwallis EAF_Cellgrowthcg , by(Treatment_cg)

Kruskal-wallis equality-of-populations rank test

Treatme~g	Obs	Rank Sum
1/2 MIC	1	4.00
1/4 MIC	1	5.00
Control	1	6.00
MIC E	1	1.50
MIC E+FCZ	1	1.50
MIC FCZ	1	3.00

chi-squared = 4.857 with 5 d.f.
probability = 0.4336

chi-squared with ties = 5.000 with 5 d.f.
probability = 0.4159

The Kruskal Wallis test was completed by comparing both the ethanolic and ethyl acetate fractions of the *G. africana* extract against *C. glabrata*.

```
. kwallis EF_Cellgrowthcg_01 , by( Treatment_ca_01 )
Kruskal-wallis equality-of-populations rank test
```

Treatment~01	Obs	Rank Sum
1/2 MIC	1	9.00
1/2 MICEAF	1	7.00
1/4 MIC	1	10.00
1/4 MICEAF	1	8.00
Control	1	12.00
ControlEAF	1	11.00
MIC E	1	2.50
MIC E+FCZ	1	2.50
MIC EAF	1	2.50
MIC EAF+FCZ	1	2.50
MIC FCZ	1	6.00
MIC FCZ EAF	1	5.00

```
chi-squared = 10.615 with 11 d.f.
probability = 0.4760
```

```
chi-squared with ties = 11.000 with 11 d.f.
probability = 0.4433
```

Kruskal- Wallis rank test completed on *C. krusei* using the ethanolic fraction of *G. africana*.

```
. kwallis EAF_Cellgrowthck , by( Treatment_ck )
Kruskal-wallis equality-of-populations rank test
```

Treatment~k	Obs	Rank Sum
1/2 MIC	1	4.00
1/4 MIC	1	5.00
Control	1	6.00
MIC E	1	1.50
MIC E+FCZ	1	1.50
MIC FCZ	1	3.00

```
chi-squared = 4.857 with 5 d.f.
probability = 0.4336
```

```
chi-squared with ties = 5.000 with 5 d.f.
probability = 0.4159
```

Kruskal- Wallis rank test completed on *C. krusei* using the ethyl acetate fraction of *G. africana*.

. kwallis EF_Cellgrowthck , by(Treatment_ck)

Kruskal-wallis equality-of-populations rank test

Treatment	Obs	Rank Sum
1/2 MIC	1	5.00
1/4 MIC	1	4.00
Control	1	6.00
MIC E	1	1.50
MIC E+FCZ	1	1.50
MIC FCZ	1	3.00

chi-squared = 4.857 with 5 d.f.
probability = 0.4336

chi-squared with ties = 5.000 with 5 d.f.
probability = 0.4159

The Kruskal Wallis test was completed by comparing both the ethanolic and ethyl acetate fractions of the *G. africana* extract against *C. krusei*.

. kwallis EF_Cellgrowthck_01 , by(Treatment_ca_01)

Kruskal-wallis equality-of-populations rank test

Treatment	Obs	Rank Sum
1/2 MIC	1	7.00
1/2 MICEAF	1	9.00
1/4 MIC	1	6.00
1/4 MICEAF	1	10.00
Control	1	12.00
ControlEAF	1	11.00
MIC E	1	2.50
MIC E+FCZ	1	2.50
MIC EAF	1	2.50
MIC EAF+FCZ	1	2.50
MIC FCZ	1	5.00
MIC FCZEAF	1	8.00

chi-squared = 10.615 with 11 d.f.
probability = 0.4760

chi-squared with ties = 11.000 with 11 d.f.
probability = 0.4433

Appendix C

Figure 39 to figure 42 shows MIC confirmation of pure compounds 1-3 and 10-5A of *G. africana* against *C. albicans* ATCC. All plates were inoculated with 30 μ l of the organism, swabbed on SDA plates and incubated for 24 h at 34 $^{\circ}$ C. Results were read as the lowest concentration at which there was no growth.

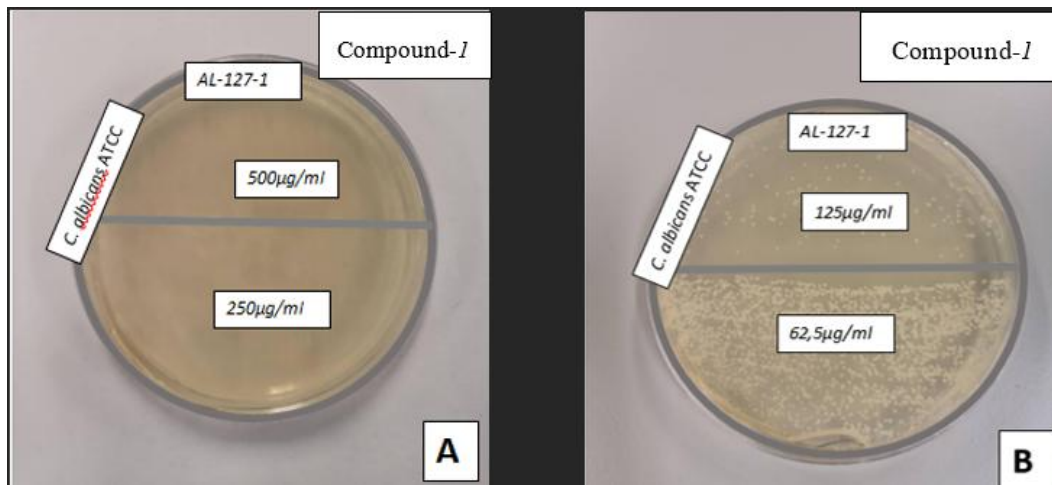
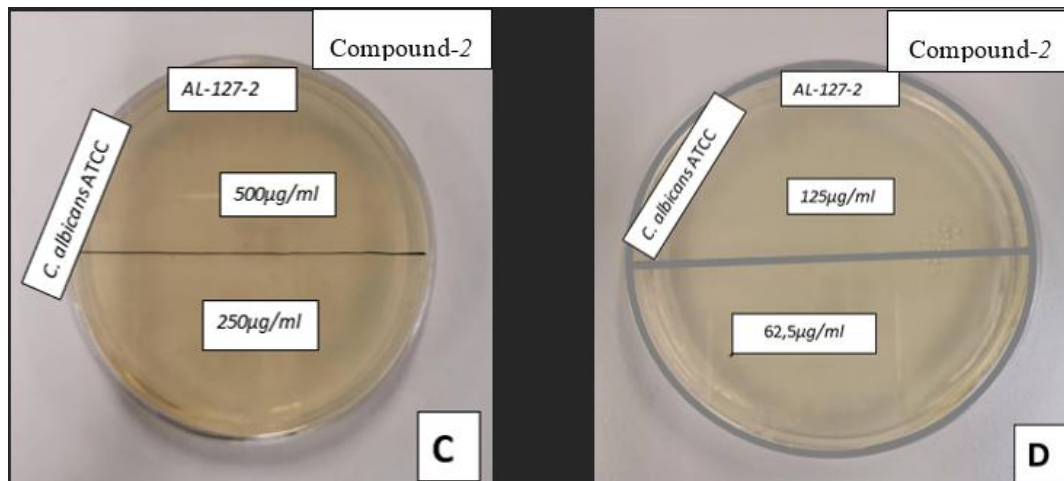


Figure 39: MIC confirmation of pure compound 1 tested against *C. albicans* (ATCC 90028)



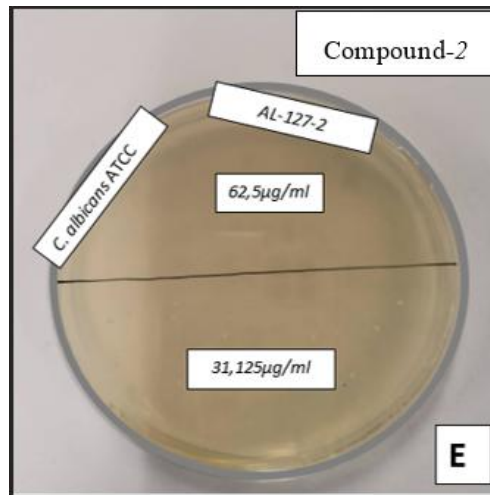


Figure 40: MIC confirmation of pure compound 2 tested against *C. albicans* (ATCC 90028)

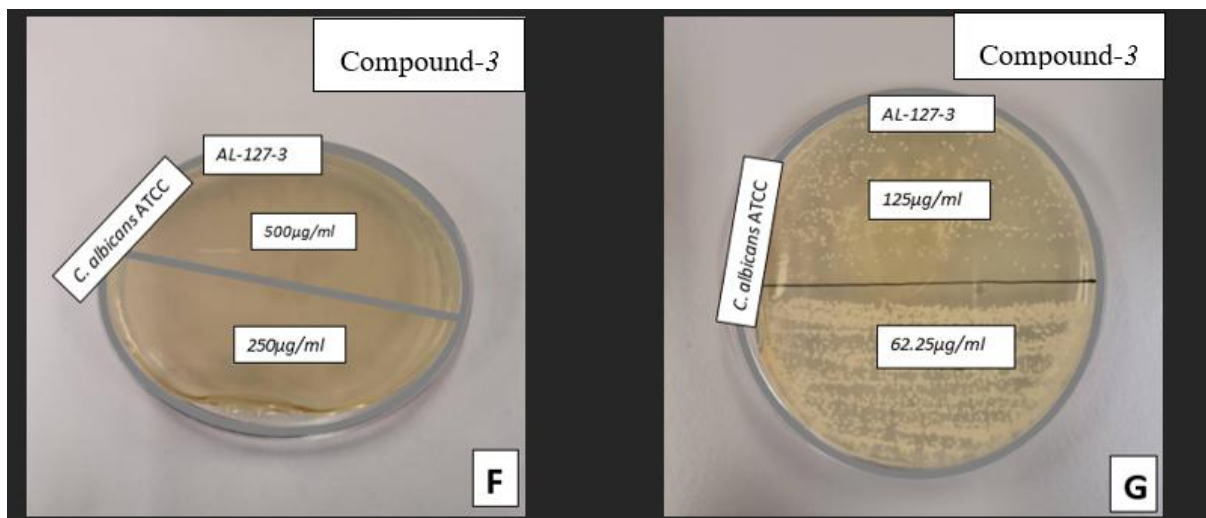


Figure 41: MIC confirmation of pure compound 3 tested against *C. albicans* ATCC (ATCC 90028)

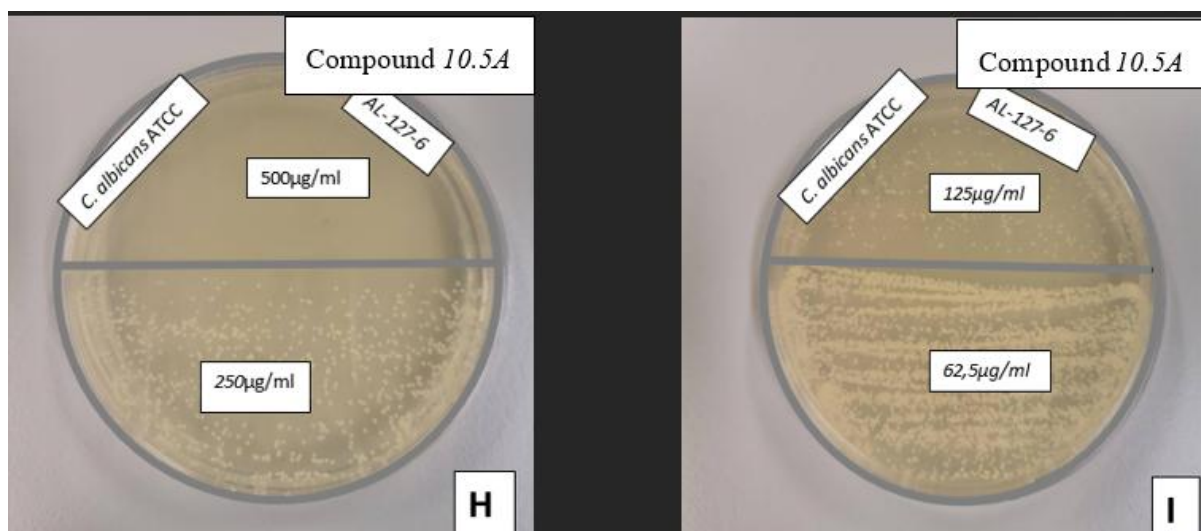


Figure 42: MIC confirmation of compound mixture 10.5A tested against *C. albicans* (ATCC 90028)

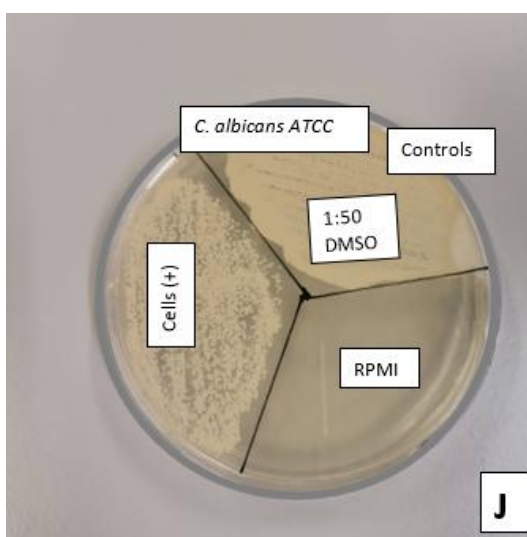


Figure 43: Controls used in MIC confirmation of pure compounds 1-3 and 10.5A against *C. albicans* (ATCC 90028)

Appendix D

Figure 42 to figure 47 shows MIC confirmation of pure compounds 1-3 and 10.5A of *G. africana* against *C. albicans* ATCC. All plates were inoculated with 30µl of the organism, swabbed on SDA plates and incubated for 24 h at 34°C. Results were read as the lowest concentration at which there was no growth.

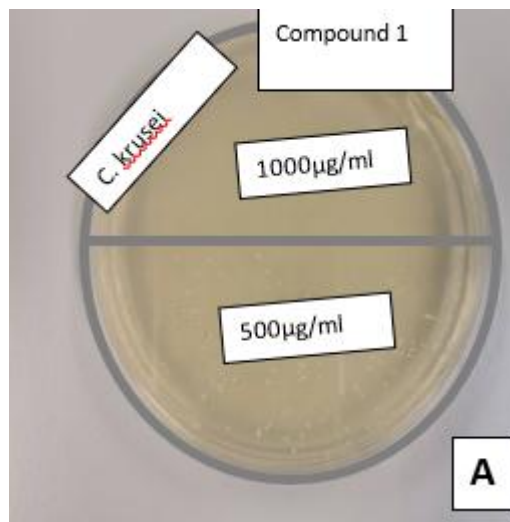
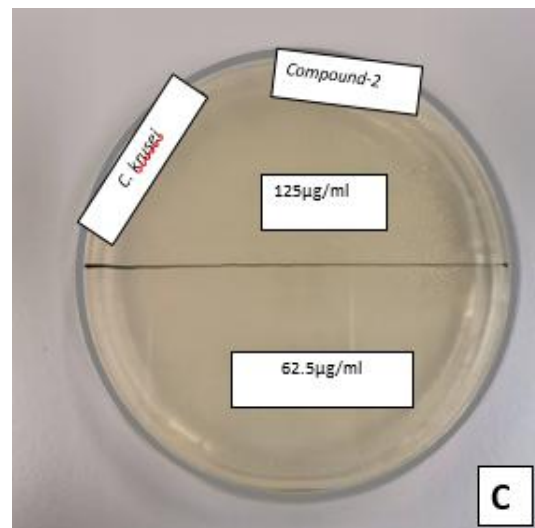
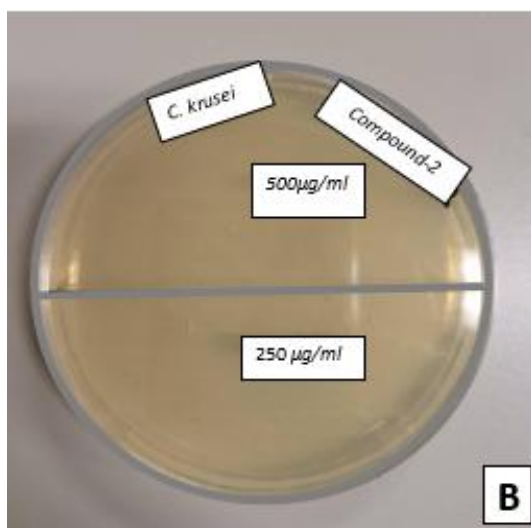


Figure 44: MIC confirmation of compound 1 tested against *C. krusei* (ATCC 2159)



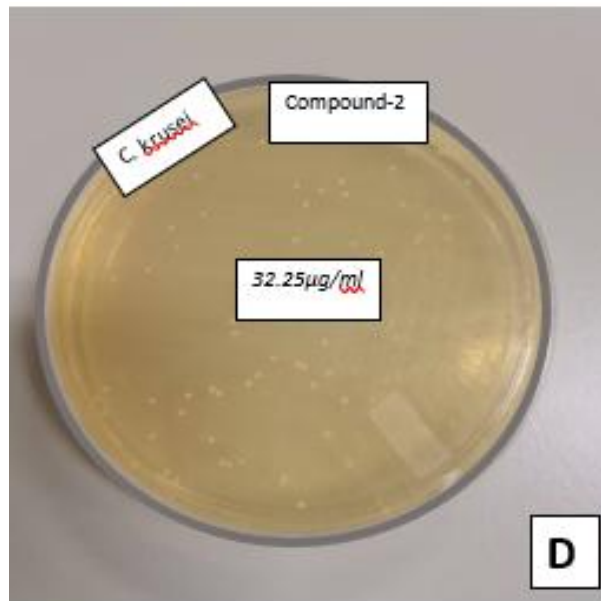


Figure 45: MIC confirmation of compound 2 tested against *C. krusei* (ATCC 2159)

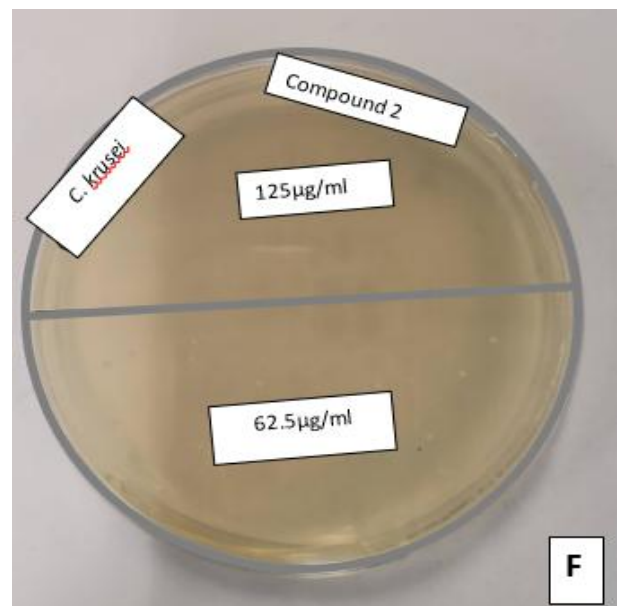
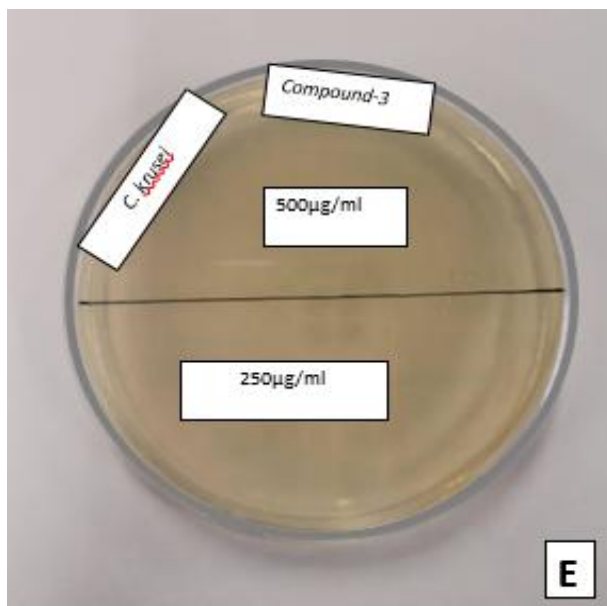


Figure 46: MIC confirmation of compound 3 tested against *C. krusei* (ATCC 2159)

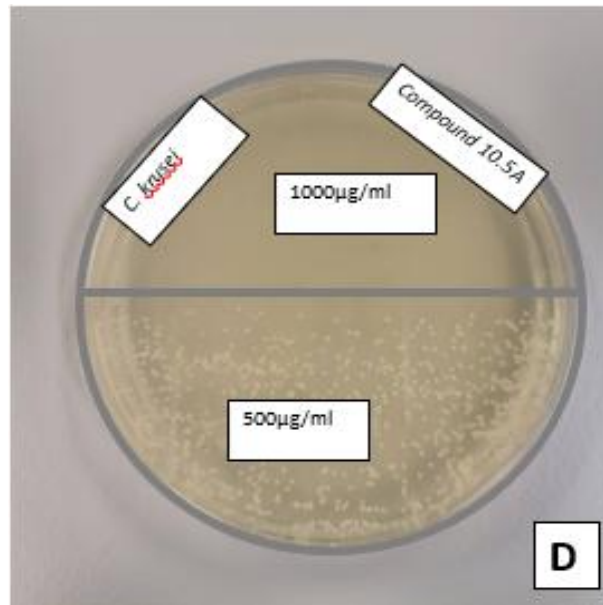


Figure 45: MIC confirmation of compound 10.5A tested against *C. krusei* (ATCC 2159)