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EFFECT OF TETRAZOLE DERIVATIVES ON APOPTOSIS AND ERGOSTEROL BIOSYNTHESIS PATHWAY IN *CANDIDA ALBICANS*

Londanani Rahulani

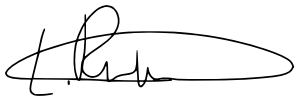


A dissertation submitted to the faculty of Health Sciences, University of Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2020

DECLARATION

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

Londanani Rahulani 

14 day of July 2020

ABSTRACT

Despite the availability of a large number of antifungal drugs used to treat *C. albicans* infections, successful treatment continues to be a challenge due to development of drug resistance and side effects. To combat this problem, a number of strategies are being explored as possible solutions. Ergosterol is an important sterol in fungi and a number of antifungal drugs in clinical use target different enzymes in ergosterol biosynthesis pathway. Despite this, ergosterol still remains a rich source of antifungal drug targets because it is synthesized in a multi-step pathway that involves a large number of enzymes. Synthetic compounds with improved pharmacological benefits have received a lot of attention as an alternative treatment. Tetrazole derivatives are aromatic hydrocarbons widely used in medicinal chemistry with a broad spectrum of activity, including antifungal activity. Apoptosis, a form of programmed cell death happens naturally in living organisms, but a number of drugs and compounds have been found to induce the process. Therefore, the aim of this study was to determine the effects of tetrazole derivatives on apoptosis and ergosterol biosynthesis pathway in *Candida albicans*.

The antifungal activity of three tetrazole derivatives (AN1, AN2 and AN3) was established against three *C. albicans* strains. Inhibitory and subinhibitory concentrations of each tetrazole derivative were selected and used for further studies. Effects of tetrazole derivatives on ergosterol biosynthesis pathway were determined by quantifying ergosterol content. Cell death in *C. albicans* in response to tetrazole derivatives was evaluated. Apoptotic cell death was studied on the basis of phosphatidylserine externalization, DNA fragmentation and release of cytochrome c oxidase, using FITC annexin V staining, TUNEL assay and cytochrome c oxidase assay, respectively.

Tetrazole derivatives possessed antifungal activity, with varying Minimum Inhibitory Concentrations (MIC). AN1, AN2 and AN3 had an MIC range of 0.39-1.56 μg/mL, 6.25-12.5 μg/mL and 0.39-1.56 μg/mL, respectively. Significant impairment of ergosterol content was induced when *C. albicans* cells were exposed to inhibitory concentrations of tetrazole derivatives. Lower concentrations of tetrazole derivatives also suppressed ergosterol in a dose-dependent manner. Treatment of *C. albicans* cells with tetrazole derivatives resulted in early apoptosis and late apoptosis and/ or necrosis, an indication of phosphatidylserine exposition and membrane damage. Cells exhibited fragmented DNA and condensed as well as irregular nuclei shapes. There was evidence of cytochrome c release to cytosol following exposure of cells to tetrazole derivatives. Inhibitory concentrations induced necrotic cells whereas apoptosis was more pronounced at subinhibitory concentrations.

In conclusion, at subinhibitory concentrations, all three tetrazole derivatives produced highly pronounced apoptotic features. In addition, they also inhibited ergosterol content; highlighting the ability of compounds to bind to or inhibit enzymes in ergosterol biosynthesis pathway. Therefore, these compounds have the ability to be developed into therapeutic agents against *Candida* infections.

DEDICATION

In loving memory of my brother

Ndivhuwo Bennedict

PRESENTATION

Poster presentation: Rahulani, L, Patel, M and Ahmad, A. Antifungal activity of novel tetrazole derivatives by disrupting ergosterol biosynthesis against *Candida albicans.* Faculty of Health Science Research Day. University of the Witwatersrand, Johannesburg. 6 September 2018.

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LIST OF ABBREVIATIONS AND ACRONYMS

• α	Alpha
• β	Beta
• %	Percent
• ° C	Degree Celsius
• AIDS	Acquired Immuno-Deficiency Syndrome
• ART	Anti-Retroviral Therapy
• ATCC	American Type Culture Collection
• ATP	Adenosine Triphosphate
• CFU	Colony Forming Unit
• CLSI	Clinical and Laboratory Standards Institute
• DMSO	Dimethyl Sulphoxide
• DNA	Deoxyribonucleic Acid
• dUTP	Deoxyuridine Triphosphate
• EUCAST	European Committee on Antibiotic Susceptibility Testing
• FDA	Food and Drug Administration
• HIV	Human Immunodeficiency Virus
• MFC	Minimum Fungicidal Concentration
• MIC	Minimum Inhibitory Concentration
• mL	Millilitre
• nm	Nanometre
• OH	Hydroxide
• pH	Potential Hydrogen

- RNA Ribonucleic Acid
- rpm Revolution per second
- TNF Tumour Necrosis Factor
- UV Ultraviolet
- WHO World Health Organization
- µl Microlitre

CHAPTER 1: INTRODUCTION

In the beginning of the 20th century, bacterial infections were the leading cause of disease epidemics, and fungal infections were as good as non-existent (Bongomin et al., 2017). This drastically changed when antibiotics were introduced and their use was in full swing; resulting in emergence of fungal infections which are now considered a global health threat, estimated to affect over 80% of the world's population (Bongomin et al., 2017). Fungi are abundant in nature, with most of them being beneficial and only a small number being pathogenic. However, even non-pathogenic fungi are turning into opportunistic pathogens, and are in the frontline of invasive fungal disease epidemics.

Common causes of fungal infections are *Cryptococcus*, *Aspergillus*, and *Candida*, with *Candida albicans* being the predominant species in the *Candida* genus reported to cause mucosal and systemic infections (Wisplinghoff et al., 2004; Pfaller and Diekema., 2007; Bongomin et al., 2017). Other *Candida* species that are also capable of causing fungal diseases are *Candida tropicalis*, *Candida glabrata*, *Candida auris*, *Candida krusei*, *Candida parapsilosis*, *Candida kefyr*, *Candida lipolytica*, *Candida famata*, *Candida rugose*, *Candida guilliermondii, Candida lusitaniae, Candida C. dubliniensis, Candida norvegensis, Candida pelliculosa* and *Candida C. inconspicua* (Chander, 2017; Deorukhkar and Shahriar, 2018). The isolation frequency of these non-*albicans Candida* species in infections varies: In European countries, incidence rates for non-*albicans* infections were reported to be 14 % for *C. glabarata* and *C. parapsilosis,* followed by 7% for *C. tropicalis* and 2% for *C. krusei* (Tortorano et al., 2006). In Chile, *C. parapsilosis* was the most frequently isolated in cases of candidaemia, followed by *C. tropicalis* and *C. glabarata* (Ajenjo et al., 2011). In South Africa, *C. parapsilosis* and *C. glabrata* were isolated in 35% and 7% of cases of blood stream infections, respectively (Govender et al., 2016). A study prior to this reported a differing

trend, with more cases of *C. glabrata* followed by *C. parapsilosis* (Arendse and Orth, 2008). This change in epidemiology is a consequence of a combination of factors, such as the use of fluconazole for prophylaxis, the increase in the number of immunocompromised patients, underlying diseases, advancements in invasive medical procedures and the use of immunosuppressants (Marr, 2004; Horn et al., 2009; Bongomin et al., 2017). Although incidences of candidaemia caused by non-*albicans species* have been reported*, C. albicans* still remains the predominant cause of fungal infections and is responsible for the high mortality rates in patients with fungal infections (Li et al., 2012; Sardi et al., 2013; Yapar, 2014).

The number of antifungal agents available for clinical use are limited because fungi are eukaryotic and closely related to humans (Onyewu et al., 2003). It is therefore challenging to develop new antifungal agents with selective toxicity (Roemer and Krysan, 2014). The current treatment options available are azoles, polyenes, echinocandins and flucytosine (Perfect, 2016). Within the azole group, fluconazole is the standard of care for *C. albicans* infections, but its fungistatic nature as well as extensive and incorrect use have resulted in the development of resistance reported in clinical settings (Sardi et al., 2013; Bhattacharya et al., 2016; Theill et al., 2016; Kangogo et al., 2017). Phenotypes of *C. albicans* resistant to fluconazole have been correlated with expression of virulence traits such as adherence proteins, biofilm formation and phenotypic switching (Molepo and Musenge, 2012; Monroy-Pérez et al., 2016; do Rosário Esteves Guimarães et al., 2019). *Candida albicans* has therefore developed fitness attributes against fluconazole and warrants the development of new drugs.

As a strategy to combat the resistance of *C. albicans* to existing antifungals, researchers are now focusing on developing new drugs that can target cellular pathways and virulence factors

that are necessary for the survival and pathogenesis of this organism (Pagan-Mercado et al., 2009; Li et al. 2015). Apoptosis, a form of programmed cell death, occurs naturally in living cells including those of fungi as part of homeostasis and regulation of damaged cells. Although it is a natural process it can also be induced by external stimuli, and certain drugs and compounds have been found to induce apoptosis in *C. albicans* (Choi and Lee, 2015; Lee and Lee, 2015). Research has led to an increase in knowledge on mechanisms, markers and mediators of apoptosis, and this has led to great interest in devising therapeutic strategies that modulate the process (Xu et al., 2019).

Tetrazole derivatives contain the tetrazole ring, whose side chains can be modified to give it desired pharmacological and biological properties (Salake et al., 2014). Studies have reported these compounds to have anticandidal activity comparable to that of standard antifungal drugs (Ostrovskii et al., 2012; Kaplancikli et al., 2014,). Ester tetrazole derivatives have been reported to attenuate certain virulence traits in *C. albicans*, but their ability to induce apoptosis and inhibit the ergosterol biosynthesis pathway, an established drug target, have not been fully explored. This was investigated in this study.

CHAPTER 2: LITERATURE REVIEW

2.1 *Candida albicans*

Candida albicans is a single-celled fungal species from the genus *Candida*. This species belongs to the kingdom Fungi, phylum Ascomycota; the largest phylum in the kingdom Fungi (Hibbett et al., 2007; Carris et al., 2012). The *Candida* genus has over 100 species, with *C. albicans* being one of the species of medical importance. *Candida albicans* is a unique species, it grows as a unicellular yeast or as filamentous cells, and this unique nature of transformation helps it thrive in different niches. *Candida albicans* is found as a commensal in about 50% of humans, inhabiting the gut, vagina, skin and oral mucosa, and the commensalism is maintained by host factors (Yapar, 2014; Kullberg and Arendrup, 2015). Changes in host immunity (immunosuppression) or diet (intake of foods that lower pH in the stomach) can however cause transition from commensalism of the fungi into pathogenic form (Yamaguchi et al., 2005; Puel et al., 2012). This is further enhanced by the numerous virulence factors possessed by *C. albicans*, that help it flourish as a pathogen. Some of the important virulence factors of *C. albicans* include adhesins, biofilm, hydrolytic enzymes and phenotypic switching (Mayer et al., 2013).

Table 2.1: Classification of *Candida albicans*

Kingdom	Phylum	Classs	Genus	Species
Fungi	Ascomycota	Saccharomycetes	*Candida*	*Candida albicans*

2.2 Cell wall of *Candida albicans*

Candida albicans cell wall is an important structure, since the cell wall is the first to interact with the host, houses numerous virulence factors and antigenic properties, and is required for

growth (Calderone and Braun, 1991; Ruiz-Herrera et al., 2006). The cell wall is composed of carbohydrates, proteins and lipids, and microscopic observations have shown the cell wall to be composed of multiple layers, as illustrated in Figure 2.1 (Calderone and Braun, 1991). These constituents of the cell wall interact with each other, giving rise to the overall structure of the cell wall. Carbohydrates form the biggest part of the cell wall; and up to 90% of the cell wall is said to be composed of carbohydrates, followed by proteins then lipids (Latgé, 2007).

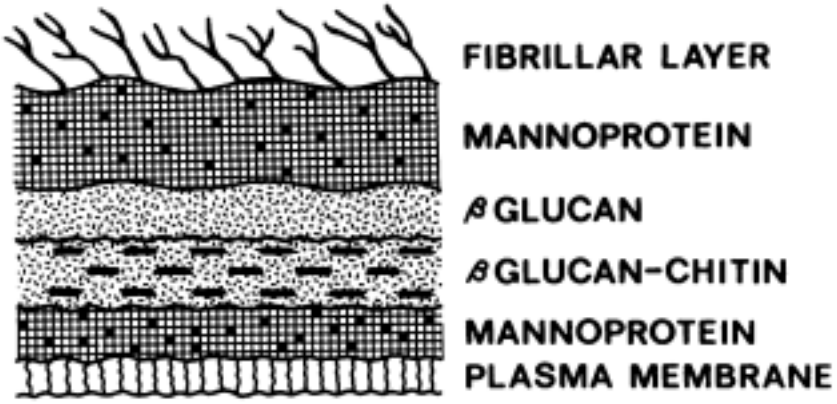


Figure 2.1: Composition of the cell wall of *C. albicans* (Calderone and Braun, 1991)

Important polysaccharides found on the cell wall are the highly branched ß 1,3- and ß 1,6-glucan, mannan and chitin (Ruiz-Herrera et al., 2006). Chitin and glucans are microfibrillar polymers located in the inner layer of the cell wall, closer to the plasma membrane, and they form a rigid skeleton for the cell and a scaffold for proteins on the outermost part of the cell wall (McKenzie et al., 2010; Wagener et al., 2012). ß 1,6-glucan is the central molecule responsible for linking other polysaccharides and proteins of the cell wall (Roman et al., 1997). Chitin folds back on itself to form anti-parallel chains that are held together by hydrogen bonds, and this bulky structure adds extra strength to the cell layer (Lenardon et al., 2010).

The outer part of the cell wall is layered with proteins, and the most important are mannoproteins. Although a carbohydrate, mannan is found linked to proteins through glycosylation (mannoproteins) to give the protein structural functions (Chaffin, 2008). Mannoproteins are highly concentrated on the outer layer, and possess antigenic properties, assisting the cell in adherence to the host and nutrient-rich substrates (Ruiz-Herrera et al., 2006).

Lipids are hydrophobic biomolecules that constitute minor components of the cell wall. They act as energy storage and serve as an environment for enzyme and other biological membrane functions (Brennan et al., 1975; van der Meer-Janssen et al., 2010). In yeast cells, lipids fall either in the phospholipid or neutral lipid class, with the phospholipid class encompassing phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol and the neutral class triglycerides, sterols and fatty acids (Mishra and Prasad, 1991). Apart from lipids having structural and metabolic functions, they play a role in the development of multidrug resistance indirectly; and the lipid composition of azole resistant and susceptible *Candida* species has been found to be different, with the most changes happening in common lipids (Singh et al., 2013).

2.3 Ergosterol biosynthesis pathway in *Candida albicans*

Ergosterol is the main sterol in fungi and an integral part of the cell membrane (Abe et al., 2009; Lv et al., 2016). In addition to ergosterol being protective to cell organelles, ergosterol harbours enzymes needed in metabolic and regulatory pathways (Daum et al., 1998). Synthesis of sterols in eukaryotes is conserved; with the same intermediates in the pathway, but different enzymes that catalyze the synthesis. In fungi, ergosterol is synthesized in a multi-step pathway with acetyl-CoA as starting material for the pathway (Figure 2.2) (Daicho et al., 2007). Thereafter, sterol intermediates are formed, and synthesis steps involve removal of methyl groups.

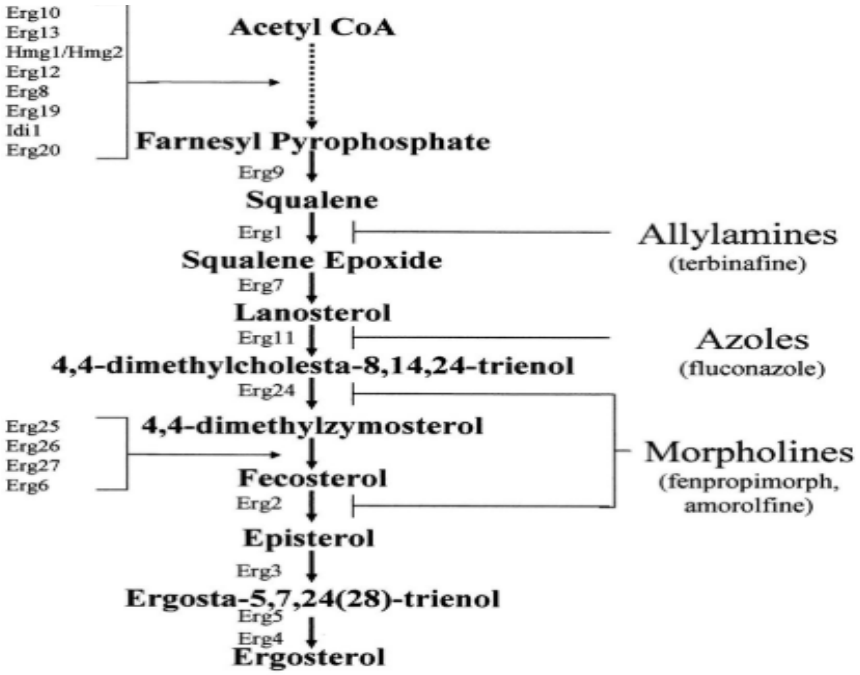


Figure 2.2: A schematic illustration of ergosterol biosynthesis pathway and drug targets (Daicho et al., 2007)

Synthesis of ergosterol involves around 20 enzymes, and each is coded by a different ergosterol biosynthetic gene (ERG) (Bhattacharya et al., 2018). The most important enzyme, probably due to its contribution as a target for azoles, is lanosterol 14α-demethylase (ERG11). Lanosterol contains a C14-α methyl group that is removed by lanosterol 14α-demethylase to eventually form ergosterol (Gachotte et al., 1997; Strushkevich et al., 2010; Tyndall et al., 2016). Another enzyme, sterol Δ5,6-desaturase (ERG3) catalyzes addition of double bond to episterol in the second last step of the pathway (Alizadeh et al., 2018). Lastly, sterol Δ22-desaturase and sterol Δ22-desaturase coded by ERG4 and ERG5, respectively, catalyze the final conversion to ergosterol (Li et al., 2018). Because there are many enzymes that catalyze the ergosterol biosynthesis pathway, ergosterol is one rich source of possible

antifungal drug targets and there is much to be explored in ergosterol as a drug target (Alizadeh et al., 2018).

Using the basis of whether the ergosterol biosynthetic gene is necessary for yeast survival, ergosterol biosynthesis genes are divided into essential and nonessential genes (Kristan and Rižner, 2012). For a gene to make a good drug target, it must be essential, because impairment of such a gene inhibits growth of the fungi (Aaron et al., 2001). Generally, when *C. albicans* cells with mutations on essential genes are exposed to antifungal drugs, they show susceptibility. Of the essential genes, ERG1 codes for squalene epoxidase, which converts squalene to oxidosqualene (Hu et al., 2017). When exposed to several antifungal drugs, a mutant of ERG1 showed susceptibility, and in the place of ergosterol, toxic sterol intermediates which do not support fungal growth accumulated. The mutant also showed reduced ability to form hyphae (Pasrija et al., 2005). Disruption of essential genes ERG27 and ERG26 also resulted in inhibition of ergosterol synthesis, and the intermediates that accumulated did not support fungal growth (Aaron et al., 2001; Pierson et al., 2004). Interestingly, some nonessential genes have also shown potential as antifungal drug targets, and one such gene is ERG24. ERG24 codes for the sterol C-14 reductase and is used as a target by the morpholine class of antifungals that are used for plant pathogens (Onyewu et al., 2003; Sant et al., 2016). ERG24 mutants accumulate ergosta-8,14,22-trienol in the place of ergosterol; and this sterol intermediate allows for mutants to grow under normal conditions. Membranes of ERG24 mutants have however been reported to be permeable, showing susceptibility to metabolic inhibitors and morpholines (Jia et al., 2002). Although not essential for growth, ERG24 is required for virulence during infections, so ERG24 mutations have anti-pathogenicity benefits (Becker et al., 2010; Luna-Tapia et al., 2015). Collectively, these findings suggest that inhibitors of essential genes and of some nonessential genes, represent a novel class of antifungal agents which will help circumvent resistance to azoles.

2.4 Virulence factors of *Candida albicans*

Candida albicans expresses a wide range of virulence attributes that help cells succeed in pathogenicity. Different virulence factors are expressed at different niches in the host, and this is the reason why *C. albicans* is able to flourish and cause a wide spectrum of diseases in different body sites. Virulence factors expressed by *C. albicans* include cell surface adhesins, phenotypic switching, biofilm formation, hydrolytic enzymes and environmental stress response (Samaranayake, 2002; Mayer et al., 2013).

2.4.1 Adherence and adhesins

Adherence to and colonization of *C. albicans* in the host is a vital step and a pre-requisite for expression of virulence (Moyes et al., 2015). Adherence requires *C. albicans* to have proteins on its surface that interact with specific ligands on the host as well as to other fungal cells or implanted objects such as catheters (Kabir et al., 2012; Lim et al., 2012). All other fungal-host interactions that follow are dependent on the success of this step. *Candida albicans* has a number of adhesins: The glycosylphosphatidylinositol-dependent cell wall proteins (GPI-CWP), non-covalent wall-associated proteins and integrin surface proteins (Int1) (Moyes et al., 2015). The Agglutinin-like sequence (ALS) protein family is the best studied and characterized group of adhesins (Tronchin et al., 2008; Mayer et al., 2013). The ALS protein family has eight proteins (ALS1-7 and ALS9) encoded by different genetic loci (Hoyer et al., 2001; Hoyer et al., 2008; Hoyer and Cota, 2016). ALS3 is reported to be more involved in epithelial adhesion than the rest of the ALS proteins (Murciano et al., 2012; Mayer et al., 2013). ALS has also been found to be upregulated and produced in large amounts on the surface of germ tubes and hyphae (Coleman et al., 2009). The hyphal wall protein (Hwp1) is another important adhesin that is hyphae-associated and is responsible for linking hyphae to the host cell (Sundstrom et al., 2002). Attenuation of this gene has been found to result in

reduced adhesion to epithelial cells, which implicates its importance in successful adhesion (Sundstrom et al., 2002).

Following adherence, *C. albicans* gains entry into the host through invasion and this is important for successful dissemination of the fungi. *Candida albicans* uses two distinct mechanisms for invasion, namely; Induced endocytosis and active penetration (Zakikhany et al., 2007; Dalle et al., 2010). Induced endocytosis depends on invasins that bind to host ligands and trigger cell engulfment; whereas in active penetration, fungal adhesins and proteases mediate the process by degrading tissue barriers on the host (Wächtler et al., 2011; Mayer et al., 2013). Whether invasion happens via an active or passive process depends largely on the cell morphology as well as the type of cell being invaded (Yang et al., 2014). *Candida albicans* faces different epithelial cells and this influences the mechanism by which fungi invades cells (Dalle et al., 2010).

2.4.2 Phenotypic switching

Candida albicans has the ability to grow in different morphological forms; from budding yeast and pseudo hyphae to true hyphae form (Kabir et al., 2012; Noble et al., 2017). Phenotypic switching is considered important because it creates heterogenaeity in the fungal population, which assists in adaptation to changing host environments. All forms of the fungi contribute to virulence in different ways (Jain et al., 2006; Shapiro et al., 2011). Isolates associated with invasive diseases switch at a higher frequency than commensal and superficial isolates (Hellstein et al., 1993; Jones et al., 1994). Phenotypic switches are controlled by environmental cues: at low pH, yeast cells are favoured and at a pH greater than 7, hyphal growth is induced. Other conditions such as starvation, physiological temperature, presence of serum and CO_2 support hyphal growth (Sudbery, 2011).

In its yeast form, *C. albicans* exists as opaque or white cells, and in hyphae form, it can either be pseudo or true hyphae. Saville et al. (2003) highlighted different roles played by hyphae and yeast in *C. albicans* infection (Saville et al., 2003). The yeast form plays a role in dissemination and mating, while the hyphae is well-suited for invasion (Miller and Johnson, 2002; Sudbery et al., 2004; Saville et al., 2003). The expression of hyphal genes has been found to be co-regulated with adhesins, both epithelial and endothelial cell adhesins, and degradative enzyme genes (Kumamoto and Vinces, 2005). With the help of co-expressed virulence genes, hyphae are able to penetrate epithelial cells and damage endothelial cells, causing deep tissue infections (Khan et al., 2010). This clearly proves that *C. albicans* is well equipped to adhere to and colonize different types of tissues and flourish as a pathogen.

2.4.3 Biofilm formation

Biofilms are microbial communities embedded in extracellular polymeric substances produced by the microorganism and adhere to biotic and abiotic surfaces (Mathé and Van Dijck, 2013; Gulati and Nobile, 2016). The increased use of indwelling medical devices such as dental implants, catheters, heart valves and artificial joints has led to a rise in the risk of infections related to biofilms because inert objects are substrates to which biofilms attach and grow (Chandra et al., 2001). For this reason, biofilms contribute a great deal in the development of *Candida* infections. Biofilms form in a sequential manner, starting with adherence of cells to a surface, proliferation, formation of hyphae, maturation and then dispersion of cells from biofilm to surrounding areas (Finkel and Mitchell, 2011; Tournu and Van Dijck, 2011; Nobile et al., 2012;). The complex polymeric structure protects the cells from drugs and host immunity, and cells within the biofilm have an altered metabolism which affects their response to drugs (Taff et al., 2012; Silva-Dias et al., 2015; Sun et al., 2015).

Although adhesion and hyphae genes are expressed in both planktonic and biofilm cells, the expression is higher in cells that form biofilms (Chandra et al., 2001; García-Sánchez et al., 2004; Nobile et al., 2012). Surface proteins ALS, specifically ALS1 and AL3, have been reported to play a role in the adherence of biofilms (Nobile et al., 2008; Roudbarmohammadi et al., 2016). *Candida albicans* cells deficient in ALS gene expression have been reported to form fragile biofilms with abnormal architecture, indicating the functional significance of ALS proteins in successful biofilm formation (Zhao et al., 2006). Expression of ALS1 and ALS3 however differs, depending on the cell morphology of *C. albicans.* In yeast and hyphal cells, ALS1 is upregulated and AL3 is upregulated in hyphal cells only (Li and Palecek, 2003; Green et al., 2005). Another cell wall protein, HCWP, is also involved in biofilm adherence, and has been reported to complement the function of ALS1 and ALS3. Together, they undergo a complementarity binding reaction which is crucial for *C. albicans* cell-to-cell adherence in biofilms of ALS1 and ALS3 (Bonhomme and d'Enfert, 2013). Transcriptional factors have been discovered to control the expression of genes involved in biofilm expression, namely; Enhanced filamentous growth transcriptional factor (Efg1), Transposon enhancement control (Tec1), Non-DiTyrosine (Ndt80) and Biofilm and cell wall regulator (Bcr1) (Nobile et al., 2000; Nobile and Mitchell, 2005; Fanning and Mitchell, 2012; Glazier et al., 2017). This regulation is a response to environmental conditions such as pH, temperature and availability of nutrients (Nobile et al., 2012). Biofilm formation has been reported to intensify at neutral and alkaline pH (Ferreira et al., 2016). Biofilms are able to grow under different temperatures: 37 ºC and 42 ºC, but the architecture at higher temperatures is affected compared to growth at 37 ºC (Pumeesat et al., 2017). Environmental conditions not only affect biofilm architecture, but they also affect the expression of biofilm genes (Kucharíková et al., 2011). Once biofilm is mature, non-adherent cells spread to initiate

biofilms in neighbouring cells, and some disseminate into the blood stream and urinary tract, contributing to the spread of an infection (Donlan and Costerton, 2002).

2.4.4 Hydrolytic enzymes

Candida albicans produces a number of hydrolytic enzymes that assist in host invasion. Hydrolytic enzymes responsible for host invasion are phospholipase, proteinase and lipases (Pawar et al., 2014). Phospholipases and proteinase have received the most attention in research, because their targets, phospholipids and proteins, are the main constituents of host cellular membranes (Ghannoum, 2000; Sachin et al., 2012; Mayer et al., 2013). Hydrolytic enzymes help *C. albicans* digest host membranes for deep tissue penetration, iron acquisition and to attack the host immune system. All these properties implicate hydrolytic enzymes to play a role in the hematogenous spread of the fungi (Staniszewska et al., 2012, Tsai et al., 2013). Because hydrolytic enzymes affect normal functioning of the host, they qualify to be considered as virulence factors (Niewerth and Korting, 2001; Pereira et al., 2015).

Phospholipases are a group of heterogenous enzymes that cleave ester linkages in glycerophospholipids (Pereira et al., 2015). Early studies reported *C. albicans* to be the only species that produces phospholipase in the *Candida* genus (Samaranayake et al., 1984; Ghannoum, 1998). However, it was later reported that other *Candida* species also produce phospholipases, although at significantly lower levels compared with *C. albicans* (Pakshir et al., 2013). Phospholipases produced by *C. albicans* are grouped according to their mode of action and substrates they target (Niewerth and Korting, 2001). Of all phospholipases identified, phospholipase A, phospholipase B, phospholipase C and phospholipase D (PLA, PLB, PLC, PLD), PLB1 and PLB5 have been shown to be responsible for virulence in animal models of candidiasis (Oksuz et al., 2007; Mayer et al., 2013).

Another important hydrolytic enzyme secreted by *C. albicans* is the Secreted aspartyl proteinase (Sap). Sap is encoded by 10 genes which produce different isoenzymes (Sap1-10) that play different roles in infection (Dutton et al., 2016). One functional difference of the isoenzymes is their different optimum pH, with studies reporting Sap1-3 to be highly active in acidic environments and Sap 4-6 at more alkaline conditions (Smolenski et al., 1997; Borg-von Zepelin et al., 1998; Aoki et al., 2011). This wide range of activity in different pH is essential when *C. albicans* infects different anatomical sites such as the vagina with a low pH and the oral mucosa with a neutral pH.

Sap contributes to virulence by degrading host cell proteins for nutrients and uncovering sites where the cell can adhere to epithelial cells (Yano et al., 2019). Sap also degrades host molecules such as keratin to assist penetration during invasion, hydrolyze immunoglobulins to evade host immune attack and break down protease inhibitors that degrade *C. albicans* (Naglik et al., 2003; Silva et al., 2011; Pawar et al, 2014). A clear role of Sap in causing infections has been demonstrated: Bernardis et al. (1990) and Lima et al. (2018) showed that *C. albicans* isolates in patients with vaginal candidiasis are more proteolytic than isolates from asymptomatic carriers (Bermadis et al., 1990; Lima et al., 2018). In another study, a high level of Sap activity was detected in isolates from children with severe early childhood caries (S-ECC) than in children that were caries free (Li et al., 2014). Although the role of individual Sap isoenzymes in infection is not clear, studies have shown that Sap1-3 expression is important in early stages of mucocutaneous infections whereas Sap4-6 play a role in systemic infections (Staniszewska et al., 2012; Pawar et al., 2014; Kumar et al., 2015).

2.4.5 Environmental stress response

During infection, *C. albicans* deals with a number of stressors from changing niches within the host. This leads to the activation of protective mechanisms that help cells to evade host

immunity and continue to proliferate as a pathogen. Within the host, phagocytes produce excessive amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in response to *C. albicans* infection (Mayer et al., 2013). To counteract stress induced by ROS, *C. albicans* produces enzymes (superoxide dismutase, catalase, glutathione reductase, etc.) that are responsible for detoxifying ROS. Genes that code for these enzymes have been found to be upregulated in the presence of neutrophils (Fradin et al., 2005). Likewise, in response to RNS, detoxifying enzymes are produced. Flavohaemoglobin-related proteins are the most important, and their effects result in restoration of the redox homeostasis, allowing *C. albicans* to continue to grow (Ullmann et al., 2004; Hromatka et al., 2005).

A number of signalling pathways have also been found to be involved in stress response. The mitogen-activated protein (MAP) kinases are important pathways that are key elements in controlling adaptation to environmental stress (Román et al., 2007). Pathways in *C. albicans* are high osmolarity glycerol response (Hog1), MAP kinase for *C. albicans* (Mkc1) and *Candida* ERK-like response (Cek1) (Alonso-Monge et al., 1999; Hohmann, 2002; Moyes et al., 2010). Signaling pathways respond by activating the transcription of different genes, and their ultimate function is to help cells resist osmolarity changes, maintain cell wall integrity, and to facilitate biofilm formation and mating when *C. albicans* encounters various stress conditions.

Although there are mechanisms that help cells escape stressful conditions in the host, there are instances were cell death occurs in response to stressful conditions. Mechanisms of cell death are apoptosis, necrosis, autopathology and pyroptosis (Fulda et al., 2010). These types of cell death are activated depending on the mode and nature of stress. Cell death helps eliminate older or damaged cells and, in this way, preserves the colony by reducing

competition for food sources (Hu et al., 2015). In an infection context, *C. albicans* cells that are fit will continue proliferating as a pathogen.

2.5 Apoptosis

Apoptosis is a form of programmed cell death (PCD) and a universal feature in living systems. It is responsible for maintenance of homeostasis and survival of the organism (Mazzoni and Falcone, 2008; Fuchs and Steller, 2011). Apoptosis ensures that cellular processes responsible for host defence against infections and those for cellular development are functioning well. Given its fundamental role, it is important for this process to be well-regulated and for it to function accordingly, as it has been noted that excessive cell death results in destruction of healthy cells, and is associated with neurodegenerative disorders; while under-expression of the process has been linked to proliferation of cancer cells and autoimmune diseases (Mazzoni and Falcone, 2008; Robertson et al., 2009).

2.5.1 Caspases

Apoptosis is driven by cysteine proteases known as caspases, with the name derived from 'Cysteine-dependent aspartic acid specific protease' (Mazzoni and Falcone, 2008; Wu and Bratton, 2013). The structure of a caspase is made up of motifs that are involved in the signal transduction of apoptosis. These proteases exist as inactive zymogens, and for them to partake in apoptosis, they must be activated through cleavage at specific sites (Shi, 2002; Julien and Wells, 2017). Caspases involved in apoptosis are grouped into two classes; the initiator caspase (caspase 2, 8, 9 and 10) and the effector caspase (caspase 3, 6 and 7) (Shi, 2002; Mazzoni and Falcone, 2008; Galluzzi et al., 2015). They have different sizes, with initiator caspases having a longer prodomain, which is important for its catalysis activity, than effector caspases (Shi, 2002; Lavrik et al., 2005). The initiator caspase, which is autoactivated, cleaves the effector caspase, the executioner of apoptosis, after a specific

aspartate residue within the effector caspase, thus activating the effector caspase (Mcllwain et al., 2013). The initiator contains a recognition motif in its structure which is responsible for its autoactivation. This cascade augments the apoptosis signal pathway and eventually leads to cell death.

Caspases are produced as inactive zymogens in cells and are activated through proteolytic cleavage (Mllwain et al., 2013). Activation of caspases occurs through the intrinsic or extrinsic pathway, depending on the origin of the stimuli (Belkacemi, 2018). In the extrinsic pathway, the signal for apoptosis is triggered by interaction between death receptors and ligands. Death receptors involved in apoptosis belong to the Tumour Necrosis Factor (TNF) superfamily and have death domains (DD) which are important for cell-death signaling (Ashkenazi and Dixit, 1998; Lavrik et al., 2005). Interaction between receptor and ligand forms a death-inducing signalling complex (DISC), to which procaspase-8 is recruited and activated into caspase-8 through dimerization (Mcllwain et al., 2013). This mature caspase is released into the cytosol to go and effect effector caspases.

The intrinsic pathway is stimulated by several stress-causing situations such as heat shock, hypoxia, DNA-damaging agents and hormones (Brenner and Mak, 2009; Choi and Lee, 2015; Lee and Lee, 2015). Cells damaged by these stressors stimulate the release of a proapoptotic protein, Bax, which opens up their mitochondrial membrane and thus cytochrome c is released out of the mitochondrion (Loreto et al., 2014). In the cytoplasm, cytochrome c interacts with apoptosis protease activating factor-1 (APAF1), forming an apoptosome to which procaspase-9 is recruited (Cain et al., 2002). When cytochrome c binds to APAF1, its conformation changes, exposing a nucleotide-binding site, to which ATP binds (Mcllwain et al., 2013). The bound ATP is hydrolyzed, then cleaves and produces an active

caspase-9, which is now able to activate effector caspases for apoptosis (Srinivasula et al., 1998).

2.5.2 Apoptosis in yeast

For a very long time, apoptosis was believed to be confined to multicellular organisms, but further research revealed that even unicellular eukaryotes such as *Trypanosoma cruzi*, *T. brucei rhodesiense, Dictyostelium discoideum* and *Tetrahymena thermophilado* undergo apoptosis (Ameisen, 1996). Apoptosis was not believed to occur in yeast cells until an apoptotic phenotype was identified by Madeo et al (1997), implying that steps leading to apoptosis do occur in unicellular organisms or yeast in particular (Madeo et al., 1997). The discovery of apoptosis in yeast made yeast the preferred tool for apoptosis research, since it has a simpler genome, is easy to handle, and is an eukaryote and as such can be used to study human diseases related to apoptosis (Ramsdale, 2006; Guaragnella et al., 2012).

In addition to apoptotic features found in yeast, apoptosis regulatory proteins that are homologous to those found in metazoans have been identified (Madeo et al., 2002; Fahrenkrog et al., 2004; Wissing et al., 2004). Initially, when apoptosis regulators homologous to metazoans were searched on the genome of *Saccharomyces cerevisiae*, none were found. However, there were studies that showed that the expression of metazoan pro-apoptotic genes in yeast cells induces a typical apoptosis phenotype, and when anti-apoptotic genes were expressed, the same apoptotic features were not elicited (Tao et al., 1997; Kang et al., 1999; Ligr et al., 2001). Apoptotic regulators that are present in metazoans and yeast are cytochrome c, Aif and endonuclease G, and the fact that these specific regulators are involved in mitochondria-dependent apoptosis implies that the mitochondrion is a universal orchestrator of apoptosis (Guaragnella et al., 2012). Lastly, although caspases are not found in yeast, a protease with a proteolytic domain has been identified which is a functional

analogue of caspases, referred to as a metacaspase (Uren et al., 2000; Madeo et al, 2002; Wasilewski and Scorrano, 2009). A metacaspase or Yca1 is activated during stress response and like caspases, it cleaves substrates that are involved in apoptosis (Wong et al., 2012; Léger et al., 2015). Given these findings, it is evident that the apoptotic mechanism is present in yeast and it occurs in a regulated manner.

2.5.3 Apoptosis markers

Cells undergo different types of cell death, but what distinguishes apoptosis from the rest is the cell morphological changes (Kroemer et al., 2005). The changes are cell shrinkage, chromatin condensation, plasma membrane blebbing, nucleus fragmentation, no ultrastructural modification of cytoplasmic organelles, and the maintenance of an intact plasma membrane until the last stage of apoptosis (Kroemer et al., 2005; Taylor et al, 2008; Wu et al., 2010; Pistritto et al., 2016).

It is said that understanding and studying the mechanism of apoptosis will open doors for new therapeutic strategies. In order for such research to be conducted, techniques and tools for identifying apoptotic cell death are important and a necessity. Because apoptosis happens in different pathways and at different cell compartments, having multiple techniques in studying the process is recommended and advantageous. A number of techniques are used in detecting the wide range of biochemical and morphological changes induced by apoptosis (Elmore et al., 2007). The most common yeast apoptotic markers targeted are phosphatidylserine exposition, cytochrome c release, DNA fragmentation, nuclear changes and production of ROS (Tian et al., 2017; Tian et al., 2017). In addition to the use of these techniques, it is advisable to do time kinetics assays, because apoptotic features are time-dependent and some morphologies are similar to those of necrosis or other cell death types (Gelles et al., 2016).

2.5.3.1 Release of cytochrome c

The release of cytochrome c from the mitochondrion is one of the key steps known to precede other apoptotic markers and is used to characterize mitochondria-dependent apoptosis (Goldstein et al., 2005). Under normal conditions, cytochrome c is contained in the mitochondrion, and there it acts as an electron carrier in the respiratory chain (Garrido et al., 2006). When apoptosis is induced, cytochrome c is released from the mitochondrion into the cytosol where it cooperates with certain proteins to activate caspases needed for execution of apoptosis (Garrido et al., 2006; Hüttemann, et al., 2011).

Proapoptotic proteins, Bid and Bax, permeabilize the outer mitochondrial membrane, creating channels for the export of cytochrome c (Von Ahsen et al., 2000). Although proapoptotic proteins cause changes in the mitochondrion, the changes are indistinct and do not affect the mitochondrial potential. Maintenance of the mitochondrial potential means that metabolic functions of the mitochondrion are preserved (Von Ahsen et al., 2000). Interestingly, it has been found that the mitochondrial potential is reduced only after export of cytochrome c and caspase activation (Bossy-Wetzel et al., 1998; Seervi et al., 2011).

Although there are debates about the exact mechanism responsible for cytochrome c release, two models have been proposed as the cause: In one model, cytochrome c is released simply through diffusion from intermembrane spaces after mitochondrial outer membrane permeabilization (MOMP) and is independent of caspase activation, whereas in the other model, other changes in the mitochondrion need to take place for it to be released (Green et al., 2004; Goldstein et al., 2005; Ripple et al., 2010). The release of cytochrome c is studied either by determining the enzymatic activity via reduced cytochrome c stimulation of oxygen consumption or by determining the enzyme content in the cytosol directly using cell

fractions. The most commonly employed methods of detection are Western blotting, fluorescence microscopy and colorimetric assays (Manickam et al., 2017).

2.5.3.2 DNA fragmentation

Chromosomal DNA fragmentation is a late step in apoptosis and is orchestrated by nucleases within the cell (Wong, 2011). Caspase-activated DNase (CAD) is a nuclease responsible for DNA fragmentation and is found in greater abundance in cells that quickly undergo apoptosis than in those that do not (Nagata et al., 2003; Crowley et al., 2016). CAD has preference for A-T-rich regions where it cleaves, unpacking the supercoiled chromatin then cleaves the exposed spacer regions' DNA, fragmenting the DNA into nucleosomal units. CAD specifically makes double-stranded breaks in chromosomal DNA and is not known to cleave single-stranded DNA or RNA (Nagata et al., 2003; Napirei et al., 2009; Koyama et al., 2016). Additionally, CAD is activated by an active caspase-3, an indication that cleavage is linked to the execution of apoptosis, and this contributes to the specificity of the process (Larsen and Sørensen, 2017). The endonuclease G is another nuclease that is responsible for DNA fragmentation during apoptosis. Contrary to CAD, endonuclease G is caspase-independent and is mitochondria-bound, travelling to the nucleus during apoptosis (Li et al., 2001; Toro-Londoño et al., 2011).

There are a number of methods used to detect fragmentation of DNA due to apoptosis. These range from commercial kits, fluorescent assays, gel electrophoresis and assays that use fluorescent microscopy and flow cytometry (Koopman et al., 1994; Kalyuzhny, 2011; Suman et al., 2012; Crowley et al., 2016; Baek et al., 2018). Detection methods have differing advantages and disadvantages, with the most prominent differences being quantitative and qualitative.

The classical method used in detecting DNA fragmentation is running DNA on agarose gel, where the DNA fragments form a ladder (Archana et al., 2013). This assay is cost-effective, and the band pattern produced by DNA fragmentation through apoptosis is distinct from that produced by necrosis, which appears as a smear (Samarghandian and Shabestari, 2013). This method has to be supplemented with a more specific method because formation of DNA fragments has been shown to be cell-dependent, so its absence does not necessarily mean there was no apoptosis (Cohen et al., 1992; Kasibhatla et al., 2006).

Alternatively, the Terminal Deoxynucleotide Transferase dUTP Nick End Labelling (TUNEL) assay is used, which is more reliable and specific. It uses flurochrome-labelled deoxynucleotides that are incorporated to the 3' OH end of the fragmented DNA for fluorometric visualization (Tinari et al., 2008; Kyrylkova et al., 2012). Because it allows visualization at a single-cell level, the TUNEL assay allows for quantification of apoptosis (Gavrieli et al., 1992). Pre-fixing of cells helps preserve apoptotic cells and prevents any further DNA damage which might be mistaken for apoptosis. Despite its advantages, Kraupp et al (1995) pointed out a few drawbacks of the TUNEL assay (Kraupp et al., 1995); Liver cells that underwent different types of cell death were found to be positive for the TUNEL assay, making the assay non-specific for apoptosis and necessitating analysis of ultrastructural changes which confirm apoptosis.

2.5.3.3 Phosphatidylserine exposition

One of the alterations that occur in the early stages of apoptosis is the migration of phosphatidylserine (PS) to the cell surface in a caspase-dependent manner (Martin et al., 1996; Suzuki and Nagata, 2014). In normal cell membrane, phospholipids are asymmetrically distributed in the plasma membrane, with the amino phospholipids in the inner leaflet of the plasma membrane and choline phospholipids in the outer leaflet (van Engeland et al., 1998;

Mariño et al., 2013). This asymmetry is maintained by the flippase enzyme (Bevers and Williamson, 2010). During apoptosis, the phospholipid distribution is scrambled, and the phosphatidylserine is transported to the outer leaflet of the plasma membrane with the help of the scramblase (Leventis and Grinstein, 2010). During apoptosis, activated caspases cleave the flippase and scramblase, activating the latter and deactivating the former (Vermes et al., 1995; Hankins et al., 2015; Segawa and Nagata, 2015). Once translocated to the outer membrane, phosphatidylserine acts as the main signal for phagocytosis (van Engeland et al., 1998; Yarden, 2011).

For detection of PS exposure in apoptotic cells, the Annexin V binding assay is used (Bundscherer et al., 2013). This assay is based on the fact that the annexin protein binds to PS in a calcium-dependent manner and it is conjugated to a fluorochrome for cytometric analysis (Logue et al., 2009; Lizarbe et al., 2013). Propidium iodide (PI), a DNA-binding dye, is also included in the assay to help distinguish apoptotic from necrotic cells. Exposure of PS happens in both apoptotic and necrotic cells. However, in apoptotic cells, the cell's intactness is maintained, unlike in necrotic cells (Vermes et al., 1995). So apoptotic cells will be negative for PI and necrotic cells will be positive, since they are permeable to PI. Membrane damage occurs post PS exposure in cells, therefore PI can be used to distinguish early and late apoptosis. However, this membrane damage does not indicate if the damage is due to apoptosis or necrosis, so it is important for other markers to be detected in order to ascertain apoptosis (Hingorani et al., 2011).

2.5.3.4 Ultrastructural changes

In apoptosis, the most dramatic changes occur in the nucleus. In addition to DNA fragmentation, other morphological changes of the nucleus are observed, and are linked to caspase-3-activation (Ajiro et al., 2010; Kitazumi et al., 2011). Caspases contribute to

ultrastructural changes by targeting proteins that are responsible for maintaining chromatin structures and also disrupting interaction of chromatin with the nuclear matrix (Casiano et al., 1996). These ultrastructural changes happen in a sequential manner, with each stage producing a different morphology.

The first change seen is chromatin condensation (Latrasse et al., 2016). Although a characteristic of apoptosis, chromatin condensation also takes place in cells undergoing mitosis. However, the chromatin in mitosis is easily distinguished from the apoptotic one because it is less dense and does not eventually form apoptotic bodies (Dobrucki et al., 2001; Toné et al., 2007). Following condensation, the nucleus aggregates to the margin of the nuclear membrane and this is followed by invagination of the nucleus. These are pinched off to form apoptotic bodies that contain cellular and nucleic contents (Atkin-Smit et al., 2017; Xu et al., 2019). Detachment of these bodies happens in preparation for phagocytosis. Ultimately, the cell shrinks as these changes take place (Ihara et al., 1998; Elmore, 2007; Toné et al., 2007). In tissues, the fate of apoptotic bodies is engulfment via phagocytosis, and this is a clean way of removing damaged tissues as there is no leakage of cell contents and thus no inflammation (Wickman et al., 2012; Poon et al., 2014). However, in the absence of phagocytosis, apoptotic cells progress to necrosis and this is characterized by plasma membrane damage (Berghe et al., 2013).

Analysis of ultrastructural changes is made possible by electron microscopy which resolves a lot of image detail and forms images with higher magnifications than light microscopy (Archana et al., 2013). Transmission electron microscopy (TEM) analysis is considered the gold standard in apoptosis analysis, and it helps distinguish type of cell death according to the morphological changes induced (Krysko et al., 2008). Additionally, TEM marries results obtained from other biochemical tests with the type of cell death causing the changes. It is

however impossible to view the entire organism using this microscope, because only thin sections of the specimen are accommodated. Although images produced by TEM have good resolution, they appear as grey, which takes away the natural state of the cell, and the sample preparation is a laborious and challenging task (Archana et al., 2013).

As illustrated above, there is evidence that yeast cells undergo apoptosis and possess an apoptotic regulatory framework. Such findings will open doors for the design of new antifungal drugs that have yeast apoptosis inducing-capabilities.

2.6 Disease spectrum

Because *Candida* species naturally inhabit different anatomical sites with different environmental pressures, it is able to thrive better than other commensals that are transient colonisers, making its disease spectrum wider than those of other colonisers (Calderone and Fonzi, 2001). *Candida albicans* causes disease known as candidiasis, and the disease spectrum ranges from superficial to serious bloodstream infections (Sardi et al., 2013).

Superficial candidiasis results from a localized overgrowth of commensal fungi as a result of flora imbalance or in people with compromised immune systems. Generally, superficial or mucosal infections do not threaten the life of the patient, with antifungal drugs sufficient for eradicating infections, provided there are no underlying illnesses. Mucosal infections are common in older patients, denture-wearers, HIV patients as well as patients with diabetes mellitus (Akpan and Morgan, 2002). An enhanced production of certain virulence features has been reported in cancer patients on treatment, suggesting that these strains have the potential to cause infections (Ramla et al., 2016). Mucosal membranes affected by *C. albicans* are the oral cavity, oesophagus and genitals (Dabas, 2013; Jabra-Rizk et al., 2016).

Oropharyngeal candidiasis (OPC) is a common opportunistic infection and is responsible for death in Human Immunodeficiency Virus (HIV) patients. HIV patients with OPC develop AIDS faster than those without. As a result, OPC is used as a marker for disease progression in HIV patients (Nielsen et al., 1994; Campo et al., 2002). When Antiretroviral Therapy (ART) was introduced, a decline in cases of OPC was reported, probably because ART improves the immune system and inhibits production of the fungal protease that contributes to virulence (Arribas et al., 2000; Patel et al., 2012; Garcia-Cuesta et al., 2014). *Candida albicans* is the most common cause of OPC, but this epidemiology is shifting towards non-*albicans* species, due to selection pressures produced by drug over-use and intrinsic resistance in emerging pathogens (Wingard et al., 1991; Barchiesi et al., 1993; Kaur et al., 2016). Although *C. albicans* remains the leading cause of OPC, *C. glabrata* and *C. dubliniensis* have also been reported in OPC cases (Patel et al., 2012).

Vulvovaginal candidiasis (VVC) is another superficial infection that affects the lower female genital tract. This is a common infection in women of child-bearing age, and they experience the infection at least once in their lifetime (Achkar et al., 2010). However, VCC can be recurrent in some patients, and, in this case, it is referred to as recurrent vulvovaginal candidiasis (RVVC). In RVVC, a patient has 4 or more episodes of VVC in a space of 12 months (Sobel et al., 1998). Although the causative agent of VCC is *C. albicans*, the number of RVCC cases caused by *C. glabrata* have been on the rise (Ray et al., 2007; Sherry et al., 2017). Interestingly, *C. albicans* isolated from RVCC cases were found to be susceptible to antifungal agents used to treat this infection. Yet persister fungi were responsible for the recurrent episodes (Sobel,1992). Although superficial infections do not generally cause death in patients, they however may worsen the condition of immunocompromised patients. For example, oral candidiasis can disseminate and cause oropharyngeal candidiasis affecting the oesophagus which can worsen the condition of the patient.

2.7 Treatment options for *Candida albicans* infections

Four classes of antifungal drugs are available for use and are classified according to their target (Perfect, 2016). Specific *Candida albicans* targets have been taken advantage of in the development of antifungal drugs and generally include ergosterol and its pathway, the cell wall and nucleic acids (Ostrosky-Zeichner et al., 2010; McCarthy et al., 2017). Current antifungal classes available for the treatment of fungal infections are polyenes, azoles, pyrimidine analogs, and echinocandins (Denning and Hope, 2010; Gulati and Nobile, 2016). Another class, allylamines, is also available, but due to intolerable effects, allylamines are only used topically for dermatophytic infections (Vandeputte et al., 2011; Campoy and Adrio, 2017).

2.7.1 Polyenes

The polyene class has over 200 drugs, and the drugs are isolated from the *Streptomyces* bacteria. Although a high number of polyene drugs are available, only three are used in clinical settings because they have the most tolerable toxicity, namely Amphotericin B (AmB), nystatin and natamycin (Vandeputte et al., 2011). For many years, AmB was the only antifungal drug that could be administered systemically and is the preferred polyene over others which are more toxic, and are limited to topical use (Odds et al., 2003; Ashley et al., 2006; Salci et al., 2018). In addition, AmB is the preferred drug due to its wide spectrum of activity, having activity against the most important pathogens causing fungal infections (*Candida, Cryptococcus* and *Aspergillus fumigatus*), and its low resistance cases (Bondaryk et al., 2013; Spampinato and Leonardi, 2013).

Polyenes are amphipathic in nature, containing a hydrophobic and hydrophilic side. Drugs in this class are unique because they do not use the typical antimicrobial mechanism of action. Instead of inhibiting enzymes, polyenes bind directly to ergosterol (White et al., 1998; Odds

et al., 2003). Through binding to ergosterol, polyenes intercalate into membranes, forming channels that destabilize the fungal plasma membrane and lead to leakage of cellular contents, potassium ions (K^+) in particular (Palacios et al., 2011). Amphotericin B (AmB) binds to ergosterol with much avidity and the opposite is seen when it interacts with cholesterol (Neumann et al., 2010). This is due to the difference in shape of the two sterols and it explains increased selectivity for ergosterol (Kotler-Brajtburg et al., 1974). However, weak interactions formed with cholesterol do cause some host toxicity, particularly inducing nephrotoxicity, which is a limitation on drug usage (Grela et al., 2018).

Despite being fungicidal and being in clinical use for many years, the toxicity profile of AmB limits its use in treatment, reserving the drug for severe cases. To combat host toxicity, lipid formulations of AmB were developed (Chandrasekar, 2011). The drug is incorporated into liposomes, which are phospholipid vesicles, and this aids in selective transfer of the drug to the target without causing toxicity to other host cells. Once AmB reaches the target site, AmB is released from the liposome by phospholipases found in human cells and in fungi. Three lipid formulations of AmB are available for use, namely AmB lipid complex (ABLC), AmB colloidal dispersion (ABCD) and Ambisome (L-AmB) (Wong-Beringer, 1998). Lipid formulations of AmB and echinocandins have been reported to penetrate biofilm better than amphotericin B deoxycholate and azoles (Kuhn et al., 2002; Tobudic et al., 2010).

2.7.2 Pyrimidine analogs

In the pyrimidine analog class, only flucytosine is approved for use (Dismukes, 2000). Flucytosine, also known as 5-Fluorocytosine (5-FC), is an analog of the cytosine nucleotide, and it targets the disruption of DNA and protein synthesis (Ghannoum and Rice, 1999; Prasad et al., 2016). Flucytosine was originally developed for use in cancer therapy, but when further research was conducted, flucytosine proved to have antifungal activity against

Candida species and *Cryptococcus* species, and thus its use as an antimycotic (Waldorf and Polak, 1983). Additionally, 5-FC has antifungal activity, although limited, against *Aspergillus, Phialophora* and even parasites such as *Leishmania* (Stiller et al., 1983).

In its original form, 5-FC has no antifungal activity. It is the conversion into different forms that gives 5-FC antifungal activity. When the drug is administered, it is taken up by the cell through the help of the permease enzyme. Once in the cell, cytosine deaminase converts it to 5-flourouracil. This is further converted into either 5-fluorouridylic acid (FUMP), which prematurely terminates protein synthesis, or 5-fluorodeoxyuridine monophosphate which disrupts DNA synthesis (Odds et al., 2003; Kanafani and Perfect, 2008; Prasad et al., 2016). Some filamentous fungi do not have the enzymes required for conversion of 5-FC. This restricts the use of 5-FC to a few pathogenic yeasts. 5-FC is used in combination with AmB or FLC due to resistance that arises when it is used as monotherapy (Morace et al., 2014; Fang et al., 2017; Drew, 2019). A few rare side effects of the drug have been observed.

2.7.3 Azoles

The introduction of azoles as antifungal agents was a major advancement in the treatment of systemic mycosis, and it is widely used in clinical settings. Miconazole was the first azole to be approved, but because it was only available in toxic intravenous formulation, it was not frequently used (Heeres et al., 1979). Azoles are divided into two classes based on the number of nitrogen atoms they have in their ring. The first class, which contains two nitrogen atoms, includes imidazoles, consisting of ketoconazole, miconazole, econazole and clotrimazole while the second class with three nitrogen atoms is triazoles, consisting of fluconazole, posaconazole, itraconazole, terconazole and voriconazole (Maertens et al., 2004; Shapiro et al., 2011). Imidazoles are used for mucosal infections whereas triazoles are used for both invasive and mucosal infections. Ketoconazole and triazoles: fluconazole and itraconazole were later introduced and used as alternatives to AmB.

Although azoles have their individual pharmacokinetic properties, they all target cytochrome P450 enzyme, lanosterol 14-α-demethylase (Maertens, 2004; Bersani et al., 2019). Lanosterol 14-α-demethylase has a heme moiety in its active site. The heme has an iron to which oxygen binds and activates demethylation of lanesterol to ergosterol. However, azoles inhibit this by binding to the iron competitively via their unsubstituted nitrogen atom on imidazole or triazole rings (Odds et al., 2003). Consequently, less ergosterol will be synthesized and toxic by-products of the ergosterol biosynthesis pathway such as 14α-methylated sterol will accumulate (Shapiro et al., 2011; Prasad et al., 2016). Replacement of ergosterol by other sterol intermediates results in change in fluidity and permeability of the cell, and ultimately inhibition of cell growth (Nigam, 2015).

2.7.4 Echinocandins

Echinocandins are synthetic derivatives of lipopolypeptides that are produced by various fungi (Vandeputte et al., 2011). This was the last group to be added to available antifungal drugs for use by clinicians. In the early 2000s, three drugs in the echinocandin class were approved for clinical use by the Food and Drug Administration (FDA): caspofungin, micafungin and anidulafungin (Negri et al., 2014). In clinical use, echinocandins show fungicidal effect against yeasts (*Candida* species) and fungistatic effect against moulds (*Aspergillus* species), but against fungi such as *Cryptococcus, Fusarium, Trichosporon* and *Scedosporium* species, these drugs do not show any antifungal activity (Odds et al., 2003).

Unlike most classes of drugs that target ergosterol and its synthesis pathway, echinocandins target the β (1-3)-D-glucan pathway which is responsible for the synthesis of β-glucan, a strengthening polysaccharide of the fungal cell wall (Roemer and Krysan, 2014). Specifically, echinocandins target the FKS1 gene, which codes for the β-glucan synthetase. Consequently, fungal cells that have been exposed to echinocandins lose their mechanical

strength, and as a result, they cannot withstand osmotic pressure; leading to cell lysis (Letscher-Bru and Herbrecht, 2003; Lorand and Kocsis, 2007). This class of drugs is highly selective for fungal cells since mammalian cells do not have β (1-3)-D-glucan. Thus, echinocandins do not cause host toxicity (Letscher and Herbrecht, 2003; Shapiro et al., 2011).

2.8 Antifungal drug resistance

The spectrum of fungi causing infections in immunocompromised patients is growing, but opposite is the case with the number of drugs available for treating diseases caused by these pathogens (Roemer et al., 2014). This compromises the success of treatment, as drugs are overused, and, in most cases, drug resistance results. Other factors have also been reported to play a role in treatment failures, such as delayed or incorrect diagnosis, the virulence of the causative agent as well as the toxicity profile of the drug (Kanafani and Perfect, 2008). It is thus advisable for clinicians to consider such factors before administering treatment, to ensure a successful outcome. As a strategy to ensure successful treatment, a guide to clinicians in choosing the appropriate drug for treatment and resistance surveillance, Antimicrobial Susceptibility Testing (AST), has been developed. Specifically, AST determines the susceptibility of a fungal strain *in vitro*, using standardized techniques to establish the Minimum Inhibitory Concentration (MIC) of drugs (Schumacher et al., 2018). These standards were created by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antibiotic Susceptibility Testing (EUCAST) in order to establish species-specific clinical breakpoints (CBP), and epidemiological cut-off values (Pfaller et al., 2010).

Resistance to drugs can either be intrinsic or extrinsic. Intrinsic resistance occurs naturally without the effect of a drug, such as in the resistance of *Candida krusei* to fluconazole and *Cryptococcus neoformans* to echinocandins (Arendrup et al., 2017). On the other hand,

extrinsic resistance develops after a microorganism that was initially susceptible to a drug develops resistance after exposure to that drug; as seen in the resistance of *C. albicans* to fluconazole (Eliopoulos et al., 2002; Kanafani and Perfect, 2008). Selective pressure allows strains that are resistant to drugs to survive in the presence of the drug, with strains becoming fit by gaining mutations. Change of resistance in microorganisms is universal, and fungi have developed different ways of fighting the effects of drugs (Cort et al., 2016). Multidrug resistance (MDR) is one form of acquired resistance (to more than one agent in two or more classes of antifungal drugs) that develops and is most commonly reported in *Candida auris* (Arendrup et al., 2017). *Candida auris* is characterized by resistance to more than one azole, polyenes and echinocandins, with one study reporting resistance in 93% and 33% of strains tested to fluconazole and voriconazole, respectively (Lockhart et al., 2017). Some isolates have shown resistance to Amphotericin B, which rarely occurs in other *Candida* species (Vincent et al., 2013; Kathuria et al., 2015; Lockhart et al., 2017). Echinocandins were introduced as alternative treatment for *C. auris* infections following resistance to azoles and polyenes. However, elevated MIC values of echinocandins were shortly treported hereafter (Vallabhaneni et al., 2016; Chowdhary et al., 2016; Lockhart et al., 2017). Resistance to fluconazole is the major point of concern, because fluconazole is the standard of treatment, and other treatment alternatives might not be accessible in all clinical settings. Lastly, *C. auris* resistance to major antifungal classes poses a challenge in treating infections as no other treatment options are available as alternatives.

2.8.1 Mechanisms of resistance to azoles

Resistance mechanisms are diverse and of different nature; also dependent on the type of drug that confers the resistance. Initially, resistance was thought to be limited to the physiological and biochemical level, until molecular studies were done that revealed how

genetics contributes to development of resistance. After resistance has developed it is maintained, even in the absence of the inducer, implying that mutations in the isolate have occurred that maintain this resistant phenotype (Wirsching et al., 2000). The most studied mechanisms of resistance are from the azole group, probably due to their importance in clinical settings, their fungistatic nature and the reports on resistance. Several mechanisms of resistance have been found to be the cause of azole resistance, namely: over-expression of efflux pumps, alterations in the target enzyme and mutations that lead to the over-expression of the ERG11 (Kanafani and Perfect, 2008; Pemán et al., 2009; Sanglard, 2016).

2.8.1.1 ERG genes alterations

Azoles inhibit ergosterol synthesis by attaching to the active site of lanesterol 14α-demethylase enzyme synthesized by the ERG11 gene (Henry et al., 2000). The result of this is depletion of ergosterol. Resistant strains of *C. albicans, Cryptococcus neoformans, Histoplasma capsulatum* and *Aspergillus fumigatus* have been reported to have reduced susceptibility to lanesterol 14α-demethylase enzyme and the role of ERG11 mutations in causing resistance has been investigated (White et al., 1998).

One way in which ERG11 expression is altered is through point mutations that cause conformational changes in the lanesterol 14α-demethylase enzyme (Parker et al., 2014). Single or multiple mutations are responsible for this resistance, and these mutations have been discovered by various studies to be highly concentrated around the catalytic and the heme site, and certain amino acid regions in these sites have been marked as hotspots for such mutations (Asai et al., 1999; Marichal et al., 1999; Song et al., 2004; Flowers et al., 2015). These changes confer resistance by preventing azoles from binding to the enzyme effectively. However, the enzyme function in ergosterol biosynthesis is preserved (Parker et al., 2014). Although azole drugs all target the product of the ERG11 gene, they bind with

different affinities and this also influences their individual responses to mutations on ERG11 (Flowers et al., 2015). Fluconazole is known to bind more avidly than voriconazole and itraconazole, and it was therefore shown that fluconazole is highly responsive to amino acid substitutions, and for voriconazole and itraconazole to be resistant, multiple mutations must have occurred first (Warrilow et al., 2010; Flowers et al., 2015). Low sensitivity to mutations by other azole drugs would imply that in cases of fluconazole resistance, other azoles can be used.

Another way in which *C. albicans* becomes resistant to azole drugs is through changes in the ergosterol biosynthetic pathway. Such changes create compensatory pathways that help the fungi circumvent antifungal inhibitions and allow fungal cells to grow even in the presence of the drug (Lupetti et al., 2002). Mutations in ERG3 have been identified as one of the changes in the ergosterol pathway that result in resistance (Lupetti et al., 2002; Martel et al., 2010). When *C. albicans* is treated with azole drugs, the resultant effect is ergosterol suppression and its replacement with the sterol intermediate, 14α-methyl-3,6-diol, which is toxic (Spettel et al., 2019). Formation of such a methylated sterol intermediate is catalyzed by the sterol α 5,6-desaturase, encoded by erg3 gene (Luna-Tapia et al., 2018). Mutations can however replace toxic sterols with a less toxic methylfecosterol which allows for cell survival and also leads to AmB resistance (Watson et al., 1989; Vale-Silva et al., 2012).

Another alteration is the upregulation of ergosterol genes, and this has been linked to the accumulation of the UPC2 transcriptional factor (Dunkel et al., 2008; Whaley et al., 2017). UPC2 is a member of fungal transcription factors that control sterol synthesis (Vik and Rine, 2001; Silver et al., 2004). Homologues of UPC2 are present in *Saccharomyces cerevisiae* and they bind to ergosterol promoters, inducing expression through feedback mechanism in cases of sterol starvation, mostly caused by drugs that affect the ergosterol biosynthesis pathway

(Davies et al., 2005; Yang et al., 2015). A gain of function mutation on UPC2 in *C. albicans* has been associated with the constitutive expression of ergosterol genes, whereas deletion mutations increase hyper susceptibility of *C. albicans* isolates (Silver et al., 2004; Dunkel et al, 2008). An interesting observation is that in the absence of drug pressure, ergosterol genes are still expressed, but at basal levels in UPC2 deletion mutants. However, when there is drug pressure, the expression of these genes is increased (Silver et al., 2004). This shows that UPC2 only plays a role in response to drug pressure and is therefore important in drug resistance development.

2.8.1.2 Drug efflux pumps

One common mechanism of antifungal resistance is the increase in drug efflux (Sanglard et al., 2009). Two types of efflux pumps are known in eukaryotes, namely: ATP-binding cassettes (ABCs) and Major facilitator superfamily (MFs) transporters (Cannon et al., 2009). ABCs get their energy from ATP hydrolysis for transportation of compounds out of cells whereas MFs use the cells' proton gradient (Sanglard et al., 1995). These proteins are responsible for the pumping of toxins out of the cell, but it is their over-expression that results in antifungal drug resistance (Cannon et al, 2009; Singh et al., 2018). In cases of resistance, their genes are upregulated, increasing the rate of drug efflux out of the cell and thus reducing the drug concentration inside the cell (Coste et al., 2004). Pumps that are important for resistance in *C. albicans* are *Candida* drug resistance 1 (CDR1), *Candida* drug resistance 2 (CDR2) and MDR (Pourakbari et al., 2017).

2.7.1.3 ABC transporters

In the ABC family, pumps involved in drug resistance are CDR1 and CDR2; and they are upregulated in resistant clinical isolates when compared to their susceptible counterparts. This upregulation is a result of increased promoter activation due to mutations (Sanglard et

al., 1995; Sanglard et al., 1997; Coste et al., 2006; Manoharlal et al., 2008). Although similar, CDR1 and CDR2 are not identical, and this is shown by their differing roles in resistance. CDR1 is expressed in susceptible and resistant strains, whereas CDR2 is only expressed in resistant strains (Holmes et al., 2008). However, in susceptible isolates, CDR1 is expressed at basal levels (Sanglard et al., 1997; Micheli et al., 2002). CDR1 mutations do not confer resistance to fluconazole only, but to other azoles as well, implicating them in multi-drug resistance (Tsao et al., 2009). CDR genes are also upregulated through gain-of-function mutations in their transcriptional activators (Coste et al., 2004). CDR promoters have a drug responsive element (DRE) in their promoter region, to which the zinc-cluster transcription factor (TCAP1) binds. Hyperactive TCAP has been found in resistant isolates, leading to their constitutive binding on the DRE and overexpression of CDR genes. Homozygous mutations are responsible for this phenotype, suggesting synergism in two mutated alleles in the conference of resistance (Coste et al., 2006).

2.8.1.4 Major facilitators

Drug efflux in *C. albicans* is also increased through upregulation of *C. albicans* Multidrug Resistance 1 gene (CaMDR1) of the MFS family (Feng et al., 2018). This upregulation results in increased mRNA levels in clinical resistant isolates and induces resistance in fluconazole only (Pourakbari et al., 2017). In susceptible isolates, MDR1 is expressed at low levels, implicating their role in fluconazole resistance (Wirsching et al., 2000). In contrast to CDR genes which are regulated by cis- and trans-acting regulatory proteins, MDR1 over-expression has been found to be under the control of trans-acting regulatory proteins (Wirsching et al., 2000; Sasse et al., 2012). Upregulation of the zinc cluster transcriptional factor Multidrug resistance regulator 1 (MRR1) has been correlated with the upregulation of MDR1 in resistant strains and inactivation of MRR1 inhibited MDR1 expression, and thus resistance. Mutations in MRR1 alleles have been identified as the cause of constitutive

activation of MDR1 in drug resistant strains (Morschhäuser et al., 2007). Although there are species-specific differences in nucleotide sequences, MDR confers resistance in different species (Wirsching et al., 2000).

2.9 Alternative treatment strategies

Available antifungal drugs have become limited in their ability to treat the numerous fungal infections, mainly due to antifungal resistance that has emerged. Mortality rates due to fungal infections have also not reduced regardless of the availability of antifungal drugs. This compels the development of new drugs or the use of alternative treatment strategies. In order for a new drug to be ideal, it must be fungicidal, improve mortality rate, be effective even in drug-resistant species, it should not induce any host toxicity and the mechanism of action should be novel (Pianalto and Alspaugh, 2016; Parente-Rocha et al., 2017).

Although there is evidence that there is a need for new antifungals, the difficulty of identifying novel drug targets and lack of interest from investors remains an obstacle that has slowed down antifungal drug development. Several strategies are being used in the development of new drugs. Firstly, metabolic and virulence factors of fungi are being targeted. Compounds with improved pharmacological profiles that share targets with available antifungal drugs and drugs used for other conditions are being explored. Lastly, signaling pathways that control fungal survival are also being explored as possible targets (Perfect, 2016).

2.9.1 Tetrazoles

Tetrazoles are aromatic heterocyclic compounds with one carbon and four nitrogen atoms (Mohammed, 2016). Tetrazoles have unique structural features that give them a broad spectrum of activity and are thus used in numerous fields. However, the most important

application of tetrazoles is in medicinal chemistry, with about 45 FDA approved drugs containing the tetrazole moiety reported by 2016 (Zhao, 2016). These compounds are structural fragments of many bioactive compounds.

2.9.2 Bioisosterism

In medicinal chemistry, tetrazolic acids are used as surrogates for carboxylic acids, following the concept of bioisosterism (Meanwell, 2011). In bioisosterism, a functional group is replaced with a different one with similar chemical and physical properties, but the biological properties are preserved (Arabi, 2017). This technique has gained popularity in medicinal chemistry because it helps improve the absorption, distribution, metabolism and excretion (ADME) properties of candidate drugs (Lima and Barreiro, 2005; Wermuth, 2011; Ballatore et al., 2013). Just like carboxylic acid, the tetrazolic acid fragment of a compound (CN_4H) is ionized at physiological pH and the two groups have similar acidity profiles (pKa values are similar to each other): a property that preserves the acidic moiety of the drug (Hansch et al., 1995). However, tetrazoles have several advantages over carboxylic acid. Firstly, they are more lipophilic, with enhanced membrane penetration of the drug (Dei et al., 2002). Secondly, the numerous nitrogen atoms in the tetrazole ring allow for more hydrogen bonding with receptors on the target, and lastly, they are more stable than carboxylic acids (Patel and Karia, 2015). Although they do not possess any biological activity, tetrazoles are resistant to biological degradation, and owing to other numerous advantages, the World Health Organization (WHO) has declared it an important ingredient in the design of new drugs following the analogue-based drug discovery method (Ostrovskii et al., 2012). Bioisosterism is therefore a well-established concept in medicinal chemistry that will continuously contribute to the drug discovery process as it sparks creativity and allows opportunity for acquiring novel intellectual property.

2.9.3 Biological activity

A high number of tetrazole derivatives have been successfully synthesized, and are used as clinical drugs to treat diseases. The best known is the antihypertensive drug Losartan and its derivatives (Bhalla et al., 2013). Another successful use of tetrazoles is the development of reverse-transcriptase inhibitors specific for HIV-1 (Li et al., 2013). These derivatives have also been shown to inhibit protein synthesis and growth in certain microorganisms, suggesting their potential as antimicrobial agents (Nguyen-Trung et al., 2013).

The antifungal activity of tetrazoles has also been reported, and is said to be dependent on the substituents on the ring, with some not inducing antifungal activity and others enhancing it. Salake et al. (2014) showed in a study how fungi respond differently to tetrazole derivatives, depending on the substituents on the ring (Salake et al., 2014). The response to tetrazoles was also shown to be species-dependent, and when a number of *Candida* species were tested, *C. albicans* was reported to be the most susceptible to tetrazoles (Kaplancikli et al., 2014). Studies have also reported tetrazole derivatives to possess better anticandidal properties than standard antifungal drugs, making them good therapeutic candidates (Matysiak et al., 2003; Upadhayaya et al., 2004). Although it has been shown that tetrazole derivatives have enhanced the biological activity of compounds, depending on the substituents on its ring, very little information is available on the biological activity of ester-disubstituted tetrazoles as well as their application (Li et al., 2013). The carrying out studies that evaluate the activity of such compounds is necessary, in the hope that they could be effective in treating diseases that are difficult to treat with standard drugs.

Bondaryk et al. (2015) assessed the antifungal activity of several tetrazole derivatives (2,5-disubstituted tetrazole derivatives) against *C. albicans*. In this study, esters containing the tetrazole ring proved to be the most effective against *C. albicans*, with the most active

compound having an MFC value of 0.03 µg/mL. When the structures were analyzed, it was concluded that the alkyl chain length and position of the acetoxy group do play a role in the activity of the compound. Furthermore, this compound was found to inhibit early stages of biofilm formation and subsequent adherence; proof that tetrazole derivatives have anti-virulence properties against *C. albicans* (Bondaryk et al., 2015). The study however did not assess whether compounds tested had ergosterol suppression capabilities, but Malik et al. (2012) showed differently synthesized tetrazoles to suppress ergosterol production (Malik et al., 2012). Lastly, commercialized antifungal drugs such as AmB have been found to induce apoptosis in *C. albicans* (Phillips et al., 2003), and we hypothesize that, given the antifungal activity of tetrazoles, they also have the ability to induce apoptosis, which can be characterized by different hallmarks.

2.10 Aim

The aim of the study was to determine the effects of tetrazole derivatives on apoptosis and ergosterol biosynthesis pathway in *Candida albicans*.

2.11 Objectives

- To determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of three newly synthesized tetrazole derivatives and select MIC, ½ MIC and ¼ MIC for further analysis.
- To study the effects of MIC, ½ MIC and ¼ MIC on ergosterol biosynthesis pathway by quantifying ergosterol content.
- To study the effects of MIC and ½ MIC on apoptosis by detecting different apoptotic markers.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cultures of *Candida albicans*

The clinical isolates used in this study were obtained from the Oral Microbiology Laboratory. These isolates were collected in previous years from immunocompromised patients that were attending different clinics at the Charlotte Maxeke Johannesburg Academic Hospital under ethical certificate number M000402 and stored in glycerol stocks in a -80 °C freezer in the Department of Oral Microbiology Laboratory. To use these strains for this study, permission was obtained from The Human Research Ethics Committee (Medical), University of the Witwatersrand, Johannesburg (Appendix A). In addition to two clinical isolates, a standard laboratory strain of *Candida albicans* ATCC 90028 was also used for this study. Each isolate was cultured on Sabouraud Dextrose (SD) agar (Sigma-Aldrich, USA) and incubated at 37 °C for 24 hours. API 20 C Aux sugar assimilation test (Biomerieux, France) followed by Germ tube assay were done to confirm the cultures as *C. albicans*. Prior to the experiments, colonies of *C. albicans* were emulsified in phosphate buffered saline (PBS) (Sigma-Aldrich, USA) and the suspension was adjusted to approximately 6 x 10^8 CFU/mL. This suspension was used as an inoculum for all experiments in the study.

3.2 Chemical compounds

Three tetrazole derivatives (AN1, AN2 and AN3) were synthesized and collected from Dr. Mohmmad Y Wani of the Texas Therapeutics Institute, Brown Foundation Institute of Molecular Medicine, The University of Texas Health Science Center at Houston, Texas, United States. Structures of the compounds are shown in Figure 3.1. Synthesis of these novel compounds was done by multistep reactions following the procedure as described in Wani et al. (2012). They were reconstituted to a stock concentration of 1000 µg/mL using 100%

dimethyl sulphoxide (DMSO) (Associated Chemical Enterprises, South Africa) and this stock preparation was used for the study.

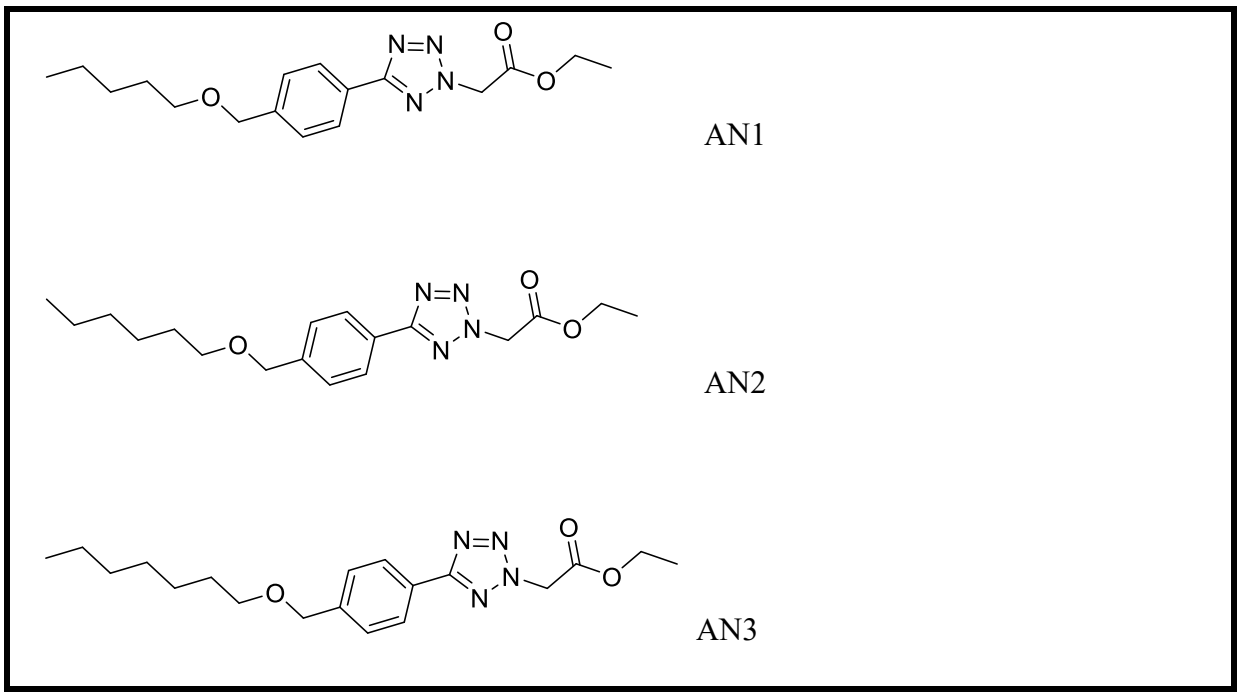


Figure 3.1: Structures of tetrazole derivatives used in the study

3.3 Minimum Inhibitory Concentration and Minimum Fungicidal Concentration

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) were obtained using the microtiter double-dilution method, to determine the antifungal activity of tetrazole derivatives against *C. albicans* as per Clinical and Laboratory Standards Institute (CLSI) M27-A3 guidelines (CLSI, 2008). This method was used because a number of concentrations can be tested simultaneously, and it is gives quantitative results. Hundred microlitres of SD broth was added to wells of a 96-well round bottom microtiter plate (Thermo Fischer Scientific, USA). The stock concentration of all three tetrazole derivatives was further diluted in 1% DMSO to 100 µg/mL and 100 µL of this was added on the first row of wells, which further diluted the compound by half. The tetrazole derivatives were further diluted serially (twofold), and the range of concentrations in the wells was 25- 0.006 µg/mL. Cell suspensions of 0.5 McFarland Standard (1 x 10^6 cells/mL) were prepared in 0.8% saline

and 100 µl of suspension was added to each well. The plates were incubated at 37 °C for 18 to 24 hours. In every experiment set, positive (fluconazole), negative (DMSO) and media (drug and culture free) controls were included. After incubation, results were read visually, and the lowest concentration with no visible growth was recorded as MIC. Dilution in each well was sub-cultured on SD agar and incubated at 37 °C for 24 hours. The lowest concentration with no growth was recorded as MFC. These experiments were done in triplicate and, for each isolate, median MIC for each test compound was calculated and used for subsequent assays.

3.4 Ergosterol quantitation assay

The effect of tetrazole derivatives on the ergosterol biosynthesis pathway was studied following a technique described by Ahmad et al. (2011) with modifications (Ahmad et al., 2011). The modification of the method included change in the test concentrations of the test compounds, depending on the MIC values. Compound concentrations used were median MIC, ½ and ¼ of that concentration. Concentrations used for AN1 were 0.1 µg/mL, 0.2 µg/mL and 0.39 µg/mL; those for AN2 were 3.13 µg/mL, 6.25 µg/mL and 12.5 µg/mL; and those for AN3 were 0.39 µg/mL, 0.78 µg/mL and 1.56 µg/mL. The experiment was done in triplicate. Sterile 50 mL Falcon tubes (BD Biosciences, USA) were prepared with 20 mL of SD broth (Merck, South Africa), MIC and sub-inhibitory concentrations of the test compounds. In one tube, fluconazole (8 µg/mL) was added instead of the test compound and in another tube only media and culture were added. Tubes with fluconazole and culture only were used as positive and negative control, respectively. To the tubes, 50 µl of cell suspension was added and the tubes were incubated for 16 hours at 37 °C in an incubator under gentle shaking (Labotec, South Africa). Cells were harvested by centrifugation at 2,700 rpm for 5 minutes, and washed with sterile distilled water. The pellets were weighed, 3 mL of 25% alcoholic potassium hydroxide was added to each pellet, and the mixture was vortexed

for 1 minute vigorously. Thereafter, the cell suspension was transferred to sterile glass screw-cap tubes and incubated for 1 hour at 85 °C in a water bath (Lasec, South Africa). After incubation, the tubes were allowed to cool to room temperature then sterol was extracted by adding a mixture of 1 mL distilled water and 3 mL n-heptane (Sigma-Aldrich, USA). The mixture was vortexed for 3 minutes then the heptane layer (top layer) was transferred to a clean 15 mL Falcon tube. The sterol extract was diluted five-fold with 100% ethanol (Sigma-Aldrich, USA) and scanned spectrophotometrically at wavelength 240-300 nm with a UV-1800 Shimadzu spectrophotometer (Shimadzu Scientific Instruments Inc, Japan). The ergosterol content was calculated using the following formulae:

1. % ergosterol + % 24(28) DHE = ($A_{281.5}$/290) x F/pellet weight
2. % 24(28) DHE = (A_{230}/518) x F/pellet weight
3. % ergosterol = % ergosterol + % 24(28) DHE - % 24(28) DHE

F is the dilution factor in ethanol, and 290 and 518 are the E values determined in percentages per centimetre for crystalline ergosterol and 24(28) DHE, respectively (Arthington-Skaggs et al., 1999).

3.5 Apoptosis study

Assays to detect apoptosis markers on yeast cells following treatment with tetrazole derivatives were done following techniques described by Khan et al. (2014). The assays were done using kits and following the manufacturer's instructions. As most of the kits used in this study were similar to the kits used by Khan et al. (2014), all the methods and calculations were done similarly. Yeast cells were treated with median MIC and ½ MIC concentrations, and thereafter protoplasts were prepared and used for apoptosis assays. Concentrations used were 0.2 µg/mL and 0.39 µg/mL for AN1, 6.25 µg/mL and 12.5 µg/mL for AN2 and 0.78 µg/mL and 1.56 µg/mL for AN3. All experiments were repeated twice for each strain.

3.5.1 Protoplast preparation

Protoplasts were prepared by digesting the cell wall with lyticase enzyme (Sigma-Aldrich) in wash steps, with different buffers (Khan et al. 2014). Ten millilitres of 6 x 10^8 CFU/mL cell suspension was centrifuged at 3,500 rpm and added to 15 mL Falcon tubes with 2 mL SD broth and test compound at varying concentrations. The tubes were incubated for 180 minutes at 37 °C in an incubator under gentle shaking. Untreated cells and cells treated with 2 µg/mL Amphotericin B (Sigma-Aldrich, USA) were included as the negative and positive control, respectively. After incubation, the cells were harvested by centrifugation at 3,500 rpm for 5 minutes then washed once with sterile distilled water. Three protoplast buffers at pH 7.4 were prepared, and used in different wash steps. The constituents of buffers used are listed in Appendix B. Cells were washed twice in 500 µl of buffer 1, with 5 minutes of incubation at 25 °C in between, then harvested by centrifugation at 1,500 rpm with a soft break, and the supernatant discarded. The pellet was washed with PBS, resuspended in 500 µl of buffer 2; then 0.002 µl of lyticase was added. This was incubated at 25 °C for 30 minutes. Buffer 2 was removed by centrifugation and cells were washed in PBS. Cells were resuspended in buffer 3 and incubated at 25 °C for 15 minutes. After removing buffer 3 by centrifugation, cells were washed in PBS, then resuspended in 200 µl of PBS. The suspension was used for apoptosis assays.

3.5.2 Annexin V-Cy3 staining

The FITC Annexin V Apoptosis Detection kit I (BD Biosciences, USA) was used to study translocation of phosphatidylserine (PS) from internal to external parts of the plasma membrane, which is a marker for early apoptosis (Khan et al. 2014). The assay was carried out following manufacturer's instructions, with some modifications. Falcon 5 mL round-bottom tubes were prepared with 100 µl 1X Annexin buffer and 10 µl of protoplast

suspension was added, followed by 2 µl of FITC Annexin and 2 µL of propidium iodide (PI). The tubes were incubated at 25 °C in the dark for 15 minutes. Thereafter, 400 µL of 1X Annexin buffer was added to tubes, and results quantified using a FACS-Calibur flow cytometer (BD Biosciences, USA). For compensation controls, heat-killed cells stained with PI only, AmB treated cells stained with FITC Annexin only and unstained protoplast were used. Flow cytometry results obtained were analysed using FlowJo.

3.5.3 Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

To detect DNA breaks caused by apoptosis, the TUNEL assay was done using the Click-iT™ Plus TUNEL Assay (Thermo Fischer Scientific, USA), following manufacturer's instructions (Khan et al. 2014). Treated yeast cells were fixed in 500 µl of 4% paraformaldehyde (Sigma-Aldrich, USA) in PBS at 37 °C for 1 hour. The cells were harvested and washed in PBS and protoplasts prepared according to instructions above. Microscope slides (Lasec, South Africa) were coated with 20 µl poly-L-lysine (Sigma-Aldrich, USA), rinsed, dried and 100 µl of protoplasts was applied onto the slides then incubated for 30 minutes at 37 °C to dry. The slides were rinsed with PBS to remove unbound cells then 100 µl of permeabilization solution (0.2% Triton-X100 and 0.1% Sodium citrate in PBS) was added to cells and incubated for 2 minutes on ice. Thereafter, slides were rinsed with PBS and TUNEL reaction was done as follows: Slides were incubated with 100 µl of deoxynucleotidyl transferase (TdT) reaction buffer at 37 °C for 10 minutes. TdT reaction buffer was removed, then 50 µl of TdT reaction mixture containing TdT reaction buffer, EdUTP and TdT enzyme was added onto each slide and incubated for 1 hour at 37 °C in a humid chamber to prevent evaporation. Slides were then rinsed in deionized water. The Click-iT™ Plus TUNEL reaction cocktail was prepared, made up of 1 x Click-iT™ Plus TUNEL reaction buffer, copper protectant, Alexa Fluor 488 picolyl azide dye and 10 x Click-iT™ Plus TUNEL reaction buffer additive. Fifty microlitres of cocktail mix was added onto each slide immediately after preparation and

slides were incubated by protecting them from light for 30 minutes at 37 °C. The slides were then rinsed with PBS and dried.

Hoechst 33342 (Thermo Fischer Scientific, USA) was used to stain the cellular nucleus for easy localization of cells under the microscope. One-hundred microlitres of Hoechst stain was added onto slides and incubated at 25 °C for 15 minutes in the dark. The stain was rinsed off with deionised water and dried, then a coverslip was placed on the slides. The cells were analysed under a Zeiss LSM 780 confocal microscope (Biosciences, Germany), with excitation/emission for Alexa flour 488 and excitation/emission for Hoechst 33342. Images were analysed using ImageJ software.

3.5.4 Release of cytochrome c oxidase

3.5.4.1 Bradford protein assay

Before the cytochrome c oxidase activity was quantified, the total protein content of samples was estimated using the Bradford method (Bradford, 1976). Bovine serum albumin (BSA) (Roche Diagnostics, Germany) was used as standard protein, and concentrations prepared ranged from 0-2 µg/mL. In a cuvette, 1.5 mL of Bradford reagent (Sigma-Aldrich, USA) was added, followed by 30 µl of standards and unknown samples. The mixture was incubated at room temperature for at least 5 minutes, then absorbance was measured at 595 nm using the UV- 1800 Shimadzu spectrophotometer. To obtain a standard curve, absorbance readings of the standards versus their respective concentrations were plotted. Protein concentration for each sample was calculated using the standard curve generated.

3.5.4.2 Cytochrome c oxidase assay

The cytochrome c oxidase activity of cells exposed to tetrazole derivatives was measured to detect mitochondrial membrane integrity which can be compromised as a result of the

intrinsic pathway of apoptosis. Cytochrome C Oxidase Assay (COX) kit (ScienceCell, USA) was used according to manufacturer's instructions. Protoplasts prepared were suspended in 2 mL of lysis buffer consisting of 50 mM Tris, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), at pH 7.5. A cell homogenizer (Sigma-Aldrich, USA) was used to disrupt the cells by whisking vigorously for 30 seconds, and this was repeated for 4 cycles while resting on ice in between. Thereafter, homogenate was centrifuged at 1000 g for 10 minutes at 4 °C. The supernatant was then collected into an Eppendorf tube and was used to measure cytochrome c content and total protein content.

In a cuvette, 930 µl Assay buffer, 10 µl n-Dodecyl β-D-Maltidose solution, 50 µl substrate solution and 10 µl of protoplast suspension was mixed and the decrease in absorbance was read using the UV- 1800 Shimadzu spectrophotometer at a wavelength of 550 nm. The kinetic program was used, and it was set to take readings at an interval of 5 seconds and a duration of 30 seconds. Enzyme activity was defined as the amount of cytochrome c oxidised per minute. The following equation was used to calculate cytochrome c oxidase activity:

$$\text{Unit/mg mitochondria} = \frac{\Delta A/\ \text{min}}{\varepsilon \text{ x mg mitochondria}}$$

where;

$\Delta A/\ \text{min}$ = (change in OD reading)/time

ε=19.6 mM^{-1} cm^{-1}: the extinction coefficient of reduced cytochrome c per minute.

3.6 Statistical analysis

The results were reported as mean ± standard deviation (SD). The student's t-test was used to compare the effect of test compounds with the control in *C. albicans* strains.

Differences in results at two concentrations were assessed using Student t test. Comparison of results at more than two concentrations were made using analysis of variance (ANOVA). P values <0.05 were considered significant.

CHAPTER 4: RESULTS

4.1 Identification of *Candida albicans* strains

Strains used for the study were *C. albicans* ATCC 90028, fluconazole resistant R1 and sensitive S1. They were confirmed as *C. albicans* by cultural characteristics; appearing as white creamy-coloured colonies on Sabouraud dextrose agar (Figure 4.1), and a series of biochemical reactions using the API 20 C AUX sugar assimilation test (Biomerieux, France) as shown in Figure 4.2.

Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008) were used to categorise the strains as fluconazole (FLC) resistant or susceptible, based on their MIC values as shown in Table 4.1. Two strains, *Candida albicans* 90028 and *C. albicans* R1 were categorised as fluconazole resistant. MIC for *C. albicans* 90028 and R1 were 0.49µg/mL and 0.78µg/mL , respectively. Strain R1 was categorised as fluconazole resistant strain R1, with MIC of 64 µg/mL.

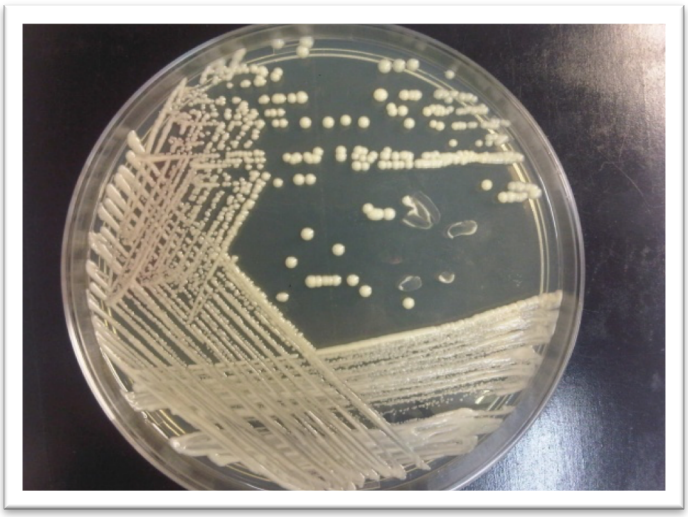


Figure 4.1: *C. albicans* colonies on Sabouraud agar plate

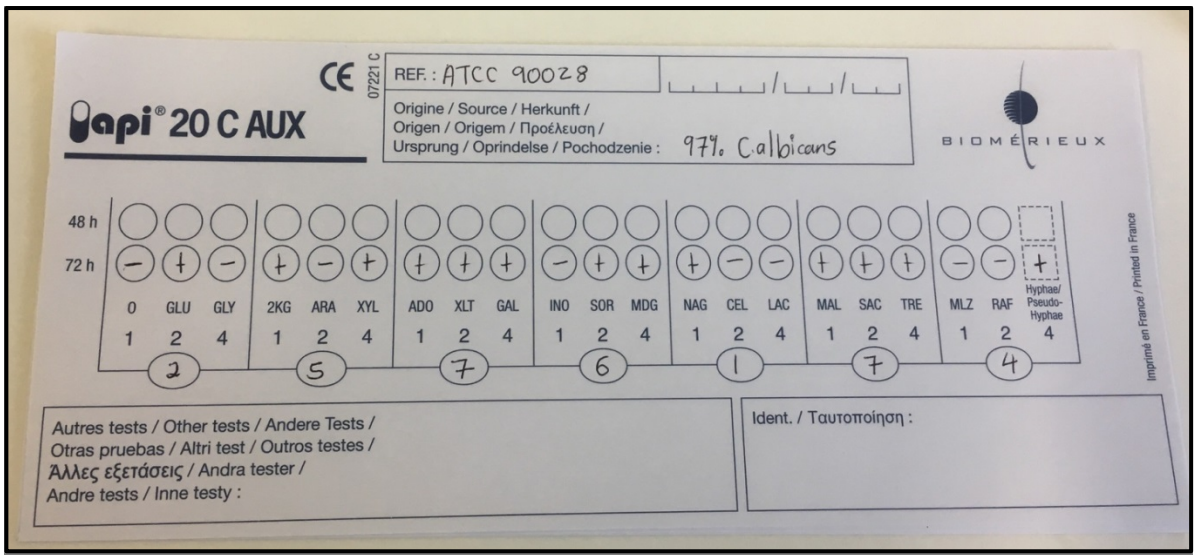


Figure 4.2: API strip for *C. albicans* ATCC 90028 strain

Table 4.1: Minimum Inhibitory Concentrations of fluconazole against *Candida albicans* strains

Compound	*C. albicans* strain	Repeats	MIC (µg/mL)	Median MIC (µg/mL)	Interpretation
Fluconazole	90028	1	0.49	0.49	Sensitive
		2	0.49		
		3	0.24		
	R1	1	62.5	62.5	Resistant
		2	62.5		
		3	62.5		
	S1	1	0.78	0.78	Sensitive
		2	0.78		
		3	0.78		

MIC=Minimum Inhibitory Concentration, *C. albicans* strains 90028 and S1 are fluconazole sensitive (MIC<64µg/mL), *C. albicans* R1 is fluconazole resistant (MIC≥ 64µg/mL).

4.2 Antifungal activity of tetrazole derivatives against *Candida albicans* strains

Three tetrazole derivatives, AN1, AN2 and AN3, were screened for their antifungal activity against three *C. albicans* strains 90028, R1 and S1. For each strain three repeats were performed for statistical purposes. All three compounds possessed antifungal activity, and the MIC and MFC values are shown in Table 4.2 and summarised in Table 4.3. The negative and

positive controls used were 1% DMSO and fluconazole, respectively. DMSO had no effect on *C. albicans,* whereas FLC inhibited growth of *C. albicans*.

AN1 and AN3 had the lowest MIC values, ranging from 0.39-1.56 μg/mL. AN2 on the other hand had a high MIC range of 6.25-12.5 μg/mL. There was no difference in the antifungal activity of the three compounds between the clinical strains and laboratory strain used. When the results were summarised (Table 4.3) it can be seen that AN1 had the lowest MIC value (0.39 μg/mL) compared to other two compounds.

The MFC values for compounds against the isolates tested varied. AN1 had MFC range of 1.56-3.13 μg/mL, AN2 had an MFC value of 25 μg/mL and lastly, the MFC of AN3 ranged between 1.56-3.13 μg/mL.

Table 4.2: Minimum Inhibitory Concentrations and Minimum Fungicidal Concentrations of tetrazole derivatives against *Candida albicans* strains

Compound	*C. albicans* strain	Repeats	MIC (μg/mL)	Median MIC (μg/mL)	MFC (μg/mL)	Median MFC (μg/mL)
AN1	ATCC 90028	1	0.39	0.39	1.56	1.56
		2	0.39		1.56	
		3	0.39		1.56	
	R1	1	1.56	1.56	6.25	3.13
		2	1.56		3.13	
		3	1.56		3.13	
	S1	1	0.39	0.39	1.56	1.56
		2	0.76		1.56	
		3	0.39		0.78	
AN2	ATCC 90028	1	6.25	6.25	25	25
		2	6.25		25	
		3	6.25		25	
	R1	1	12.5	12.5	25	25
		2	12.5		25	
		3	12.5		25	
	S1	1	12.5	12.5	25	25
		2	12.5		12.5	
		3	12.5		25	
AN3	ATCC 90028	1	1.56	1.56	3.13	3.13
		2	1.56		3.13	
		3	1.56		3.13	
	R1	1	0.78	0.39	1.56	1.56
		2	0.39		0.78	
		3	0.39		1.56	
	S1	1	1.56	1.56	3.13	3.13
		2	1.56		3.13	
		3	0.78		3.13	

MIC=Minimum Inhibitory Concentration; MFC=Minimum Fungicidal Concentration, *C. albicans* strains 90028 and S1 are fluconazole sensitive, *C. albicans* R1 is fluconazole resistant.

Table 4.3: Summary of Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of tetrazole derivatives against *Candida albicans*

	Median (range) n=9	
Compound	**MIC (μg/mL)**	**MFC (μg/mL)**
AN1	0.39 (0.39-1.56)	1.56 (1.56-3.13)
AN2	12.5 (6.25-12.5)	25 (25)
AN3	1.56 (0.39-1.56)	3.13 (1.56-3.13)

MIC=Minimum Inhibitory Concentration; MFC=Minimum Fungicidal Concentration, MIC and MFC were reported as the median of three readings, n=total number of repeats

4.3 Effect of tetrazole derivatives on ergosterol content in *Candida albicans*

For this assay, depending on the MIC values, three concentrations for each of the test compounds were selected (Table 4.4). Cell suspensions of *C. albicans* were grown for 16 hours, to allow cells to reach stationary phase, with varying concentrations (outlined in Table 4.4) of tetrazole derivatives, to determine if compounds inhibit fungal growth through inhibition of the ergosterol biosynthesis pathway, which results in suppression of ergosterol content in cells. Fluconazole was used as a positive control because it is known to inhibit *C. albicans* growth by interfering with the ergosterol biosynthesis pathway.

Table 4.4: Outline of concentrations used for ergosterol quantitation assay

Concentrations used (μg/mL)	**AN1**	**AN2**	**AN3**
0.1	**X**		
0.2	**X**		
0.39	**X**		**X**
0.78			**X**
1.56			**X**
3.13		**X**	
6.25		**X**	
12.5		**X**	

4.3.1 Comparison of ergosterol content between test compounds and controls

The mean ergosterol content was calculated from three repeats in each concentration and this was used to calculate percentage decrease in ergosterol content in treated cells. Table 4.5 and Figure 4.3 show ergosterol content in cells treated with three tetrazole derivatives, in comparison to FLC treated cells. There was a significant reduction ($p<0.05$) in ergosterol content by all three test compounds compared to the negative control (Figure 4.3).

Table 4.5: Effect of tetrazole derivatives on ergosterol in *Candida albicans* cells

C. albicans strains	Repeats	Ergosterol content in *C. albicans* strains n=9									
				Compounds (µg/mL)							
		Control	FLC	AN1			AN2			AN3		
				0.1	0.2	0.39	3.13	6.25	12.5	0.39	0.78	1.56
ATCC 90028	1	0.0165	0.00167	0.0168	0.0161	0.01527	0.00598	0.00533	0.00276	0.01825	0.0152	0.0152
	2	0.0182	0.00117	0.01528	0.0165	0.01534	0.00515	0.0531	0.00209	0.01298	0.01537	0.011
	3	0.01769	0.0022	0.01707	0.01628	0.01208	0.00718	0.0523	0.00293	0.01829	0.01445	0.0122
	Mean	**0.01758**	**0.00168**	**0.01638**	**0.01629**	**0.01423**	**0.0061**	**0.00529**	**0.00259**	**0.0165**	**0.015**	**0.0128**
	±SD	**0.00068**	**0.00052**	**0.00097**	**0.0002**	**0.00186**	**0.00102**	**0.00005**	**0.00044**	**0.00305**	**0.00049**	**0.00216**
R1	1	0.03105	0.02437	0.02398	0.01621	0.01222	0.01809	0.01335	0.00909	0.02549	0.023	0.01709
	2	0.03128	0.02402	0.02406	0.0235	0.02091	0.01609	0.01197	0.0124	0.02612	0.02344	0.01812
	3	0.0322	0.025	0.02445	0.022	0.02245	0.0181	0.0151	0.00796	0.02571	0.0226	0.01937
	Mean	**0.03151**	**0.02446**	**0.02416**	**0.02057**	**0.01853**	**0.01743**	**0.01347**	**0.00982**	**0.02577**	**0.02301**	**0.01819**
	±SD	**0.0006**	**0.0005**	**0.00025**	**0.00385**	**0.00555**	**0.0012**	**0.0016**	**0.0023**	**0.0003**	**0.0004**	**0.0011**
S1	1	0.01556	0.00145	0.01445	0.01223	0.01344	0.00623	0.00437	0.00441	0.00928	0.00901	0.00608
	2	0.01729	0.00147	0.01479	0.013	0.00903	0.0094	0.00309	0.00276	0.01091	0.00622	0.0061
	3	0.017	0.00112	0.01263	0.01349	0.00927	0.00845	0.00321	0.00309	0.00934	0.0075	0.00622
	Mean	**0.01662**	**0.00135**	**0.01396**	**0.01291**	**0.0106**	**0.00803**	**0.00356**	**0.00342**	**0.00984**	**0.00758**	**0.00613**
	±SD	**0.00093**	**0.00019**	**0.00116**	**0.00064**	**0.00248**	**0.00163**	**0.00071**	**0.00087**	**0.00092**	**0.0014**	**0.00008**
	Combined mean	**0.0219**	**0.00916**	**0.01817**	**0.01659**	**0.01445**	**0.01052**	**0.00744**	**0.00528**	**0.01737**	**0.0152**	**0.01237**

Ergosterol content expressed as a percentage of the cell weight, ±SD=Standard deviation, compound concentrations(µg/mL) used for AN1= 0.1, 0.2 and 0.39, AN2= 3.13, 6.25 and 12.5, AN3= 0.39, 0,78 and 1.56

The mean ergosterol content for the negative control was 0.02 and for the FLC it was 0.009. At the highest concentrations, ergosterol content for the test compounds AN1, AN2 and AN3 was 0.01445, 0.00528 and 0.01237, respectively (Table 4.5)

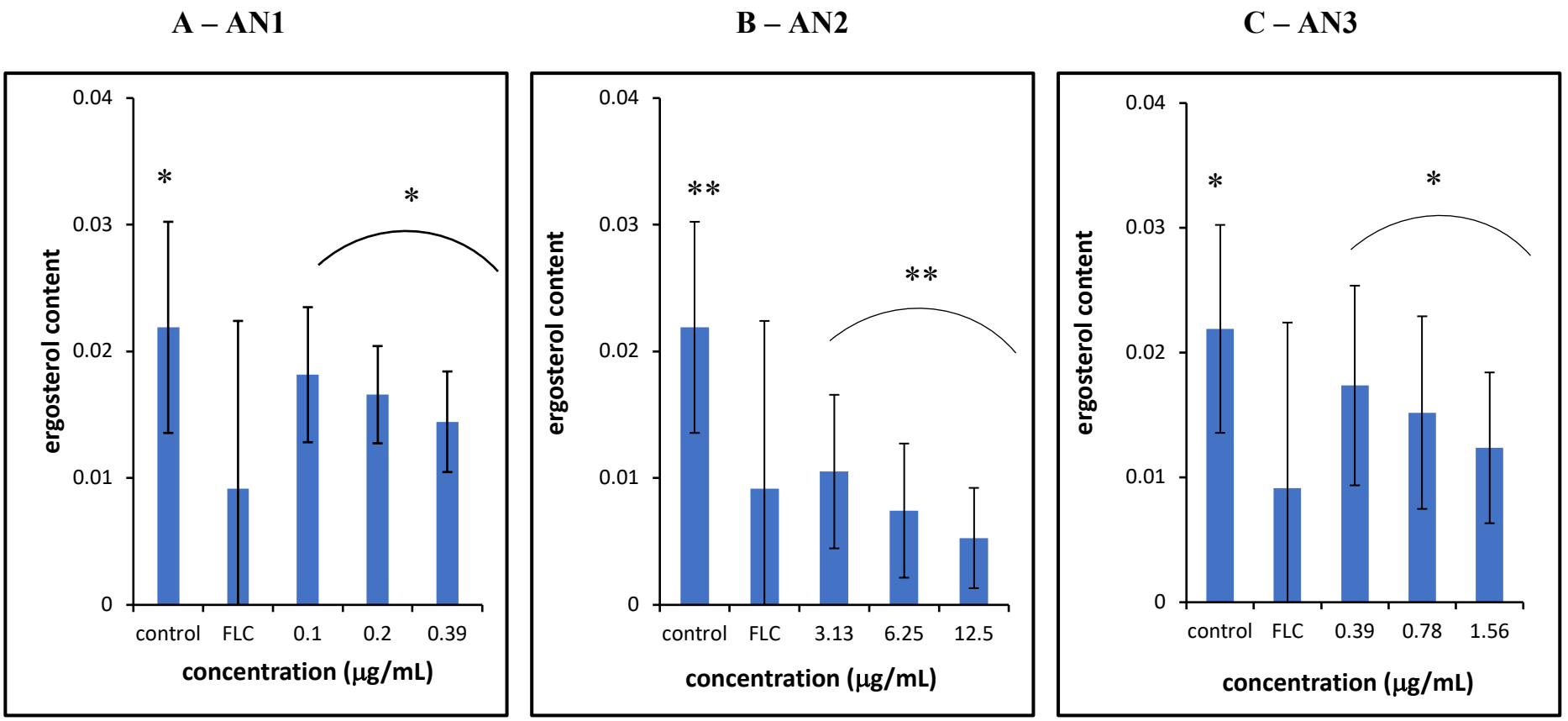


Figure 4.3: Effect of AN1 (**A**), AN2 (**B**) and AN3 (**C**) on ergosterol content of *C. albicans* cells. Three concentrations of each compound were used as well as the negative and positive (FLC) control.

- *, **: Comparison between ergosterol content of negative control and treated *C. albicans* cells using ANOVA
- *, **: p value < 0.05
- Error bars represent standard deviations of the mean value

4.3.2 Comparison of ergosterol percentage reduction

The three compounds tested exhibited different percentage reduction in ergosterol content in the three concentrations used (Table 4.6). Compound AN1 reduced ergosterol content by 14.4 to 32.2% and AN3 by 22.1 to 44.5%. However, AN2 reduced ergosterol content by a higher percentage (53.9 to 68.5%) when compared to other tetrazoles. Overall, there was a dose-dependent decrease in ergosterol content in *C. albicans* cells grown in the presence of all three tetrazole derivatives.

When the results of ergosterol reduction of test compounds were compared to the results of FLC, AN1 showed a significant difference in ergosterol reduction, with a p value of <0.05 (Figure 4.4). This means that although AN1 had some anti-ergosterol activity, it was not as good as fluconazole. However, ergosterol reduction in AN2 and AN3 was not different from that of FLC ($p>0.05$). This means that their antifungal activity is comparable, and as good as commonly used antifungal agent fluconazole.

When the results of test strains were compared, it was seen that on average, cells treated with FLC had a reduction of 68.2% in ergosterol content, compared with untreated cells. Fluconazole resistant strain (R1) showed a decrease of 22% and FLC susceptible strains (90028 and S1) showed a decrease of over 90%. For resistant strain R1, all test compounds performed better than FLC (FLC: 22.3% vs AN1, AN2, AN3: 18.2-68.8%). However, for FLC sensitive strain S1, only AN2 proved to be superior and comparable to FLC (FLC: 92% vs AN2: 80%). This suggests that overall AN2 performed best in causing reduction in the ergosterol content in *C. albicans*.

Detailed statistical results are shown in Table 4.7.

Table 4.6: Percentage decrease of ergosterol content in *Candida albicans* cells treated with tetrazole derivatives

***C. albicans* strains**	**FLC**	**% decrease of ergosterol content**								
		Compounds (μg/mL)								
		AN1			**AN2**			**AN3**		
		0.1	**0.2**	**0.39**	**3.13**	**6.25**	**12.5**	**0.39**	**0.78**	**1.56**
ATCC 90028	90.4	6.8	7.3	19	65.3	69.9	85.3	7.4	15.8	28.2
R1	22.3	23.3	34.7	41.2	44.7	57.3	68.8	18.2	27	42.3
S1	91.8	16	22.3	36.3	51.7	78.6	79.4	40.8	54.4	63.1
Combined mean	**68.2**	**15.4**	**21.4**	**32.2**	**53.9**	**68.5**	**77.8**	**22.1**	**32.4**	**44.5**

Percentage reduction in the mean ergosterol content of cells treated with fluconazole and three tetrazole derivatives compared to the ergosterol content in untreated cells

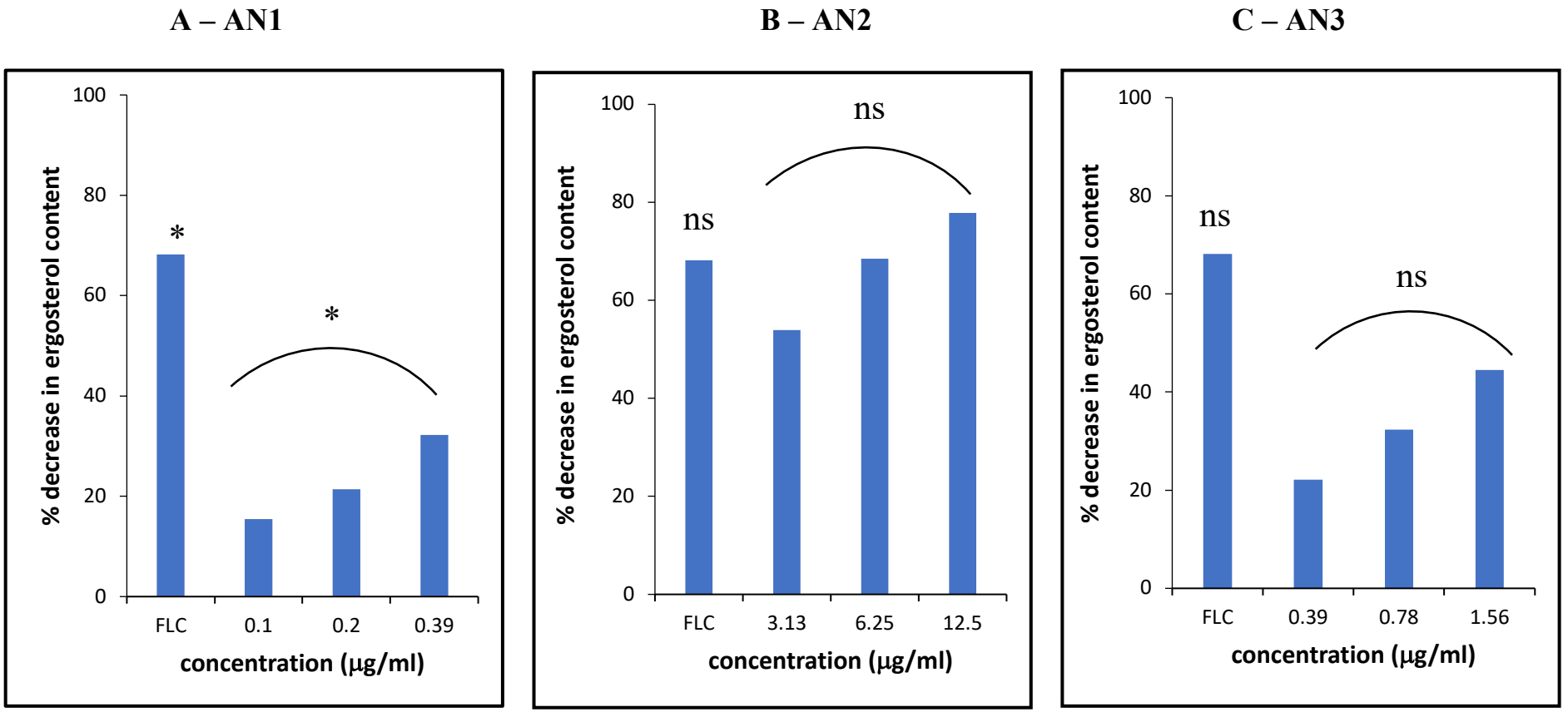


Figure 4.4: Percentage decrease of ergosterol in *C. albicans* cells treated with AN1 (**A**), AN2 (**B**) and AN3 (**C**) in comparison to cells treated with FLC.

- *, **ns**: comparison of ergosterol reduction percentage between *C. albicans* cells treated with FLC (positive control) and tetrazole derivatives using ANOVA
- *: p value < 0.05

Table 4.7: Statistical analysis of data obtained in ergosterol quantitation assay

Compounds	Comparison	p value
AN1	FLC to all concentrations	0.049
	Control to all concentrations	0.04
	Control to 0.1 μg/mL	0.14
	Control to 0.2 μg/ mL	0.03
	Control to 0.39 μg/ mL	0.01
AN2	FLC to all concentrations	0.17
	Control to all concentration	<0.001
	Control to 3.13 μg/ mL	0.008
	Control to 6.25 μg/ mL	0.05
	Control to 12.5 μg/ mL	<0.001
AN3	FLC to all concentrations	0.17
	Control to all concentrations	0.035
	Control to 0.39 μg/ mL	0.39
	Control to 0.78 μg/ mL	0.05
	Control to 1.56 μg/ mL	0.02

4.4 Effect of tetrazole derivatives on apoptosis

Apoptosis assays were done to detect different apoptotic markers following exposure of *C. albicans* cells to different concentrations of tetrazole derivatives. Each experiment was repeated twice. Concentrations used for all apoptosis assay are tabulated in Table 4.8. *Candida albicans* cell suspensions were incubated with test compounds and negative and positive control (AmB) for 160 minutes. Thereafter, protoplasts were prepared from treated cells using lyticase enzyme at an optimized concentration of 0.02 mg/mL. This was the concentration that showed no apoptosis or necrosis which could possibly yield false-positive results. All apoptosis assays were performed on protoplasts.

Table 4.8: Tetrazole derivatives concentrations used for apoptosis study

Concentration used (µg/mL)	**AN1**	**AN2**	**AN3**
0.2	**X**		
0.39	**X**		
0.78			**X**
1.56			**X**
3.13			
6.25		**X**	
12.5		**X**	

4.4.1 Effects of tetrazole derivatives on early apoptosis, late apoptosis and necrosis

To assess if antifungal activity of tetrazole derivatives is through induction of apoptosis, annexin V-Cy3 assay was done on *C. albicans* protoplasts treated with AN1, AN2 and AN3.

As shown in Table 4.9, all three compounds showed a similar pattern of activity, with all three compounds inducing early apoptosis in 10 to 14% of the cell population. Generally, AN2 had the most effect on *C. albicans* protoplasts when compared to other compounds.

Results obtained show that both apoptosis and necrosis account for tetrazole-induced cell death; indicated by a positive population in Annexin V^+PI^- quadrant (early apoptosis), Annexin V^+PI^+ quadrant (late apoptosis), Annexin V^-PI^+ quadrant (necrosis) and Annexin V^-PI^- quadrant (live). So in addition to inducing early apoptosis, the compounds also caused membrane damage. Population of cells with membrane damage was however higher than population of early apoptosis, ranging from 23 to 50% and 8 to 11%, respectively (Table 4.10 and Figure 4.5-4.7). Although cells in the Annexin V^+PI^+ quadrant showed membrane damage, it cannot be easily deduced if this is a result of late apoptosis or necrosis, since late apoptotic cells also have compromised membranes that take up PI stain. Treatment with the compounds also resulted in dead cells that were positive for PI stain only.

When treated cells were compared to positive control (AmB), there was a significant difference in the number of cells in the early apoptosis quadrant (Figure 4.5, 4.6 and 4.6). AmB induced early apoptosis in 32%, whereas the compounds collectively induced early apoptosis in 7 to 10% of the *C. albicans* cell population (Table 4.10). Therefore, AmB was more effective than tetrazole derivatives in inducing early apoptosis

Statistical comparison between concentrations of all three compounds with untreated controls, showed that the compounds significantly induced early apoptosis in *C. albicans* cells. Similarly, the same trend was observed in late apoptosis population, except in cells treated with 6.25µg/mL AN2, which showed no statistical significance (Table 4.11).

Table 4.9: Effects of tetrazole derivatives on cell death mode in *C. albicans*

Compound	*C. albicans* strain	Concentration (μg/mL)	Repeats	Percentage of cell death mode (%)			
				Live	Early apoptosis	Late apoptosis	necrotic cells
Control	ATCC 90028	0	1	99.8	0.085	0.014	0.099
			2	99.8	0.0084	0.003	0.11
	R1		1	99.5	0.13	0.003	0.0034
			2	99.6	0.0058	0.0034	0.29
	S1		1	99.1	0.65	0.058	0.24
			2	99.6	0.073	0.006	0.27
AmB	ATCC 90028	2	1	69	18.2	9.57	3.29
			2	69.5	18	9.11	3.33
	R1		1	48.4	51.2	0.28	0.16
			2	57.8	41.3	0.65	0.19
	S1		1	54.5	42.7	2.55	0.24
			2	67.2	19.1	9.92	3.72
AN1	ATCC 90028	0.2	1	43.5	7.5	40.9	8.14
			2	49.8	10.7	30.6	8.88
		0.39	1	32	7.26	51.4	9.34
			2	30.2	5.87	51.8	12.1
	R1	0.2	1	65	10.7	20.5	3.87
			2	65	8.42	18.1	8.46
		0.39	1	64.3	7.03	22.5	6.13
			2	60.8	7.62	25.9	5.73
	S1	0.2	1	61.2	14	21	3.78
			2	63.5	14.4	18.8	3.26
		0.39	1	54.1	14	28.1	3.72
			2	60.7	14.3	21.4	3.67
AN2	ATCC 90028	6.25	1	55.1	10.3	26.5	8.09
			2	45.4	6.91	33.5	14.1
		12.5	1	24.6	6.59	56.7	12.1
			2	28.2	3.92	53.2	14.7
	R1	6.25	1	45.4	12.4	34.4	7.75
			2	52.2	13.5	28.6	5.64
		12.5	1	16.8	9.79	63.7	9.66
			2	24	9.74	56.5	9.76
	S1	6.25	1	67.8	11.5	16	4.68
			2	71.9	10.4	11.4	6.24
		12.5	1	49.4	7.77	35.9	6.88
			2	48.7	7.64	36.2	7.49
AN3	ATCC 90028	0.78	1	52.1	9.82	28.8	9.24
			2	52.9	10.8	27.4	8.95
		1.56	1	42.5	10.2	37.8	9.56
			2	33.1	8.61	48.5	9.82
	R1	0.78	1	67.7	10.2	18.9	3.29
			2	63.1	9.68	21.8	5.49
		1.56	1	63	9.68	23.1	4.21
			2	62.8	9.73	19.5	7.92
	S1	0.78	1	68.8	11.1	15.6	4.51
			2	65	11.3	19.8	3.88
		1.56	1	64.3	3.93	24.4	7.38
			2	58.9	4.59	27.9	8.65

Table 4.10: Summary of the results of cell death mode caused by various concentrations of tetrazole derivatives in *C. albicans*

Compound	*C. albicans* strain	Concentration (μg/mL)	Mean percentage of cell death mode (%) n=6		
			Live	Early + Late apoptosis = Total apoptosis	necrotic cells
Control	ATCC 90028	0	99.8	0.06	0.1
	R1		99.55	0.07	0.15
	S1		99.35	0.36	0.26
	Mean		**99.57**	**0.16**	**0.17**
	±SD		**0.26**	**0.28**	**0.11**
AmB	ATCC 90028	2	69.25	27.34	3.31
	R1		53.1	46.72	0.18
	S1		60.8	37.14	1.98
	Mean		**61.07**	**37.1**	**1.82**
	±SD		**8.79**	**19.65**	**1.79**
AN1	ATCC 90028	0.2	46.65	44.85	8.51
	R1		65	28.86	6.17
	S1		62.35	34.1	3.52
	Mean		**58**	**35.93**	**6.07**
	±SD		**9.12**	**11.84**	**2.68**
	ATCC 90028	0.39	31.1	58.17	10.72
	R1		62.55	31.53	5.93
	S1		57.4	38.9	3.7
	Mean		**50.35**	**42.87**	**6.78**
	±SD		**15.28**	**17.98**	**3.33**
AN2	ATCC 90028	6.25	50.25	38.61	11.1
	R1		48.8	44.45	6.7
	S1		69.85	24.65	5.46
	Mean		**56.3**	**35.91**	**7.75**
	±SD		**11.24**	**11.67**	**3.36**
	ATCC 90028	12.5	26.4	60.21	13.4
	R1		20.4	69.87	9.71
	S1		49.05	43.76	7.19
	Mean		**31.95**	**57.95**	**10.1**
	±SD		**13.75**	**13.8**	**2.92**
AN3	ATCC 90028	0.78	52.5	38.41	9.1
	R1		65.4	30.29	4.39
	S1		66.9	28.0	4.2
	Mean		**61.6**	**32.53**	**5.89**
	±SD		**7.33**	**5.8**	**2.59**
	ATCC 90028	1.56	37.8	52.56	9.69
	R1		62.5	31.01	6.07
	S1		61.6	30.41	8.02
	Mean		**54.1**	**37.99**	**7.92**
	±SD		**13.1**	**13.71**	**2.04**

±SD= Standard deviation, n=total number of repeats

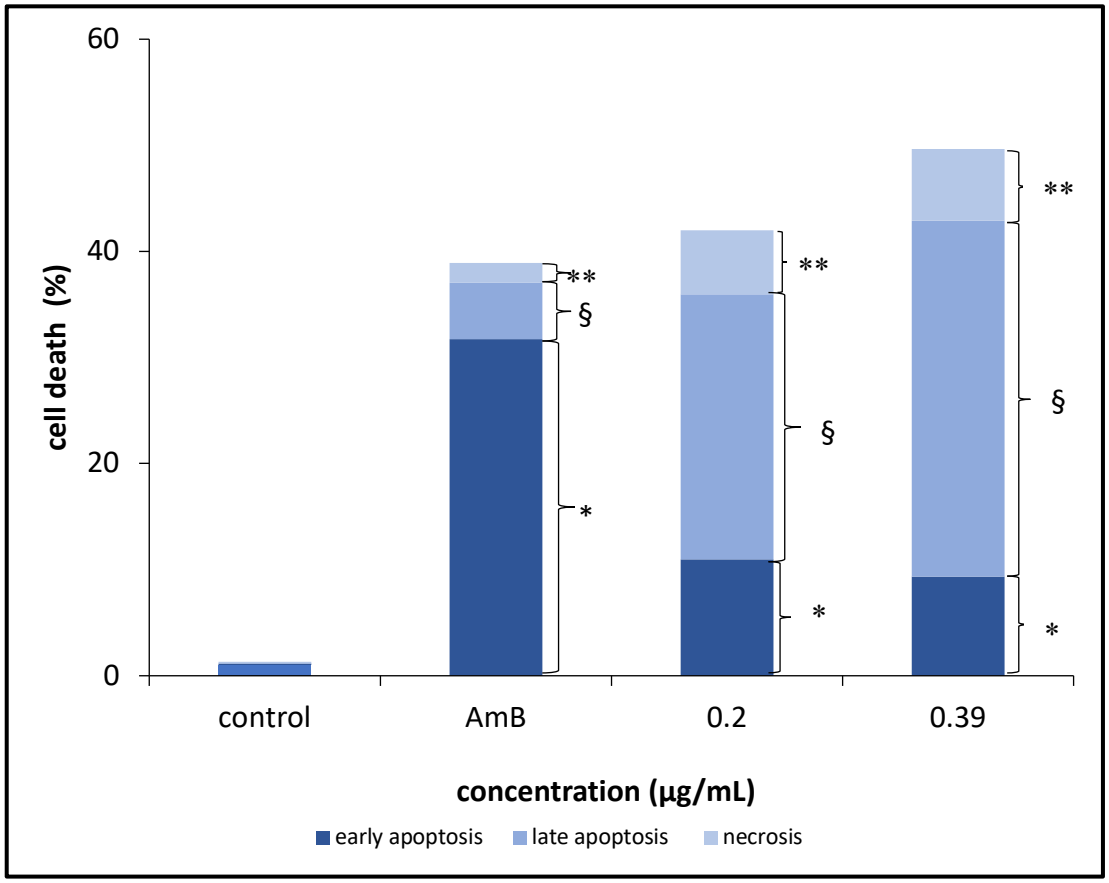


Figure 4.5: Effects of AN1 on early apoptosis, late apoptosis and necrosis on *C. albicans* cells. *, **, §: p <0.01

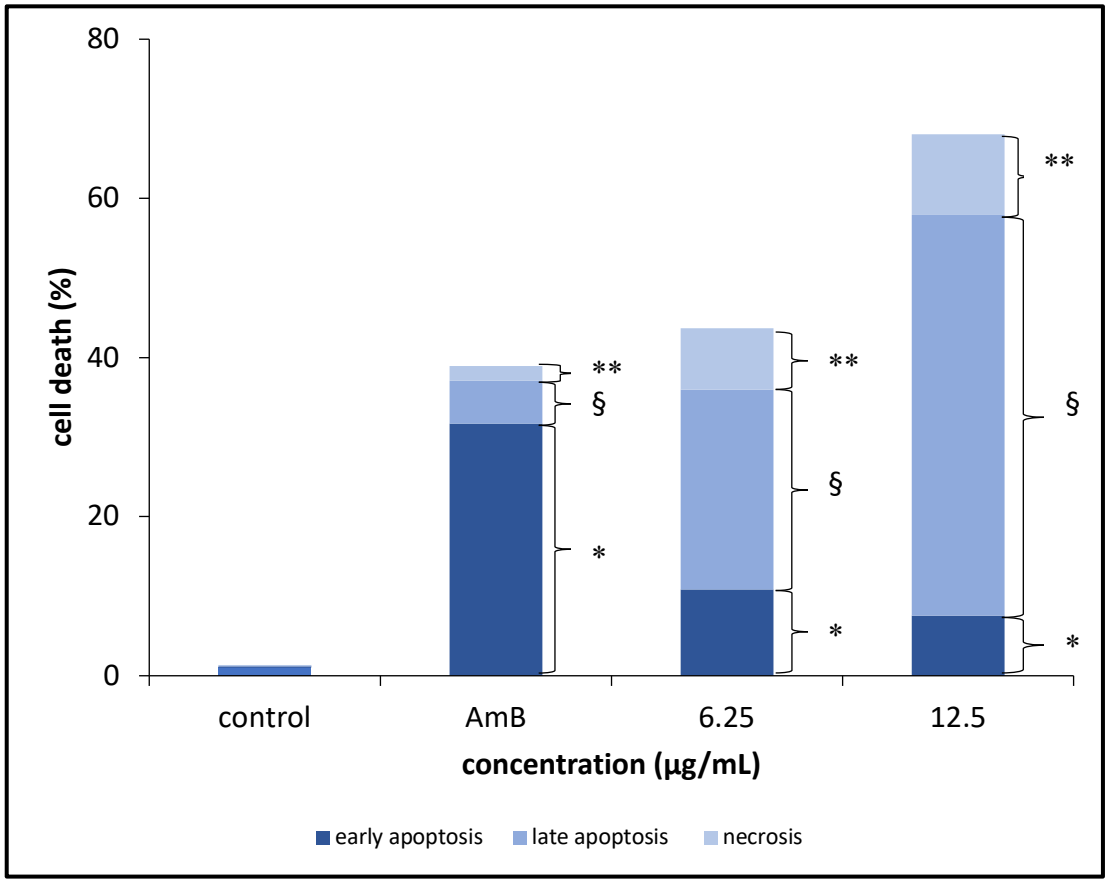


Figure 4.6: Effect of AN2 on early apoptosis, late apoptosis and necrosis on *C. albicans* cells. *, **, §: p <0.01

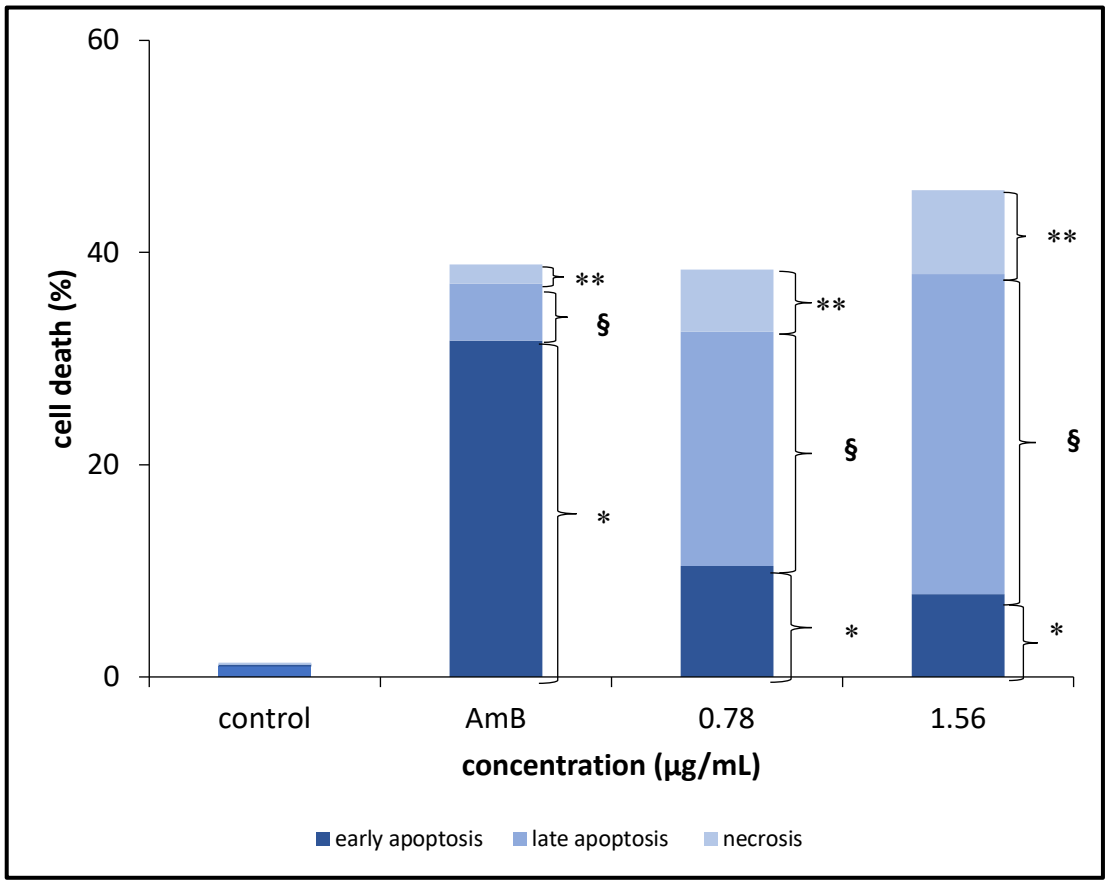


Figure 4.7: Effect of AN3 on early apoptosis, late apoptosis and necrosis on *C. albicans* cells. *, **, §: p <0.01

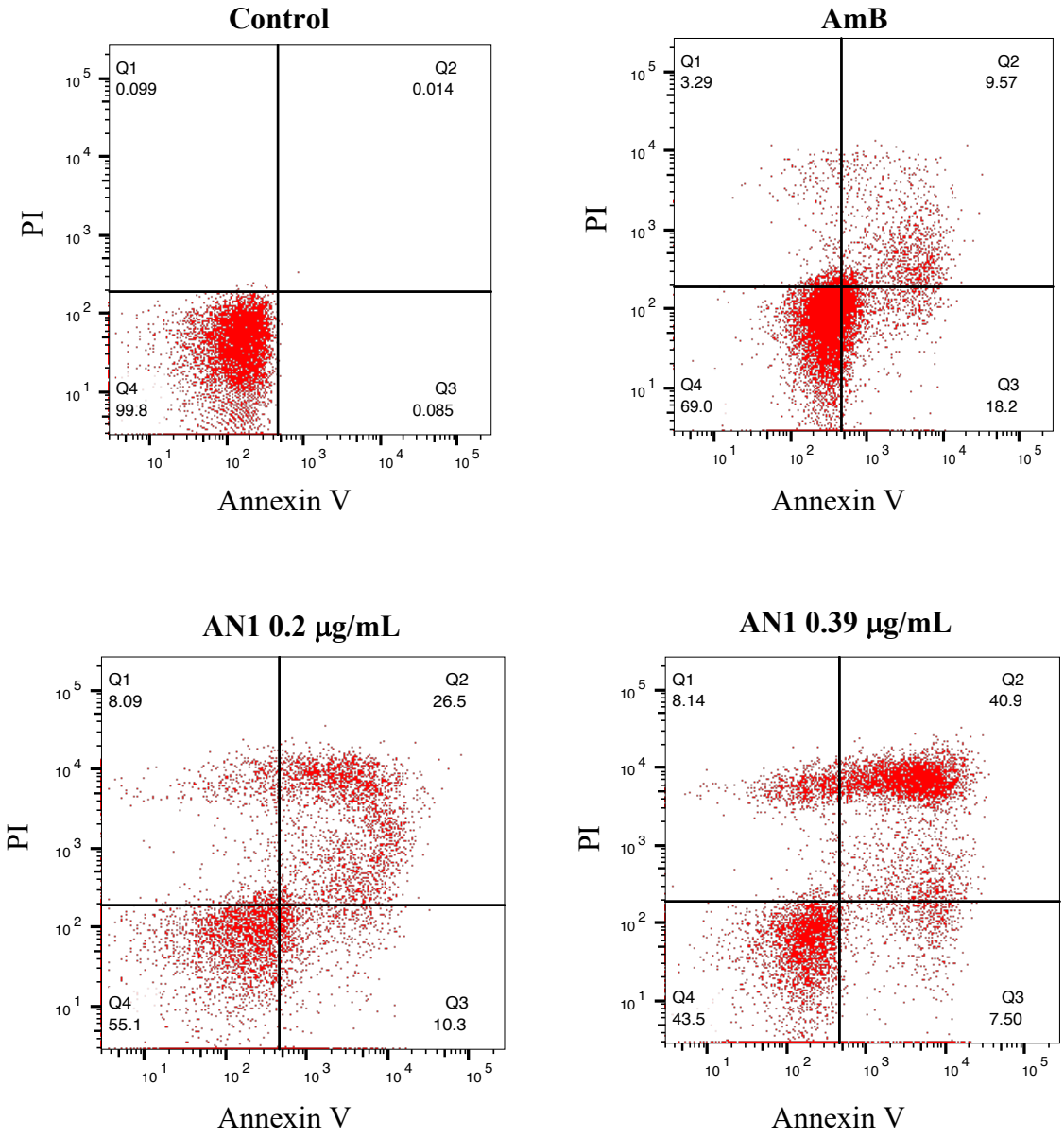


Figure 4.8: Cytograms of Annexin V-Cy3 assay using *C. albicans* ATCC 90028 cells treated with AN1. Quadrant Q1 shows PI^+ cells only (necrosis); quadrant Q2 shows Annexin V^+PI^+ cells (late apoptosis); quadrant Q3 represents Annexin V^+PI^- cells (early apoptosis); and quadrant Q4 represents Annexin V^-PI^- cells (live).

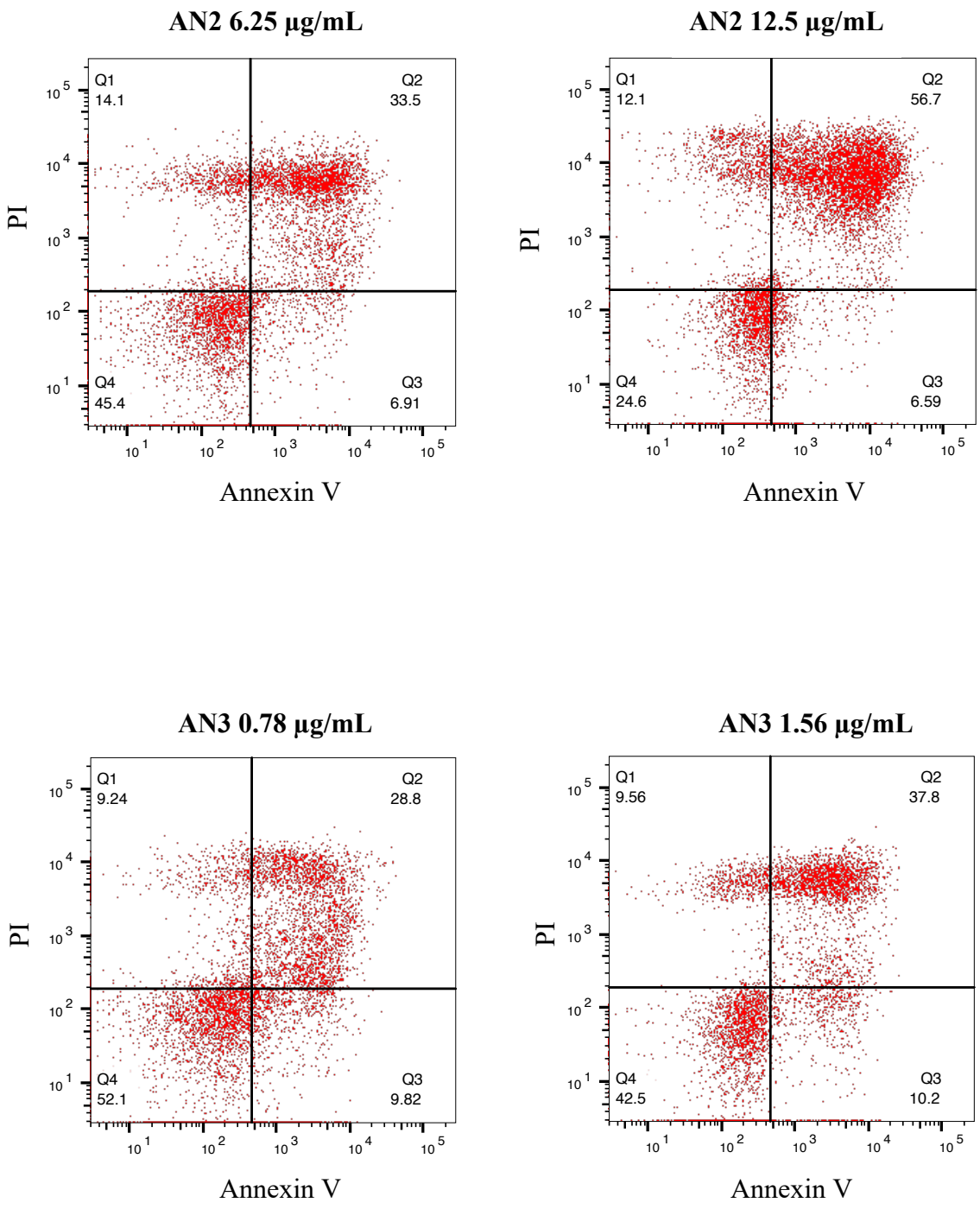


Figure 4.9: Cytograms of Annexin V-Cy3 assay using *C. albicans* ATCC 90028 cells treated with AN2 and AN3. Quadrant Q1 shows PI^+ cells only (necrosis); quadrant Q2 shows Annexin V^+PI^+ cells (late apoptosis); quadrant Q3 represents Annexin V^+PI^- cells (early apoptosis); and quadrant Q4 represents Annexin V^-PI^- cells (live).

Table 4.11: Statistical analysis of data obtained for Annexin V-Cy3 assay used to detect the effect of tetrazole derivatives on the apoptosis in *C. albicans*

Compound	Comparison	p value		
		Early apoptosis	**Late apoptosis**	**Necrosis**
AN1	AmB to all concentrations	0.001	<0.001	<0.001
	Control to all concentrations	<0.001	<0.001	<0.001
	Control to 0.2 µg/ mL	0.002	0.03	0.02
	Control to 0.39 µg/ mL	0.02	0.001	0.004
AN2	AmB to all concentrations	<0.001	<0.001	0.02
	Control to all concentrations	<0.001	<0.001	<0.001
	Control to 6.25 µg/ mL	0.03	0.1	0.05
	Control to 12.5 µg/ mL	0.06	<0.001	0.04
AN3	AmB to all concentrations	<0.001	<0.001	0.04
	Control to all concentrations	<0.001	<0.001	0.001
	Control to 0.78 µg/ mL	<0.001	0.03	0.01
	Control to 1.56 µg/ mL	0.06	0.001	0.02

4.4.2 Effect of tetrazole derivatives on DNA fragmentation in *Candida albicans* cells

Fluorescence microscopy was used to investigate whether tetrazole derivatives induce DNA damage, which is a marker for late apoptosis. TUNEL assay was carried out, where Alexa Fluor 488 (green fluorescence) was used to stain cells with fragmented DNA. Thereafter, cell nuclei were counter-stained with Hoechst 33342 (blue fluorescence) to assess changes in nuclei morphology of cells. The results were analysed qualitatively and are shown in Figures 4.10 to 4.13.

The results of controls are shown in Figure 4.10. It can be seen that the negative control has no fragmented DNA. Whereas, AmB treatment shows cells that underwent apoptosis, as well as cells with distorted nuclei. Cells treated with tetrazole derivatives were all positive for Alexa Fluor 488. The results of AN1 are shown in Figure 4.11. It can be seen that treatment with AN1 resulted in DNA fragmentation at both concentrations (0.2 μg/ mL and 0.39 μg/ mL). Treatment in AN2 and AN3 also resulted in DNA fragmentation, as shown in Figure 4.12 and 4.13 respectively. Generally, there were more cells with DNA fragmentation in lower concentrations of tetrazole derivatives.

Hoechst-stained cells showed different cell morphology as well as nucleus structure. The untreated control had few granular nuclei and no condensation was observed as seen in figure 4.10. On the other hand, cells exposed to compounds showed different nuclear morphologies following staining, with the majority of cells having condensed nuclei and appearing bright blue. Hoechst staining highlighted cells with a crescent-shaped nuclei and with nuclei with an irregular shape (grey arrows) in cells treated with AN2 12.5 μg/mL (Figure 4.12). In Figure 4.13, it can be seen that treatment with AN3 resulted in some stained DNA having long, tubular structures (red arrow, Figure 4.13), a typical abnormality associated with apoptosis. Scale bar: 50 μm

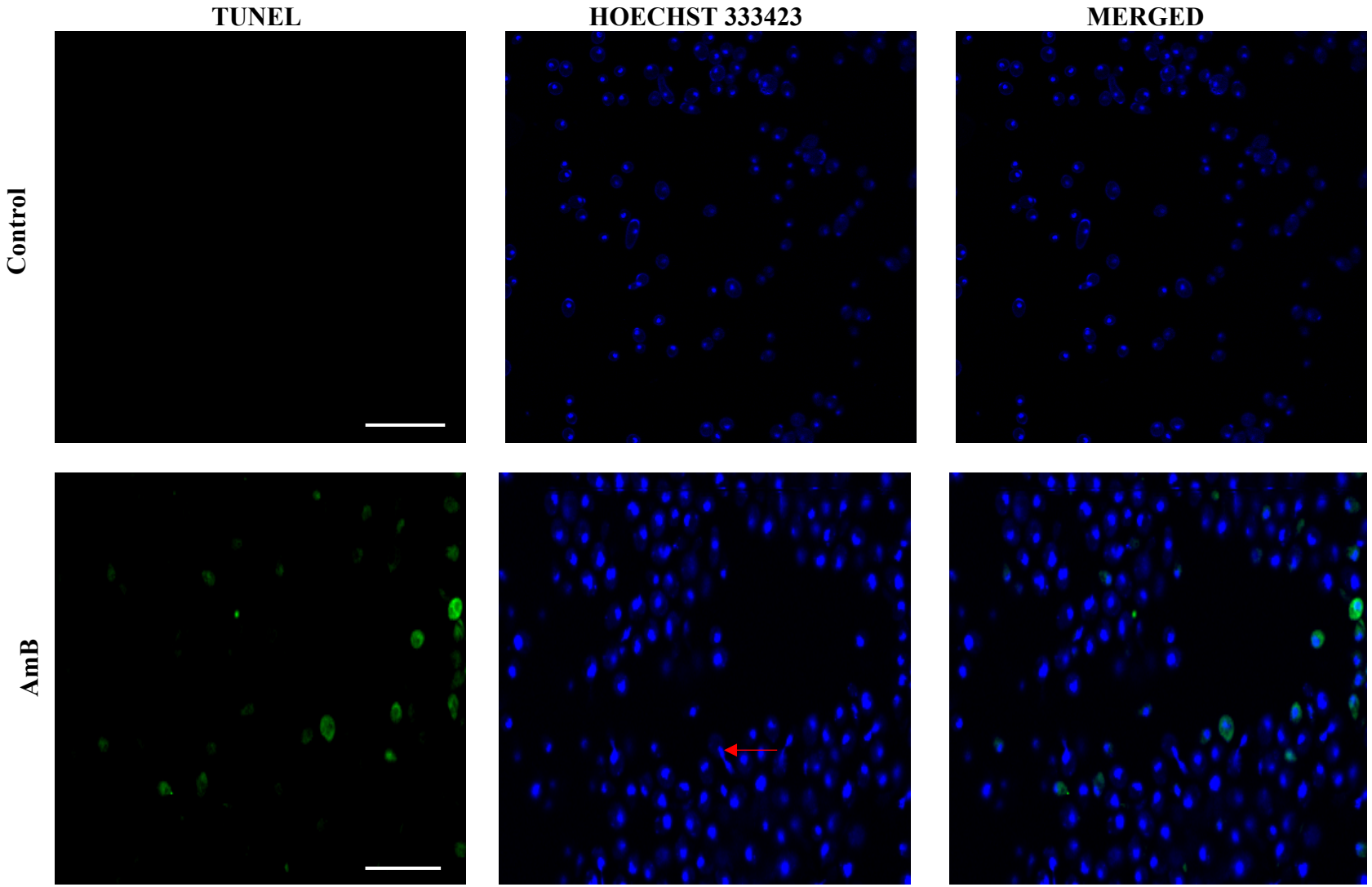


Figure 4.10: Fluorescence microscopy images of *C. albicans* (control and AmB). Arrow shows long, tubular nucleus.

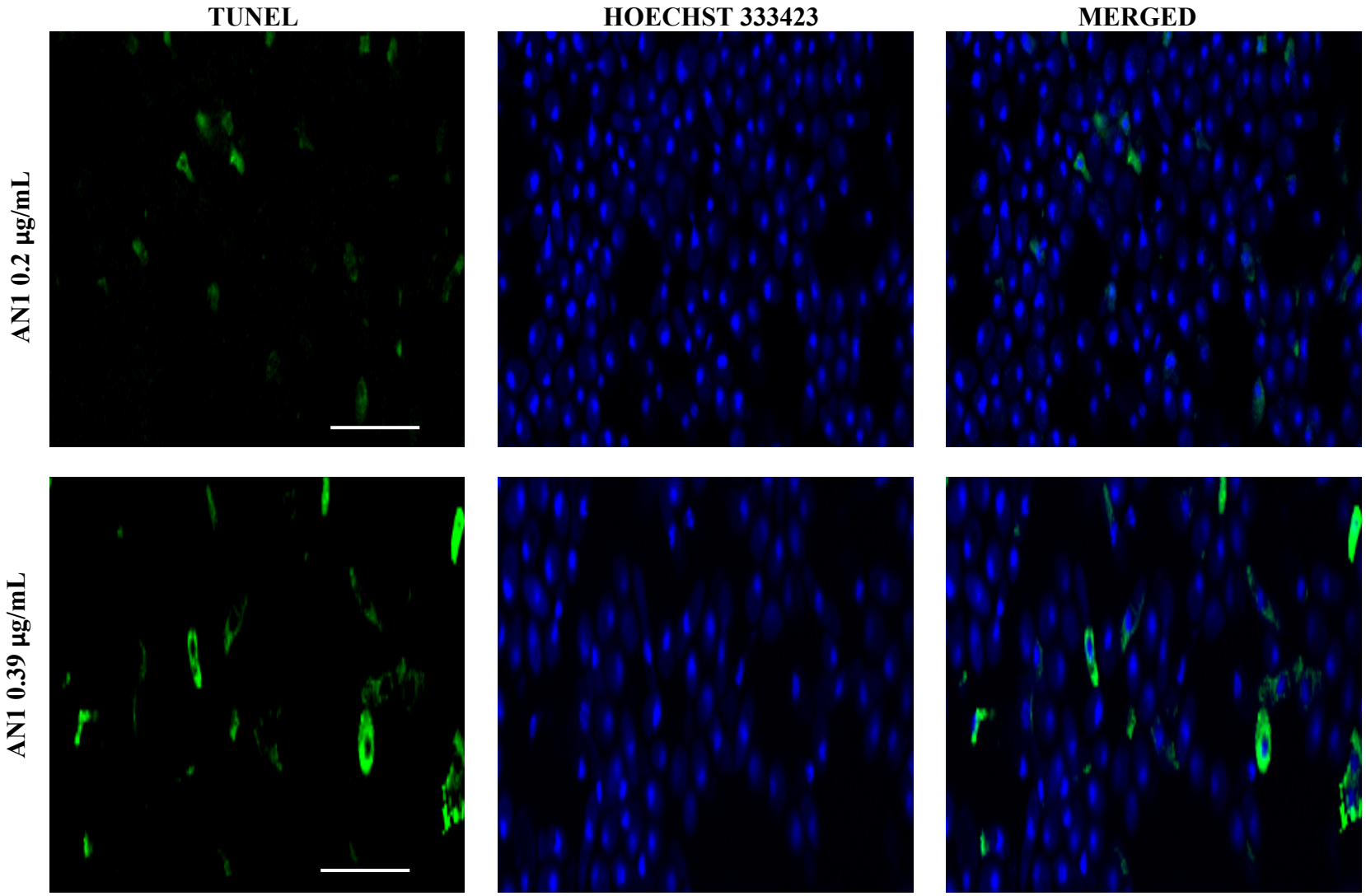


Figure 4.11: Fluorescence microscopy images of S1 *C. albicans* cells treated with AN1

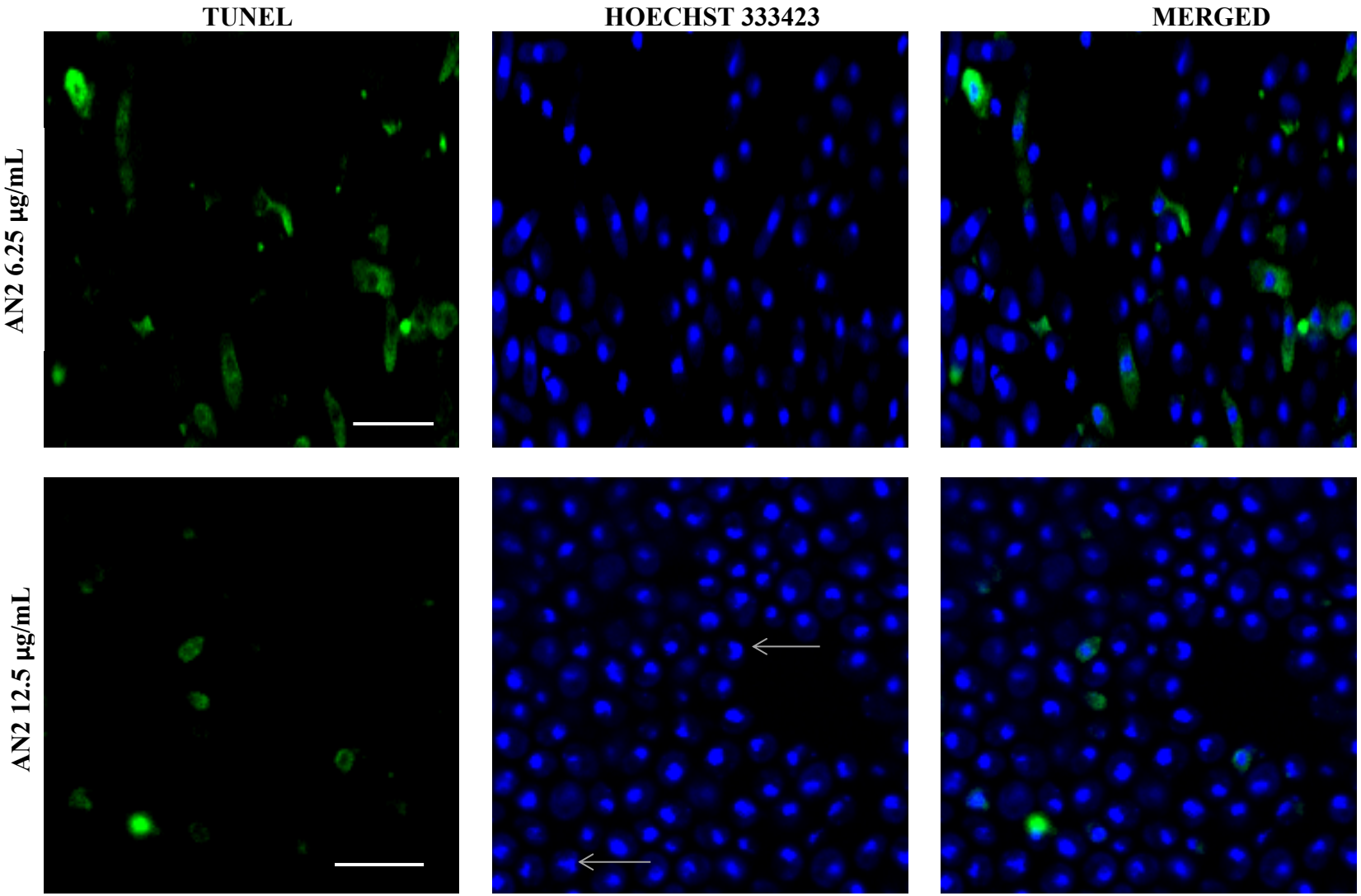


Figure 4.12: Fluorescence microscopy images of AN2 treated cells, following TUNEL assay. Arrow shows distorted nucleus

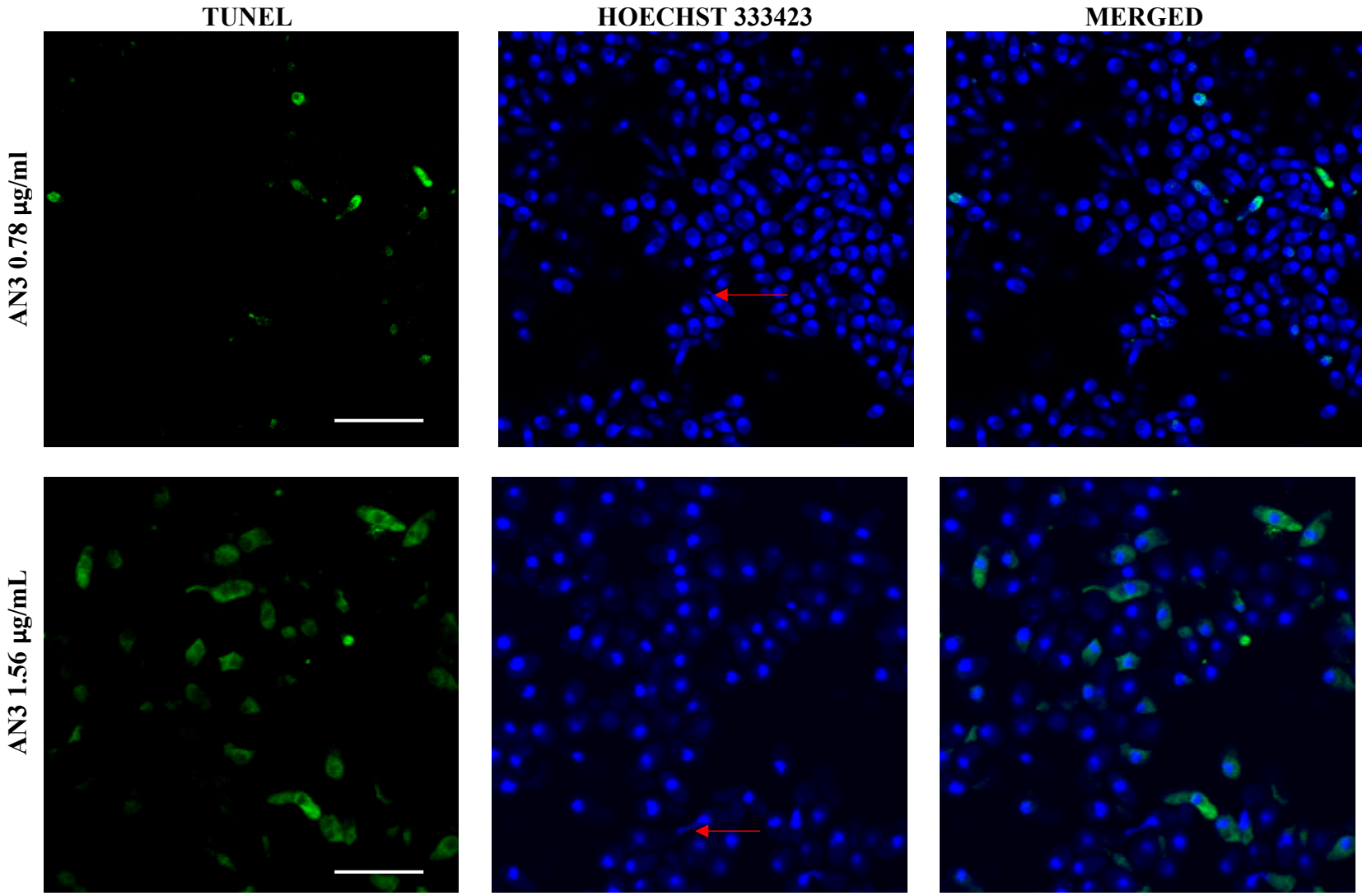


Figure 4.13: Alexa Fluor staining (green) and Hoechst 333423 (blue) of AN3 treated cells. Red arrow shows elongated nucleus

4.4.3 Release of cytochrome c oxidase enzyme in *Candida albicans* treated with tetrazole derivatives

4.4.3.1 Protein content estimation

Prior to cytochrome c oxidase content evaluation, the protein content of cells was quantified using the Bradford method, and a standard curve was generated using absorbance readings obtained from different concentrations of BSA (0-2 mg/mL). The protein concentrations of samples were estimated using linear equation obtained in the standard curve in Figure 4.14, and were used to calculate cytochrome c oxidase content. The high R^2 value obtained indicates how closely the standard curve represents the data obtained, with a 93% correlation.

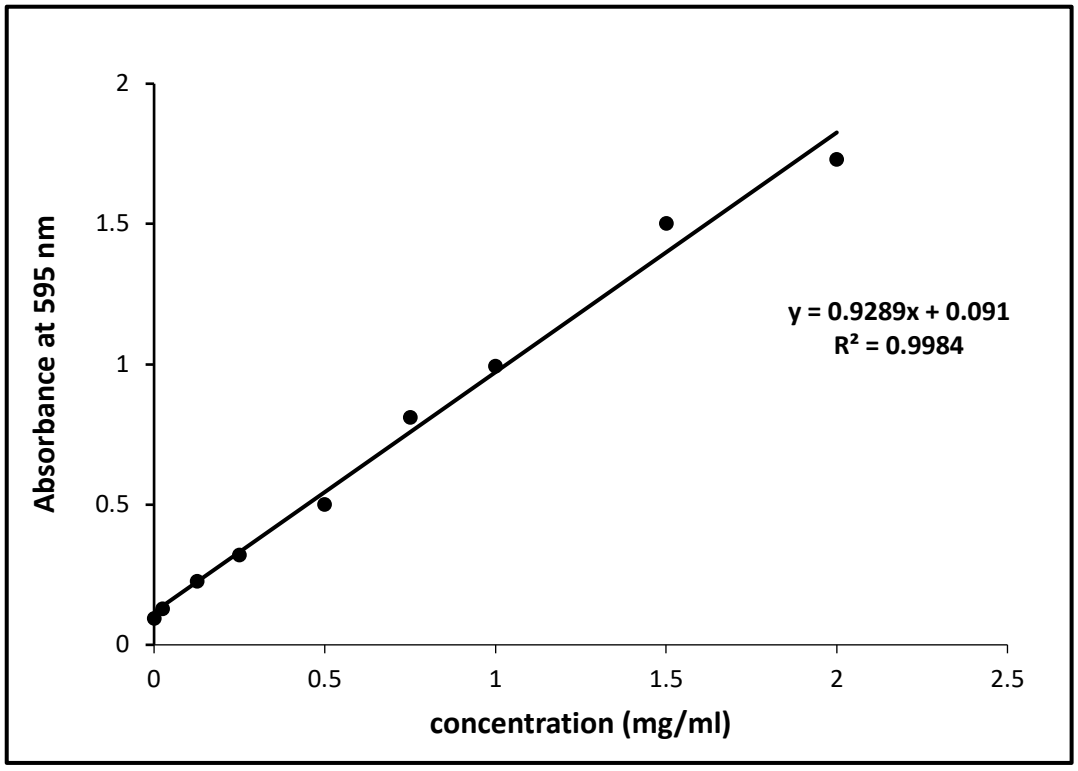


Figure 4.14: Standard curve generated from BSA for the protein content

4.4.3.2 Cytochrome c oxidase activity

The release of cytochrome c oxidase enzyme from mitochondrion into the cytosol is a crucial step in the execution of apoptosis following the intrinsic pathway. Protoplasts were evaluated for their cytosolic content of cytochrome c oxidase, following homogenisation.

Table 4.12 and Table 4.13 show the effect of tetrazole derivatives on *C. albicans.* Cytochrome c oxidase activity was detected in all treated cells. In Figure 4.15, it can be seen that the difference in enzyme activity between treated cells and the untreated control was statistically significant. In comparison to AmB control, tetrazole derivatives induced cytochrome c oxidase content modestly, and the difference between the two was statistically significant (Table 4.14 and Figure 4.16). At the highest concentration tested, AN1 (0.39 µg/mL) and AN3 (0.78 µg/mL) showed the highest activity, followed by AN2 (12.5 µg/mL). Lower concentrations of all three tetrazole derivatives yielded the same (amount) of enzyme activity. With all three compounds, the cytochrome c oxidase content decreased with increasing concentration.

Table 4.12: Assessment of cytochrome c release in *Candida albicans* treated with tetrazole derivatives

Compound	*C. albicans* strains	Concentration (µg/mL)	Repeats	Cytochrome c oxidase content (µmol/min/mg protein)
Control	ATCC 90028	0	1	0.33
			2	0.57
	R1		1	0.492
			2	0.446
	S1		1	1.1
			2	0.56
AmB	ATCC 90028	2	1	29.74
			2	18.6
	R1		1	83.8
			2	60.0
	S1		1	35.97
			2	26.08
AN1	ATCC 90028	0.2	1	19.78
			2	20.4
		0.39	1	12.84
			2	14.76
	R1	0.2	1	12.36
			2	14.14
		0.39	1	7.29
			2	7.65
	S1	0.2	1	9.0
			2	12.56
		0.39	1	7.98
			2	8.6
AN2	ATCC 90028	6.25	1	8.19
			2	8.32
		12.5	1	2.37
			2	4.41
	R1	6.25	1	10.37
			2	12.84
		12.5	1	10.0
			2	8.9
	S1	6.25	1	12.0
			2	14.21
		12.5	1	8.6
			2	11.0
AN3	ATCC 90028	0.78	1	15.2
			2	18.0
		1.56	1	12.0
			2	12.88
	R1	0.78	1	18.91
			2	12.67
		1.56	1	10.0
			2	12.0
	S1	0.78	1	9.9
			2	9.86
		1.56	1	7.68
			2	6.2

Table 4.13: Summary results of effect of tetrazole derivatives on cytochrome c oxidase activity

Compound	*C. albicans* strains	Concentration (µg/mL)	Cytochrome c content (µmol/min/mg protein) n=6
Control	ATCC 90028	0	0.45
	R1		0.47
	S1		0.83
	mean		**0.58**
	±SD		**0.27**
AmB	ATCC 90028	2	24.17
	R1		71.9
	S1		31.03
	mean		**42.4**
	±SD		**24.7**
AN1	ATCC 90028	0.2	20.09
	R1		13.25
	S1		10.78
	mean		**14.7**
	±SD		**4.5**
	ATCC 90028	0.39	13.8
	R1		7.47
	S1		8.25
	mean		**9.9**
	±SD		**3.1**
AN2	ATCC 90028	6.25	8.26
	R1		11.61
	S1		13.11
	mean		**11**
	±SD		**2.5**
	ATCC 90028	12.5	3.39
	R1		9.45
	S1		9.8
	mean		**10.5**
	±SD		**0.71**
AN3	ATCC 90028	0.78	16.6
	R1		15.79
	S1		9.88
	mean		**14.09**
	±SD		**3.9**
	ATCC 90028	1.56	12.44
	R1		11
	S1		6.94
	mean		**10.13**
	±SD		**2.7**

±SD= Standard deviation, n=total number of repeats

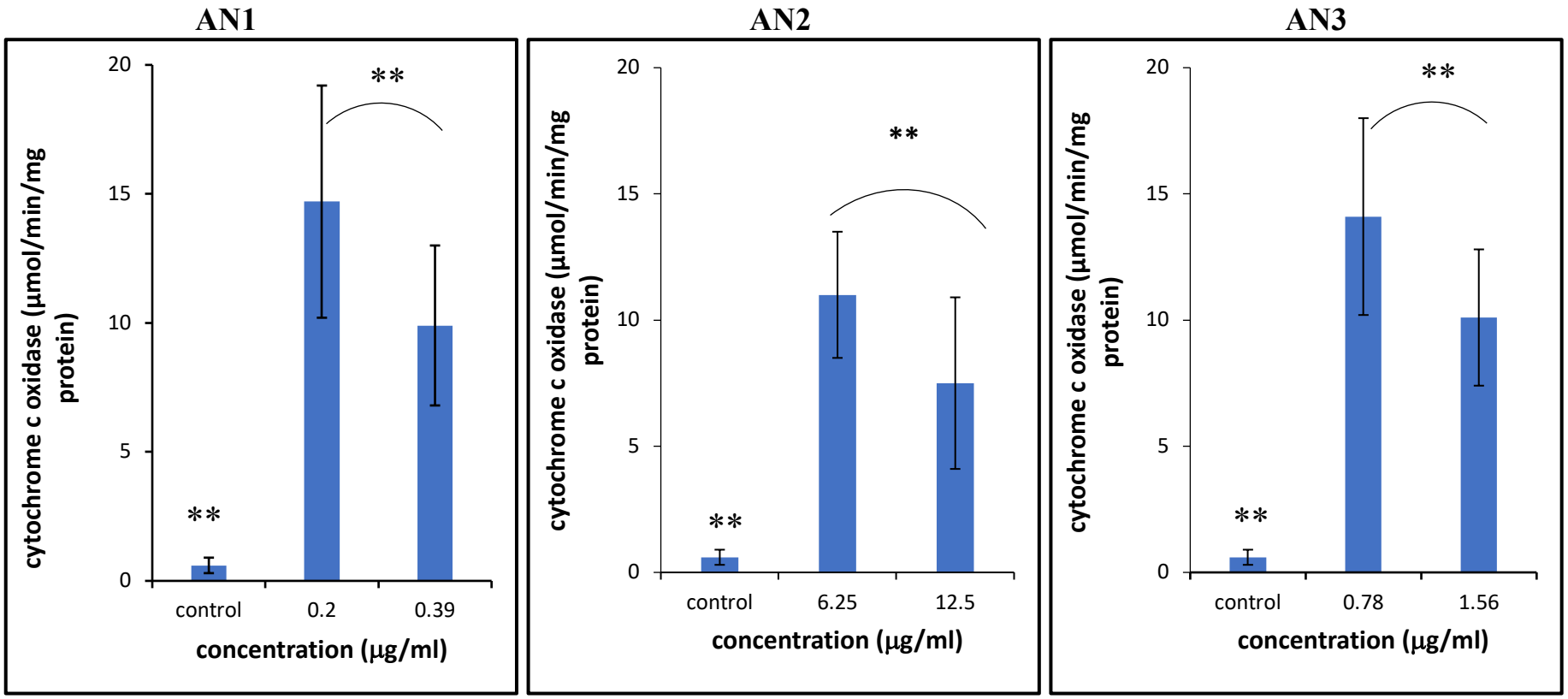


Figure 4.15: Cytochrome c oxidase released in *C. albicans* cells treated with AN1, AN2 and AN3 in comparison to untreated cells.
**: comparison of cytochrome c oxidase content between cells treated with tetrazole derivatives and untreated cells.
**: p value < 0.01

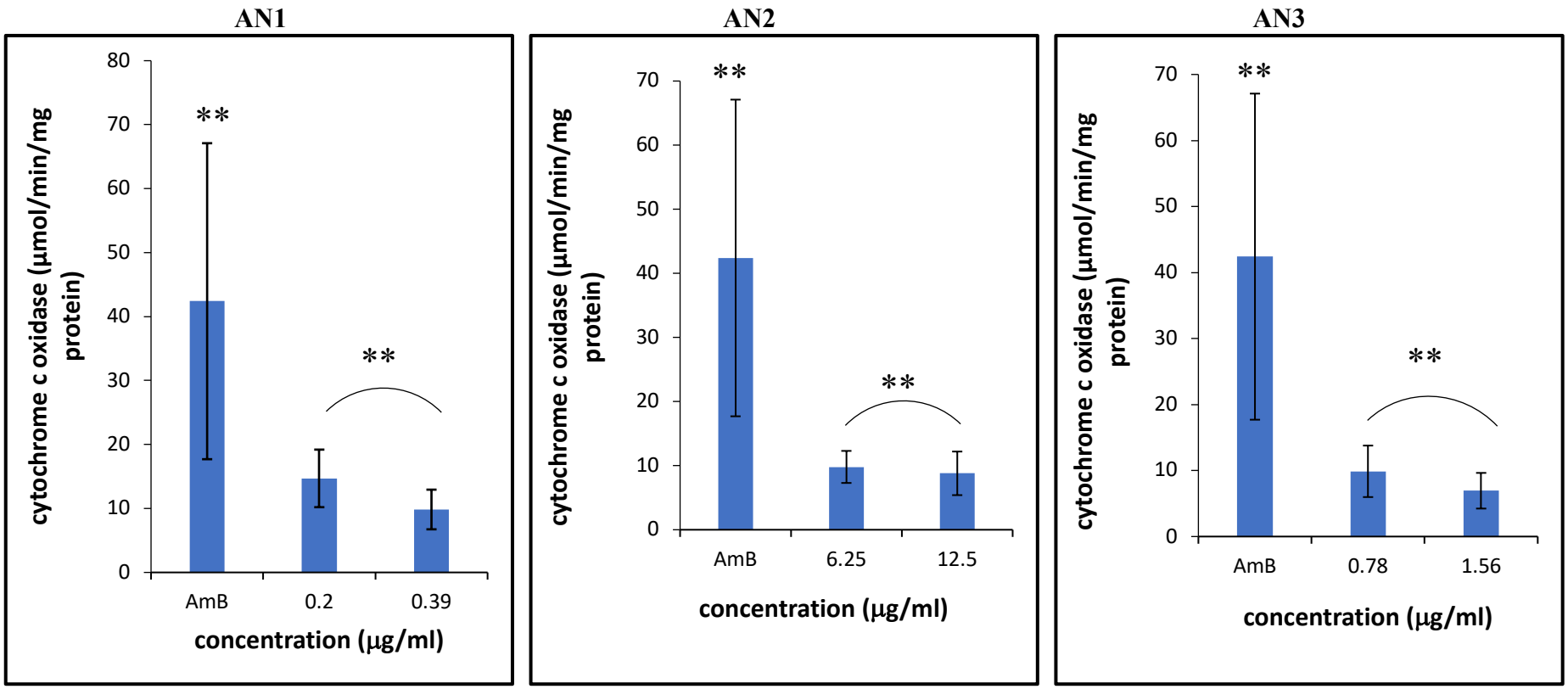


Figure 4.16: Cytochrome c oxidase activity in *C. albicans* cells treated with tetrazole derivatives and AmB.
**: comparison of cytochrome c oxidase content between cells treated with tetrazole derivatives and AmB control.
**: p value < 0.01

Table 4.14: Statistical data analysis of the effect of tetrazole derivatives on *Candida albicans* cells on cytochrome c oxidase

Compound	Comparison	p value
AN1	AmB to all concentrations	<0.001
	Control to all concentrations	<0.001
	Control to 0.2 μg/mL	0.001
	Control to 0.39 μg/mL	0.04
AN2	AmB to all concentrations	<0.001
	Control to all concentrations	<0.001
	Control to 6.25 μg/mL	0.001
	Control to 12.5 μg/mL	0.03
AN3	AmB to all concentrations	<0.001
	Control to all concentrations	<0.001
	Control to 0.78 μg/mL	<0.001
	Control to 1.56 μg/mL	0.03

CHAPTER 5: DISCUSSION

Following an increase in population of immunocompromised patients, the number of *C. albicans* infections has also been on the rise (da Silva et al., 2016). This has resulted in the over-use of antifungal drugs and consequently the emergence of antifungal drug resistance, which continues to be a major challenge. This problem necessitates the development of new antifungal agents and treatment strategies. Although there have been great investments in research dedicated to the development of new antifungal agents, there is still a limited number of antifungal drugs to treat various fungal infections. Therefore, this study investigated the potential use of three structurally different tetrazole derivatives as antifungal agents against *C. albicans,* and also established their mechanism of action through studying their effect on ergosterol content and their ability to induce apoptosis.

5.1 Antifungal activity of tetrazole derivatives

Tetrazoles have contributed to the success of pharmaceutical chemistry greatly. The tetrazole ring is used as a drug precursor in pharmaceuticals because of its ability to improve a drug's pharmacokinetic profile (Crawford et al., 2012). In recent years, an increase in the number of studies on new drugs and other biological compounds containing the tetrazole moiety has been witnessed, which led to the WHO and FDA declaring the tetrazole moiety as an important structural unit in the design of new drugs (Ostrovskii et al., 2012; Neochoritis et al., 2019). Amongst other benefits, a number of differently synthesized tetrazole derivatives have been reported to possess antifungal and anti-*Candida* activity, deeming them valuable because of the ever-increasing number of fungal infections (Matysiak et al., 2003; Upadhayaya et al., 2004; Rajasekaran et al., 2006; Malik et al., 2012; Ostrovskii et al., 2012; Kaplancikli et al., 2014,).

In a study by Matysiak et al. (2003), the MIC of an ester containing a tetrazole ring was found to be in the range 25-50 µg/mL, which is relatively high compared to the activity of the tetrazole derivatives in the present study. In another study, a series of esters bearing the tetrazole ring were tested for their antifungal activity, and the MIC ranged from 4-0.03 µg/mL (Bondaryk et al., 2015). Lastly, De Farias et al. (2016) found most active ester tetrazole to have an MIC of 512 µg/mL. In this present study, the median MIC of AN1 and AN3 were 0.39 µg/mL and 1.56 µg/mL, respectively, and AN2 had an MIC of 12.5 µg/mL (Table 4.2). The tetrazole derivatives were also active against a FLC resistant isolate, highlighting compromised membranes which have increased fluidity in resistant strains that allow passive diffusion of compounds (Mukhopadhyay et al., 2002). Given these findings, it can be concluded that variations in MIC values are a clear indication that substituents and position of substituents are important for the antifungal activity of a compound.

In ester compounds, position of the ester group and length of the alkyl chain between the tetrazole ring and ester group contribute primarily to the antifungal activity of the entire compound (Bondaryk et al., 2015). Compounds with shorter alkyl chains have increased activity when compared to compounds with longer chains. Additionally, in the case where the compound has an aromatic ring, substituents on the aromatic ring in ortho position improve the compound's activity as opposed to substituents in para position (Bondaryk et al., 2015; Bouchal et al., 2019). In this present study, AN1, AN2 and AN3 are similar in structure, substituents as well as position of substituents, with the only difference being the length of alkyl chain on the ether group attached to the aromatic ring. The antifungal activity of tetrazole derivatives has been reported to increase with increasing alkyl chain length (Łukowska-Chojnacka and Mierzejewska, 2014). Contrary to this observation, AN1, that had the shortest chain, was the most active, followed by AN3 and then AN2. So in this present study, antifungal activity of tetrazole derivatives increases with decreasing length of the ether

alkyl chain. Additionally, the weak activity or deviation of AN2 may be due to impurities in the compound or its instability which might have a negative effect on its antifungal activity. This clearly suggests further optimization and characterisation of synthesized compounds for an in-depth understanding of structure-activity relationship. Overall, tetrazole derivatives used in this study showed better activity than what has been reported in literature.

5.2 Inhibition of ergosterol biosynthesis pathway by tetrazole derivatives

Ergosterol is an essential lipid in fungi, mainly responsible for the architecture of the plasma membrane (Seemi et al., 2017). Three classes of antifungal drugs in clinical use, i.e., azoles, allylamines and morpholines, have already been developed to inhibit ergosterol enzymes lanosterol 14-a demethylase, squalene epoxidase and sterol C-14 reductase, respectively (Polak, 1988; Saag et al., 1988; Abdel-Rahman et al., 1997). As a result, enzymes in the ergosterol biosynthesis pathway have proven to be good and effective targets for antifungal agents. This study therefore investigated the possibility of the ergosterol biosynthesis pathway being a target of tetrazole derivatives.

Growth in the presence of inhibitory and various sub-lethal concentrations of tetrazole derivatives led to highly significant alterations of sterol patterns in *C. albicans* cells. This pattern of activity was also evident in the FLC resistant strain, regardless of its resistance to ergosterol suppression when treated with the FLC control. These findings have been supported by previous findings which suggest that tetrazole derivatives are able to circumvent the resistance problem (Ahmad et al., 2011; Ahmad et al., 2017).

Despite AN2 having the lowest antifungal activity, it was the most effective compound against ergosterol biosynthesis; inhibiting by up to 78% when treated with the inhibitory concentration, followed by AN3 which inhibited up to 45%, and the least effective was AN1,

which inhibited ergosterol by an average of 32% when treated with inhibitory concentrations (Table 4.6). Sub-lethal concentrations also inhibited ergosterol significantly, in a dose-dependent manner. However, inhibition by the lowest concentrations for all the tetrazole derivatives tested did not show significant inhibition (Table 4.6). Previous study by Malik and co-workers supported this finding, where they reported anticandidal activity of acyl-hydrazones with tetrazole rings, with inhibition of ergosterol biosynthesis in the range 86-100% at MIC concentrations in FLC susceptible and resistant strains (Malik et al., 2012). Warrilow et al (2014) also reported effects of a tetrazole derivative on the ergosterol biosynthesis pathway of *C. albicans*. In their study, they used a tetrazole-based antifungal candidate drug, VT-1161, which is an oral preparation that is being developed against recurrent vulvovaginitis and is currently in phase III of a clinical trial (Warrilow et al., 2014). The effects were more pronounced than what was found in this present study, with the compound reducing ergosterol by 92% at inhibitory concentration (0.002 μg/mL). This effect correlated with high affinity binding of the compound to fungal CYP51, providing a clear indication of the exact mechanism of action of tetrazole against ergosterol. It can be speculated that tetrazole derivatives used in this study bind to enzymes with slightly less affinity (hence their inability to suppress ergosterol completely even at inhibitory concentrations). Lastly, despite VT-1161 binding to CYP51 with high affinity, it failed to interact with the human enzyme. This makes tetrazoles a good substitute for azole drugs that interact with human enzymes, producing side-effects. Overall, the findings emphasize the ability of tetrazoles to disrupt the ergosterol biosynthesis pathway and their contribution in overcoming the challenge of antifungal resistance.

5.3 Pro-apoptotic effects of tetrazole derivatives

Although there are no studies on ester tetrazole derivatives that trigger apoptosis, various pharmacological agents and compounds possessing anti-*Candida* activity (Amphotericin B,

lycopene and resveratrol) have been reported to trigger yeast apoptosis (Phillips et al., 2003; Choi and Lee, 2015; Lee and Lee, 2015). Because apoptosis is characterised by different cell changes, multiple key markers were used conclusively to implicate apoptosis as the mechanism of action of tetrazole derivatives.

5.3.1 Phosphatidylserine exposition

The exposition of phosphatidylserine to the outer leaflet of the plasma membrane is one of the early markers of apoptosis. Under normal conditions, the phosphatidylserine is located in inner leaflet of the plasma membrane, and this orientation is maintained by the flippase enzyme (Suzuki and Nagata, 2014). However, during apoptosis, activated caspase or metacaspase deactivates the flippase enzyme that maintains this orientation, and activates the scramblase enzyme that translocates phosphatidylserine to outer surface (Vermes et al., 1995; Hankins et al., 2015; Segawa and Nagata, 2015).

Phosphatidylserine exposition has been found by studies to be a typical marker of apoptosis in *C. albicans* cells treated with various apoptosis inducers (Phillips et al., 2003; Khan et al., 2014; Jia et al., 2018). In this present study, there was evidence of phosphatidylserine exposition in *C. albicans* cells treated with tetrazole derivatives, as summarised in Table 4.10. At inhibitory concentrations, both AN3 (1.56 µg/mL) and AN2 (12.5 µg/mL) induced early apoptosis in 9% of cell population, followed by AN1 (0.39 µg/mL), which led to phosphatidylserine exposition in 10% of the population (Table 4.10). In addition to early apoptosis, treatment with tetrazole derivatives also resulted in two different types of necrotic population: late apoptosis population (stained positive for Annexin V and propidium iodide) and necrotic population (stained positive for propidium iodide only). Although cells that take up both Annexin V and PI stain are labelled late apoptotic, it cannot be ascertained whether these cells died by apoptosis or necrosis, as cells in advanced stages of apoptosis also have

damaged membranes, and will take up the PI stain (Ludovico et al., 2001; Wlodkowic et al., 2011). A study by Hao et al. (2013) assessed cell death in *C. albicans* cells treated with caspofungin at inhibitory and sub-inhibitory concentrations (Hao et al., 2013). On average, caspofungin induced early apoptosis in 20-25% of the population, late apoptosis and/ necrosis in 5-7% of the population and necrosis in 26-47% of the population. Caspofungin produced a larger population of early apoptotic cells than tetrazole derivatives used in the present study. However, similarly to the findings of the present study, caspofungin showed a dual effect, inducing apoptosis in some cells and necrosis in other cells. Amphotericin B was also found to produce the same effect, showing a higher proportion of apoptotic cells at low concentrations and more necrotic cells at fungicidal concentrations (Phillips et al., 2003). The extent of cell damage was also found to be concentration-dependent; with the number of early apoptotic cells decreasing with increasing concentration, and the number of necrotic cells increasing with increasing concentration. A similar trend was noted in this present study, highlighting that tetrazole derivatives induce both apoptosis and necrosis, by showing characteristics such as phosphatidylserine externalization and membrane disruption at subinhibitory and inhibitory concentrations, respectively.

5.3.2 Intrinsic pathway in apoptosis

Apoptosis occurs via two pathways: the extrinsic and intrinsic pathway (Belkacemi, 2018). The extrinsic pathway is triggered by the death receptor-ligand complex while the intrinsic pathway is triggered by environmental forces (Guicciardi and Gores, 2009; Wong, 2011; Zhang et al., 2018). In the intrinsic pathway, the mitochondrion acts as the main switch for apoptosis. The mitochondrion is permeabilized, following a stimulus, by proapoptotic proteins Bax and Bak, encoded by the Bcl2 gene family (Roufayel, 2016). This allows cytochrome c to be released from the mitochondrial intermembrane space into the cytosol, where it activates the apoptosis-activating factor 1 (Apaf1). In its active form, Apaf1 cleaves

and activates the metacaspase, thereby inducing apoptosis (Srinivasula et al., 1998; Loreto et al., 2014).

Given the nature of our stimuli, initiation of the intrinsic pathway was studied. Here, treatment of *C. albicans* cells with tetrazole derivatives resulted in permeabilization of the mitochondrion, and as a result, cytochrome c was present in supernatant fractions at varying concentrations, as shown in Table 4.12 and 4.13. On average, the cytosolic concentration of cytochrome c detected following treatment with AN1 (0.39 µg/mL) was 12.3 µmol/min/mg protein, while AN3 (1.56 µg/mL) treated cells had 12.1 µmol/min/mg protein. AN2 (12.5 µg/mL) showed the least activity of the compounds, at 10.8 µmol/min/mg protein. Hwang et al. (2016) also linked the release of cytochrome c in *C. albicans* cells treated with hibicuslide C to apoptosis (Hwang et al., 2016). In another study, *C. albicans* cells treated with a flavone and thiourea were found to exhibit varying results (Hwang et al., 2012). Cytochrome c release was detected in cells treated with the flavone, but co-treatment with thiourea suppressed this release and no mitochondrial membrane damage was detected. Because thiourea is a hydroxyl radical scavenger, it inhibits the permeabilisation of mitochondrion in the presence of an apoptosis inducer, and subsequently cytochrome c release. This clearly highlights the importance of reactive oxygen species (ROS) production in apoptosis. Reactive oxygen species damage mitochondrial membranes and this leads to the release of cytochrome c during apoptosis. Although tetrazole derivatives induced release of cytochrome c, it is possible that this was a secondary response to the compounds' antifungal activity (considering the low quantities of cytochrome c detected in this study), with the possibility of metacaspase being activated by other mitochondrial proteins such as ROS, as previously reported (Cao et al., 2009; Wu et al., 2010). Generally, there is conflicting evidence on the role of cytochrome c release in activating yeast apoptosis: Some studies state that the inhibition of cytochrome c release prevents apoptosis from happening (Silva et al., 2005) and

other studies state that the expression of Bax proteins responsible for sensitizing the mitochondrial membrane does not necessarily lead to release of cytochrome c, and, in turn, this does not prevent apoptosis from happening (Roucou et al., 2000). In summary, mechanisms of apoptosis induction differ according to the inducer, and because of the complexity of the process, studying the pattern of pro-apoptotic protein expression following apoptosis induction would be beneficial (Khan et al., 2014).

5.3.3 Nucleic changes

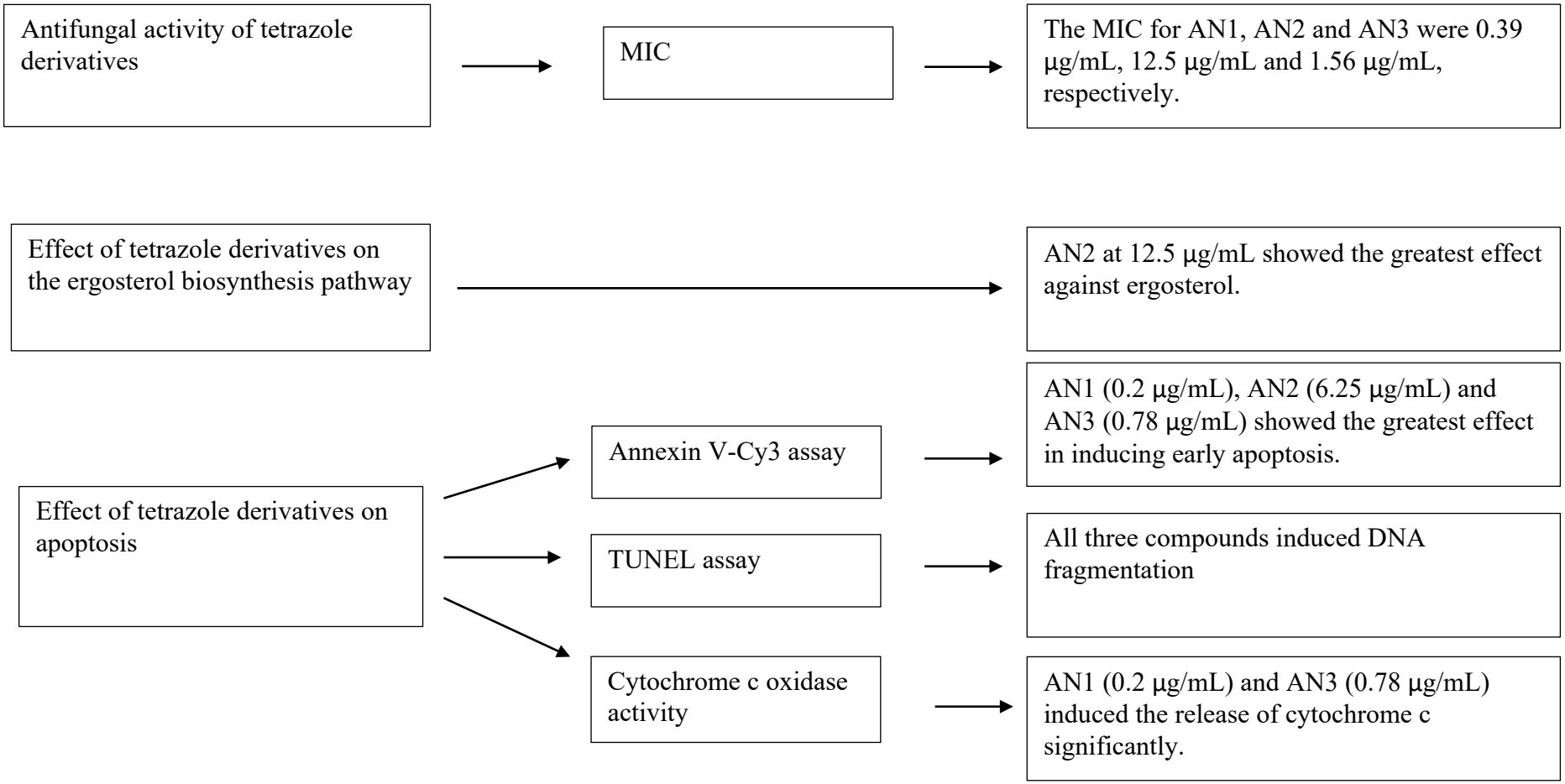
Lastly, the most dramatic changes that occur during apoptosis are in the nucleus. In the late stages of apoptosis, DNA is fragmented and chromatin condenses due to proteolysis by nuclear proteins (Dobrucki et al., 2001). In this study, DNA fragmentation was assessed qualitatively using TUNEL assay, followed by evaluation of chromatin structure using Hoechst 333423 staining. Cells exposed to tetrazole derivatives exhibited TUNEL-positive nuclei (Figure 4.10 to Figure 4.12). When the same apoptotic marker was assessed in *C. albicans* cells treated with plant essential oils by Khan et al. (2014), a similar trend was found, with the most pronounced effect at sub-inhibitory concentrations (Khan et al., 2014). However, when Hao et al. (2013) quantified TUNEL-positive cells, it was noted that inhibitory concentrations were more effective in inducing DNA fragmentation than sub-inhibitory concentrations were. The contradictory results are a clear indication that the nature of the compound inducing apoptosis determines the final outcome.

As an additional confirmation of nuclei changes, cells exposed to tetrazole derivatives were counter-stained with Hoechst 333423. Cells exposed to tetrazole derivatives showed a deep blue fluorescence, which confirms that cells in late stages of apoptosis have permeabilised membranes that allow easy uptake of the dye (Hao et al., 2013). Hoechst staining revealed a large number of cells with chromatin condensation, when compared to TUNEL-positive cells.

This difference is confirmation that during apoptosis, changes in the chromatin precede DNA fragmentation (Ludovico et al., 2001). Nuclei with abnormal margins in some cells were also highlighted. Some cells had crescent-shaped nuclei and others long tubular structures. In agreement with these findings, *C. albicans* cells treated with lactoferrin were reported to have half-ring shapes and images of tubular structures were observed (Andrés et al., 2008). In another study, only chromatin condensation was reported when *C. albicans* cells were treated with flavone Baicalein (Dai et al., 2009). These differences in nuclear morphology indicate that nuclei changes caused by apoptosis are not uniform as cells do not undergo apoptosis all at the same time.

The incorporation of a tetrazole ring in compounds helps improve its pharmacological benefits, and, most importantly, prolongs drug action. The atoms on the ring give the compound improved binding to membranes, and the lipophilic nature of tetrazoles improves penetration through biological membranes (Patel and Karia, 2015; Zhao, 2016), a plausible explanation for its affinity to ergosterol in fungal membranes. Overall, this study showed how tetrazole derivatives exhibit necrotic and apoptotic effects on *C. albicans*. Collectively, data obtained in this study demonstrate that tetrazole derivatives interact with and disrupt the biosynthesis of ergosterol, and this stress on the cell wall also acts as a trigger for apoptosis. At inhibitory concentrations, the necrotic effect of the compounds is more pronounced, which could be a result of the inhibition of ergosterol. On the other hand, sub-inhibitory concentrations are more effective in inducing apoptosis. Figure 5.1 summarises the different effects tetrazole derivatives had on both ergosterol and apoptosis.

Figure 5.1 Study design and results of the effect of tetrazole derivatives on *C. albicans* cells



5.5 Clinical implications

This study has established that tetrazole derivatives possess antifungal activity against *C. albicans.* Therefore, tetrazole derivatives can be developed into antifungal drugs that can be used in clinical settings to treat *Candida* infections. The use of tetrazole derivatives would be advantageous, because required doses are low and minimise host toxicity. When administered at MIC, tetrazole derivatives will effectively inhibit proliferation of the pathogen. Additionally, when the drug level drops below MIC within the body, low concentrations will effectively inhibit the ergosterol content, which contributes to membrane integrity and cell survival. Low concentrations will also induce apoptosis at a high frequency in surviving cells, rendering them non-viable. In this way, tetrazole derivatives will effectively reduce the burden of *Candida* infections.

CHAPTER 6: CONCLUSION, LIMITATIONS AND FUTURE RESEARCH

6.1 Conclusion

In conclusion: this study has established that tetrazole derivatives possess antifungal activity against FLC resistant and susceptible *C albicans*, making it the perfect solution to the antifungal resistance crisis. All three tetrazole derivatives inhibited ergosterol content, highlighting the ability of compounds to bind to or inhibit enzymes in the ergosterol biosynthesis pathway. The study of apoptotic markers revealed that tetrazole derivatives promoted apoptosis at subinhibitory concentrations, and, at the same time, inhibitory concentrations damaged cells and eventually led to necrosis. Collectively, findings in this study provided insight into mechanism by which tetrazole derivatives exert their antifungal action on *C. albicans* and highlighted the multiple-effects of tetrazole derivatives. Tetrazole derivatives therefore have the potential to be developed as antifungal drugs for treatment of fungal infections.

6.2 Limitations to the study

Owing to time and financial constraints, the following were not studied:

1. Ergosterol biosynthesis inhibition by tetrazole derivatives was confirmed. However, the exact mechanism of action or which enzyme in the pathway the compounds inhibit, was not established
2. Time kinetics assays. These would help clearly to establish the type of cell death tetrazole derivatives induce, as apoptotic features are time-dependent, and some morphologies are similar to those of necrosis or other cell death types.

3. Investigation into the presence of reactive oxygen species (ROS) in apoptotic cells. ROS have been found in abundance in yeast cells undergoing apoptosis, with the role of activating metacaspase for the execution of apoptosis. The investigation would help confirm apoptosis induction and explain the activation of the apoptotic cascade even in low levels of cytochrome c oxidase.
4. Analysis of ultrastructural features using transmission electron microscopy (TEM). This is an important aspect of apoptosis studies, as morphological changes are clearly seen. It also confirms apoptosis by correlating ultrastructural changes with results obtained from other assays.

6.3 Future studies

1. Synthetic compounds often show good antimicrobial activity in laboratory studies, but when tested in humans they show toxicity. Conducting cytotoxicity studies on human cells will help determine the cytotoxicity profile, or what doses of the tetrazole derivatives are safe for use on humans.
2. Experimental results obtained *in vitro* might vary from *in situ* because of the differences in environmental conditions. This can be confirmed by conducting clinical trials on humans.
3. The number of apoptotic cells was seen to increase with decreasing concentrations. It would be beneficial to test more concentrations, to determine the concentration that triggered necrosis and the most effective concentration for apoptosis
4. Because the induction of apoptosis is mainly controlled by expression of different proteins, expression profiles of these proteins could also be studied, to give a detailed sequence of events of the entire process.

CHAPTER 7

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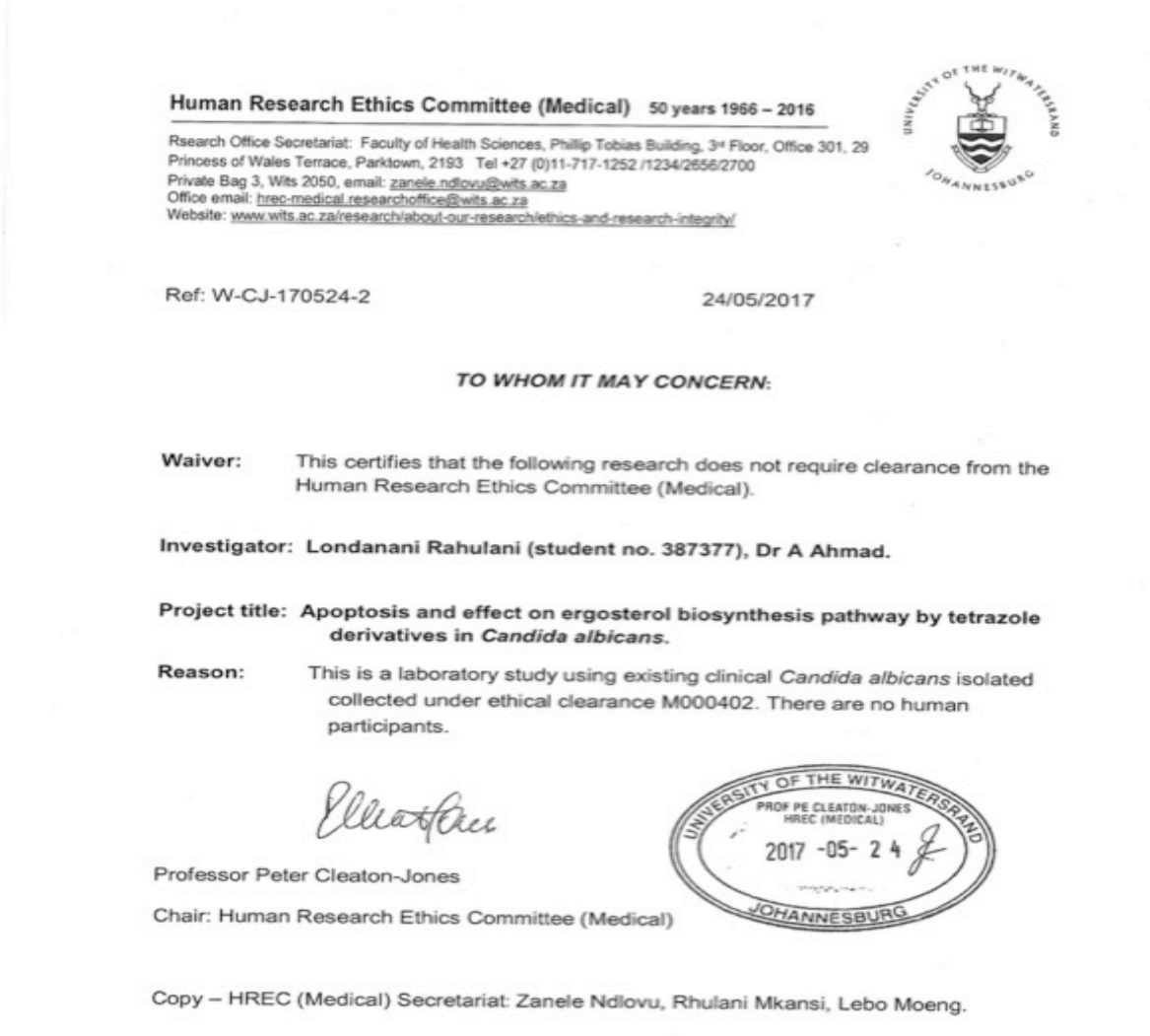
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CHAPTER 8

APPENDICES

Appendix A: Ethical clearance certificate



Human Research Ethics Committee (Medical) 50 years 1966 – 2016

Rsearch Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 301, 29 Princess of Wales Terrace, Parktown, 2193 Tel +27 (0)11-717-1252 /1234/2656/2700
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Ref: W-CJ-170524-2 24/05/2017

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Londanani Rahulani (student no. 387377), Dr A Ahmad.**

Project title: **Apoptosis and effect on ergosterol biosynthesis pathway by tetrazole derivatives in *Candida albicans*.**

Reason: This is a laboratory study using existing clinical *Candida albicans* isolated collected under ethical clearance M000402. There are no human participants.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2017 -05- 24
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi, Lebo Moeng.

Appendix B: Preparation of media, buffers and solutions used

25% Alcoholic potassium hydroxide

Potassium hydroxide (Merck, South Africa)	25 g
Absolute ethanol (Merck, South Africa)	50 mL
Sterile distilled water	35 mL

The constituents were mixed well and after the KOH pellets had dissolved, the volume was adjusted to 100 mL by adding ethanol.

Fluconazole (5mg/mL)

Fluconazole (Sigma-Aldrich, USA)	100 mg
DMSO (Associated Chemical Enterprises, South Africa)	5 mL

FLC powder was dissolved in DMSO to make a stock solution and stored between 2-4 °C.

4% Paraformaldehyde

Paraformaldehyde (Sigma Aldrich, USA)	4 g
PBS	100 mL

PBS was added to a glass beaker on a heating plate in a ventilated hood. Paraformaldehyde was added to the heated PBS and NaOH was added dropwise until the solution cleared. The solution was cooled and aliquoted then stored at -2 °C until use.

1X PBS

PBS tablets (Sigma-Aldrich, USA)	5 tablets
Distilled water	1 L

The tablets were dissolved, and the pH adjusted to 7.4. The solution was autoclaved at 121°C for 15 minutes.

Permeabilization solution

1. **0.2% Triton-x-100**

Triton-X-100	100 µL
1X PBS	50 mL

Triton-X-100 was added to PBS and stirred until completely mixed.

2. **0.1% sodium citrate**

Sodium citrate	1.47 g
Sterile distilled water	100 mL

The mixture was stirred until the powder dissolved.

To make the permeabilization solution 0.2% Triton-X-100 (50 mL) and 0.1% sodium citrate (50 mL) were mixed before use.

Protoplast buffers reagents

A stock concentration of 1M of all the protoplast buffer reagents was prepared and further diluted according to the concentrations needed for each buffer:

1. **1 M D-sorbitol**

D-sorbitol (Sigma-Aldrich, USA)	18.2 g
Sterile distilled water	100 mL

The solution was mixed until the sorbitol dissolved and stored at room temperature.

2. **1 M DTT**

DTT powder (Thermo Fischer Scientific, USA)	15.45 g
Sterile distilled water	100 mL

DTT powder was dissolved in sterile distilled water then stored at -20 °C until use.

3. **1 M Magnesium chloride**

$MgCl_2$ (Sigma-Aldrich, USA)	23.8 g
Sterile distilled water	1 L

Magnesium chloride powder was dissolved in distilled water then autoclaved and stored at room temperature. This was used as a stock solution.

4. **1 M tris base**

Tris base (Merck, South Africa)	12.1 g
Sterile distilled water	100 mL

Tris base was dissolved in distilled water and the pH was adjusted to 6.5. The solution was kept at 4 °C.

Sabouraud Dextrose broth

Sabouraud Dextrose broth powder (Merck, South Africa)	30 g
Distilled water	1 L

The mixture was dissolved and autoclaved at 121°C for 15 minutes.

Sabouraud Dextrose Agar

Sabouraud Dextrose Agar powder (Sigma-Aldrich, USA)	65 g
Distilled water	1 L

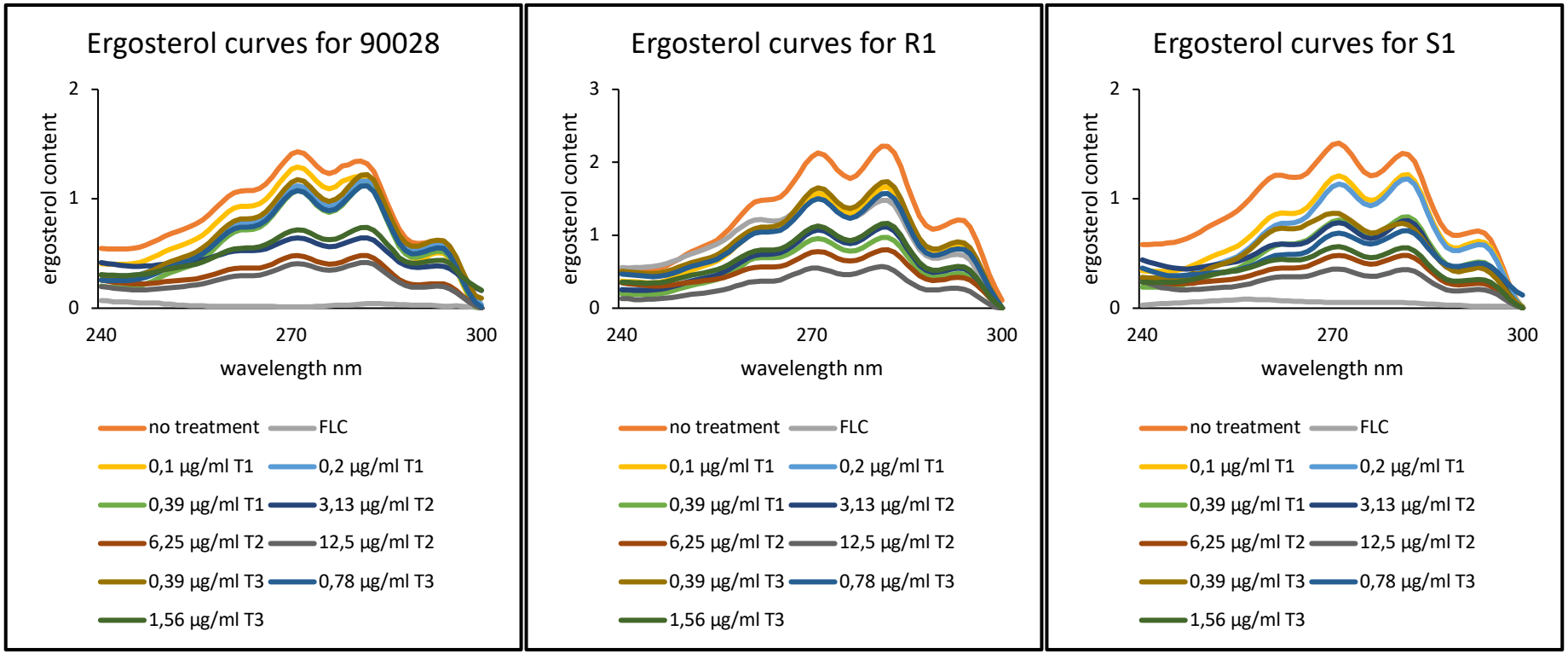
The powder was dissolved in water with stirring on a hot plate. After the powder had dissolved, the solution was autoclaved at 121 °C for 15 minutes then poured into sterile petri dishes which were then kept at 4 °C until use.

0,8% saline

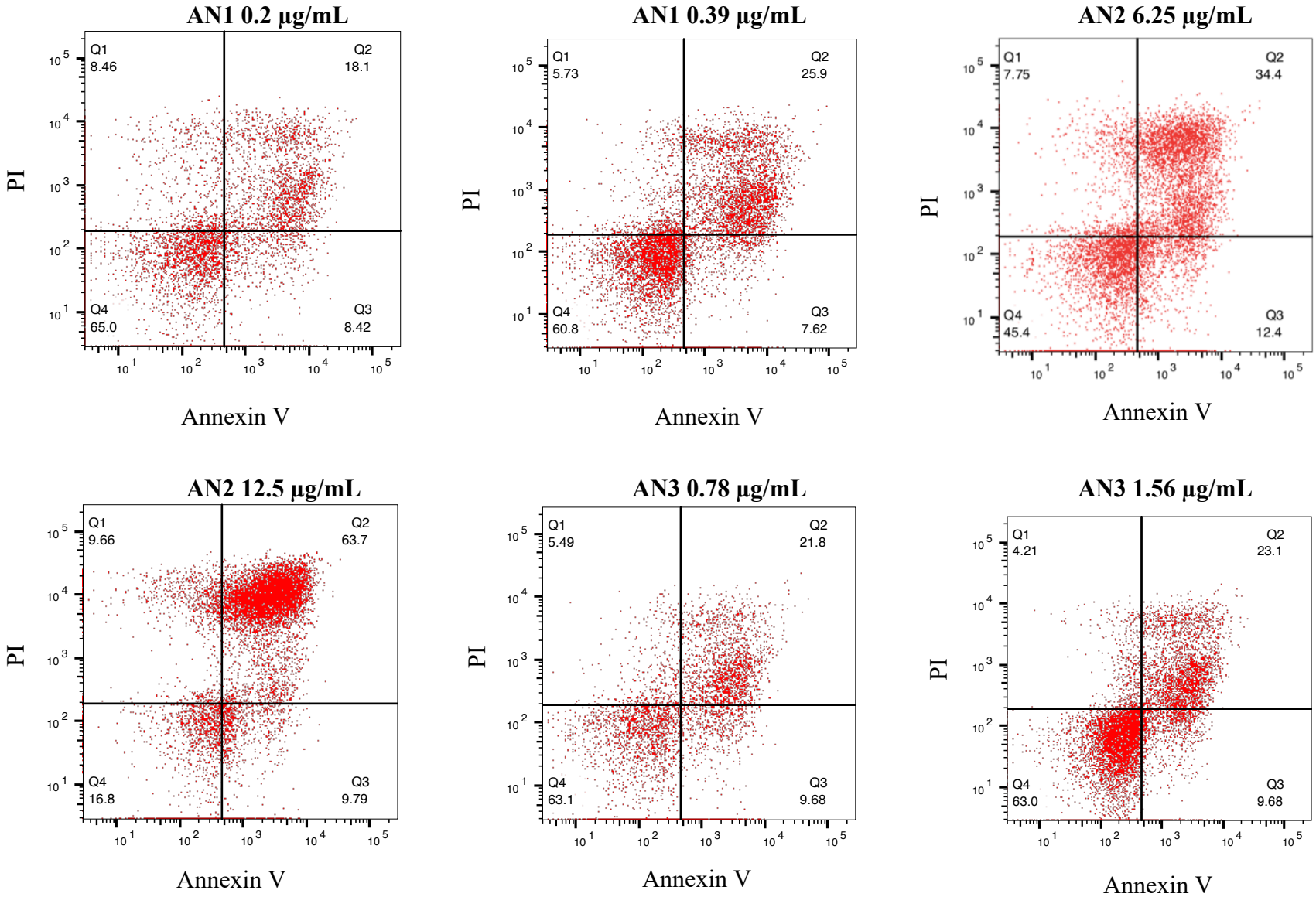
Sodium chloride (Sigma-Aldrich, USA)	8 g
Distilled water	1 L

The solution was stirred using a stirring rod until the sodium chloride dissolved and then autoclaved at 121 °C for 15 minutes then stored at room temperature.

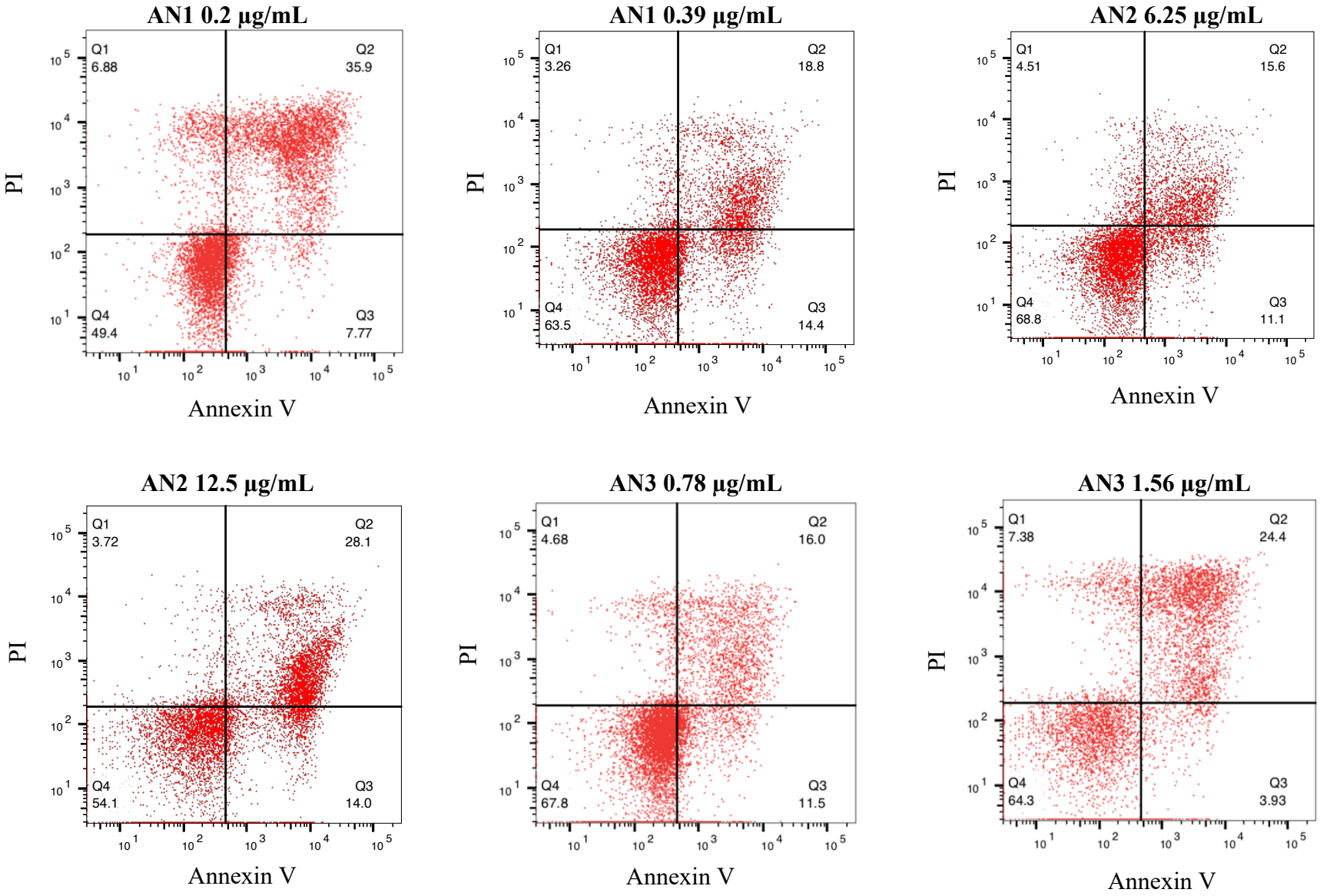
Appendix C: Ergosterol curves



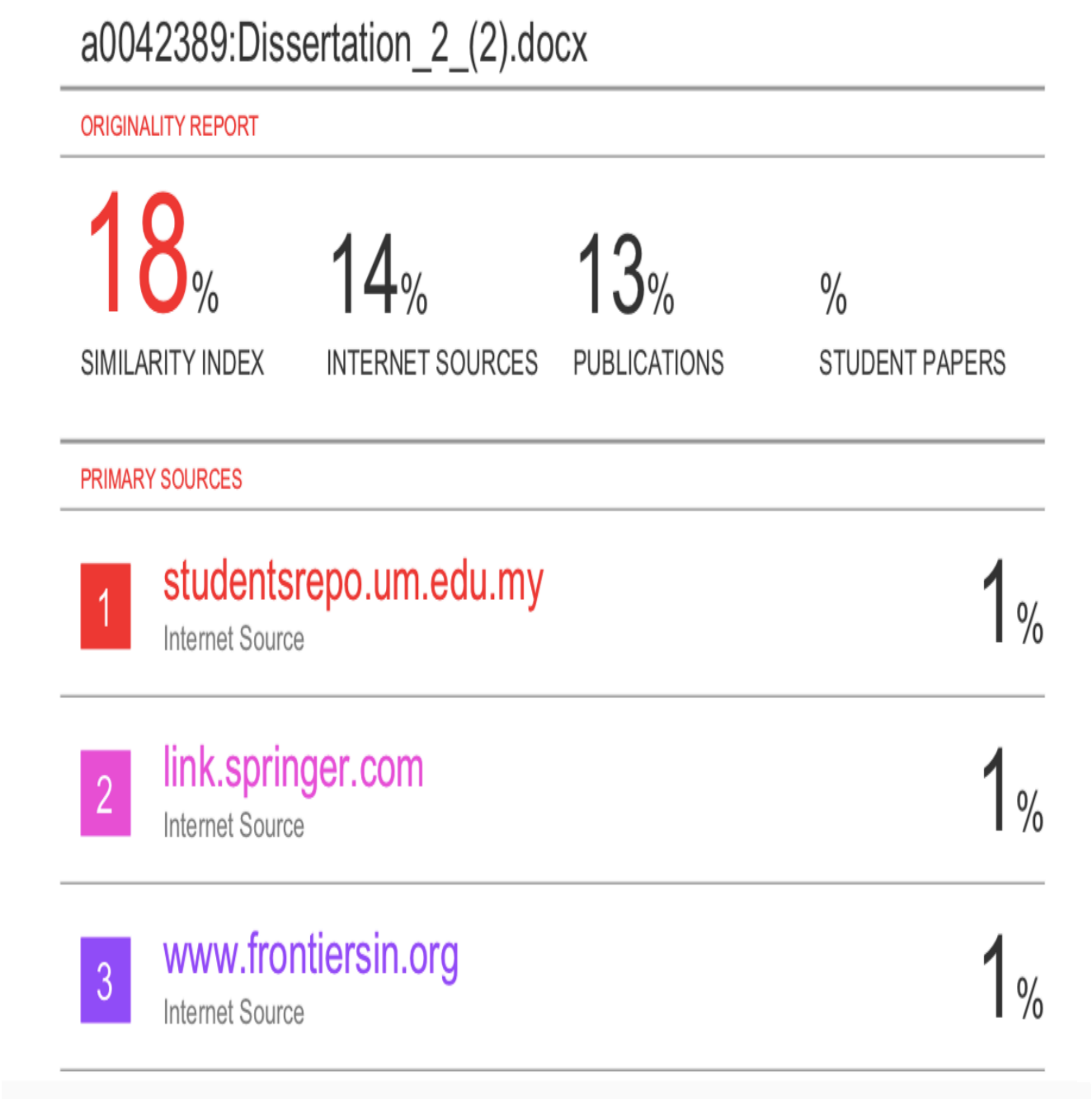
Appendix D: Cytograms of FLC resistant *C. albicans* after exposure to ½ MIC and MIC values of the test compounds



Appendix E: Cytograms of FLC susceptible *C. albicans* after exposure to ½ MIC and MIC values of the test compounds



Appendix F: Turnitin report



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