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Combination interaction of fluconazole with efflux pump inhibitors against antifungal resistant Candida parapsilosis

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Combination interaction of fluconazole with efflux pump inhibitors against antifungal resistant *Candida parapsilosis*

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Degree of Master of Science in Medicine by research only

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2023.

DECLARATION

I, Marwah Omar Alati, hereby certify that this dissertation is entirely original. It is being submitted to the University of the Witwatersrand in Johannesburg for consideration for the degree of Master of Science in Medicine. It has never before been submitted to this University or any other University for a degree or examination.



…….. (Signature of candidate)

…22…… day of ……March….

DEDICATION

To my husband, who was at my side through each and every step of the journey, and for my daughters, who were the unseen source of strength that God provided for me.

To my wonderful and supportive family, without whom I would not have been able to get to this point in my life.

In honour of my father, who has never lost trust in me.

For my mum, who no matter how many times I fell, she was there to pick me up and help me get back up again.

ABSTRACT

Introduction

C. parapsilosis is the most common fungal pathogen recovered in neonatal intensive care units and is associated with a higher mortality rate due to its multidrug resistance. This study investigated the virulence factors and reversal of efflux activity by using a well-known antifungal drug (fluconazole) in combination with two efflux pump inhibitors (pantoprazole and omeprazole). In addition the effect of most active combination on pathogenicity markers of *C. parapsilosis* has studied.

Materials and methods

The microbroth dilution method was used to obtain antifungal susceptibility tests of conventional antifungal drugs (fluconazole and amphotericin B) against twelve clinical isolates of *C. parapsilosis*, as well as antifungal susceptibility tests of two efflux pump inhibitors (EPI). In addition, the combination of efflux pump inhibitors (pantoprazole and omeprazole) with fluconazole, an antifungal drug, was determined by calculating the fractional inhibitory concentration index (FICI) based on zero-interaction theory of Loewe additivity. a time-kill kinetics assay was used to study the antimicrobial activity of fluconazole and efflux pump inhibitors against *C. parapsilosis*. The virulence factors in *C. parapsilosis* isolates were determined using in vitro virulence assays. The effects of the efflux pump inhibitors on virulence factors were also studied, and comparison of percent reduction of virulence factors was determined in both fluconazole-resistant and susceptible *C. parapsilosis*. The effects of Pantoprazole and Omeprazole on efflux pump were also investigated using two assays (Rhodamine 6G accumulation and Hoechst 33342 accumulation).

Results

The efflux pump inhibitors showed enhanced antifungal activity in combination with antifungal agents against *C. parapsilosis*. When pantoprazole was combined with fluconazole, the median Minimum inhibitory concentration for fluconazole and pantoprazole decreased from 125 to 31.25 µg/ml and from 620 to 310 µg/ml respectively. In the time-kill kinetics assay, fungistatic activity of Fluconazole was changed to fungicidal by addition of efflux pump inhibitors (pantoprazole and omeprazole) at their ½MIC values. On the other hand, this study demonstrated that *C. parapsilosis* had a variety of virulence characteristics, including the ability to adhere to epithelial cells and secretion of proteinase hydrolytic enzyme. Pantoprazole and omeprazole reduced the ability of *C. parapsilosis* cells to adhere to epithelial cells. Adhesion was reduced in a concentration-dependent manner. The reduction was significant ($p < 0.001$) at all the test concentration. Proteinase synthesis in *C. parapsilosis* strains, was also significantly reduced at test concentrations. The reduction was significant $P = < 0.01$ However, the results also showed that pantoprazole and omeprazole and their combinations was significantly reduced efflux pump activity.

Conclusion

Candida resistance has increased, particularly to azoles, and advent of *C. parapsilosis* as most prevalent non *albicans Candida* species poses a significant threat to the future. Combination therapy is shown to be therapeutically efficacious, and MIC values of fluconazole decreased when it was combined with omeprazole and pantoprazole.

Further study is needed to understand the epidemiology, microbiology, genetics, and antibiotic susceptibility of this pathogen. The ability to express different virulence factors, such as adherence, proteinase, and phospholipase production, is strain-dependent and the widespread use of antifungal agents leads to drug resistance in *C. parapsilosis*.

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LIST OF ABBREVIATIONS AND ACRONYMNS

AST: Antifungal susceptibility testing

BEC: Buccal Epithelial Cells

BMD: Broth microdilution

BSA: Bovine serum albumin

CFU/ml: Colony Forming Units per millilitre

CLSI: The Clinical and Laboratory Standards Institute

DMSO: Dimethyl sulfoxide

ECDC: The European Centre for Disease Prevention and Control

AIDS: Acquired Immuno-Deficincy Syndrome

FICI: Fractional inhibitory concentration index

g: Gram

GPI: Glycosylphosphatidylinisotol

GRSA: Generally recognized as safe

HIV: Human Immunodeficiency Virus

HWP1: Hyphal wall protein 1

ITS: Internal Transcribed Region

MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight

MDR: Multi-drug resistant

MFC: Minimum Fungicidal Concentration

mg/ml: Milligram per millilitre

MIC: Minimum Inhibitory Concentrations

ml: Millilitre

mm: Millimetre

µl: Microlitre

µm: Micrometre

NAC: Non-Albicans *Candida*

NAG: N-Acetyl Glucosamine

NICD: The National Institute for Communicable Diseases

NTP: National Toxicology Program

OPC: Oropharyngeal/esophageal candidiasis

rpm: Revolutions per minute

SAP: Secreted Aspartyl Proteinase

SD: Standard Deviation

SDA: Sabouraud dextrose agar

SDB: Sabouraud dextrose broth

VVC: Volvovaginal candidiasis

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Fungi have become important sources of human illness since the 1980s, particularly among immuno-compromised people and hospitalized patients with serious underlying diseases (Tortorano *et al*. 2006). This includes cancer patients on chemotherapy, transplant recipients, HIV patients, diabetic patients, and premature babies, especially those with low birth weight (Banerjee *et al*, 1992; Fidel *et al*, 1999). Candidiasis is a fungal infection that can develop as a less serious superficial infection of the skin, oral cavity, and vagina. More serious infections can be invasive systemic infections in the form of septicemia and candidemia (Cortegiani *et al*, 2018). C*andida* bloodstream infections (BSI) are prevalent in hospitalised patients usually hospital-acquired illnesses that can be fatal (Tortorano *et al*, 2006).

Azoles are frequently used as broad-spectrum antifungal drugs, particularly as a first-line therapy for all types of *Candida* infections. It has advantages over other antifungal drugs, such as being cheaper, having high solubility, and being less toxic than other drugs. *Candida albicans* is now thought to be the most common cause of *Candida* infections in health-care places. On the other hand, non-*albicans*-*Candida* (NAC) species such as *C. glabrata, C. krusei, C. parapsilosis, and C. tropicalis,* have been found to cause invasive fungal infections (Cortegiani *et al*, 2018). *Candida parapsilosis* has become more common in recent years. It is the second most prevalent *Candida* species identified from blood cultures (Costa-de-Oliveira *et al*, 2008). Furthermore, *Candida parapsilosis* has demonstrated resistance to the three major classes of antifungal drugs, and it is difficult to detect using standard laboratory techniques (Tóth *et al*, 2019).

Therefore, it is important to find new strategies to prevent and treat *C. parapsilosis* infections. This encompasses a wide range of strategies, such as developing novel antifungal drugs, combining therapies to boost the efficacy of conventional antifungal agents, and targeting known virulence factors. In this study, the effects of efflux pump inhibitors, which are known to have antifungal activity were studied for their combination activity, with antifungal agents and their effects on the virulence factors of *C. parapsilosis*.

1. LITERATURE REVIEW

Numerous eukaryotic organisms that make up the kingdom of fungi are recognised by the presence of chitin in their cell walls. Multicellular or unicellular, fungi are heterotrophic creatures. There is over 250,000 different kinds of fungi described (Mueller and Schmit, 2007). About 300 of these fungal species are known to cause infections in humans (Taylor and Berbee, 2006). *Candida species*, which comprise many species, are a natural part of human flora (Dixon *et al*, 1980), and they are considered to cause opportunistic infections.

1.1. Fungal infections

There are two types of fungal infection- the opportunistic and the true infection. However, reduced host immune resistance to infection determines how quickly a disease may appear as well as how to classify the type of infection. *Aspergillus*, *Cryptococcus* and *Candida* species are the most common fungi involved in opportunistic infections that affects the immunocompromised patients (Pfaller and Diekema, 2007). In fact, since 1979, the yearly incidence of fungal infections has increased. Most people will get superficial fungal infections throughout their lifetimes, which are usually easy to treat. Most of these infections occur on the skin, mucosa, and nails (Figure 1.1), which impact 25% (1.7 billion) of the global population (Havlickova *et al*, 2008). But millions of people around the world will get serious

invasive fungal infections which are far more difficult to diagnose as well as to treat even if its frequency is still lower than superficial infections (Brown *et al*, 2012).

Oral and vaginal mucosal infections caused by *Candida* species are the most prevalent fungal infections.

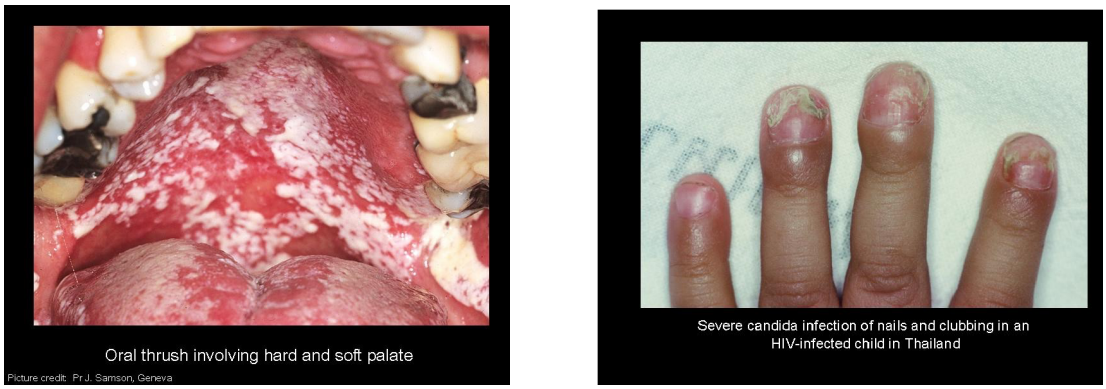


Figure 1.1: Superficial fungal infections: mucosal candidiasis and *Candida* infection of nails. A: oral thrush involving soft and hard palate, B: Sever *Candida* infection of nails in an HIV-infected child in Thailand. Source of photos: infectiousdiseaseadvisor.com was first indexed by Google in February 2014 https://www.infectiousdiseaseadvisor.com/home/decisionsupport-in-medicine/infectious-diseases/candida/

Vulvovaginal candidiasis affects 50%-75% of women in their reproductive years (Sobel, 2007). Oral fungal infections are more common in young children due to their low immunity, elderly patients who wear dentures and, have poor oral and denture hygiene, or in individuals with risk factors such as diabetes mellitus. Transplant patients, and HIV patients are also vulnerable to these infections (Brown *et al*, 2012; Salerno *et al*, 2014; Samaranayake *et al*, 2009). On the other hand, *Candida* infections can range in severity from a superficial, localised infection to a deep-seated, widely distributed systemic infection. Etiologically invasive candidemia is when *Candida* enters the blood causing bloodstream infection (Haynes, 2001).

Such invasive fungal infection causes significant damage and increases the rate of mortalities and morbidity noting 0.4 deaths per 100,000 populations (Brown *et al*, 2012; Enoch *et al*, 2006; Husain *et al*, 2003; Kibbler *et al*, 2003). *Candida* species are now the fourth biggest cause of nosocomial infections in hospitals (Arendrup *et al*, 2005; EspinelIngroff *et al*, 2009; Sardi *et al*, 2013). Each year, there are 24 infections per 100,000 people (Pfaller and Diekema, 2007). The *Candida* genus is made up of a diverse collection of fungal species. *Candida parapsilosis, Candida albicans, Candida glabarata, Candida krusei,* and *Candida tropicalis* are among the more than 17 *Candida* species known to cause fungal infections in humans (Pfaller *et al*, 2008). However, *Candida* infections are most commonly caused by *Candida albicans*, but there are certain non-*C. albicans* (NAC) species that cause opportunistic infections with clinical importance. This might be related to the widespread use of broad-spectrum antibiotics, an increase in the number of immunocompromised people, and the use of implants and medical devices (Pfaller *et al*, 2014). Furthermore, NAC species have been demonstrated to be less susceptible to antifungal drugs, particularly fluconazole (Khan *et al*, 2007; Kim *et al*, 2009). As a result, the fast spread of NAC fungal infections poses a serious threat to human health. Among the NAC species, there are a few that stand out. Studies have shown *C. parapsilosis* to be the second most commonly isolated *Candida* species from blood cultures in Latin America and eastern Asia (Kim *et al*, 2018). *C. parapsilosis* has a number of alarming characteristics, including its ability to cause nosocomial epidemics. In addition, it is the most common fungal pathogen recovered in many neonatal intensive care units (NICUs).

1.2. Emergence of *C. parapsilosis*

C. parapsilosis is an emerging species of *Candida* genus. Exactly in 1928, Ashford identified *C. parapsilosis* in the faeces of a patient with diarrhoea in Puerto Rico as a *Monilia* species incapable of digesting maltose (Ashford *et al*, 1928). And to distinguish it from the more frequent isolated species, it was reclassified as *C. parapsilosis* in 1932 (Langeron and Talice, 1932). As the *Candida albicans*, also was known as "*Moniliapsilosis".* Despite being initially considered to be non-pathogenic, later, *C. parapsilosis* was found to be a causal agent of a fatal case of endocarditis in an intravenous drug user (Joachim, and Polayes, 1940). From 2005, *C. parapsilosis* was classified as I, II, and III groups. Additional genetic investigations discovered enough variations to separate the groups into closely related, different species such as *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* (Tavanti *et al*, 2005). Nonetheless, *C. parapsilosis* is responsible for majority of clinical illnesses, and few medical microbiology laboratories discriminate between these species, especially when more accurate commercial systems are not available. On the other hand, *C. parapsilosis* is well-known for its ability to thrive in total parenteral nutrition and form biofilms on catheters and other implanted devices, as well as for nosocomial dissemination by hand cart, and for perseverance in a hospital environment (Ferreira *et al*, 2009).

In the neonatal intensive care unit (NICU), infections of bloodstream (BSI) caused by species of *Candida* are less common than those Gram-positive or Gram-negative bacteria; nonetheless, their rates are greater regarding morbidity and mortality. Among infants, 4−8% of infants with an exceptionally low birth weight (1000 g) will have candidemia, which has a 30% death rate in this patient group (Benjamin *et al*, 2014; Kelly *et al*, 2015). Indeed*, C. parapsilosis* is absolutely dangerous in critically ill or premature neonates (Leibovitz E,

2012). accounting for more than one-quarter of all invasive fungal infections in low-birthweight newborns in the UK (Clerihew *et al*, 2007). Additionally, in North America, up to one-third of *Candida* bloodstream infections in new-borns are caused by this pathogen (Fridkin *et al*, 2006). Neonatal candidemia risk factors include preterm, use of central venous catheters (CVCs), intubation, parenteral feeding, and broad-spectrum antibiotics. Furthermore, a study in Spain comprised of 175 *C. albicans* infections and 78 of *C.*

parapsilosis bloodstream infection showed that the factors that independently predicted *C. parapsilosis* infection were a neonatal age, transplant recipient status, prior antifungal therapy (mostly fluconazole), and receiving nutrition by intravenous catheter. In addition, compared to patients with *C. albicans* infections, those with *C. parapsilosis* had a decreased mortality rate (Almirante *et al*, 2006; Rodriguez *et al*, 2010). In a prospective population-based study, adults hospitalised in medical and surgical ICUs across Spain showed a lower mortality rate from *C. parapsilosis* infection. In this series, *C. parapsilosis* infection caused 7% of deaths within 7 days, compared to 56% for *C. albicans* (Puig-Asensio *et al*, 2014). Since the 1980s, there has been a substantial rise in bloodstream infections caused by *Candida* species other than *Candida albicans*, specifically *C. glabrata* in the United States and *C. parapsilosis* and *C. tropicalis* in Europe, Canada, and Latin America (Almirante *et al*, 2006). Although *C. parapsilosis* is assumed to be less virulent than *C. albicans,* it has shown the highest increase in frequency since 1990. The prevalence of *C. parapsilosis* has significantly increased over the last two decades.

1.2.1. Genomic features of *C. parapsilosis*

Outbreaks of *C. parapsilosis* infections in intensive care units reported in hospitals from geographically different parts of the world highlight the disease's growing epidemiological significance (Diaz Granados *et al*, 2008; Brillowska-Dabrowska *et al*, 2009). This yeast may

also be isolated from a variety of environmental sources such as soil, fresh and salt water, and insects (Sampaio, 2005; Medeiros and Suh, 2008). Sexual reproduction in *C. parapsilosis* isolates has not been detected. As a result, the teleomorphic form of *C. parapsilosis* was postulated as Loddero myceselongisporus producing asci (Kurtzman, 2011; Nakase *et al*, 1979; Hamajima *et al*, 1987). Indeed, *Lodderomyces elongisporus* strains and *C. parapsilosis* type strains had identical G + C content in nuclear DNA, substantially conserved rDNA sequences, and physiological characteristics in cultivation/substrate assimilation tests. This species' isolates were thought to be L-arabinose non-utilizing form II of *C. parapsilosis*. Several laboratory experiments have conclusively proven that these yeasts belong to two different species (James *et al*, 1994; Lin *et al*, 1995; Rycovska *et al*, 2004; Lockhart *et al*, 2008). Significantly, cumulative evidence from examination of several molecular markers, which is well confirmed by multi locus sequence typing, the groups were classified as three different species, namely *C. parapsilosis* [group I isolates], *C. orthopsilosis* [group II], and *C. metapsilosis* [group III] (Tavanti *et al*, 2005). Furthermore, a search of isolates from Brazil and Japan identified strains that differed significantly from the three groups, leading to the proposal of a [group IV] as an intermediate isolate between groups II and III. This shows that the '*psilosis*' complex contains more species (Iida *et al*, 2005).

C. parapsilosis strains had much lower sequence diversity than *C. orthopsilosis* and *C. metapsilosis* strains. This led to the hypothesis that the species separated recently, most likely within the last million years, and that they co-evolved with humans (Fundyga *et al*, 2004). This fascinating theory is consistent with the species extensive global distribution and might be related to the species adaptability to humans and relative ease of human-to-human transmission (Tavanti *et al*, 2005; Pfaller *et al*, 2008). Several studies have reported that alleles in *C. parapsilosis* isolates are also found in *C. orthopsilosis* and *C. metapsilosis* strains, but no *C. orthopsilosis* or *C. metapsilosis*, specific alleles have been found in *C.*

parapsilosis. Recombination between three species has been suggested (Fundyga *et al*, 2004). As a result, *C. parapsilosis* might have developed lately as a genetically homogenous subpopulation diverged from a common ancestor or as a subpopulation that was favourably selected because of a particular morphological trait (s). One hundred and seventy million years ago, *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* group diverged from other hemiascomycetes (Kurtzman and Robnett 1997; Diezmann *et al*, 2004; Massey *et al*, 2003). And includes clinically significant pathogenic yeasts such as *Candida albicans*, *Candida dubliniensis*, and *Candida tropicalis*, as well as many other species involved with insect digestive tracts (Suh et al, 2008). These species' cytosolic translation machines read the 'CUG' codon in nuclear gene transcripts as a serine rather than a leucine (Nakase *et al*, 1979). In the *Candida* genomes, 99% of ancestral 'CUG' codons were functionally changed, according to whole genome study of the leucine to serine shift (Butler *et al*, 2009).

1.2.1.1 Unique features of the mitochondrial genome

Despite the fact that mitochondrial genome of baker's yeast (Saccharomyces cerevisiae) consists mostly of polydisperse linear DNA molecules lacking distinct terminal structures, genetic and physical techniques have produced a circular map of mitochondrial DNA (mtDNA; reviewed in Williamson 2002). This mtDNA design is now known as a "circularmapping mitochondrial genome" (Jacobs *et al*, 1996). Several yeast species' mitochondrial genomes have been extensively sequenced (Sekito *et al*, 1995; Kerscher *et al*, 2002; Bullerwell *et al*, 2003; Jones *et al*, 2004) and likely have the same structure. In comparison, mtDNA of the yeast *C. parapsilosis* has a very distinct molecular architecture. According to restriction enzyme analysis, mitochondria of *C. parapsilosis* contain around 30 kb of homogeneous, linear DNA molecules (Kovac *et al*, 1995). Furthermore, it has been demonstrated that linear mtDNA

retains its functional and structural integrity in the presence of ethidium bromide and acridine orange, a characteristic that may account for *C. parapsilosis* ability to grow in the presence of high concentrations of these intercalating agents (Maleszka, 1994). In silico analysis it reveals that the mitochondria of *C. parapsilosis* contain a highly compact genome that, despite its distinct molecular architecture, shares a number of characteristics with the mtDNAs of other yeasts (Nosek, *et al*, 2004). As such, full sequencing of *C. parapsilosis* mtDNA offers a foundation for solving issues associated with the replication and development of linear genomes in yeast mitochondria (Nosek *et al*, 1995).

1.2.2. Identification of *C. parapsilosis*

It is critical to identify *Candida* species timeously and accurately during infections in order to select adequate treatment strategies (Chowdhary *et al*, 2017). Typically, a fungal blood culture or culture of another bodily fluid is usually used to diagnose *Candida* infections (Tortorano *et al*, 2013). Traditional biochemical approaches to nucleic acid-based technologies are utilized to identify *Candida* species (Sardi *et al*, 2013). Commercial systems such as API20, BD Phoenix, and vitek2 are used in conventional identification approaches
(Kim *et al*, 2016).

These identification systems include nitrogen and carbon compound growth assays (Chowdhary *et al*, 2017). The rising number of NAC species other than *C. albicans* causing nosocomial infections has made it difficult for diagnostic laboratories to offer reliable and speedy diagnosis, particularly for closely related species. This is significant for the species that comprised the *C. parapsilosis* complex, which cannot be distinguished using current biochemical methods. Although the clinical significance of *C. metapsilosis and C.*

orthopsilosis has yet to be discovered, precise identification of the three species within the complex is required in order to acquire epidemiological data.

Several DNA-based approaches to identify members of the *C. parapsilosis* complex, include restriction profiles of secondary alcohol dehydrogenase gene segments. It can identify *C. orthopsilosis, C. metapsilosis, and C. parapsilosis* (Tavanti *et al*, 2005; Mirhendi *et al*, 2010). Amplification fragment length polymorphism (Borst *et al*, 2003; Tavanti *et al*, 2007; Hensgens *et al*, 2009), Microarray-based System (Campa *et al*, 2008), Pyrosequencing (Borman *et al*, 2016), as well as polymerase chain reaction amplification and sequencing of the ribosomal internal transcribed spacer (ITS) region are also used. Matrix-assisted laser desorption/ionization time-off light mass spectrometry (MALDI-TOF-MS) has also been developed to rapidly identify fungal species (Eggimann *et al*, 2011).

Commercially available MALDI-TOF-MS facilitates species identification using mass spectrum analysis of crude cell extract, which is predominantly composed of numerous proteins. As such the resulting spectra are compared to the reference spectra, and the instrument database contains species-specific patterns. The main advantage of this method is its speed while the entire procedure takes only a few minutes to finish. So, this method might potentially be utilised for intraspecific typing (Qian *et al*, 2008; Carolis *et al*, 2012).

1.2.3. Biological characters of *C. parapsilosis*

Generally the cells of *C. parapsilosis* have oval, circular, or cylindrical. *C. parapsilosis* colonies are white, creamy, and lustrous, they could be smooth or wrinkled when growing on Sabouraud dextrose agar (Figure 1.2 and 1.3). On a chrogenic agar the colonies are pink/red in colour (Figure 1.3).

C. parapsilosis, unlike *C. albicans* and *C. tropicalis* does not develop a real hyphae and instead lives in either a yeast phase or a pseudohyphal form (Laffey *et al*, 2005).

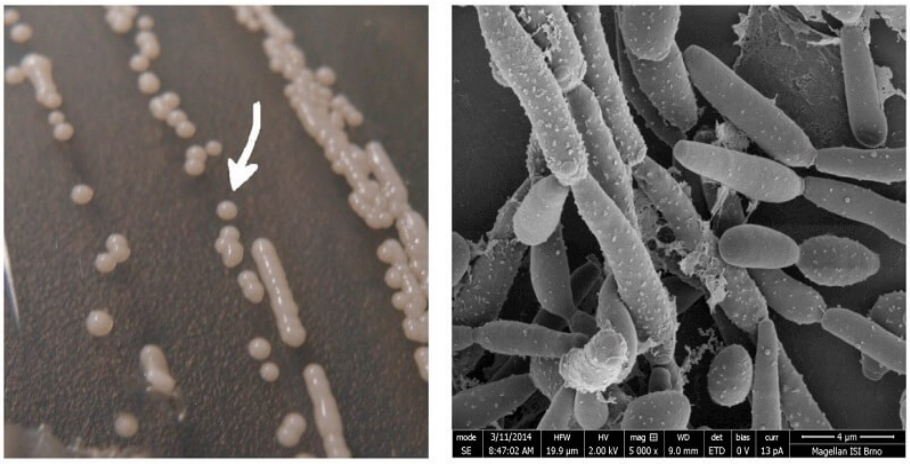


Figure1.2: (A) *Candida parapsilosis* colonies grown on agar for 48 hours. Figure: (B) SEM (Scanning electron microscopy) picture of *C. parapsilosis* (cultivation for 48 hours on a glass substrate). Image Source: (Samek *et al*, 2014). https://www.mdpi.com/14220067/15/12/23924

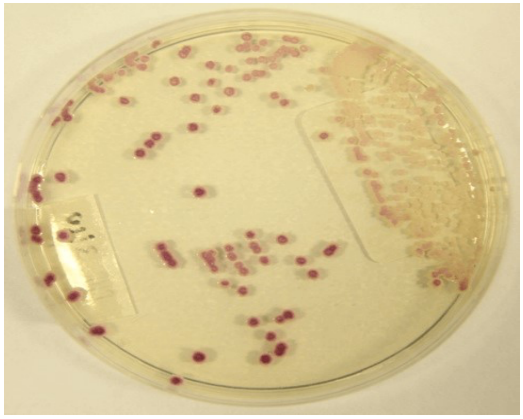


Figure 1.3: *Candida parapsilosis* on CHROMAgar™. Image Source: http://www.lifeworldwide.org/fungal-diseases/candida-parapsilosis

Light microscopy has been used to identify pseudohyphae found during growth on cornmeal agar. It has been suggested that the production of pseudohyphae in *C. parapsilosis* is connected to a specific set of amino acids (Kim *et al*, 2006).

According to transport studies, amino acid-mediated morphogenesis does not need ligands transport through the plasma membrane. This suggests that amino acids may be detected by receptor molecules, which trigger signal transduction pathways that govern cell differentiation. In a study, comparing two different *C. parapsilosis* strains. the first strain CLIB214 produced four stable and heritable colony morphologies [crepe, concentric, smooth, and crater] while, the second strain SR23 develops [smooth, stalk, wrinkled, super wrinkled, and concentric colonies has similar morphologies] (Butler *et al*, 2006). Furthermore, the dimorphic transition in cell morphology, which fluctuates between single-cell form and pseudohyphal filaments, is linked to variations in colony shape (Figure 1.4). Environmental cues trigger particular cell differentiation pathways, according to studies of *C. albicans* (Gancedo, 2001).

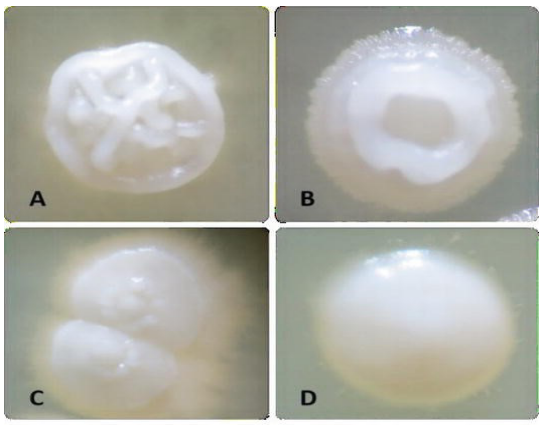


Figure 1.4: *C. parapsilosis* phenotypes in domestic environment. (A) Crepe phenotype, (B) concentric phenotype, (C) crater phenotype and (D) smooth phenotype. Image Source: https://www.intechopen.com/chapters/63742#F1

The production of biofilms on diverse surfaces is closely linked to the molecular pathways implicated in morphological switching and dimorphic transition. Biofilms have a complicated three-dimensional architecture that influences nutrition and waste product fluxes as well as cell susceptibility to antifungal medications (Douglas, 2003; Ramage *et al*, 2005). When *C.*

parapsilosis cells thrive on medium with high glucose and fat concentrations, biofilms develop easily, which has been closely related to an increased frequency of bloodstream infections in patients receiving parenteral nutrition. Biofilm growth boosts the microbe's capacity to attach catheters and intravascular lines, suggesting their preference for medical plastics (Weems, 1992; Trofa *et al*, 2008).

1.2.4. Survival of *C. parapsilosis*

The fact that *C. parapsilosis* is one of the few species in the genus not exclusively associated with people may explain why it is detected in nature more frequently than other *Candida* species. Due to its isolation from nonhuman sources like domestic animals, insects, water and soil, *C. parapsilosis* infections is not only found in humans (Trofa *et al*, 2008). Similar to other pathogenic yeasts*, C. parapsilosis* cells can live inside phagocytes and endothelial cells (Toth *et al*, 2014). Induced exocytosis, pseudohyphae development, and intracellular budding were also detected. In addition, absorbed yeast cells prevented the host cells from going through mitosis (Toth *et al*, 2014). Internalized yeasts in endothelial cells are resistant to the host cell's acidity and are shielded from neutrophil cell killing (Glass *et al*, 2015).

It has been proposed that the species secreted lipases and extracellular proteases which may play a role in the survival within developing phagosomes, despite the fact that the underlying molecular processes governing the aforementioned events, remain mostly unknown (Horváth *et al*, 2012; Holland *et al*, 2013). However, *C. parapsilosis* is one of the fungi that is frequently isolated from the human hands and is also a typical human commensal. Many people disagree on whether *C. parapsilosis* is a pathogen or a bystander in some diseases because of its temporary colonization of human integument.

1.2.5. Geographic distribution

Although there is geographical diversity, *C. parapsilosis* is the second most prevalent species among children (Warris *et al*, 2020). However, *C. parapsilosis* is also the predominant species of *Candida* on the skin and is consequently connected with outbreaks (Pinhati *et al*, 2016; Arastehfar *et al*, 2021). Furthermore, fluconazole-resistant *C. parapsilosis* isolates have been indicated in a number of countries, including but not limited to South Africa (Govender *et al*, 2016), Kuwait (Asadzadeh *et al*, 2017), South Korea (Choi *et al*, 2018), India (Singh *et al*, 2019), Italy (Castanheira *et al*, 2020), Mexico (Corzo-Leon *et al*, 2021), and Finland (Sarvikivi *et al*, 2005). In addition, between 2001 and 2005, the prevalence of *C. parapsilosis* increased from 4.8 % to 6.6 %. Furthermore, higher frequencies of *C. parapsilosis* isolation were identified in a study of 5346 clinical *Candida* isolates from 91 medical facilities between 2001 and 2006. Significant proportions of *Candida* species were found in Asia Pacific (15.97%), Latin American (18.62%), European (10.63%), and North American (14.04%) regions (Pfaller *et al*, 2008). However, *C. parapsilosis* was identified from blood and implantable medical devices at a proportionally higher rate (34.3%) than *C. albicans* (8.5 %) (Kojic and Darouiche, 2003). Therefore, underlying clinical status of the patients has a substantial impact on geographic variation in incidence of invasive *C. parapsilosis* infections.

1.2.6. Specific distribution in South Africa

While there are regional variations in overall incidence trends of *Candida* blood stream infection (BSI), there are few studies on the species distribution among blood stream infection (BSI) in patients in sub-Saharan Africa. *C. parapsilosis* has been reported as a predominant pathogen in the South African private sector, and it was the second most frequent pathogen in public sector (Pammi *et al*, 2013). Three retrospective, single-center studies were done in South

Africa bloodstream infection (BSI). At Tygerberg Hospital, *C. albicans* was the most frequently isolated species in 2005 (69%), *C. glabrata* came in second in the Western Cape Province in public sector (10%), *C. parapsilosis* (10%), and various additional species (11%) (Arendse and Orth, 2008). An investigation at a public hospital in Soweto, Gauteng province studied the species distribution among adults with candidemia (Kreusch and Karstaedt, 2013). Although *C. albicans* was the most prevalent species overall, *C. tropicalis* was the most prevalent species in the early time. In more recent years, *C. parapsilosis* and *C. glabrata* each accounting for 20% of isolates emerged as significant pathogens. Contrarily, a single unit study from a public Johannesburg hospital (2007–2011) that examined neonatal BSIs revealed that *C. parapsilosis* was the predominant pathogen (Ballot *et al*, 2013).

1.2.7. Virulence factors of *C. parapsilosis*

There are three primary steps through which pathogenic *Candida* species infect human hosts and cause disease: adhesion, invasion, and damage (Polke *et al*, 2015). Numerous characteristics referred as "virulence factors" make these actions easier. The microbiological characteristics that a pathogen exhibits to infect a host are known as virulence factors (Casadevall and Pirofski 2001). Extensive research has been done on *C. albicans* pathogenicity but limited data is available on NAC. Regarding the virulence determinants of *C. parapsilosis*, not much is known. This is a serious hindrance to the diagnosis, treatment, and prevention of *C. parapsilosis*-caused illnesses and infections.

1.2.7.1. Adhesion

Adhesion to epithelial surfaces occurs through direct physical attachment, via interaction with colonizing microorganisms, and through contact with abiotic surfaces (Richardson *et al*, 2018). This process is mediated by cell wall components and proteins found on the surface of the cell (Ener and Douglas, 1992; Hazen, 1989; Hazen *et al*, 1991). The adherence of organism to the surface of host or of a medical device is the initial stage of a *Candida* infection, which frequently results in creation of biofilms (Chandra *et al*, 2001). The amount of adhesion is therefore determined by microbial, host, and abiotic surface features, such as cell surface hydrophobicity and cell wall composition. Adhesion is thus an important stage in the infection process (Silva *et al*, 2011; Henriques *et al*, 2002). Attachment to epithelial cells can activate the yeast to hyphae morphological transition in some *Candida* species. Hence, two major adhesins in *C. albicans* are hypha associated Agglutinin like sequence proteins (Als) and hyphal wall protein 1(hwp1). Als are a family of cell surface proteins that mediate adhesion to epithelial cells (Hoyer and Cota, 2016). These proteins have an intrinsic ability to form amyloids, which has been shown to be an essential component of yeast adhesion proteins (Dehullu *et al*, 2019). Although it is currently a common cause of Candidaemia and is strongly associated to healthcare personnel inadequate hand hygiene, *C. parapsilosis* is generally regarded as one of the least virulent yeast species (Trofa et al, 2008).

C. parapsilosis colonization and infection are reliant on its adherence capacity to host cells and tissues, specifically mucosal surfaces. The reconstructed human epithelium (RHE) study showed a high capacity for colonization by *C. parapsilosis* despite having a lesser capacity to enter this tissue (Gácser *et al*, 2007). Furthermore, *C. orthopsilosis* also causes similar damage to RHE, whereas *C. metapsilosis* had comparatively reduced virulence. However, adherence to implantable medical devices causes the development of biofilm and provide an entry point

to the body of host. Initial *C. parapsilosis* adhesion to surfaces has been connected to the hydrophobicity of cell surfaces (Panagoda *et al*, 2001). *C. parapsilosis* propensity to adhere to plastic catheters has been linked to the production of slime (Branchini *et al*, 1994). In the comprehensive investigation of the adhesion of 24 isolates of *C.*

parapsilosis and 12 isolates of *C. albicans*, it was found that *C. parapsilosis* had a 20.6% higher avidity for buccal epithelial cells (BEC) and a 143.7% higher adherence to acrylic material (Panagoda *et al*, 2001). However, other, smaller investigations reported an 80% to

95% higher potential for *C. albicans* adherence to BEC compared to *C. parapsilosis* (BarrettBee *et al*, 1985). The avidity for BEC was 51.5 % higher in *C. parapsilosis* isolates from superficial infection than in systemic infections (Panagoda *et al*, 2001). In addition, different strains of *C. parapsilosis* with comparable pathogenicity in an experimental vaginal infection had different levels of ability to adhere to plastic (Cassone *et al*, 1995).Therefore, adherence in vivo may be relevant for infection but adhesion to plastic is not a clear-cut virulence factor related to vaginopathic potential or systemic infection (Bernardis *et al*, 1999).

1.2.7.2. Secretion of hydrolytic enzymes (Proteinase and phospholipase)

It is well known that one of the key aspects of pathogenicity in *Candida* species is the development of extracellular hydrolytic enzymes. Lytic enzymes appear to be essential for the adhesion and penetration of host tissues (Polke *et al*, 2015). Additionally, the production of hydrolytic enzymes promotes *Candida* survival by deteriorating host tissues in order to obtain nutrients. As such, hemolysins, phospholipases, proteinases, and lipases are a few of the prominent hydrolytic enzymes (Polke *et al*, 2015). Secretion enzymes in microbial pathogens have gained much attention for their role in pathogenesis and potential targets for designing novel drugs to treat infections. Two of the major hydrolytic enzymes in *C. albicans* are

phospholipases and secreted aspartyl proteinases (SAPS) and phospholipase (Niewerth and Korting, 2001).

Aspartic proteinases (Sap1p to Sap10p) are secreted *by C. albicans*, and this is a key factor in determining its pathogenicity (Figure 1.5) (Monod *et al*, 2002). Furthermore, SAPs damage the mucous membranes of host tissues by facilitating invasion and colonization (Rüchel *et al*,1992). Destroying crucial immune system and structural defence proteins, including collagen, fibronectin, C3 protein, immunoglobulin G heavy chains, and lactoperoxidase (Pichová *et al*, 2001).

Secreted aspartyl proteinases (SAP) activity is significantly lower in *C. parapsilosis* compared to *C. albicans* (Rüchel *et al*, 1986). In *C. parapsilosis*, three SAPs have been found, but only two of them have been substantially described (Merkerová and Dostál, 2006). The biochemistry of the Sapp1p isoenzyme has been described (Dostál and Dlouha, 2005; Fusek *et al*, 1994). Although, SAPP2P, which was first categorised as a pseudogene, produces a functioning proteinase called Sapp2p, which makes up roughly 20% of the SAPs that can be extracted from a culture supernatant (Fusek *et al*, 1993). On the other hand, Pepstatin A, a particular aspartic proteinase inhibitor, prevents *C. albicans* and *C. parapsilosis* from initially penetrating mucosal surfaces and lessens histological changes during experimental cutaneous Candidiasis (Gácser *et al*, 2007; Schaller *et al*, 2003). Among isolated strains of *C. parapsilosis*, SAP production varies, and its role in pathogenesis is still unknown. Developing new drugs which can target the production of saps can lead to development of novel classes of drugs with different mode of antifungal action.

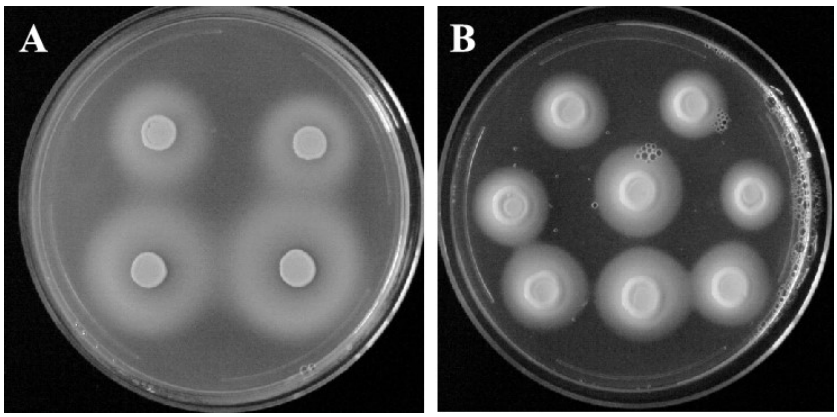


Figure 1.5: Culture of *Candida albicans* in specific media for the induction of proteinase (A) e phospholipase (B). Growth of 4 strains of *Candida albicans* in proteinase agar (A) and 8 strains of *C. albicans* in phospholipase agar (B). After incubation period, zones of degradation (A) and/or precipitation (B) could be seen around the yeast colonies.

It has been demonstrated that *C. albicans* produces a number of phospholipases, including lysophospholipase, lysophospholipas etransacylase, and phospholipase A-D (Niewerth and Korting, 2001). Increasing *C. albicans* pathogenicity has been related to higher phospholipase activity. In fact, invasive *C. albicans* strains have higher secretion of phospholipase than noninvasive strains (Niewerth and Korting, 2001). Although it is thought that phospholipases have a role in the breakdown of host membranes during infection, their exact function is not fully understood. Less is known about the function of phospholipases in the pathogenesis of *C. parapsilosis*. There have been conflicting results, with some researchers stating that up to 51% of *C. parapsilosis* strains exhibit phospholipase activity (Ghannoum, 2000). Others discovering no activity (Kantarcioğlu and Yucel, 2002; Samaranayake *et al*, 1998; Shimizu *et al*, 1996). Moreover, only one of the four isolated *C. parapsilosis* strains that caused Candiduria in Sao Paulo, Brazil, displayed phospholipase activity (Silva *et al*, 2015).

Furthermore, differences in the generation of phospholipases have been discovered when comparing systemic versus superficial isolates, with some researchers only detecting

phospholipase activity in bloodstream isolates (Dagdeviren *et al*, 2005). Additionally, others stating that superficial *C. parapsilosis* isolates have noticeably higher activity than systemic ones (Fernanado *et al*, 1999). Despite the function of phospholipase in *C. parapsilosis* pathogenesis is not clear yet, it has already shown that this enzyme is an emerging drug target to develop antifungal drugs (Ahmad *et al*, 2016).

1.2.7.3. Biofilm formation

Microorganisms grouped into organized colonies and encased in an exopolymeric matrix make up biofilms. Biofilms shield pathogenic cells from antifungal therapy and human immune system's response, leading to development of pathogens that are resistant to antibiotics. Biofilms exhibit resistance to antifungal therapies through a variety of mechanisms, including a rise in cell density, a slowing of growth and nutrition depletion, sequestering of antifungal medications, and a rise in gene expression encoding pump efflux (Sherry *et al*, 2017).

A large number of infections in humans are related to biofilm formation in *Candida* spp (Donlan, 2001). There are two distinct layers in *C. albicans* biofilm that are formed: a thin, a yeast layer and a hyphal layer that is thicker and less compact than the base yeast layer. The formation of biofilm is related to dimorphic switch from yeast to hyphal development (Baillie and Douglas, 1999). Contrarily, compared to *C. albicans*, *C. parapsilosis* strains produce biofilm that is both numerically and architecturally less complex (Hawser and Douglas, 1994; Kuhn *et al*, 2002). Prior to formation of biofilm, organisms attach to tissues or medical devices, which is likely what causes a change in shape and behaviour of the organism. *C. parapsilosis* biofilms can grow on medical equipment such as intra cardiac prosthetic devices, prosthetic joints, hemodialysis and peritoneal dialysis catheters, peripheral and central venous catheters (Ramage *et al*, 2006). The organism can come into contact with medical equipment before or while it is being used by a patient because it is a commensal of human skin, especially in

medical settings where poor hand hygiene is present. It is noteworthy that outbreaks have been linked to *C. parapsilosis* isolates with higher biofilm ability (Kuhn *et al*, 2004). Since it provides significant resistance to antifungal agents by limiting the penetration of drugs through matrix and shielding cells from host immunological responses, biofilm development is a powerful pathogenicity factor for a variety of *Candida* species. In comparison to patient isolates unable to form biofilm, biofilm-forming *C. albicans, C. parapsilosis, C. tropicalis,* and *C. glabrata* isolates have been strongly related to significantly higher mortality rates in patients at an Italian university hospital (Tumbarello *et al*, 2007). The mortality rate for isolates of *C. parapsilosis* that formed biofilm in vitro was 71.4 %, compared to 28 % for isolates that did not produce biofilm. Depending on how well they can form biofilms, different *C. parapsilosis* isolates may differ in their potential to wreak illness in distinct tissues. According to one study, biofilm formation was possible in 86 percent of *C. parapsilosis* blood isolates compared to 47 percent of isolates from other body areas (Shin *et al*, 2002). In another study, biofilm was generated by 59% of isolates from bloodstream and only 39% of those from skin (Růžička *et al*, 2007). In contrast, a study reported that only 21.8% of blood isolates could develop biofilm (Tumbarello *et al*, 2007). The circumstances employed to measure the creation of biofilms, as well as how long and how strains were stored before the study, may have contributed to the variations in result.

It is well known that *Candida* species in biofilm are resistant to antimycotic drugs (Christophe d′Enfert, 2006). Despite having a less complicated structure, *C. parapsilosis* biofilm is almost as resistant to amphotericin B and azole compounds as *C. albicans* biofilm is to these drugs (Katragkou *et al*, 2010; Růžička *et al*, 2007). However, metabolic activity of *C. parapsilosis* in biofilm can be inhibited by echinocandins at therapeutic doses (Cocuaud *et al*, 2005; Katragkou *et al*, 2015). Lipid amphotericin B formulations have demonstrated

effectiveness against biofilm of *C. parapsilosis* (Kuhn *et al*, 2002). Further research is required to understand the biofilms formation in different pathogens including *C. parapsilosis.*

1.3. Antifungal therapy

Development of antifungal drugs is slower than antibacterial drug development for a number of reasons. The major structural and metabolic differences between fungi and bacteria include the fact that most drugs target fungal cell membranes, whereas bacteria's cellular structure allows a variety of targets (Ghannoum and Rice, 1999). Only three main families of antifungal drugs including polyenes, azoles, and echinocandins, are now available for treatment of fungal infections (Mohr *et al*, 2008). Recently, other medications such as griseofulvin, allylamines, and flucytosine have been added to antifungal drug list, to treat some invasive fungal infections (Chandrasekar, 2010). Each of the three antifungal drug classes targets different fungal cell wall components, but their antifungal efficacy, side effects, and availability vary, necessitating thorough understanding of pharmacological features of each drug class (Thompson *et al*, 2009).

1.3.1. Antifungal drugs

1.3.1.1 Polyenes

Polyenes, which have been identified from Streptomyces species, are the first family of antifungal drug. Nystatin and amphotericin B are the only polyenes that are readily available right now. Ergosterol is a crucial component of yeast cell membrane that preserves integrity and function of the membrane. Polyenes work by attaching to ergosterol in membranes of fungi. This triggers a rise in membrane permeability, metabolic disruption, cytoplasmic contents leaking out from cell, and finally cell death (Mathew and Nath, 2009).

ERG (25 identified) enzymes control the synthesis of ergosterol. Due to its significant contribution to the persistence of fungi, ergosterol synthesis has emerged as a key target in the development of antifungal drugs (Dupont *et al*, 2012). Furthermore, amphotericin B, which is often administered intravenously, has long been regarded as gold standard treatment for severe fungal infections. The broad antifungal spectrum and better response to treatment with use of amphotericin B accounts for its wide spread use despite the possibility of nephrotoxicity risk (Ellis, 2002). It is important to remember that this polyene antifungal agent has a high risk of toxicity, including side effects such as fever, chills, headaches, nausea, and vomiting (Gallis *et al*, 1990). However, compared to traditional form, new amphotericin B formulations, such as lipid and liposomal formulation, have demonstrated better potency (Sanglard and Odds, 2002; Moen *et al*, 2009). On the other hand, the initial polyene antifungal that was created is nystatin which has wider antifungal efficacy than amphotericin B, but due to its high toxicity, it is restricted to be used as a topical treatment only (Semis, Rita *et al* 2013).

1.3.1.2 Azoles

The azole antifungal agents consists of two drug classes: triazole group, which contains fluconazole, itraconazole, and voriconazole, and imidazole group, which includes clotrimazole, miconazole, and ketoconazole. In both systemic and superficial fungal infections, only the triazoles and ketoconazole are applicable (Mathew and Nath, 2009).

Generally, comparing with the polyenes which are fungicidal drug, the azoles are fungistatic drugs. Even when both of them are targeting fungal cell membrane, the mechanism of them are completely different, Azoles depletes cytochrome P450 dependent enzyme (14-α lanosterol demethylase), which interpose with conversion of lanosterol to ergosterol in fungal cell membrane, resulting in inhibition of ergosterol and prevention of fungal growth and replication (Cronin *et al*, 2010). Fluconazole is one of the most commonly used azoles because

of its cheap cost, high solubility, less toxicity and other side effects. Furthermore, Fluconazole is a triazole antifungal drugs and has long been used as part of treatment plan for immuno compromised individuals (Debruyne and Ryckelynck, 1993).

1.3.1.3 Echinocandins

Echinocandins, which include caspofungin, micafungin, and anidulafungin, are semi synthetic lipopeptide molecules that represent the newest class of antifungal drugs. Echinocandins reduce the production of glucan, which is crucial for formation and operation of fungal cell wall. Blocking the pathway of (1, 3)-D- glucan synthase enzyme can cause cell death (Cappelletty *et al*, 2007). These antifungal drugs family has a potentially fungicidal activity against most C*andida s*pp and it is always used intravenously (Odds *et al*, 2003). The first drug in this class that was developed, is capsofungin. However, comparative studies have indicated that caspofungin and micafungin are safer for patients with kidney disease and have equal efficacy and fewer side effects to amphotericin B in the treatment of invasive Candidiasis (Villanueva *et al*, 2002; DeWet *et al*, 2004).

1.4. Resistance to antifungal drugs

The widespread use of antifungal agents leads to drug resistance in different *Candida* species (Pfaller *et al*, 1994). Antifungal substances interact with particular cellular targets to prevent fungal survival or growth. However, fungi can interfere with the effects of antifungal agents through a number of methods. When MIC of a certain antifungal drug rises in comparison to MIC of same species, this is a sign of antifungal resistance. (Sanglard and Odds, 2002). Resistance is categorized as either primary or secondary. Primary resistance, also known as inherent resistance, is seen before receiving therapy either in *vivo* or in *vitro* (Sanglard and Odds, 2002). On the other hand, repeated use of antifungal drugs may result in secondary

resistance. Secondary resistance may be acquired as a result of genetic changes or it may be reversible when the organism briefly adapts to antifungal alterations (Sanglard and Odds, 2002). Compared to intrinsic resistance, acquired resistance is less frequently documented, this is possibly due to the fact that fungi do not exchange their resistance genes via plasmids which is common in bacteria. Consequently, during the course of an antifungal treatment, an individual selects for resistance genes (Arendrup and Patterson, 2017). It has been shown that patients with HIV/AIDS and bone-marrow transplants who received repeated azole treatment for *Candida* infections acquired antifungal resistance (Marr *et al*, 1997; White *et al*, 1998). Resistance to polyenes (amphotericin B) is uncommon, however significant number of studies demonstrate resistance to azole and echinocandins in *Candida* species. Currently, the first-line treatment for invasive candidiasis is echinocandins. Echinocandins are fungicidal against the majority of *Candida* species, including those that exhibit resistance to azole and polyene antifungals (Pfaller *et al*, 2019). Nevertheless, over the years (1997 to 2016) fluconazole resistance has not changed much according to the ARTEMIS DISK study. However, *C. parapsilosis* has shown an increase in fluconazole resistance over time. The use of Fluconazole as first-line therapy in the past may have contributed to this development in fluconazole resistance (Pfaller et al. 2019). There is currently no agreement on the best way to treat invasive *C. parapsilosis* disorders, although the standard therapeutic approach involves removing any removable foreign bodies and administering a systemic antifungal. Amphotericin B has traditionally been the most widely used antifungal but the problem is nephrotoxicity might make it difficult to administer amphotericin B, so the dosage must be decreased (Walsh *et al*, 2002) or abandon the treatment (Moudgal *et al*, 2005). The high potential for toxicity is applicable, particularly in those with renal impairment (Baley *et al*, 1990). This has resulted in the development of lipid formulations of amphotericin B with comparable effectiveness and decreased nephrotoxicity (Shohet *et al*, 2001; Linder *et al*, 2003; Oppenheim *et al*, 1995).

There are specific case reports that show that infectious strains are resistant to amphotericin B (Tunkel *et al*, 1993). And Studies reveal a 2–3% in vitro resistance rate for *C. parapsilosis* to amphotericin B (Ostrosky-Zeichner *et al*, 2003). Between 0.13 and 1μg/ml and, 0.5 and 1μg/ml, respectively, are documented average values of *C. parapsilosis* MIC50 and MIC90, determined using different approaches (Fleck *et al*, 2007; Marco *et al*, 2003; Merkerová *et al*, 2006). Therefore, despite resistance developing among specific strains of *C. parapsilosis* during treatment, the fundamental drawback of standard amphotericin B therapy is still the poor solubility in water and toxicity (Ellis, 2002). On the other hand, the alternative to amphotericin B antifungal drug that is mostly used is fluconazole. Non-*albicans Candida* species, especially *C. glabrata* and *C. krusei*, have been shown to exhibit in vitro resistance to fluconazole (Fleck *et al*, 2007). Also whether or not the use of fluconazole has caused a shift toward Non-*albicans Candida* species producing candidemia, is a subject of debate. While some support the claim that it has changed the epidemiology of non-albicans infections (Baran *et al*, 2001). There has been very little general variance in fluconazole susceptibility (Pfaller and Diekema, 2004). Nevertheless, *C. parapsilosis* has reportedly developed clinical resistance (Sarvikivi *et al*, 2005; Tumbarello *et al*, 2007). Fluconazole resistance has been detected at rates ranging from 0 to 4.6 percent in in vitro susceptibility studies (Arias *et al*, 1994; Ostrosky-Zeichner *et al*, 2003; Pfaller and Diekema, 2007) with typical MIC50 and MIC90 values between 0.5 and 1μg/ml and 1 to 2μg/ml, respectively (Fleck *et al*, 2007; Kuhn *et al*, 2002; Merkerova, 2006).

Interestingly, fluconazole-resistant strain that caused cross-infections over a 12-year period emerged as a result of the prolonged use of fluconazole to treat *C. parapsilosis* bloodstream infections in a Finland NICU (Sarvikivi and Saxen, 2005). However, there have not been any fluconazole-resistant *C. parapsilosis* found in other prophylactic study, despite the fact that they only lasted 14 to 30 months (Kaufman *et al*, 2001). A study including 384 newborns

discovered that targeted short-course fluconazole prophylaxis for neonates with very low birth weight was effective and affordable (Soghier *et al*, 2006). Only 2 (1.1%) of the 178 newborns receiving prophylaxis for *C. albicans* and *C. lusitaniae* had invasive fungal infection, compared to 13 (6.3%) of the 206 infants receiving no prophylaxis; 9 (69%) of these 13 diseases were brought on by *C. parapsilosis*, demonstrating the efficacy of fluconazole as a prophylactic drug against this frequent newborn pathogen. Prior to implementation of fluconazole prophylaxis, non-*C. albicans* species were responsible for 5 (26%) of 19 cases of *Candida* infections at NICU at the Woman's Hospital in Texas between 2000 and 2001; *C. parapsilosis* accounted for 3 infections (Healy *et al*, 2008).

Nine (41%) of 22 infections that occurred between 2002 and 2006 as a result of introduction of fluconazole prophylaxis were brought on by *non-C. albicans species*, and six were brought on by *C. parapsilosis*. These trials have led to a widespread acceptance of fluconazole targeted prophylaxis among neonatologists for newborns under 1,000 grams or under 27 weeks (Kaufman, 2008).

As such, understanding *C. parapsilosis* resistance mechanisms may assist in developing combination treatment that reverse fluconazole resistance. The resistance of *Candida* species to fluconazole has been recognized by Centre for Disease Control and Prevention as a serious threat to human health. On the other hand, biofilm formation is not the only mechanism responsible for antifungal drugs resistance, there are several mechanisms responsible for azole resistance. The most common one was failure of cells to accumulate drug due to overexpression of certain transporter proteins known as efflux pumps (Parkinson *et al*, 1995).

1.4.1. Mechanisms of antifungal resistance

Development of antifungal agents has lagged behind that of antibiotic development. This might be ascribed to a number of things, such as intrinsic difficulties in identification of a chemical that effectively affects eukaryotic fungus. The mechanism of antifungal resistance can either be inherent (present without prior exposure to the antifungal) or acquired, where resistance develops in an organism that was previously susceptible after exposure to the agent for a period of time. This depends on drug and species of *Candida*. These drugs are currently categorized based on the target of activity. Because they have the capacity to interact with ergosterol component of cell membrane to create pores, polyene antifungals like amphotericin B are fungicidal (Kanafani and Perfect, 2008). Additionally, it has been hypothesized that elevated catalase activity shields fungal cell from oxidative damage, facilitating amphotericin B resistance (Sokol-Anderson *et al*, 1986).

Furthermore, synthesis of -1, 3-D glucan, an essential component of fungal cell wall, is inhibited by echinocandins (Pfaller, 2012). Point mutations in FKS genes of 1, 3-D glucan synthase complexes are the main cause of echinocandin resistance (Rocha, 2007). Another probable mechanism of resistance to echinocandins comprises adaptive stress response system (Walker *et al*, 2010; Wiederhold *et al*, 2016). On the other hand, azoles are a different group of antifungal agents that work by interfering with fungal lanosterol demethylase enzyme to prevent the formation of ergosterol. This enzyme's conversion of lanosterol to ergosterol is one of its primary tasks, and its blockage causes the sterol in fungal cell membrane to be depleted. The most widely used azole antifungals are fluconazole and itraconazole. They have fungistatic or fungicidal activity against *Candida* species (Kanafani and Perfect, 2008).

One of the azole resistance mechanisms is failure of cells to accumulate the drug due to overexpression of certain transporter proteins known as efflux pumps (Liu *et al*, 2014; Holmes *et al*, 2016). There are studies that indicate that multidrug resistance in *Candida* spp. is caused by multidrug-efflux systems (Arendrup and Patterson, 2017). Based on above information, resistance mechanisms are summarized here.

Resistance may occur in one of the following ways (Coste *et al*, 2006; Morschhäuser *et al*, 2007) :

- Mutations of point in ERG11 gene, leading to reduced azole binding capacity.
- Overexpression of drug target (ERG11) gene which encodes for 14α demethylase.
- Efflux overexpression of gene switch is present on specific channels (efflux pumps) encoding multidrug transporters [ATP binding cassettes (ABC) and major facilitator superfamily (MFS)].
- Overexpression of CDR1 and CDR caused by activating mutations in transcription factor genes TAC1 leading to efflux of all azole antifungal agents.
- Overexpression of an MFS transporter (*MDR1*) caused by activating mutations in the transcription factor genes *MRR1* leading to efflux of fluconazole and voriconazole.
- Mutations in ERG3 gene resulting in inactivation of sterol desaturase.

1.5. Combination treatments

Combining two or more antimicrobial agents with a synergistic mode of action can increase their efficacy and broaden their spectrum of activity, as well as resolve antimicrobial resistance and reduce toxicity of these agents. Combination therapy has been effectively used in

antibacterial therapy and in antiretroviral treatment (Ghannoum *et al*, 1995). Furthermore, HIV/AIDS and TB are two infectious diseases that are treated with combination medication. Therefore, evidence points to the possibility of using combination therapies to manage invasive fungal infection (Spitzer *et al*, 2017). Although in a few instances where this combination has been demonstrated to be superior to monotherapy, it is unusual in clinical practice (Mukherjee *et al*, 2005). For instance, monotherapy with flucytosine and amphotericin B was found to be ineffective for treating cryptococcal meningitis. In randomised clinical studies, fluconazole plus flucytosine boosted survival of individuals with *Cryptococcus neoformans* meningitis (Bennett *et al*, 1979). On the other hand, increased bioavailability of medications may have contributed to their synergism. At many situation, a combination drug's diverse targets in the same protein or biological pathway may result in an additive boost in therapeutic effectiveness. For example, combination of terbinafine and azoles, both of them target ergosterol production pathway, has improved antifungal effectiveness against *C. albicans* (Perea *et al*, 2001).

Moreover, when combined with iKIXI in *vivo*, fluconazole's antifungal efficacy improved (Nishikawa *et al*, 2016). These findings show that azole-resistant infections can be effectively treated by focusing on efflux activities (Chen *et al*, 2021).

Generally, combination antifungal medication is becoming more popular as a method of treating refractory *Candida* infections, however, controlled clinical trials are still required to rule out any potential antagonistic effects and/or toxicity of any of these combinations.

1.6 Mechanism of efflux pump

The efflux pumps are transport protein channels which move components across *Candida* cell membrane, including antifungal drugs. Conceptually, simplest mechanism of resistance is one that reduces intracellular drug accumulation. A reduction in intracellular drug levels leads to a

reduction in the amount of drug that can reach a target. There are two ways to determine intracellular accumulation of drug and if the inhibition of efflux pumps is working or not. They are Rhodamine 6G (R6G) and fluorescent Hoechst 33342 dyes. Accumulation of these dyes into fungal cells means efflux pump is inhibited by the test drug whereas escape of these dyes from fungal cells would represent intact efflux pump activity. Overexpression of plasma membrane efflux pumps is the primary cause of high levels of azole resistance in clinical *Candida* isolates (Holmes *et al*, 2012; Rogers and Barker, 2003). Therefore, blocking these transporter pumps opens up possibilities for overcoming resistance to azoles related to ABC efflux pumps.

1.6.1 Classes of efflux pumps

ABC proteins and MFS proteins are two main families of efflux pumps. Using various energy sources, these membrane proteins actively translocate molecules across cell membranes. ABC proteins are major transporters that utilise ATP hydrolysis. The MFS proteins are secondary transporters that use proton-motive force to move molecules across plasma membrane (Dawson and Locher, 2006). Both types of transporters have different protein domains that confer substrate specificity: nucleotide binding domains (NBDs) in ABC pumps and transmembrane domains (TMDs) in both efflux pump families (Figure 1.6).

1.7 Efflux pump inhibitors

Since efflux pumps are the primary cause of expulsion of antifungal drugs, using efflux pump inhibitors (EPIs) can be a novel therapeutic approach. Along with its effect in lowering antifungal minimal inhibitory concentration (MIC), use of azoles and EPIs with antifungal drugs can lessen the invasiveness of *Candida* species. These inhibitors have potential to boost *C. parapsilosis* susceptibility to fluconazole. It is obvious that efflux pumps contribute to this antifungal drug resistance. Therefore, blocking these pumps holds the potential to increase

intracellular drug concentration, restore treatment efficacy against resistant bacteria, and reduce the emergence of new resistant strains. The efflux pumps underlying mechanism must first be understood. The efflux pumps can be impeded by various mechanisms (Figure 1.7) including: 1- interference with regulatory steps needed for expression of efflux pump, 2- changing antifungal structure and design, 3- inhibition of functional assembly, 4- inhibition of antifungal binding by competitive or noncompetitive binding, 5- blocking outer membrane channels which are responsible for efflux of antifungal drugs, 6- last step collapses the energy required for pump activity. EPIs and fluconazole may be utilised in combination therapy in order to suppress virulence factors and reverse fluconazole resistance in *C. parapsilosis.*

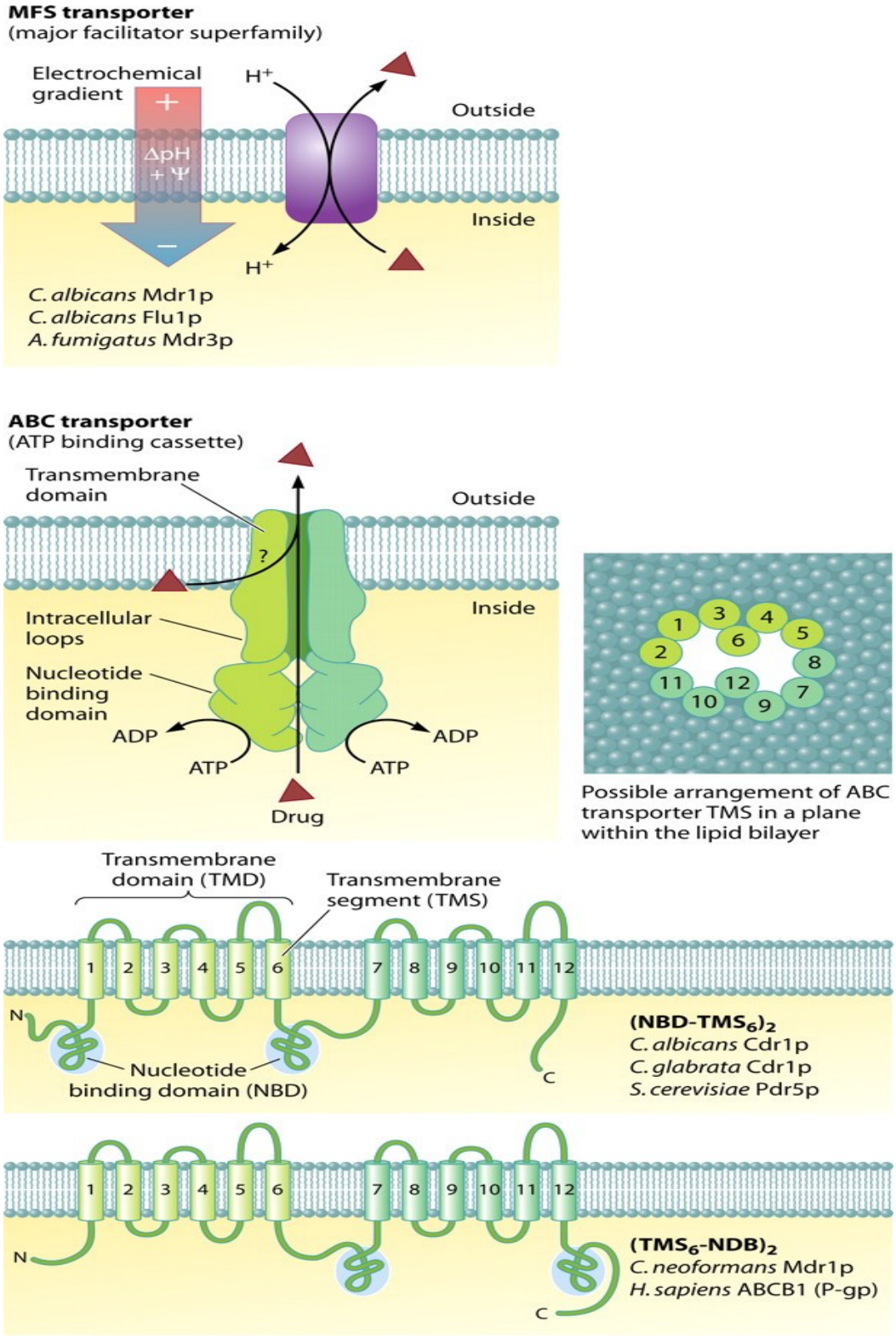


Figure 1.6: The domain configurations of the ABC and MFS transporters. The schematic membrane model of the ABC TMS is derived from the crystal structure of Sav1866 (Dawson and Locher, 2006). Source of image: https://journals.asm.org/cms/10.1128/CMR.0005108/asset/8e3124bf-509a-423c-9c16-f3f2dc335d60/assets/graphic/zcm0020922790001.jpeg

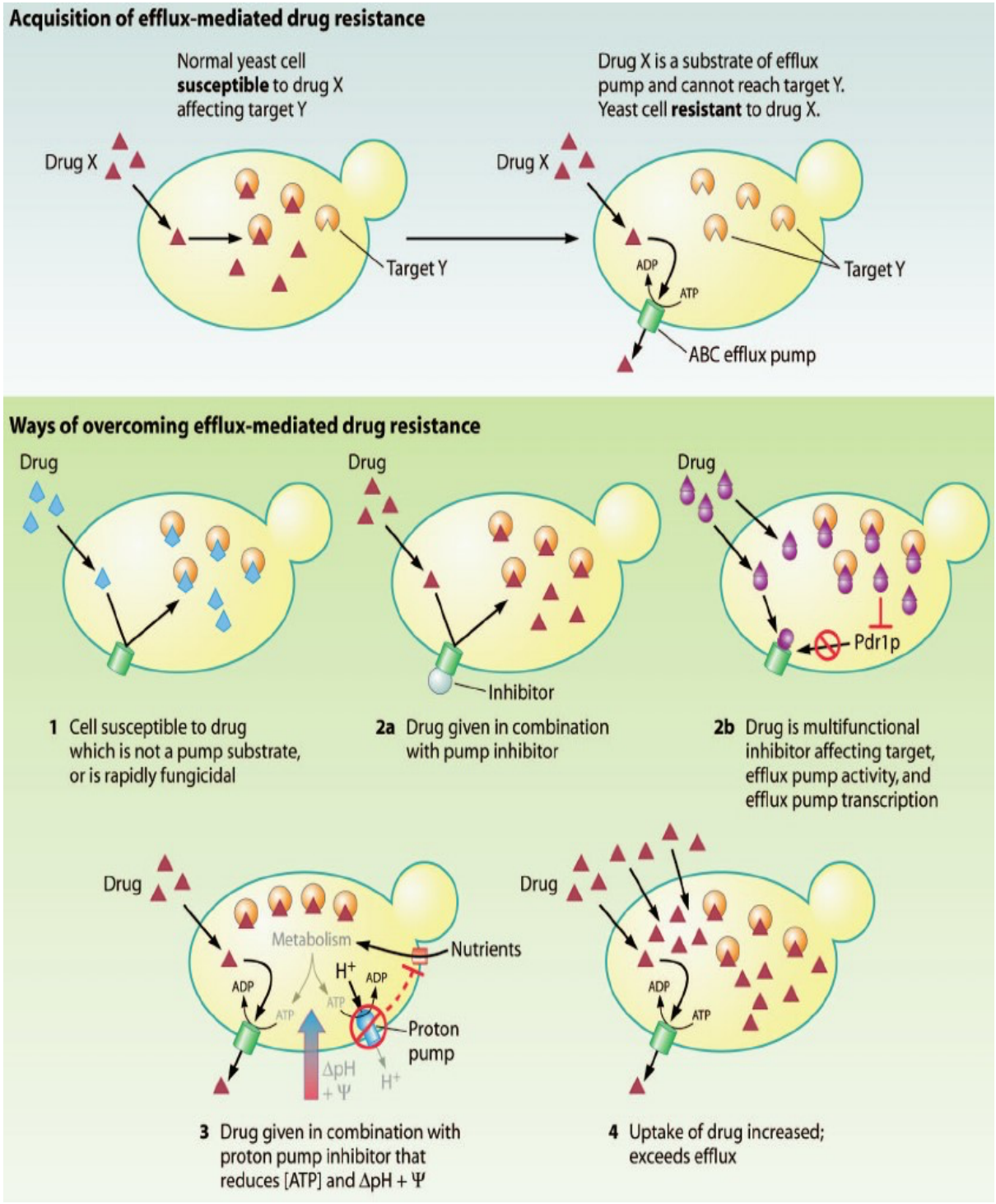


Figure 1.7: Possible ways of overcoming efflux-mediated fungal drug resistance. Source of image: (Cannon, Richard, *et al*, 2009).https://www.researchgate.net/profile/Richard-Cannon2/publication/24278060/figure/fig5/AS:277003174465536@1443054111069/Possible-waysof-overcoming-efflux-mediated-fungal-drug-resistance.png

1.7.1 Use of efflux pumps inhibitors to target virulence and antifungal resistance

A class of medications known as proton pump inhibitors reduces the amount of stomach acids produced. As a result, they are widely chosen as first-line treatment for illnesses related to acid production, such as gastroesophageal reflux disease and stomach, esophagus, and duodenal ulcers (Malfertheiner *et al*, 2017; Scarpignato *et al*, 2016). They may prevent acid formation by lowering the activity of H+/K+ -ATPase in cell membrane. In clinical settings, proton pump inhibitors (PPI) like esomeprazole, ilaprazole, lansoprazole, pantoprazole, omeprazole, and rabeprazole are frequently utilized (Lu *et al*, 2020). Furthermore, EPIs have been shown in numerous investigations to exhibit antifungal efficacy against *Candida* species. In addition, they are also efflux pump inhibitors. Omeprazole, for instance, reduced the development of susceptible *C. albicans* isolates at a dose of 10 μM (Liu and Köhler, 2016). Additionally, it was discovered that lansoprazole, at a concentration of around 200 μM, might prevent sensitive *C. albicans* from developing hyphae (Biswas *et al*, 2001). However, when EPIs and fluconazole were combined in vitro, it has been found that there was no synergistic antifungal action. On the other hand, it was demonstrated that the use of EPIs massively reduced the activity of the efflux pump. Similar to this, *C. albicans* isolates had lower phenotypic switching and phospholipase synthesis (Lu *et al*, 2020). According to these findings, fluconazole resistance in *C. parapsilosis* may be overcome by suppressing virulence factors when administered in combination with EPIs.

Pantoprazole and omeprazole are substituted benzimidazoles that inhibit gastric H+, K+ATPase selectively, preventing the final step in acid secretion (Kromer *et al*, 1999). Similar to other drugs of same class, pantoprazole and omeprazole inhibit pentagastrin-stimulated gastric acid secretion in humans after single intravenous or oral dosing (Simon et al, 2011).

Therefore, numerous drugs targeting gastric pump have been developed and are known as proton pump inhibitors, or PPIs. In this study, we used pantoprazole and omeprazole as efflux pump inhibitors in *C. parapsilosis.*

1.8 Justification for this study

C. parapsilosis has remarkable clinical significance due to its increasing resistance to multiple antifungal drugs, causing high mortality and morbidity rates especially between neonatal intensive care units and paediatric population. Associations of *C. parapsilosis* with a wide range of hospital outbreaks and increased resistance to azoles has gained this pathogen a notable clinical significance. Resistance to azoles, particularly against fluconazole is directly linked to over expression of efflux pumps. This is a major concern as fluconazole is the frequently used drug to treat such infections. As such, there are some studies which aimed at finding novel strategies to reverse drug resistance in fungal pathogens. Some of these approaches include using antifungal agents in combination and targeting virulence factors. Furthermore, very few studies elucidate the mechanisms of targeting efflux pumps to reverse drug resistance. Therefore, studying the combination of efflux pump inhibitors and azoles against *C. parapsilosis* would provide important information, which may support the use of this approach to treat fungal infections in near future.

1.9 Aim of this study

The aim of this study is to determine the combination interaction of fluconazole with efflux pump inhibitors against antifungal resistant *C. parapsilosis.*

1.10 Objectives

- To determine the antifungal activity of fluconazole and efflux pump inhibitors separately against *C. parapsilosis* by measuring MICs and MFCs

- To study the effect of combination of fluconazole and efflux pump inhibitors on *C. parapsilosis*

- To study the effect of efflux pump inhibitors in the reversal of fluconazole drug resistance in *C. parapsilosis*

- To evaluate the effect of fluconazole and efflux pump inhibitors on virulence factors of *C. parapsilosis*

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals, reagents and media

The studies were carried out with molecular or analytical grade reagents. The reagents, the composition of media, buffers and solutions are all listed in Appendix A page 128. All reagents were obtained from Sigma Aldrich in South Africa.

2.2 Antifungal agents and efflux pump inhibitors

As an antifungal agents, fluconazole (Sigma-Aldrich, South Africa) and amphotericin B (Sigma-Aldrich) were used in this study. Stock solutions of 1000 µg/ml fluconazole and 10 µg/ml amphotericin B, were prepared in 1% dimethyl sulfoxide (DMSO) and kept at -20 °C until required.

Efflux pump inhibitors, pantoprazole (Sigma-Aldrich, South Africa) and omeprazole (SigmaAldrich, South Africa) were also used in this study. Stock solutions of 10 mg/ml of pantoprazole and 20 mg/ml of omeprazole were prepared using 1% dimethyl sulfoxide (DMSO) and stored at -20 °C until needed.

2.3 Candida strains

This study included 12 clinical isolates of *C. parapsilosis* and a well-studied control laboratory strain of *C. albicans* SC5314. The clinical isolates were obtained from National Institute of Communicable Diseases (NICD), Johannesburg, South Africa, which were identified using Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). A list of *Candida* strains is provided in Table 2.1. The Department of Clinical Microbiology and Infectious Diseases kept *Candida* isolates at -80 °C in glycerol.

The Human Research Ethics Committee at University of the Witwatersrand granted permission and provided ethical waiver to utilise the isolates (clearance number W-CBP210602-01) in Appendix B page 130. On requirement, preserved *Candida* isolates, were revived by cultivating on Sabouraud dextrose agar (SDA) plates at 37 ± 1°C for 24-48 hours. To prepare an inoculum for experiments, a colony was inoculated into 5 ml of Sabouraud dextrose broth (SDB), incubated shaking for 18 hours at 37°C and the culture was used in all the experiments throughout the study. It contained approximately 1.5 $\times 10^{!}$ cfu/ml of *Candida.*

Table 2.1: A list of *Candida* isolates used in this study

Candida species	*Candida* strains (Original ID no.)	ID no. given for this study
C. albicans	SC5314	none
*C. parapsilosis*01	MRU 4315	CP1
*C. parapsilosis*02	MRU 4282	CP2
*C. parapsilosis*03	MRU 4090	CP3
*C. parapsilosis*04	MRU 4112	CP4
*C. parapsilosis*05	MRU 4120	CP5
*C. parapsilosis*06	MRU 4334	CP6
*C. parapsilosis*07	MRU 4033	CP7
*C. parapsilosis*08	MRU 4108	CP8
*C. parapsilosis*09	MRU 4036	CP9
*C. parapsilosis*10	MRU 4086	CP10
*C. parapsilosis*11	MRU 5473	CP11
*C. parapsilosis*12	MRU 5503	CP12

2.4 Antifungal susceptibility test

The broth microdilution method (BMD) was used to determine minimum inhibitory concentrations (MIC) and minimum fungicidal concentration (MFC) of fluconazole, amphotericin B, and efflux pump inhibitors (pantoprazole and omeprazole) against each of 12 *C. parapsilosis* isolates. Method described by Clinical and Laboratory Standards Institute (CLSI) guidelines reference document M27-A3 was followed with some modifications (CLSI, 2008).

To prepare an inoculum, *Candida* colonies were suspended in Sabouraud dextrose broth (SDB) and incubated at 37 °C for 24 hours. MIC/MFC assays were performed in 96-well microtitre plates. Sabouraud dextrose broth (100 µl) was added to each well, followed by the addition of 100 µl of each antifungal drug/ efflux pump inhibitors from initial concentration of test compounds of first row of the plate. Thereafter, a serial 2-fold dilutions were prepared. To each of these dilutions, 100 µl of standardised inoculum of each *Candida* isolate was added, giving a final volume of 200 µl.

The range of resulting concentrations of antifungal agents was as following:
Fluconazole concentrations ranged from 250 to 1.95 µg/ml, amphotericin B concentrations from 2.5 to 0.019 µg/ml, omeprazole concentrations from 5 to 0.03 mg/ml, and pantoprazole concentrations from 2.5 to 0.01 mg/ml.

In every set of experiment, a culture control well "C*andida* cell with no drug", a media control well "media with no C*andida* cell" and a negative control well "C*andida* cell with 1% DMSO" were added into the plates to confirm the viability and sterility of experiments. The 96-well plates were then incubated for 24 hours at 37 °C. The MIC and MFC were then determined.

The MIC was defined as the lowest concentration of antifungal agent that entirely prevented clearly visible apparent fungal growth as compared to growth control. The isolates were classified as resistant or susceptible using MIC values.

Following the determination of MIC values, minimum fungicidal concentrations (MFCs) were determined by culturing 5 µl of mixture from all wells in microtitre plates that did not show apparent growth on SD agar plates at 37°C for 24 to 48 hours. MFC was determined as concentration in first well with no growth on the plate. To confirm the results, all experiments were performed three times for each strain. Furthermore, median MIC and MFC were calculated. One fluconazole resistant and one fluconazole susceptible isolates with the highest and lowest MICs, were selected for further investigations which were *C. parapsilosis* (CP2) and (CP8) respectively.

2.5 Combination interaction

Previously reported approach was used to determine the combination interaction between fluconazole and efflux pump inhibitors (Ahmad et al. 2015). Two selected *C. parapsilosis* (CP2) and (CP8) strains and *C. albicans* SC5314 strain were used to determine MIC values of combination of Efflux pump inhibitors (pantoprazole and omeprazole) with fluconazole which were combined in a 1:1 volume ratio at their respective MIC values. One hundred microliters of SDB was added to each wells in a 96-well microtitre plate. To the first well, 50µl of efflux pump inhibitor + 50µl of fluconazole were added. The compound combinations were serially double diluted. The standard inoculum (1.5×10^{l} cfu/ml) was then added to the wells (100 µl) and after a 24-hour incubation period at 37°C, MIC values were determined. These experiments were repeated three times.

The fractional inhibitory concentration index (FICI) was determined for each combination using zero-interaction theory of Loewe additivity (LA) model (Loewe, 1953), which is standard model for evaluating the effects of medication combinations. The FICI value was determined by adding FICs of the test agents. Establishing the MIC (a / b) (MIC in combination) and dividing it by MIC of test agent acting alone yielded FIC for each agent (MIC tested alone). The following formula were used to determine the FICI:

$$\text{FICI} = \text{FICa} + \text{FICb} = \frac{\text{MICa in combination}}{\text{MICa tested alone}} + \frac{\text{MICb in combination}}{\text{MICb tested alone}}$$

Where MICa and MICb were the MIC of efflux pump inhibitors (pantoprazole, omeprazole) and antifungal drug (fluconazole) respectively.

The FICI data were interpreted using the following criteria:

- Synergy was indicated by FICI ≤0.5
- An additive effect was indicated by FICI >0.5 ≤1.0
- Indifference was indicated by FICI >1.0 ≤4.0
- Antagonism was indicated by FICI >4.0

Synergy occurs when combined drugs produce an effect that is greater than sum of their individual effect. An additive effect occurs when combination effect is consistent with potency of drugs individually. Indifference denotes that the effect of combination is equivalent to potency of most effective drug. Antagonism signifies that combination is less efficient compared to potency of most effective drug.

2.6 Time-kill kinetics

Time-kill kinetics assay was used to study antimicrobial activity of fluconazole and efflux pump inhibitors against *C. parapsilosis* over time.

Both strains of *C. parapsilosis*, one resistant (CP2) and one susceptible (CP8), were subcultures on SDA and grown overnight in SDB at 37 °C.

The inoculum of isolates was diluted in SDB at a 1:100 volume ratio. Each isolate was treated with MIC of fluconazole, omeprazole and pantoprazole alone as well as treated with combination of ½ MIC fluconazole and pantoprazole and with combination of ½ MIC fluconazole and omeprazole. At predetermined time periods (0, 2, 4, 6, 8 and 24 hour) and to get compound-free cells, 100 µl aliquots were collected and transferred to Eppendorf tubes, centrifuged (3900g at 4 °C for 1 minute), and washed twice with 0.9 mL of sterile distilled water. An appropriate volume (5 µl) aliquots were spread on SDA plates. The SDA plates were then incubated at 37 °C for 48 hours. Thereafter, plates were evaluated for number of surviving *Candida* cells. The cfu/ml was determined as follows:

$$cfu/ml = \frac{(number\ of\ colonies \times dilution\ factor)}{volume\ spread\ on\ plate}$$

The results were analysed by constructing a time-kill curve, which was obtained by plotting log10 cfu/ml against incubation time. The time-kill curve was used to observe fungicidal/fungistatic activity of fluconazole and efflux pump inhibitors in combination.

2.7 Pathogenicity markers

The effect of efflux pump inhibitors (pantoprazole and omeprazole) on pathogenicity markers was determined. This was studied by exposing *C. parapsilosis* isolates with ½ MIC, MIC, and MFC concentrations of drugs. The experiments were repeated three times.

2.7.1 Effects of efflux pump inhibitors on *Candida parapsilosis* adherence

Selected two *C. parapsilosis* strains (fluconazole resistant CP2) and (fluconazole susceptible CP8), and *C. albicans* were subjected to adherence assay using a method described by Patel et al (2009) with modifications. Oral epithelial cells (from investigator – Marwah Omar) were collected by gently rubbing a swab onto oral mucosa and emulsifying in sterile distilled water. Cells were washed three times and suspended in sterile distilled water. Using a hemocytometer the oral epithelial cell density was adjusted to $1 \times 10^{"}$ cells/ml. *C. parapsilosis* cells were grown in SD broth for 18 hours at 37 °C in shaking incubator at 200 rpm, washed three times in distilled water and re-suspended in 2 ml SDB containing three different concentration (½ MIC, MIC, and MFC) of pantoprazole/ omeprazole. Two mililiter of oral epithelial cells were mixed with 2 ml treated *C. parapsilosis.* Controls without efflux pump inhibitor was also included. The mixture was incubated for 2 hours in a 200 rpm shaker at 37 °C. Thereafter, non-adherent C*andida* cells to epithelial cells were removed using a nylon filter with a 20 µm pore size. The epithelial cells were retrieved from filter, washed and re-suspended in distilled water. Glass slides were prepared and gram stained. Smears were examined under a light microscope at 1000X magnification. Adherence was measured as the number of adherent *Candida* cells per 100 epithelial cells. The experiment was performed triplicate by preparing three slides for each isolate. Results of the unexposed controls were compared to exposed test results using a student t-test. *P-value* of ≤ 0.05 was considered significant.

2.7.2 Screening for production of proteinase

Using a previously established technique (Shaban *et al*, 2020) proteinase assays were performed. Twelve *C. parapsilosis* isolates were initially screened for extracellular proteinase activity. Isolates of *C. parapsilosis* were added to flasks containing 5 ml SDB and cultured for

18 hours at 37 °C. The cells were re-suspended in new fresh media after being centrifuged for 5 minutes at 3000 rpm. The fresh culture was adjusted to $10^{\#}$ cfu/ml. Aliquots of 2 µl were placed at equidistant points on BSA (Bovine serum albumin) medium plates (2 % agar, 2 g bovine serum albumin, 20 g glucose, yeast base without amino acids). The plates were then incubated for 5-7 days at 37 °C. As a positive control, strain of *C. albicans* SC5314 was used. Diameter of colony and zone of degradation was measured. Proteinase activity was determined by calculating proteinase activity index (P_Z) as follows:

$$Proteinase\ activity\ index\ (P_Z) = \frac{diameter\ of\ colony}{diameter\ of\ colony + zone\ of\ degradation}$$

The P_Z values were interpreted (Kantarcioğlu and Yücel 2002) as follows:

- Very strong proteinase activity (++++) was indicated by P_Z=0.69
- Strong activity (+++) was indicated by P_Z = 0.70-0.79
- Medium activity (++) was indicated by P_Z= 0.80-0.89
- Weak activity (+) was indicated by P_Z= 0.90-0.99
- No activity (-) was indicated by P_Z = 1.00

2.7.3 Drug combinations on proteinase activity

Proteinase production in two selected *C. parapsilosis* (FLU resistant) and (FLU susceptible) strains was evaluated after exposure to efflux pump inhibitors pantoprazole and omeprazole at their MFC, MIC and ½ MIC concentration values for 2-3 hours. As described in previous section, aliquots of 2 µl were placed at equidistant points on BSA agar plates. The cells without any treatment were used as a control. The plates were then incubated for 4-5 days at 37 °C. Thereafter, P_Z value of isolates was determined and interpreted.

The experiment was done three times. Results of unexposed controls were compared to exposed test results using a student t-test. *P-value* of ≤ 0.05 was considered significant.

2.7.4 Screening for production of phospholipase

Using a previously described method (Shaban *et al*, 2020), phospholipase assays were performed. All *C. parapsilosis* isolates were initially screened for extracellular phospholipase activity. Colonies of *C. parapsilosis* were added to flasks containing 5 ml SDB and cultured for 18 hours at 37 °C. The cells were re-suspended in new fresh media after being centrifuged for 5 minutes at 3000 rpm. The fresh culture was adjusted to $10^{\#}$ cfu/ml. Aliquots of 2 µl were carefully placed at equidistant points on egg yolk agar plates (2 % agar, 10g peptone, 30g glucose, 57.3g NaCl, 0.55g $CaCl_2$ and 10% egg yolk emulsion to 900 ml of distilled water), This suspension was allowed to get dry and plates were incubated at 37 °C for 7

days.

After incubation, plates were examined for phospholipase activity; this was deemed positive when precipitation zones could be seen surrounding colonies. A *C. albicans* SC5314 positive control was also included, diameters of colony and opaque zone were measured an average of

three readings were calculated. By comparing diameters of the colonies to the diameters of colonies plus precipitation zone around colonies, phospholipase activity index (the $P_Z$value) was calculated

$$Pz = \frac{\text{Diameter of colony}}{\text{Diameter of colony} + \text{Precipitation zone}}$$

2.7.5 Efflux pump inhibitors (pantoprazole and omeprazole) on phospholipase activity

The effect of efflux pump inhibitors on phospholipase activity in *C. parapsilosis* isolates has not been done since all *C. parapsilosis* strains tested in this study did not show positive phospholipase activity.

2.8 Efflux activity

2.8.1 Pantoprazole and Omeprazole on efflux pump using Rhodamine 6G assay

ABC-type efflux activity was determined by measuring glucose-induced efflux of Rhodamine 6G (R6G), using a method described by Ahmad et al. (2013). Both strains of *C. parapsilosis*, one (fluconazole resistant CP2) and one (fluconazole susceptible CP8), and *C. albicans* SC5314 were sub-cultured on SDA for 24 hours at 37 °C. Cultures were grown in 50 ml SDB for further 8 hours at 37°C. The cells were collected by centrifugation (4400 rpm for 2 minutes), washed twice and re-suspended in PBS (pH 7.4). Furthermore, 5 mM deoxyglucose and 5mM dinitrophenol were added to de-energise cells for 45 minutes in PBS. After de-energizing, the cells were centrifuged, washed twice and re-suspended in PBS. The cells were treated with fluconazole, test compounds, and their combinations at the MIC values. After adding 10 M R6G to cells, suspensions were incubated at 37 °C for 60 minutes. After 0, 10, 20, 30, 40, 50,

and 60 minutes of incubation, samples aliquots were removed. Centrifugation was used to separate cells for a further two minutes at 9000 rpm. The supernatants that resulted were tested for absorbance at 527 nm. After 25 minutes of incubation, 0.1 M glucose was added to assess the energy-dependent R6G efflux, and data were collected up to 60 minutes, controls were prepared with glucose-free PBS.

For the purpose of calculating actual concentration of R6G effluxed, a standard concentration curve of R6G was created (figure 2.1). The experiments were carried out in triplicates.

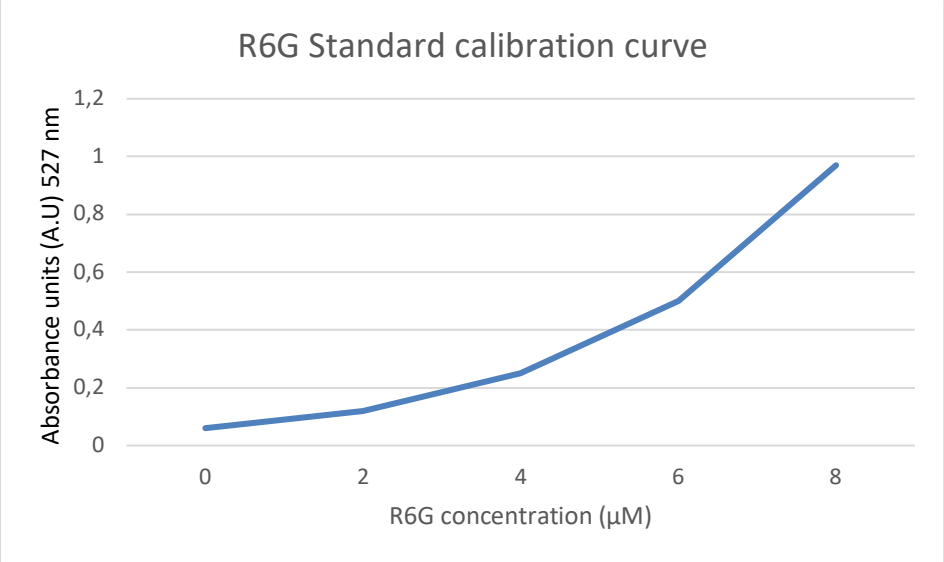


Figure 2.1: R6G Standard calibration curve (Standard solutions of R6G at concentrations of 2 µM, 4µM, 6µM, 8 µM and 10µM were prepared. The absorbance of each R6G solution was measured at 527 nm).

2.8.2 Hoechst 33342 accumulation

ABC-type efflux activity was also determined by measuring accumulation of fluorescent dye Hoechst 33342 bisbenzimide using fluorometric assay as described previously (Ahmad *et al*, 2013). In absence or presence of test compounds (pantoprazole and Omeprazole) and their combinations with fluconazole (at MIC values), these assays were performed. Two selected

strains of *C. parapsilosis*, one (fluconazole resistant CP2) and one (fluconazole susceptible CP8), and *C. albicans* SC5314 were sub-cultured on SDA for 24 hours at 37°C. These cultures were used to inoculate fresh medium of SDB that was incubated for a further 8 hours at 37°C. Cells were then collected by centrifugation at 3000g and resuspended in PBS. The optical densities of all suspensions were adjusted to 1.8 at 595 nm and aliquots were taken and analysed by Fluostar Optima plate reader with excitation/emission (nm): 350/461 while Hoechst 33342 (100μM) was added to each sample. Each experiment was repeated three times.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Since all experiments were performed in three or two replicates, results were reported as mean standard deviation. A student's t-test was used to compare controls and tests to determine the effect of drug combinations on *C. parapsilosis* across the experiments. Statistical significance was denoted by *p values* of ≤ 0.05.

CHAPTER 3: RESULTS

3.1 Antifungal activity of antifungal agents and efflux pump inhibitors against *C. parapsilosis*

Minimum inhibitory concentration and MFC values of fluconazole, amphotericin B were obtained against 12 strains of *C. parapsilosis* and one strain of *C. albicans.* The results are shown in (Table 3.1). For *C.*

parapsilosis, median MIC of fluconazole and amphotericin B, were 125 µg/ml (range 31.25-250 µg/ml) and 0.3 µg/ml (range 0.03-0.6 µg/ml) respectively. The MFC values of fluconazole against all *C. parapsilosis* strains and *C. albicans* were reported as FS (Fungistatic) since the drug did not show 99-99.5% killing activity. In contrast, amphotericin B displayed fungicidal activity against *C. parapsilosis* strains, with a median MFC value of 0.6 µg/ml (range 0.3- 1.25 µg/ml).

Minimum inhibitory concentration and MFC were performed on two selected *C. parapsilosis* strains (one fluconazole resistant and one fluconazole susceptible) as well as one *C. albicans* strain against two efflux pump inhibitors. Median MIC of Pantoprazole and Omeprazole were found to be 620 µg/ml

(range 620-3125 µg/ml) and 1250 µg/ml (range 1250-15625 µg/ml) respectively. Median MFC of Pantoprazole and Omeprazole were found to be 1250 µg/ml (range 1250-6250 µg/ml) and 2500 µg/ml (range 2500-31250 µg/ml) respectively. The results are shown in (Table 3.2).

Table 3.1: Antifungal activity of fluconazole and amphotericin B against *C. parapsilosis* and *C. albicans*

***Candida* strains**	**Fluconazole (µg/ml)**			**Amphotericin B (µg/ml)**			
	Repeats MIC	**Median MIC**	**MFC**	**Repeats MIC**	**Median MIC**	**Repeats MFC**	**Median MFC**
CP1	62.5	62.5	FS	0.19	0.19	0.39	0.78
	62.5			0.19		0.78	
	125			0.39		0.78	
CP2	125	125	FS	0.19	0.19	0.39	0.39
	125			0.19		0.39	
	250			0.39		0.78	
CP3	62.5	62.5	FS	0.15	0.15	0.3	0.6
	62.5			0.15		0.6	
	62.5			0.3		0.6	
CP4	62.5	62.5	FS	0.15	0.3	0.3	0.6
	62.5			0.3		0.6	
	125			0.3		0.6	
CP5	62.5	62.5	FS	0.07	0.15	0.15	0.3
	62.5			0.15		0.3	
	125			0.3		0.3	
CP6	125	125	FS	0.3	0.6	0.6	1.25
	125			0.6		1.25	
	250			0.6		1.25	
CP7	125	125	FS	0.3	0.3	1.25	1.25
	125			0.3		1.25	
	250			0.6		1.25	
CP8	31.25	31.25	FS	0.15	0.15	0.6	1.25
	31.25			0.15		1.25	
	62.5			0.3		1.25	
CP9	125	125	FS	0.03	0.07	0.3	0.3
	125			0.07		0.3	
	125			0.15		0.6	
CP10	125	125	FS	0.3	0.3	0.6	0.6
	125			0.3		0.6	
	250			0.3		1.25	

CP11	62.5	62.5	FS	0.15	0.3	0.6	0.6
	62.5			0.3		0.6	
	62.5			0.3		1.25	
CP12	125	125	FS	0.3	0.3	1.25	1.25
	125			0.3		1.25	
	250			0.3		1.25	
C. albicans	3.9	7.8	FS	0.3	0.15	0.62	0.62
	7.8			0.15		0.62	
	7.8			0.15		2.5	

FS: fungistatic (antifungal agent that inhibit the growth of fungus without killing it), CP: *C. parapsilosis* strains.

Table 3.2: Antifungal activity of efflux pump inhibitors (pantoprazole and omeprazole) against selected C*. parapsilosis* and *C. albicans* strains.

Candida strains	Pantoprazole (µg/ml)				Omeprazole (µg/ml)			
	Repeats MIC	**Median MIC**	**Repeats MFC**	**Median MFC**	**Repeats MIC**	**Median MIC**	**Repeats MFC**	**Median MFC**
CP2	620	620	1250	1250	1250	1250	2500	2500
	620		1250		1250		2500	
	620		1250		1250		2500	
CP8	620	620	1250	1250	1250	1250	2500	2500
	620		1250		1250		2500	
	620		1250		1250		2500	
CA	3125	3125	6250	6250	15625	15625	31250	31250
	3125		6250		15625		31250	
	3125		6250		15625		31250	

CP2: fluconazole resistant *C. parapsilosis* strain (MRU 4282), CP8: fluconazole susceptible *C. parapsilosis* strain (MRU 4108), CA: *C. albicans* (SC5314) strain used as positive control.

3.2 Effects of combination of the Fluconazole with Pantoprazole and Omeprazole on *C. parapsilosis*

Fluconazole, pantoprazole and omeprazole were combined in 1:1 volume ratios at their MIC values to study the combination effect on two selected *C. parapsilosis* strains. a *C. albicans* strain. When pantoprazole was combined with fluconazole, the median MIC for fluconazole and pantoprazole decreased from 125 to 31.25 µg/ml and from 620 to 310 µg/ml respectively (Table 3.3). The combination exhibited additive activity in fluconazole resistant *C. parapsilosis* strain with FICI between 0.5 and 1.0. indifferent activity in *C. albicans* strain and fluconazole susceptible *C. parapsilosis* strain with FICI between 1.0 and 4.0. When omeprazole was combined with fluconazole, median MIC for fluconazole and omeprazole decreased from 125 to 15.6 µg/ml and from 1250 to 310 µg/ml, respectively. The combination exhibited synergistic activity in fluconazole resistant *C. parapsilosis* strain, and indifferent activity in *C. albicans* strain and in fluconazole susceptible *C. parapsilosis* strain. (Table 3.4). Furthermore, MIC values of efflux pump inhibitors were significantly reduced in both resistant and susceptible *C. parapsilosis* strains when combined with fluconazole (Table 3.5). While the comparison was not done for MFC values, since fluconazole did not show 99-99.5% killing activity because it has a Fungistatic activity.

Table 3.3: Effect of fluconazole in combination with pantoprazole on *C. parapsilosis and C. albicans*

Candida strains	Median MIC values (n=3)				FICI	Interpretation
	Alone		In combination			
	FLU (µg/ml)	PTP (µg/ml)	FLU (µg/ml)	PTP (µg/ml)		
CP2	125	620	31.25	310	0.9	ADD
CP8	31.25	620	31.25	310	1.5	IND
CA	15.6	3125	15.6	195	1.1	IND

FLU: fluconazole, PTP: pantoprazole, FICI: Fractional Inhibitory Concentration Index, ADD: additive, IND: indifference, CP2: fluconazole resistant *C. parapsilosis* strain (MRU 4282), CP8: fluconazole susceptible *C. parapsilosis* strain (MRU 4108), CA: *C. albicans* (SC5314) strain used as positive control

Table 3.4: Effect of fluconazole in combination with omeprazole on *C. parapsilosis and C. albicans*

Candida strains	Median MIC values (n=3)				FICI	Interpretation
	Alone		In combination			
	FLU (µg/ml)	OMP (µg/ml)	FLC (µg/ml)	OMP (µg/ml)		
CP2	125	1250	15.6	310	0.36	SYN
CP8	31.25	1250	31.25	620	1.49	IND
CA	15.6	3125	15.6	195	1.1	IND

FLU: fluconazole, OMP: omeprazole, FICI: Fractional Inhibitory Concentration Index, SYN: Synergism, IND: indifference, CP2: fluconazole resistant *C. parapsilosis* strain (MRU 4282), CP8: fluconazole susceptible *C. parapsilosis* strain (MRU 4108), CA: *C. albicans* (SC5314) strain used as positive control.

Table 3.5: Comparison of MIC values of efflux pump inhibitors alone and in combination with fluconazole in fluconazole resistant and fluconazole susceptible *C. parapsilosis* strains

C. parapsilosis	MIC Pantoprazole (µg/ml)		MIC Omeprazole (µg/ml)	
	Alone	**In combination With fluconazole**	**Alone**	**In combination With fluconazole**
Flu-resistant	620	310	1250	310
Flu-susceptible	620	310	1250	620

MIC values of efflux pump inhibitors were significantly reduced when combined with fluconazole in both resistant and susceptible *C. parapsilosis* strains. Which is mean the efflux pump inhibitors will be more effective against *C. parapsilosis* cells when it is in combination with fluconazole.

3.3 Effects of combination of fluconazole and efflux pump inhibitors on time-kill kinetics

Fluconazole resistant and susceptible strains of *C. parapsilosis* were selected to study the effects of combination of fluconazole and efflux pump inhibitors (pantoprazole and omeprazole) on time-kill kinetics. Figures 3.1 and 3.2 depict time-kill curves for fluconazole, pantoprazole, and omeprazole alone at their MIC values and in combination at their ½ MIC values against fluconazole-susceptible and fluconazole-resistant *C. parapsilosis.*

When susceptible *C. parapsilosis* cells were left untreated (control), proliferation of *Candida* cells slightly increased from 3.2 to 3.6 (CFU/ml) after 24 hours, however when cells were exposed to fluconazole at MIC, the (CFU/ml) decreased from 3.15 to 1.97 during same period. Similar results were observed for pantoprazole at MIC value when comparing to fluconazole effects. On the other hand, proliferation of *Candida* cells was reduced from 3 to 1.5 (CFU/ml) when treated with MIC concentration of omeprazole.

The growth of resistant *C. parapsilosis* cells untreated (control) was same as when treated with fluconazole at MIC value; with an increase in *Candida* cells from 3 to 6 log (CFU/ml) during study time period. While exposure to pantoprazole and omeprazole at their respective MIC levels had no effect on *Candida* cell proliferation. On the other hand, fungistatic activity of Fluconazole was changed to fungicidal by addition of efflux pump inhibitors (pantoprazole and omeprazole) at their ½MIC values. After 24h of incubation, pantoprazole-fluconazole combination as well as omeprazole- fluconazole combination yielded a decrease of 2- log (CFU/ml) in comparison of fluconazole alone.

Therefore, combination of fluconazole with efflux pump inhibitors at their half MIC values dramatically reduced the growth of *Candida* cells compared to the fluconazole and efflux pump alone at their MIC values.

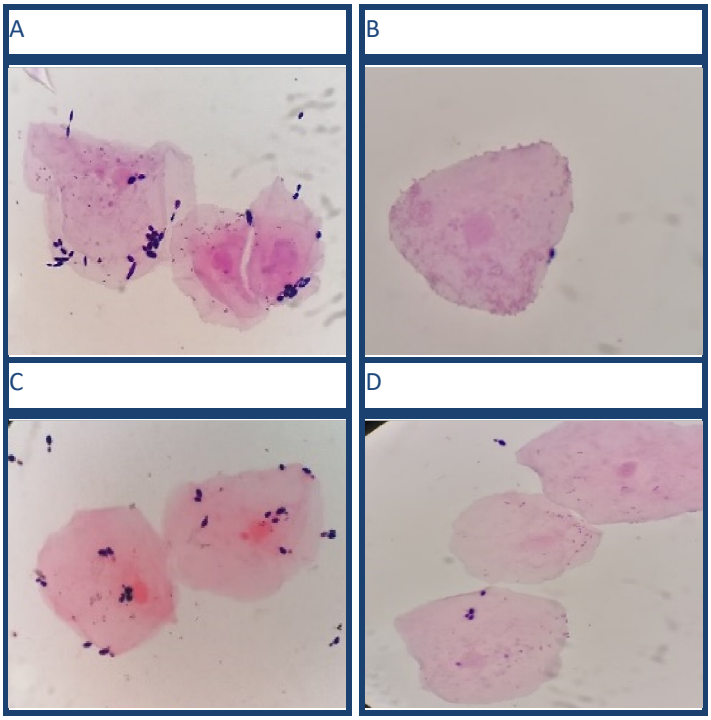


Figure 3.3: Micrograph of adherence ability of *C. parapsilosis* cells to epithelial cells when exposed and unexposed to the efflux pump inhibitors. A: Control *C. parapsilosis* strain (fluconazole resistant) not exposed to efflux pump inhibitors. B: *C. parapsilosis* (fluconazole resistant) exposed to omeprazole at MIC concentration. C: Control *C. parapsilosis* strain (fluconazole susceptible) not exposed to efflux pump inhibitors. D: *C. parapsilosis* (fluconazole susceptible) exposed to pantoprazole at MIC value.

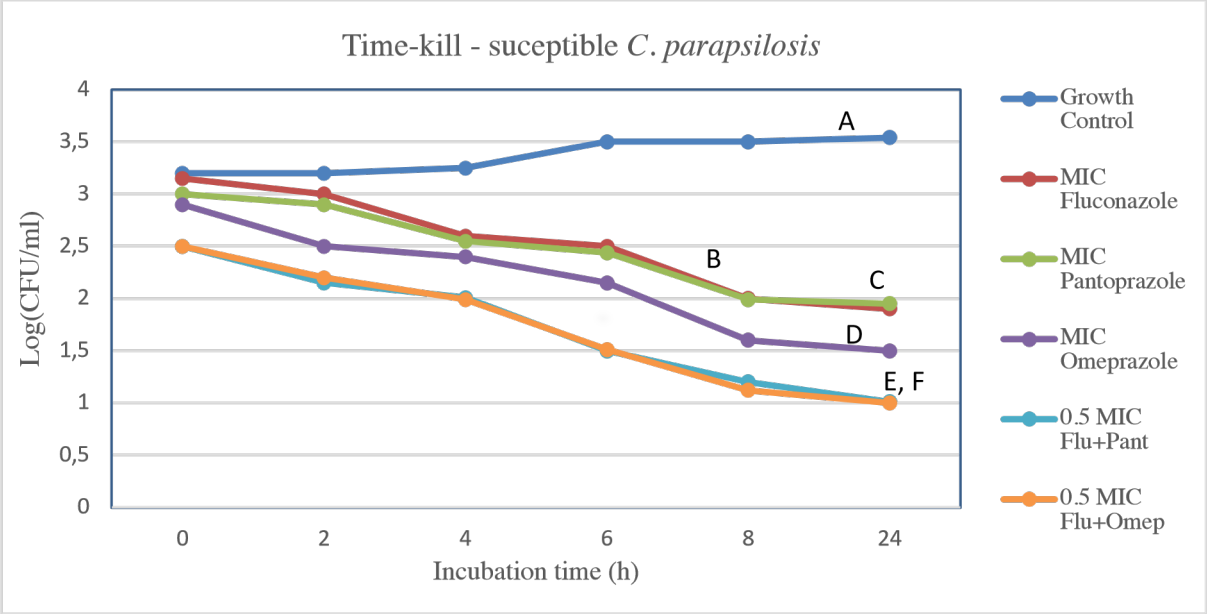


Figure 3.1: Representative time-kill curves of fluconazole susceptible *C. parapsilosis* strain. (A): untreated cells (control), (B): exposure to MIC of fluconazole, (C): exposure to MIC of pantoprazole, (D): exposure to MIC of omeprazole, (E): exposure to½ MIC of fluconazole combined with ½ MIC of pantoprazole, (F): exposure to½ MIC of fluconazole combined with ½ MIC of omeprazole.

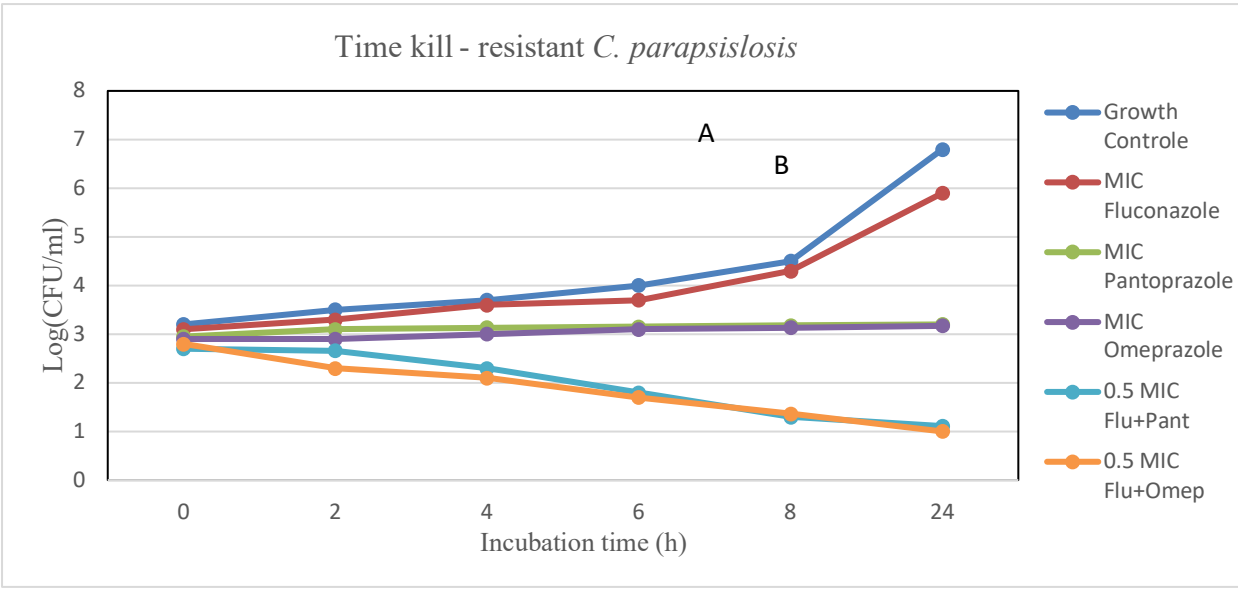


Figure 3.2: Representative time-kill curves of fluconazole resistant *C. parapsilosis* strain. (A): untreated cells (control), (B): exposure to MIC of fluconazole, (C): exposure to MIC of pantoprazole, (D): exposure to MIC of omeprazole, (E): exposure to½ MIC of fluconazole combined with ½ MIC of pantoprazole, (F): exposure to½ MIC of fluconazole combined with ½ MIC of omeprazole.

3.4 Anti-virulence assay

3.4.1 Effect of efflux pump inhibitors on *C. parapsilosis* adherence

Two strains of *C. parapsilosis* (fluconazole resistant and fluconazole susceptible) were selected for adherence study, as well as *C. albicans* strain was used as a control. For this experiment, adherence activity was measured by quantity of *Candida* cells attached to each of 100 buccal epithelial cells. The experiment were performed in presence of efflux pump inhibitors at three concentration (half MIC, MIC and MFC) which were 310, 620 and 1250 (µg/ml) for pantoprazole and 625, 1250 and 2500 (µg/ml) for omeprazole respectively. Control experiment were performed without test drugs (Table 3.6, 3.7 and 3.8 and Figures 3.3, 3.4, 3.5 and 3.6). Pantoprazole and omeprazole reduced adherence of *Candida* cells to epithelial cells. The reduction in adherence was concentration dependent and it was significant (p <0.001) at all the test concentration.

On the other hand, % reduction in the adherence at ½ MIC and MIC values were significant when the two resistant and susceptible *C. parapsilosis* strains were compared. This suggest that the resistant strain of *C. parapsilosis* requires higher concentration of these compounds to reduce adherence. Therefore at MFC values, no significant difference between two strains was noted (Table 3.9).

3.4.2 Determination of phospholipase and proteinase activity of *C. parapsilosis*

All *C. parapsilosis* isolates were tested initially for proteinase and phospholipase secretions using a plate assay containing bovine serum albumin (BSA) and egg yolk respectively.

C. albicans SC5314 was used as a positive control.

Table 3.6: Effect of efflux pump inhibitors on the adherence of *C. parapsilosis* (fluconazole resistant strain) to epithelial cells

C. parapsilosis		**Pantoprazole** Attached yeast cells /100 epi cells			**Omeprazole** Attached yeast cells /100 epi cells		
	Control	0.5 MIC	MIC	MFC	0.5 MIC	MIC	MFC
	240	60	16	0	220	43	0
	244	63	16	1	225	40	0
CP2	241	61	14	0	222	41	0
Mean	241	61	15.3	0.33	222	41	0
SD	2.1	1.5	1.2	0.6	2.5	1.5	0
P value		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

CP2: fluconazole resistant *C. parapsilosis* strain (MRU 4282), SD: stander deviation, epi: epithelial cells, CONT: control: reading without treatment. *P value*: significance compared to the control

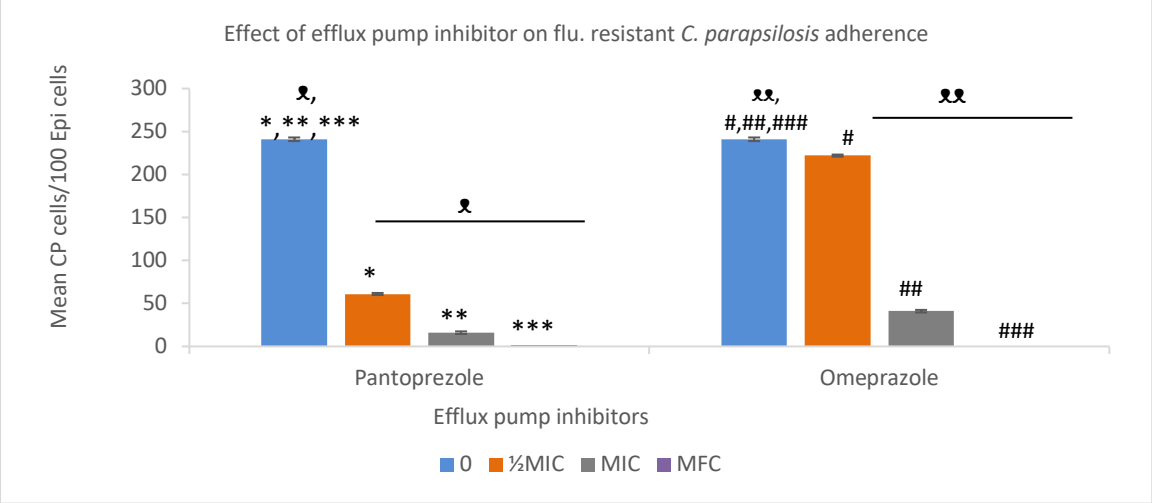


Figure 3.4: The effect of various concentration of efflux pump inhibitors (pantoprazole and omeprazole) on adherence in fluconazole resistant *C. parapsilosis.* Bars represent the means (with standard deviations) number of yeast cells adherent to 100 buccal epithelial cells, which represents the adherence ability of *C. parapsilosis*. : comparison of control to various concentration of pantoprazole, *: comparison of control to half MIC of pantoprazole. **: comparison of control to MIC of pantoprazole, ***: comparison of control to MFC of pantoprazole $P = < 0.001$ (strong significance); : comparison of control to various concentration of omeprazole. #: comparison of control to half MIC of omeprazole, ##: comparison of control to MIC of omeprazole, ###: comparison of control to MFC of omeprazole $P = < 0.001$ (strong significance).

Table 3.7: Effect of efflux pump inhibitors on the adherence of *C. parapsilosis* (flu susceptible strain) to epithelial cells

C. parapsilosis		**Pantoprazole** Attached yeast cells /100 epi cells			**Omeprazole** Attached yeast cells /100 epi cells		
	Control	0.5 MIC	MIC	MFC	0.5 MIC	MIC	MFC
CP8	160	20	1	0	50	32	0
	155	22	3	0	51	30	0
	156	25	0	2	53	35	0
Mean	157	22	1.3	0.67	51.3	32.3	0
SD	2.6	2.5	1.5	1.2	1.5	2.5	0
P value		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

CP8: fluconazole susceptible *C. parapsilosis* strain (MRU 4108), SD: stander deviation, epi: epithelial cells, control: reading without treatment. *P value*: significance compared to the control.

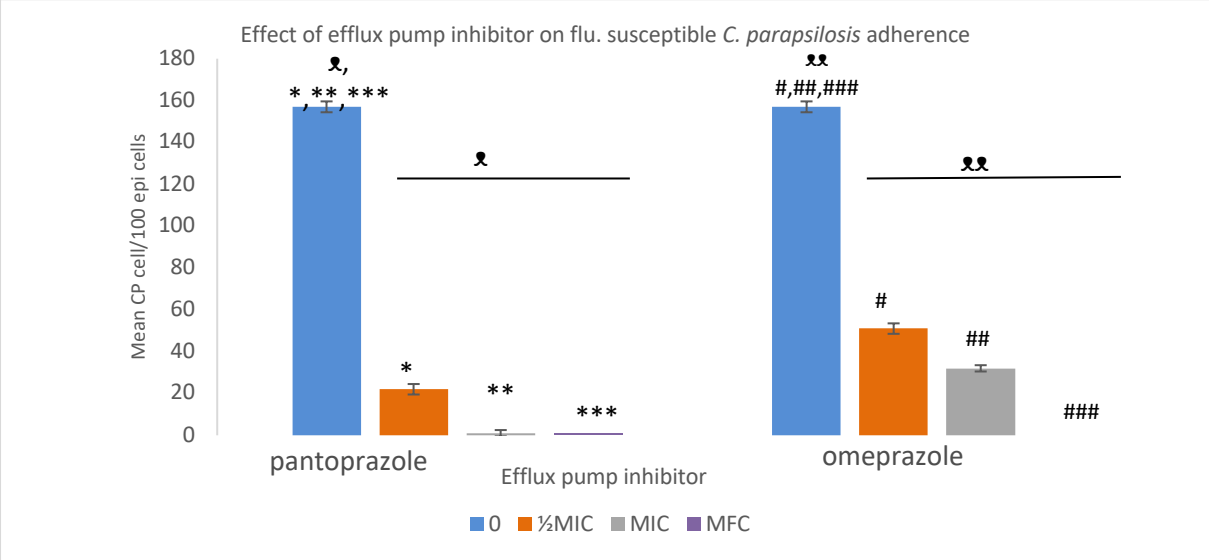


Figure 3.5: The effect of various concentration of efflux pump inhibitors (pantoprazole and omeprazole) on adherence in fluconazole susceptible *C. parapsilosis.* Bars represent the means (with standard deviations) number of yeast cells adherent to 100 buccal epithelial cells, which represents the adherence ability of *C. parapsilosis*. ♟: comparison of control to various concentration of pantoprazole, *: comparison of control to half MIC of pantoprazole. **: comparison of control to MIC of pantoprazole, ***: comparison of control to MFC of pantoprazole $P = < 0.001$ (strong significance); ♟♟: comparison of control to various concentration of omeprazole. #: comparison of control to half MIC of omeprazole, ##: comparison of control to MIC of omeprazole, ###: comparison of control to MFC of omeprazole $P = < 0.001$ (strong significance).

Table 3.8: Effect of efflux pump inhibitors on the adherence of *C. albicans* to epithelial cells

C. albicans		**Pantoprazole** Attached yeast cells /100 epi cells			**Omeprazole** Attached yeast cells /100 epi cells		
	Control	0.5 MIC	MIC	MFC	0.5 MIC	MIC	MFC
	324	75	18	2	132	33	0
	316	65	17	1	100	30	0
C. albicans	292	72	15	0	95	38	0
Mean	311	71	17	1	109	34	0
SD	16	5	1.5	1	20	4	0
P value		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

C. albicans strain (SC5314), SD: stander deviation, epi: epithelial cells, control: reading without treatment. *P value*: significant compared to the control.

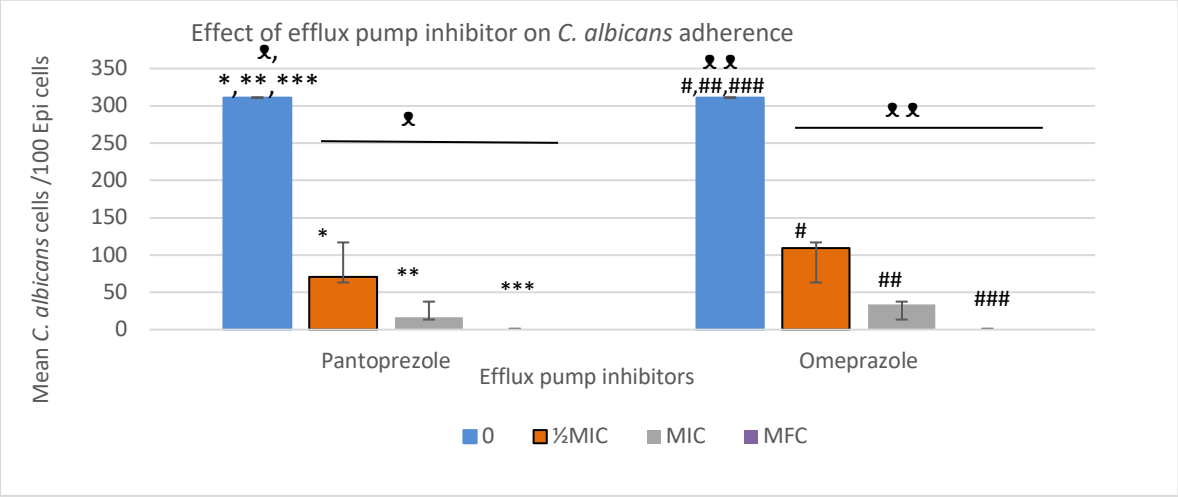


Figure 3.6: The effect of various concentration of efflux pump inhibitors (pantoprazole and omeprazole) on adherence in *C. albicans*. Bars represent the means (with standard deviations) number of yeast cells adherent to 100 buccal epithelial cells, which represents the adherence ability of *C. albicans*. ♟: comparison of control to various concentration of pantoprazole, *: comparison of control to half MIC of pantoprazole. **: comparison of control to MIC of pantoprazole, ***: comparison of control to MFC of pantoprazole $P = < 0.001$ (strong significance); ♟♟: comparison of control to various concentration of omeprazole. #: comparison of control to half MIC of omeprazole, ##: comparison of control to MIC of omeprazole, ###: comparison of control to MFC of omeprazole $P = < 0.001$ (strong significance).

Table 3.9: Comparison of % reduction of the adherence ability in both *C. parapsilosis* fluconazole (resistant and sensitive) strains when exposed to the efflux pump inhibitors

	% Reduction by Pantoprezole		% Reduction by Omeprezole	
	CP2 (resistant)	**CP8 (susceptible)**	**CP2 (resistant)**	**CP8 (susceptible)**
Conc.				
½MIC	75	87.5	8.33	68.75
	74.18	85.81	7.79	67.10
	74.69	83.97	7.88	66.03
mean	**74.62304152**	**85.7602702**	**8.001345336**	**67.29080507**
SD	**0.41**	**1.76**	**0.29**	**1.37**
p value	**0.0002 ****		**0.0000 ****	
MIC	93.33	99.38	82.08	80
	93.44	98.06	83.61	80.65
	94.19	100	82.99	76.28
mean	**93.65560922**	**99.14650538**	**82.89248086**	**78.97573752**
SD	**0.47**	**0.99**	**0.77**	**2.35**
p value	**0.0005 ****		**0.0260 ***	
MFC	100	100	100	100
	99.59	100	100	100
	100	98.72	100	100
mean	**99.86338798**	**99.57264957**	**100**	**100**
SD	**0.24**	**0.74**	**0.00**	**0.00**
p value	**0.2761**		**>0.05**	

CP2: fluconazole resistant *C. parapsilosis* strain, CP8: fluconazole susceptible *C. parapsilosis* strain, Conc.: concentration of the efflux pump inhibitors at ½MIC, MIC and MFC which were 310, 620 and 1250 (µg/ml) for pantoprazole and 625, 1250 and 2500 (µg/ml) for omeprazole; *P value* = (< 0.01) the % reduction in the adherence was significantly different for the fluconazole resistant and sensitive strains, * $P = < 0.05$ (significant), ** $P = < 0.01$ (strong significance).

3.4.2.1 Phospholipase activity in *C. parapsilosis* and C. albicans SC5314

All *C. parapsilosis* isolates tested showed negative for phospholipase activity (P_z = 1), where no visible white degradation zone was seen around fungal colony. In contrast, the control strain *C. albicans* SC5314 showed very strong phospholipase activity P_z = 0.66. The results are shown in Table 3.10 and Figure 3.7.

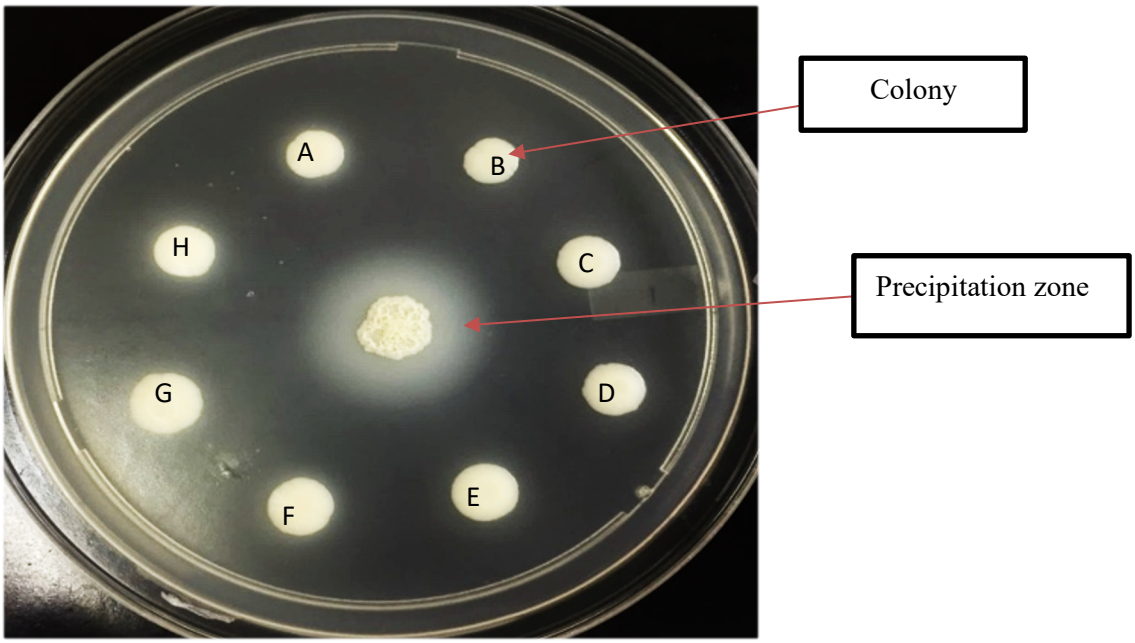


Figure 3.7: Phospholipase activity in *C. parapsilosis* and *C. albicans* SC5314 on egg yolk agar plates. Precipitation zones around the colonies indicate positive phospholipase activity. A, B, C, D, F, G, and H: are *C. parapsilosis* stains

Table 3.10: Phospholipase activity of *C. parapsilosis* isolates

***Candida* sp**	**Strains**	**Phospholipase production (*Pz* value)**				**Phospholipase activity level**
		1	**2**	**3**	**Mean ± SD**	
C. parapsilosis	CP1	1.00	1.00	1.00	1.00 ± 0.00	-
	CP2	1.00	1.00	1.00	1.00 ± 0.00	-
	CP3	1.00	1.00	1.00	1.00 ± 0.00	-
	CP4	1.00	1.00	1.00	1.00 ± 0.00	-
	CP5	1.00	1.00	1.00	1.00 ± 0.00	-
	CP6	1.00	1.00	1.00	1.00 ± 0.00	-
	CP7	1.00	1.00	1.00	1.00 ± 0.00	-
	CP8	1.00	1.00	1.00	1.00 ± 0.00	-
	CP9	1.00	1.00	1.00	1.00 ± 0.00	-
	CP10	1.00	1.00	1.00	1.00 ± 0.00	-
	CP11	1.00	1.00	1.00	1.00 ± 0.00	-
	CP12	1.00	1.00	1.00	1.00 ± 0.00	-
C. albicans	SC5314	0.70	0.60	0.67	0.66 ± 0.04	++++

Activity level: (−) P_z = 1 (no phospholipase activity). (++++) P_z = = <0.69 (very strong phospholipase activity).

3.4.2.2 Proteinase production in *C. parapsilosis* and *C. albicans*

Plates were examined for proteinase activity. The isolates showing a halo around the colonies were considered to be positive for proteinase production. The quantity of extracellular proteinase activity (P_z value) was represented by halo around the fungal colony on the BSA plates (Figure 3.8). The results are shown in (Table 3.11).

The results showed that all *C. parapsilosis* strains produced some degree of proteinases with P_z value ranging from (0.36 to 0.86) was comparable to reference strain *C. albicans* SC5314 (P_z = 0.60) proteinase production. Two selected *C. parapsilosis* strains depending on their susceptibility to fluconazole (sensitive and resistant) were used to study the effects of efflux pump inhibitors on proteinase production.

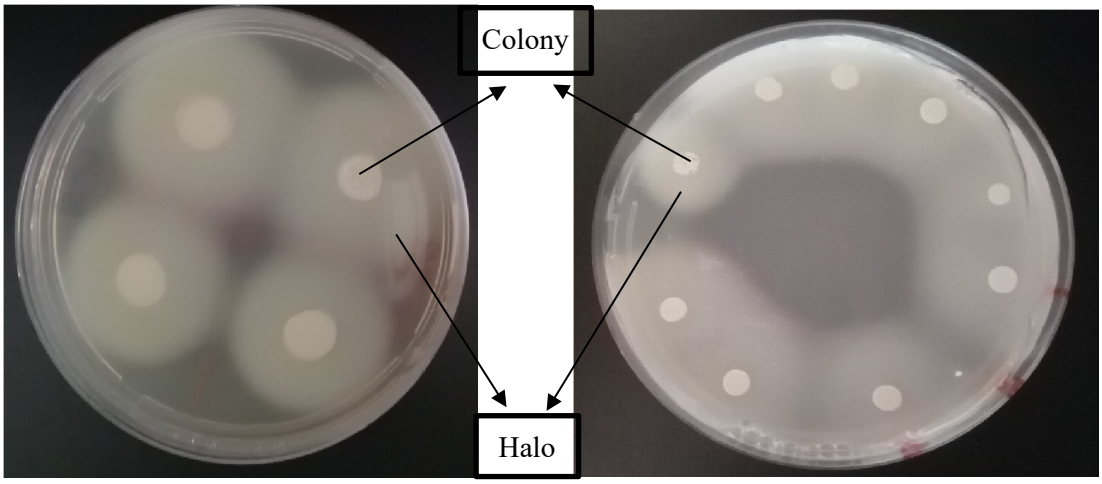


Figure 3.8: Proteinase activity of *C. parapsilosis* and *C. albicans* SC5314 on BSA plates. Zone of halo around the colonies indicate positive proteinase activity.

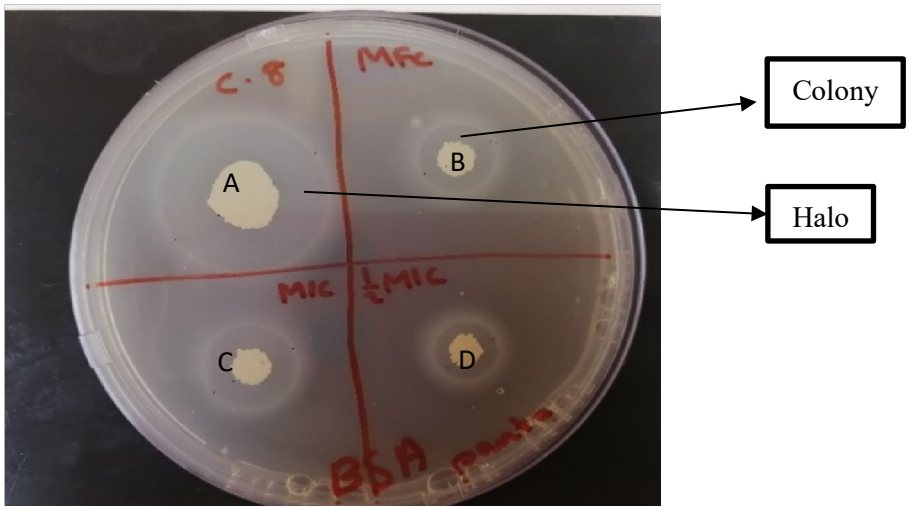


Figure 3.9: Effect of different concentrations of efflux pump inhibitors on the proteinase production on *C. parapsilosis* strain on BSA plates. A: untreated (control) + treated B, C and D.

Table 3.11: Proteinase activity of *C. parapsilosis* isolates

Candida sp	Repeats / Strains	proteinase production (*Pz* value)				proteinase activity level
		1	2	3	Mean ± SD	
C. parapsilosis	CP1	0.39	0.39	0.38	0.38 ±0.004	++++
	CP2	0.38	0.39	0.37	0.38 ± 0.01	++++
	CP3	0.4	0.39	0.41	0.4 ± 0.01	++++
	CP4	0.72	0.73	0.77	0.74 ± 0.02	+++
	CP5	0.8	0.78	0.79	0.79 ± 0.01	+++
	CP6	0.78	0.88	0.79	0.81 ± 0.05	++
	CP7	0.4	0.42	0.41	0.41 ± 0.01	++++
	CP8	0.36	0.37	0.37	0.36± 0.004	++++
	CP9	0.39	0.35	0.39	0.37 ± 0.02	++++
	CP10	0.33	0.36	0.35	0.35 ±0.02	++++
	CP11	0.45	0.43	0.42	0.43 ± 0.02	++++
	CP12	0.78	0.76	0.65	0.73 ± 0.07	+++
C. albicans	SC5314	0.37	0.38	0.38	0.37±0.004	++++

Activity level: ++, Pz = 0.80 to 0.89 (medium proteinase activity); +++, Pz = 0.70 to 0.79 (strong proteinase activity); ++++, Pz = <0.69 (very strong proteinase activity).

3.4.2.3 Effect of efflux pump inhibitors (pantoprazole and omeprazole) on the proteinase production on selected *C. parapsilosis* strains

Two *C. parapsilosis* strains as well as *C. albicans* were exposed to various concentration (½ MIC, MIC and MFC) of pantoprazole and omeprazole to determine the anti-proteinase activity. The results are depicted in (Table 3.12) and (Figures 3.9, 3.10 and 3.11). Pantoprazole exposed *C. parapsilosis(CP2)* proteinase activity was 0.4, 0.71 and 0.87 (*Pz*), while *C. parapsilosis(CP8)* proteinase activity was 0.48, 0.7 and 0.81 (*Pz*). On the other hand, omeprazole exposed *C. parapsilosis(CP2)* proteinase activity was 0.43, 0.71 and 0.76 (*Pz*), while *C. parapsilosis(CP8)* proteinase activity was 0.46, 0.7 and 0.86 (*Pz*) respectively.

The control showed *Pz* of 0.38. For *C. albicans* SC5314, proteinase activity was reduced only at pantoprazole concentration of 1250 µg/ml with mean Pz value (0.39) and reduced at all concentration of omeprazole with mean Pz value (0.4) when compared to untreated cells (Pz = 0.37). On the other hand, percentage reduction in the proteinase production at half MIC, MIC, and MFC values of pantoprazole and omeprazole were non-significant when the two resistant and susceptible *C. parapsilosis* strains were compared (Table 3.13). This indicate that proteinase synthesis of *C. parapsilosis* strains can be reduced or even stopped at any concentration of these compounds.

Table 3.12 the effect of efflux pump inhibitors on the proteinase production of *C. parapsilosis* and *C. albicans*

	Control (no treatment)	**Proteinase production (*Pz*) At various concentrations of pantoprazole (µg/ml)**			**Proteinase production (*Pz*) At various concentrations of omeprazole (µg/ml)**		
Strains		**0.5 MIC -310**	**MIC -620**	**MFC -1250**	**0.5 MIC 625**	**MIC 1250**	**MFC 2500**
CP2	0.38	0.39	0.75	0.92	0.5	0.76	0.72
	0.37	0.4	0.71	0.83	0.4	0.71	0.69
	0.39	0.42	0.66	0.86	0.4	0.68	0.88
MEAN	0.38	0.4	0.71	0.87	0.43	0.71	0.76
SD	0.01	0.01	0.04	0.04	0.05	0.03	0.08
pvalue		0.045*			0.095		
			0.0001***			7.5375E-05 ***	
				2.7425E-05 ***			0.001 **
CP8	0.36	0.5	0.75	0.83	0.43	0.75	0.92
	0.37	0.5	0.66	0.73	0.46	0.66	0.85
	0.37	0.46	0.71	0.89	0.5	0.71	0.83
MEAN	0.36	0.48	0.7	0.81	0.46	0.7	0.86
SD	0.004	0.01	0.03	0.06	0.02	0.03	0.03
pvalue		0.0004***			0.004 **		
			0.0001***			0.0001 ***	
				0.0003 ***			2.6858E-05 ***
C. albicans	0.37	0.35	0.34	0.39	0.39	0.5	0.4
	0.38	0.36	0.35	0.39	0.4	0.4	0.41
	0.37	0.36	0.34	0.4	0.41	0.41	0.39
MEAN	0.37	0.35	0.34	0.4	0.4	0.43	0.4
SD	0.004	0.004	0.004	0.004	0.01	0.04	0.01
pvalue		0.012*			0.008**		
			0.0015**			0.05*	
				0.006**			0.008 **

CP2: fluconazole resistant *C. parapsilosis* strain, CP8: fluconazole susceptible *C. parapsilosis* strain,* P = < 0.05 (significant), ** P = < 0.01 (strong significant), *** P = < 0.001 (very strong significant)

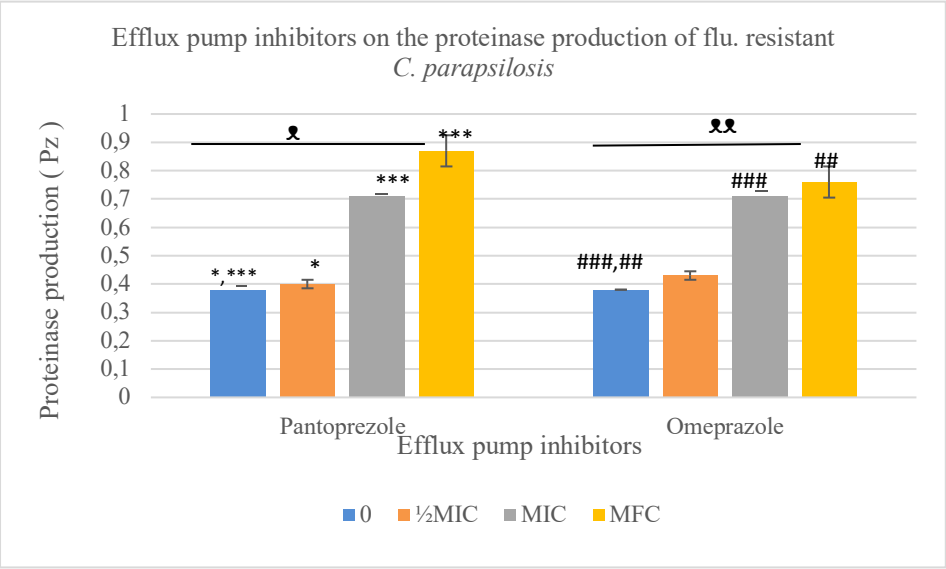


Figure 3.10: The effect of various concentrations of efflux pump inhibitors (pantoprazole and omeprazole) on the proteinase production of fluconazole resistant *C. parapsilosis*. Bars represent the means (with standard deviations) of Pz values, which represents proteinase activity of *C. parapsilosis*. ♟ P = control to all concentration was significant, * P = < 0.05, *** P = <0.001 for pantoprazole. ♟♟ P = control to all concentration was significant, ##, ### P = <0.001 for omeprazole.

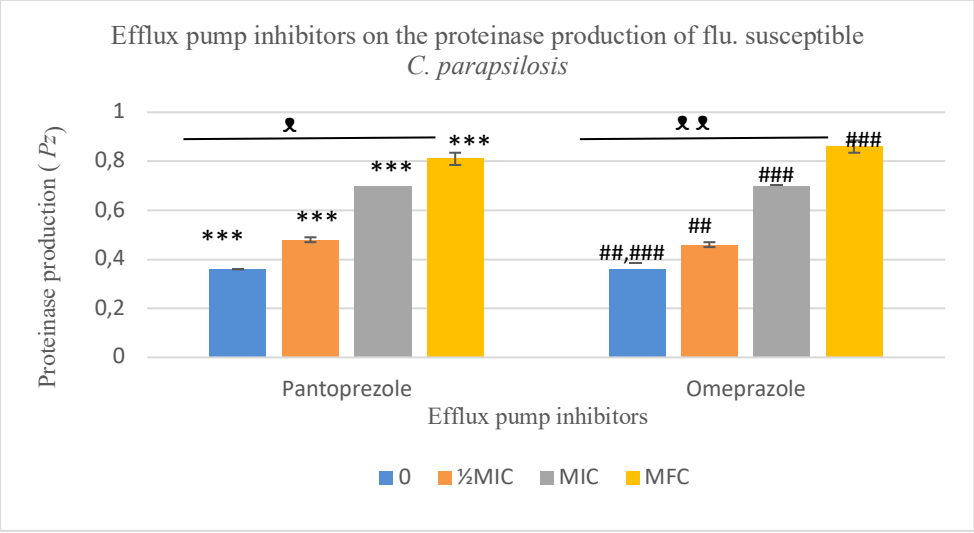


Figure 3.11: The effect of various concentrations of efflux pump inhibitors (pantoprazole and omeprazole) on the proteinase production of fluconazole susceptible *C. parapsilosis*. Bars represent the means (with standard deviations) of Pz values, which represents proteinase activity of *C. parapsilosis*. ♟ P = control to all concentration was significant, *** P = < 0.001, for pantoprazole. ♟♟ P = control to all concentration was significant, ##, ### P = <0.001 for omeprazole.

Table 3.13: Comparison of % reduction of Proteinase production in both *C. parapsilosis* fluconazole (resistant and sensitive) strains when exposed to the efflux pump inhibitors

	Proteinase production in *C. parapsilosis*			
Concentration	**% reduction by Pantoprazole**		**% reduction by Omeprazole**	
	CP2 (resistant)*	**CP8 (Susceptible)**	**CP2 (resistant)**	**CP8 (Susceptible)**
½MIC	-2.63158	-38.889	-31.578947	-19.4444
	-8.10811	-35.135	-8.1081081	-24.3243
	-7.69231	-24.324	-2.5641026	-35.1351
mean	**-6.144**	**-32.783**	**-14.083719**	**-26.3013**
SD	**3.048941**	**7.5619**	**15.4028**	**8.029992**
***P* value**	**0.0024**		**0.145**	
MIC	-97.3684	-108.33	-100	-108.333
	-91.8919	-78.378	-91.891892	-78.3784
	-69.2308	-91.892	-74.358974	-91.8919
mean	**-86.1637**	**-92.868**	**-88.750289**	**-92.8679**
SD	**14.91781**	**15.001**	**13.106022**	**15.00131**
***p* value**	**0.30**		**0.36**	
MFC	-142.105	-130.56	-89.473684	-155.556
	-124.324	-97.297	-86.486486	-129.73
	-120.513	-140.54	-125.64103	-124.324
mean	**-128.981**	**-122.8**	**-100.53373**	**-136.537**
SD	**11.52478**	**22.641**	**21.794792**	**16.69122**
***p* value**	**0.34**		**0.042**	

CP2: fluconazole resistant *C. parapsilosis* strain, CP8: fluconazole susceptible *C. parapsilosis* strain, Conc.: concentration of the efflux pump inhibitors at ½MIC, MIC and MFC which were 310, 620 and 1250 (µg/ml) for pantoprazole and 625, 1250 and 2500 (µg/ml) for omeprazole; $P = < 0.05$ (significant)

3.5 Efflux inhibitory activity of pantoprazole and omeprazole and their combination with fluconazole on *C. parapsilosis*

3.5.1 Effects of efflux pump inhibitors on Rhodamine 6G efflux

In this experiment, R6G efflux was evaluated in fluconazole resistant *C. parapsilosis* strain, in absence and presence of efflux pump inhibitors (pantoprazole and omeprazole) alone and in combination with fluconazole. When *Candida* cells were cultured in a glucose-free PBS buffer, absorption of R6G into cells increased quickly. where by, the measured extracellular concentrations of R6G showing a steep drop. Untreated *Candida* cells exhibited an energy and time-dependent outflow of R6G dye upon addition of glucose. This was evident from constant increase in extracellular R6G dye concentration as shown by the data in Figure 3.12, 3.13. In contrast, pantoprazole and omeprazole (at their MIC) and their combination with fluconazole inhibited these energy-dependent efflux pumps. When comparing control strain with treated strains after 60 minutes of incubation the effect of pantoprazole and omeprazole and their combinations significantly reduced the extracellular refluxed R6G. These findings suggested that pantoprazole and omeprazole acts as efflux pump inhibitors. The complete data is listed in (Appendix C, Page 131).

3.5.2 Effects of efflux pump inhibitors (pantoprazole and omeprazole) on Hoechst 33342 intracellular accumulation

In this experiment, the Hoechst 33342 accumulation was evaluated in fluconazole resistant *C. parapsilosis*. In the absence and presence of efflux pump inhibitors (pantoprazole and omeprazole) alone and in combination with fluconazole at their MIC values.

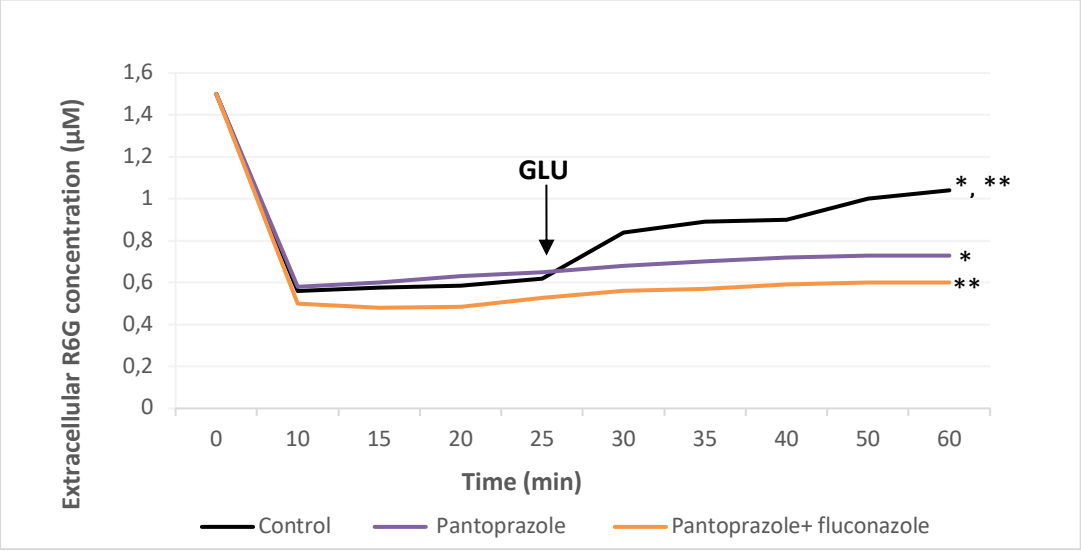


Figure 3.12: The effect of Pantoprazole and its combination with fluconazole on efflux pump studied using extracellular accumulation of Rhodamine 6G assay. The results depicts extracellular dye. * & ** represents comparison of test results (60 min) to the control. * & **: p <0.01

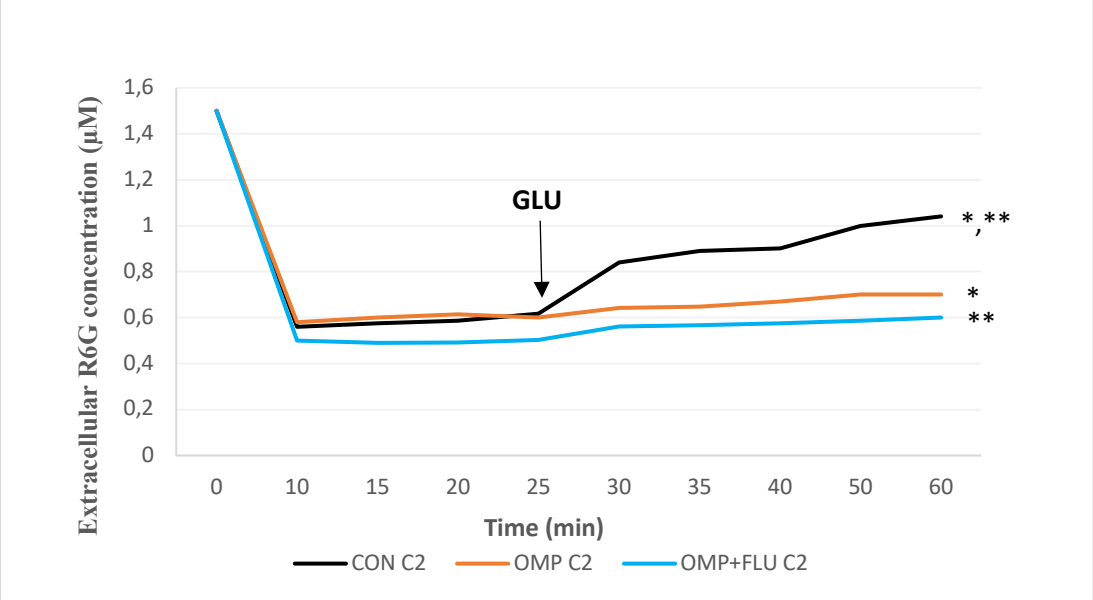


Figure 3.13: The effect of Omeprazole and its combination with fluconazole on efflux pump studied using extracellular accumulation of Rhodamine 6G assay. The results depicts extracellular dye. * & ** represents comparison of test results (60 min) to the control. * & **: p <0.01

The resistant *C. parapsilosis* strain which was treated with pantoprazole and omeprazole and their combination, showed significant increase of intracellular Hoechst 33342 dye accumulation compared with *C. parapsilosis* stain which is not exposed to the test drugs. Hence this indicates inhibition of efflux pump activity. Results are showed in Figure 3.14. Efflux pump inhibition was better with combination of fluconazole and pantoprazole than combination with omeprazole.

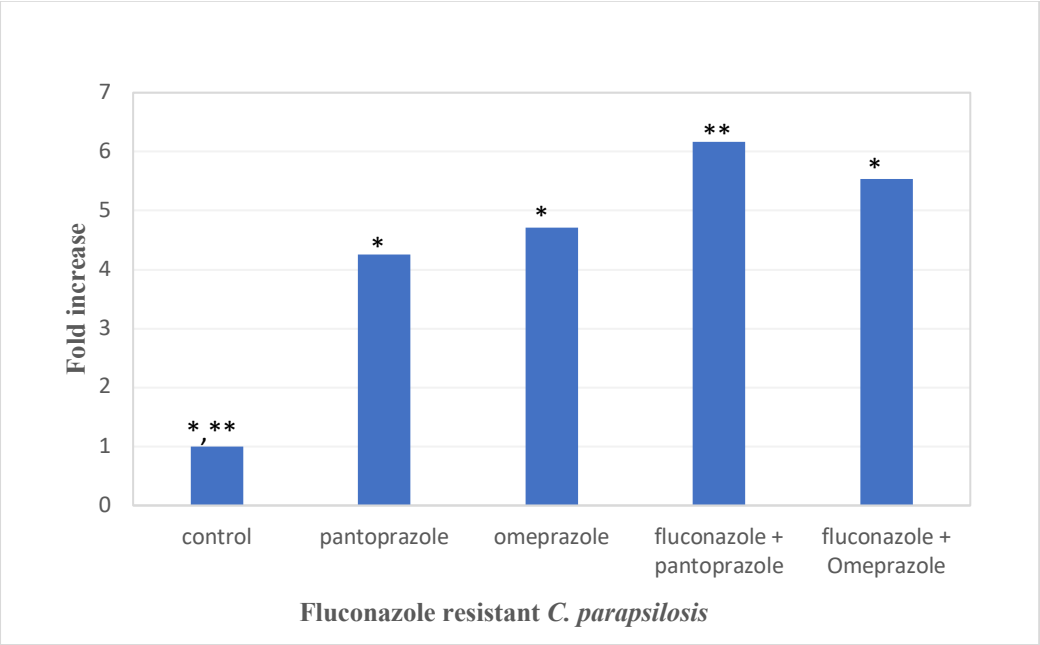


Figure 3.14: The effect of proton pump inhibitors and their combination with fluconazole on efflux pump studied using intracellular accumulation of Hoechst 33342 assay. The results were converted to fold increase using the control results. * & ** represents comparison of test results to the control. *: $p < 0.01$, **: $p = 0.05$

CHAPTER 4: DISCUSSION

Over a billion individuals worldwide are afflicted by fungal diseases, resulting in high morbidity and mortality coupled with expensive medical costs (Bongomin *et al*, 2017). Despite the widespread availability of antifungal drugs, candidiasis death rates are believed to be as high as 45 percent (Cheng *et al*, 2005). As a result of broad and prolonged use, drug resistance in *Candida* has increased, particularly to azoles (Marak and Dhanashree, 2018). The advent of *C. parapsilosis* as the most prevalent non *albicans Candida* species poses a significant threat to the future, and incidence of *C. parapsilosis* infections may continue to increase. The rising use of immunomodulatory drugs for a variety of purposes (e.g., autoimmune illnesses, cancer, infectious diseases, etc.) will raise the risk of infection with *C. parapsilosis*. This pathogen has a strong preference for parenteral nutrition, is often found on hands of healthcare workers, and forms biofilm on prosthetic surfaces and central venous catheters. Identification of *C. parapsilosis* as a major global cause of several ailments, particularly in neonates and hospitalised patients, epidemiology, microbiology, genetics, and antibiotic susceptibility of this pathogen warrant further study. Furthermore, *C. parapsilosis* is associated with an unacceptable level of morbidity and mortality, necessitating development of more effective therapies. For this reason, discovery of new antifungal drugs or strategies that act on novel targets is essential for survival of patients. On the other hand, *C. parapsilosis* is intrinsically resistant to several common antifungal drugs, making therapy difficult (Lockhart *et al*. 2017). Inadequate antifungal treatment may also increase hospitalisation duration and related expenses (Arnold *et al*, 2010). Combination therapy is shown to be therapeutically efficacious (Spitzer *et al*, 2017). Results in this study showed that MIC values of fluconazole decreased when it was combined with omeprazole and pantoprazole, which indicates synergistic activity of these efflux pump inhibitors.

This study indicated that pantoprazole and omeprazole enhance antifungal activity of fluconazole against 12 *C. parapsilosis* clinical isolates obtained from South Africa. The combination of omeprazole with fluconazole displayed more synergistic activity than combination of pantoprazole with fluconazole which was shown an additive activity against *C. parapsilosis*. Otherwise, the effects of pantoprazole and omeprazole were almost the same against both resistant and susceptible *C. parapsilosis* strains. This suggest that combination can be used in treatment for resistant and susceptible *C. parapsilosis* and similar outcome will be obtained.

The ability to express different virulence factors, such as adherence, proteinase, and phospholipase production, was also studied. The ability of *C. parapsilosis* to express these virulence factors is strain-dependent. There was a statistically significant difference between the vaginal and hematologic isolates in terms of proteinase production in vitro according to a study on *C. parapsilosis* isolates taken from Italy and Spain (Cassone *et al*, 1995). The present results demonstrated that *C. parapsilosis* had a strong ability to adhere to buccal epithelial cells and to produce the proteinase enzyme. But none of the tested isolates could synthesise the phospholipase enzyme. These results will be further discussed in this section. Moreover, the widespread use of antifungal agents leads to drug resistance in *C. parapsilosis*. Fluconazole, in particular, should not be used for an extended period of time to treat *C. parapsilosis* infections (Sarvikivi and Saxen, 2005). Failure of cells to accumulate the drug due to overexpression of efflux pumps is the most common mechanism responsible for azole resistance. As a new therapeutic strategy in this study, pantoprazole and omeprazole as efflux pump inhibitors in combination with fluconazole, as conventional antifungal agents against *C. parapsilosis* was used. While efflux pump inhibitors (omeprazole and pantoprazole) inhibit efflux pumps on *Candida* cell membrane, the antifungal drugs like fluconazole will accumulate inside *Candida* cell and kill the cell. In this study, two methods to determine accumulation of

drug were used: Rhodamine 6G (R6G) as extracellular and fluorescent Hoechst 33342 as intracellular dyes. the results of this study showed that pantoprazole and omeprazole effectively inhibited the efflux pump activity making fluconazole resistant *C. parapsilosis* cells susceptible, to the combination, as well to the individual drugs.

4.1 Antifungal susceptibility tests in *C. parapsilosis*

Antifungal susceptibility testing *in vitro* has been utilised to find optimal therapeutic concentration for a particular fungus and to identify antifungal drug resistance. This study made use of microdilution techniques. The Clinical Laboratory Standards Institute (CLSI) has standardised a MIC assay for antifungal susceptibility testing, where growth of a pathogen is evaluated with a series of drug concentrations over time. It has also established MIC breakpoints for several antifungal agents to classify resistant and susceptible strains. Even though clinicians rely on these methods to select the best antifungal drug for fungal infections, they are not always reliable predictors of *in vivo* response to drug treatment, especially when this method used to determine amphotericin B MIC values. Other commonly employed techniques include Etest, Vitek® 2, and Sensititre™ YeastOne™ (Lone and Ahmad 2019). The CLSI has proposed specific susceptibility breakpoints for all *Candida* species, these MIC breakpoints for *C. parapsilosis* were for fluconazole (S ≤8 ug/ml; R ≥64), amphotericin B (S ≤1 ug/ml; R>1). Similarly in our study, MIC values of fluconazole, amphotericin B, pantoprazole, and omeprazole were obtained using microdilution techniques.

4.1.1 Antifungal susceptibility of fluconazole and amphotericin B

Results from this study showed that 50% (n = 6/12) of *C. parapsilosis* isolates were fluconazole resistant (MIC ≥ 64μg/ml) and 100% susceptible to amphotericin B (MIC ≤ 1 μg/ml). These findings are consistent with results from other studies. At Baragwanath Academic Hospital in South Africa it was 78% (n = 57/73) were fluconazole resistant and all were sensitive to amphotericin B (Magobo *et al*, 2020). Similarly, in a more comprehensive study done in Spain that included 104 isolates of *C. parapsilosis*, fluconazole resistance was found in 87 (84%) while all isolates were fully susceptible to amphotericin B (Alcoceba *et al*, 2022). According to global reports of tested isolates, *C. parapsilosis* is extremely resistant to fluconazole. These results demonstrated that fluconazole alone is ineffective against *C. parapsilosis* infections. Current susceptibility information suggested that *C. parapsilosis* is frequently characterised by high MICs for fluconazole, although susceptibility to other antifungal drugs, including amphotericin B, is variable. Otherwise, *C. parapsilosis* is globally recognised as a multidrug- resistant (resistant to two or more classes of antifungals) pathogen (Lockhart *et al*, 2017). *C. parapsilosis* isolates used in this investigation could not be classified as multidrug-resistant because other drugs were not tested.

4.1.2 Antifungal susceptibility of pantoprazole and omeprazole

The antifungal effect of proton pump inhibitors against *Candida* species has been demonstrated (Liu and Kohler, 2016). In this study, it was determined that MIC values for proton pump inhibitors (pantoprazole and omeprazole) were much higher than those for antifungal agents (amphotericin B and fluconazole). This suggested that pantoprazole and omeprazole had a negligible antifungal effect on both resistant and susceptible *C. parapsilosis* strains. Furthermore, high doses of proton pump inhibitors showing antifungal activity against *C. albicans* has been described by other investigators. On the other hand, in this study, MIC values for omeprazole and pantoprazole are greater than MIC values reported by Biswas et al (2001)

and El-Ganiny et al (2022), those results in our study indicate that *C. parapsilosis* isolates are more resistant to omeprazole and pantoprazole than *C. albican* (Biswas *et al*. 2001; El-Ganiny *et al*. 2022). However, the aforementioned studies suggested that omeprazole and pantoprazole alone cannot be used to treat *Candida* infections due to high doses necessary to suppress yeast growth.

4.1.3 Combination interaction of Pantoprazole and Omeprazole with Fluconazole

Combination therapy is one of the method that can be used to reduce toxicity by decrease dose of the drug while increasing antifungal efficacy of currently available antifungal drugs (Ghannoum *et al*, 1995). Furthermore, combination of antifungal drugs is a significant alternative to traditional monotherapy. In difficult-to-treat situations, combination of echinocandins with additional antifungal agents against multidrug-resistant isolates has been advocated (Cappelletty D *et al*, 2007). Proton Pump Inhibitors (PPIs) are the most effective drugs for reducing gastric acids, they are used to treat acid-related diseases. PPIs have showed some antifungal activity (Monk *et al*, 1995). PPIs have various clinical applications, including proposed combined therapy with fluconazole against numerous *Candida* infections (Biswas *et al*, 2001).

In the current study, it was discovered that the fluconazole and omeprazole combination had a synergistic effect against fluconazole resistant *C. parapsilosis*, while combination of fluconazole and pantoprazole showed an additive effect against fluconazole resistant *C. parapsilosis.* Furthermore, MIC values of fluconazole were significantly reduced in both resistant and susceptible *C. parapsilosis.* These findings suggested that pantoprazole and omeprazole increased the sensitivity of fluconazole-resistant *C. parapsilosis* isolates.

Similarly Ganiny *et al*, (2022) has reported that pantoprazole and haloperidol reduced fluconazole resistance in *C. albicans* as well as in other *Candida* species include: *C. krusei*, *C.*

tropicalis, *C. parapsilosis*, *C. dubliniensis*, and *C. glabrata* (EL-Ganiny *et al*, 2022). Lu *et al*. (2020) were the first to demonstrate that proton pump inhibitors can act synergistically with fluconazole against *C. albicans* isolates resistant to fluconazole. In combination with pantoprazole, MIC values for fluconazole dropped from > 512 g/ml to 1 g/ml (Lu *et al,* 2020). in this study, MIC values of fluconazole dropped from 125 µg/ml to 31.25 µg/ml in combination with pantoprazole, and it dropped from 125 µg/ml to 15.6 µg/ml in combination with omeprazole.

Azole resistance is caused by multiple mechanisms, including increased efflux activity and overexpression of target gene erg11. In the current study, substantial reduction in fluconazole MIC values when combined with omeprazole and pantoprazole may have been due to inhibition or blocking of *C. parapsilosis* efflux pump activity. According to these findings, proton pump inhibitors may also function as efflux pump inhibitors. This hypothesis is based on prior results showing that synergistic drug combinations that lowered fluconazole resistance also reduced efflux pump activity in *C. albicans* (El-Ganiny *et al*, 2022). This suggested that additional combination interaction investigations needs to be conducted for other *Candida* species in order to develop novel treatment options.

4.2 Time-kill kinetics

Time-kill curves are utilised to measure the effect of antimicrobials over time by providing data on growth rate of organisms (Mukherjee *et al*, 2005). In addition, time-kill curves can be utilised to assess the effect of drug combinations on cell viability (Mukherjee *et al*, 2005). In the current study, it was found that fluconazole at its MIC values decreased the viability (cfu/ml) of a susceptible *C. parapsilosis* (MRU 4108), to approximately 1.2 folds.

Similar results were observed for pantoprazole at MIC value. However, omeprazole at MIC level inhibited *Candida* cell growth (cfu/mL) by two fold. On the other hand, it was discovered

that fluconazole at its MIC value did not have an effect on viability (cfu/ml) of a resistant *C. parapsilosis* representative strain (MRU 4282). In contrast, cell viability decreased when cells were exposed to pantoprazole and omeprazole at their MIC values. The fungistatic activity of fluconazole changed to fungicidal by addition of efflux pump inhibitors (omeprazole and pantoprazole) at their half MIC values, by yielding a highest decrease in cell viability. It was further demonstrated that the combination was synergistic against susceptible and resistant *C. parapsilosis* isolates. Therefore, the time-kill assay was successfully used to confirm interaction effects between fluconazole, pantoprazole and, omeprazole against resistant *C. parapsilosis*.

In contrast to the findings of present study, discrepancies have been noted between time-kill curves and FICI interpretations in *C. albicans* Li *et al*, (2008) reported inconsistencies in FICI values and time-kill curves for interaction of azoles and cyclosporine A against sensitive *C. albicans.* This suggested that multiple methods should be used to investigate combination interaction and its effects on *Candida* species which will provide more accurate data.

4.3 Virulence assays

The microbiological characteristics that a pathogen exhibits to infect a host are known as "virulence factors" (Casadevall and Pirofski 2001). To develop an infection in human hosts, pathogenic *Candida* species require virulence factors. Consequently, it is essential to discover and characterise virulence characteristics in clinically relevant *Candida* species. Numerous virulence factors in *C. albicans*, including adhesion, hyphae and biofilm formation, phospholipase, and proteinase synthesis, have been well characterised (Ahmad et al, 2016). Adhesion to host cells is the initial essential step in *Candida* invasion. Nonetheless, adhesion is also crucial for the commensal transport of these species. Adhesions contribute to the ability of *Candida* cells to adhere to other microbes, host cells, and abiotic materials such as medical

devices (Mayer *et al*, 2013). Proteinase destroy several host cell proteins, including keratin, collagen, and mucus. Additionally, these enzymes are accountable for breakdown of antibodies, complements, and cytokines (Deorukhkar *et al*, 2014). Sap1–Sap10 aspartic proteinase are proteinase with finest characterization. These enzymes can either attach to cell membrane or be released into surrounding environment (Mayer *et al*, 2013). Aspartic proteinase breakdown mucosal membranes of host tissues and facilitate degradation of immune protection proteins (Sun *et al*, 2015). It is believed that a higher Sap gene expression level is related to a rise in *Candida* virulence and, by extension, to clinical signs of candidiasis (Sardi *et al*, 2013). Phospholipases are responsible for the destruction of cell membrane by hydrolysing glycerophospholipid ester bonds (Deorukhkar *et al*, 2014). Classes A–D have thus far been identified; however, only a few members of class B are secreted to environment (Mayer *et al*, 2013). Moreover, phospholipase synthesis is strain dependent (Cafarchia *et al*, 2008; Furlaneto-Maia *et al*, 2008; Galán-Ladero *et al*, 2010). It has been demonstrated that *C. parapsilosis* expresses a multitude of virulence factors, including adhesion factors and hydrolytic enzyme secretion.

4.3.1 Adherence

Adherence of yeast cells to host tissue is the initial stage in *Candida* colonisation and subsequent infection progression (Haynes, 2001). Adhesion to host surfaces, such as buccal epithelium, prosthetic dentures, or plastic catheters, is necessary for the onset of either superficial or systemic illness (Segal *et al*, 1988). Consequently, the degree of adherence of *Candida* species to biological surfaces may suggest their pathogenic potential. *C. parapsilosis* and other *Candida* species have been previously shown to adhere to acrylic and other biological surfaces (Tobgi, 1989; Nikawa *et al*, 1997).

In our investigation, we discovered that sensitive and resistant *C. parapsilosis* isolates were able to adhere to oral epithelial cells. On the other hand, reference strain of *C. albicans* attached in greater numbers than *C. parapsilosis* cells to epithelial cells (241 yeast cells adherent to 100 BECs) compared to reference strain *C. albicans* SC5314 (311 yeast cells adherent to 100 BECs). This result is consistent with findings of Repentigny *et al*, (2004), who discovered that *C. albicans* is more adherent than *C. parapsilosis*. Furthermore, although ALS proteins have an important function in *C. albicans* adhesion, it was discovered that Agglutinin-Like Sequence (ALS) Gene gene adhesion-encoding proteins in *C. parapsilosis* were less expressed than those in *C. albicans* (Zhao *et al*, 2007). As such, the reduced adhesion ability of *C. parapsilosis* in comparison to *C. albicans* is also attributable to absence of true hyphal production in *C. parapsilosis* (Laffey *et al*, 2005; Nobile and Mitchell, 2006).

4.3.2 Effects of omeprazole and pantoprazole on *C. parapsilosis* adherence

Currently, only three drug classes are utilised to treat fungal infections in humans, and it remains difficult to create novel antifungal treatments. Virulence factors are attractive as prospective therapeutic targets since reducing their activity decreases drug resistance. However, a proton pump inhibitors have antifungal activity as well as anti-virulence efficacy against *Candida* species. For instance, lansoprazole can inhibit the development of hyphae in *C. albicans* (Biswas et al. 2001). In this investigation, it was discovered that omeprazole and pantoprazole showed anti-virulence efficacy against sensitive and resistant *C. parapsilosis* isolates. It was discovered that varying concentrations of both proton pump inhibitors at ½ MIC, MIC, and MFC values decreased the adherence ability of *C. parapsilosis* to epithelial cells (*p values* < 0.001). However, % reduction in the adherence at different concentration of pantoprazole and omeprazole on resistant and susceptible *C. parapsilosis* strains were done in

this study. There were significant difference in % reduction between resistant and susceptible *C. parapsilosis* at low concentration of pantoprazole and omeprazole indicating that drugs were exclusively effective against the susceptible strain and this suggest that resistant strain of *C. parapsilosis* requires higher dose of these compounds to reduce the adhesion. Therefore at MFC values, no significant difference between two strains were noted. These findings indicated that high concentration of pantoprazole and omeprazole, can play a role in preventing initial stage of an infection. These results could not be compared with any other studies because none were identified in literature.

4.3.3 Phospholipase activity

Phospholipases are one of the most abundant extracellular hydrolytic enzymes, and various pathogenic *Candida* species have been reported to produce them. Phospholipases assist the invasion of host mucosal epithelia by hydrolyzing one or more ester linkages in glycerophospholipids, which are thought to be responsible for breaking host cell membranes (Sachin et al, 2012). According to findings of this study, none of the 12 *C. parapsilosis* strains produced phospholipase. In contrast, according to a study by Hadrich et al. (2017), among the 172 isolates of *C. parapsilosis* strains 63.5 % were phospholipasepositive with moderate activity (Hadrich *et al*, 2017). Moreover, studies comparing isolates from systemic and superficial infections revealed contradictory results. Some researchers identified only positive activity in blood isolates (Treviño -Range et al, 2013), whereas others found a greater rate of activity in isolates from superficial infections (Ge YP et al, 2011). These distinctions could be attributable to regional variances. Therefore, it is necessary to investigate large number of *C. parapsilosis* strains isolating from different types of infections for their capacity to produce phospholipases. In this study although these strains of *C. parapsilosis* were isolated from clinical samples, the data on infections was not available. Nevertheless, none of them produced

phospholipase and therefore the effect of proton pump inhibitors (pantoprazole and omeprazole) on phospholipase could not be studied.

4.3.4 Proteinase activity

An additional essential group of extracellular hydrolytic enzymes, proteinases have been demonstrated to be produced by *C. albicans* and several other species of pathogenic *Candida* Proteinase synthesis has been identified as a significant virulence factor that contributes to pathogenicity of *Candida* species (Trofa *et al*, 2008; Chow *et al*, 2012). These enzymes were thought to play a role in breakdown of structural and immunologically significant proteins in host tissue. In addition, they damage host epithelial and mucosal membrane proteins, allowing fungal cells to adhere and hence invade the host (Ahmad et al, 2016). In this study, it was demonstrated that all tested *C. parapsilosis* strains (100%) produced proteinases, which is consistent with findings of Ramos *et al* (2015), who found that the vast majority of *C. parapsilosis* isolates (93.75%) produced proteinases (Ramos Lde *et al*, 2015). Ninety percent of the *C. parapsilosis* strains exhibited extremely strong (++++) proteinase activity, which was similar to proteinase activity of *C. albicans* SC5314 (reference strain). These findings imply that *C. parapsilosis* is a potent generator of proteinases, which are likely to contribute to pathogenicity and rapid dissemination of this infection. However, little is known about gene responsible for production of this enzyme, in *C.*

parapsilosis.

4.3.5 Effect of (pantoprazole and omeprazole) on the proteinase production in *C. parapsilosis* strains

Pantoprazole and omeprazole at different concentration, significantly decreased proteinase activity (*P value* < 0.05) in both sensitive and resistant *C. parapsilosis* isolates. The effect of omeprazole at ½ MIC values decreased the degree of proteinase secretion from "very strong activity" to "strong activity", at MIC value, proteinase activity level was reduced to "medium activity", and at MFC value it was reduced to "low activity". On the other hand, pantoprazole had same strong effect, decreased the amount of proteinase secretion from "strong activity" to "medium activity" at ½ MIC values, and reduced level of proteinase activity to "low activity" at MIC and MFC values. In this study we compared resistant and susceptible *C. parapsilosis* strains after exposed to varied concentration of pantoprazole and omeprazole, percentage reduction of proteinase production in both *C. parapsilosis* strains did not reach statistical significance. This implies that proteinase synthesis in *C. parapsilosis* strains can be inhibited or halted at both high and low concentrations. These findings suggest that proton pump inhibitors (omeprazole and pantoprazole) also play role to inhibit the hydrolytic activity of SAPs in *C. parapsilosis*. Therefore, it can be argued that these proton pump inhibitors can limit epithelial cell penetration. Since SAPs have numerous functions, omeprazole and pantoprazole's reduction of proteinase activity may also disrupt other molecular processes that enable *C. parapsilosis* pathogenicity.

4.4 Effect of pantoprazole and omeprazole and their combination on Rhodamine 6G efflux activity

Increased activity of multidrug efflux pumps is primarily responsible for azole-resistance in fungi (Liu *et al*, 2014). The efflux pumps are transport protein channels which move components across *Candida* cell membrane, including antifungal drugs. Azole resistance is frequently associated with overexpression of efflux pumps. MFC (Major Facilitator Superfamily) and ABC (ATP-binding cassette) proteins are two major families of transporters involved in antifungal resistance. Conceptually, the simplest mechanism of resistance is one that reduces intracellular drug accumulation. A reduction in intracellular drug levels leads to a reduction in the amount of drug that can reach a target. Fluorescent dyes like R6G and Hoechst 33342 can be used to detect energy-dependent efflux activity. Due to the fact that these dyes are substrates for several MDR efflux pumps (Maesaki *et al*, 1999; Sharma et al, 2009).

In this study, R6G dye was utilised to monitor efflux pump activity. We discovered that resistant *C. parapsilosis* cells could accumulate R6G in the absence of glucose without being exposed to test drugs as a (control reading). This is consistent with other findings, which has shown that extracellular concentration of R6G significantly reduced under glucose-free conditions (Ahmad *et al*, 2013). As an energy-dependent efflux activity, glucose is used to stimulate R6G efflux. The addition of glucose raised extracellular R6G content in resistant *C. parapsilosis* cells. These findings suggest that activating an energy-dependent efflux activity, *C. parapsilosis* will be able to reduce accumulation of fluconazole within their cells. Therefore, it was determined that efflux pump activity was one of the mechanisms responsible for fluconazole resistance in *C. parapsilosis* isolate studied. Other investigations had demonstrated that efflux pump inhibition can restore azole sensitivity in different resistant *Candida* isolates (Mane *et al*, 2017). In this study, it was discovered that pantoprazole, omeprazole, and their

combination with fluconazole significantly inhibited energy-dependent efflux activity of a resistant *C. parapsilosis* isolate. Based on the fact that, intracellular concentration of fluconazole was not reduced rapidly in *C. parapsilosis* cells after exposed to efflux pump inhibitors and their combination. This shows that combination prolonged the interaction period between antifungal drugs and therapeutic targets in fluconazole-resistant *C. parapsilosis* cells. Since there are no other studies available, these results could not be compared.

4.5 Effects of pantoprazole and omeprazole and their combination on Hoechst 33342 intracellular accumulation

In this study, intracellular accumulation of Hoechst 33342, a fluorescent dye and substrate of several efflux pumps, has been used as a marker for efflux activity in fluconazole-resistant *C. parapsilosis.* It was detected that Hoechst 33342 accumulation in resistant *C. parapsilosis* cells was increased about 3 folds when exposed to pantoprazole alone at MIC value, and increased about 3.5 folds when treated with omeprazole at MIC value. Whereas, intracellular accumulation of Hoechst 33342 was raised when resistant *C. parapsilosis* was treated with combination of omeprazole and fluconazole or pantoprazole and fluconazole at MIC values of around 4.5 and 5.2 folds respectively, Hence is showed reduced efflux activity of resistant *C. parapsilosis* strain. These findings demonstrated that pantoprazole, omeprazole, and their combination with fluconazole can block or inhibit the function of efflux pump in fluconazole-resistant *C. parapsilosis*, thereby lowering efflux activity and boosting intracellular antifungal drug accumulation.

Overall, these data indicated that proton pump inhibitors (pantoprazole and omeprazole) decreased ABC-type efflux activity. Accordingly, they can be utilised as efflux pump inhibitors. Corroborating our findings, Verampil, which is a proton pump inhibitor, has also

demonstrated to decrease efflux activity in *C. albicans*. In addition, verampil and fluconazole exhibited synergistic antifungal efficacy against *C. albicans* (Vega-Chacón *et al*, 2021). Furthermore, El-Ganiny et al. (2022) found that pantoprazole considerably reduced expression of CDR1 and MDR1 efflux pumps in *C. albicans* (El-Ganiny *et al*, 2022).

4.6 Clinical implications

Summary of the results are depicted in figure 4.1.

This study demonstrated that proton pump inhibitors (pantoprazole and omeprazole) have antifungal action against *C. parapsilosis*, and their antifungal activity is concentration dependent. At high concentrations, pantoprazole and omeprazole will kill susceptible as well as resistant *C. parapsilosis* cells, therefore reducing the number of cells. In addition, high concentrations of these proton pump inhibitors would reduce adherence of *C. parapsilosis* to host cells, which is crucial in pathogenesis of infection, rendering this pathogen avirulent. At low concentrations, they will inhibit proteinase secreted from surviving cells, thereby reducing their pathogenicity. Furthermore, combination of pantoprazole and omeprazole with fluconazole presented a potentiated antifungal effect on *C. parapsilosis*; these combinations will reduce required dose of the two drugs and restore sensitivity of resistant *C. parapsilosis* strains to the drugs. Overall, fluconazole and proton pump inhibitors (pantoprazole and omeprazole) reduced ABC-type efflux activity in fluconazole-resistant *C. parapsilosis*. By blocking efflux pumps, fluconazole accumulates within *Candida* cells and kills them.

This study showed that these proton pump inhibitors have preventive and therapeutic potential against *C. parapsilosis* colonisation and infections. However, further research is necessary.

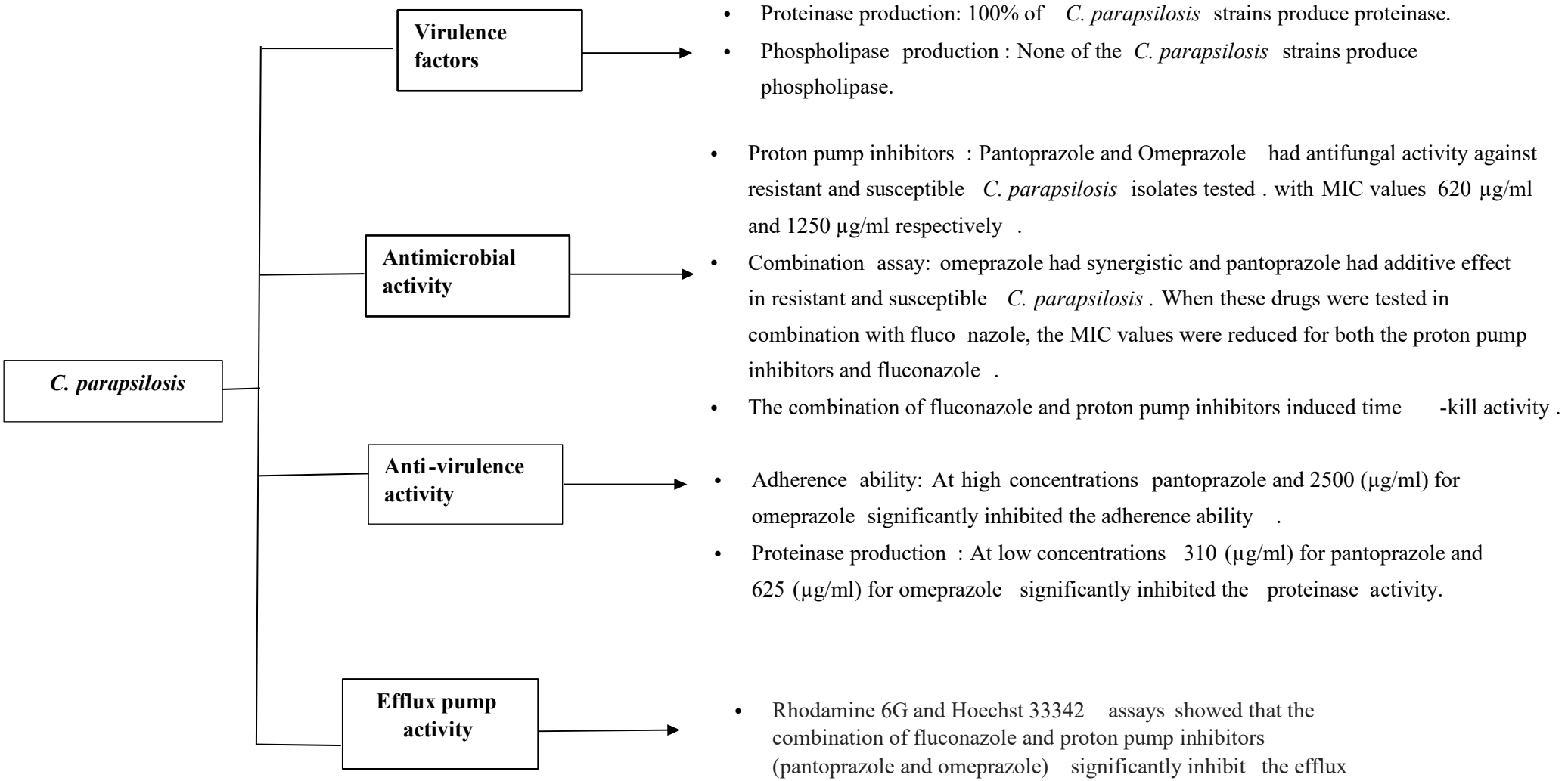


Figure 4.1: Diagram illustrating the summary of results.

CHAPTER 5: CONCLUSION, FUTURE RESEARCH AND LIMITATIONs TO THE STUDY

5.1 Conclusion

This study showed that fluconazole resistant and susceptible strains of *C. parapsilosis* have adherence abilities to epithelial cells and produce proteinase. None of the study strains produced phospholipase. At high concentrations of proton pump inhibitors, pantoprazole (MIC- 620 µg/ml) and omeprazole (MIC- 1250 µg/ml) showed anti- *C. parapsilosis* activity. However, when these drugs were tested in combination with fluconazole, MIC values were reduced for both proton pump inhibitors and fluconazole, showing additive (pantoprazole) and synergistic (omeprazole) activity. Furthermore, combination of fluconazole and proton pump inhibitors induced time-kill activity. The results also showed that at various concentrations, the test drugs significantly reduced adherence ability of *C. parapsilosis* to epithelial cells. In addition, these concentrations also reduced production of proteinase, which suggests that proton pump inhibitors can reduce virulence activity in *C. parapsilosis*. When the effect of these test drugs on efflux pump activity was studied, both assays using Rhodamine 6G and Hoechst 33342 accumulation showed that combination of fluconazole and proton pump inhibitors (pantoprazole and omeprazole) reduced efflux activity in fluconazole resistant *C. parapsilosis*. This suggests that these drugs can reverse fluconazole resistance, and increase retention of intracellular concentration of drugs.

5.2 Limitation to the study

1. A limited number of isolates, particularly resistant strains, was primary limitation of the present study.
2. The experimental strains were obtained from previously frozen isolates, which may have resulted in less virulent fungal pathogens compared to initial isolation of these pathogens.
3. Additional *in-vitro* investigations with a larger number of isolates are required, to establish efficiency of efflux pump inhibitors on resistant *C. parapsilosis*, as well as effect of these drugs on organism and its virulence while actively infecting animals.

These limitations, however, give opportunities for future study.

5.3 Future research

- Large number of different *Candida* species could be used to study the effects of different types of proton pump inhibitors on efflux pump activity.
- Compare the performance of proton pump inhibitors with other available antifungal drugs and to determine the best dose for clinical application.
- The efficacy and safety of these combinations must be determined using animal models and clinical trials. In addition, it is necessary to decode method of action of these synergistic combinations.
- It is necessary to conduct both *in-vivo* and *in-vitro* tests to determine efficiency of proton pump inhibitors against *C. parapsilosis* from various infection sites. The possibility of studying not only the effects of compounds on *C. parapsilosis*, but also how the patient reacts to these compounds.

CHAPTER 6 REFERENCES

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CHAPTER 7 APPENDICES

Appendix A
Composition and preparation of Media, Buffers and Stains

- **Sabouraud Dextrose Agar**
    - Sabouraud Agar (Sigma-Aldrich, South Africa) 60 g
    - Distilled water 1 L
    - The ingredients were stirring until completely dissolved. Thereafter, the solution was autoclaved at 121°C for 30 min. The solution was allowed to cool rapidly to 40-45°C and mixed well. It was aseptically poured into sterile petri dishes.

- **Sabouraud Dextrose Broth**
    - Sabouraud Broth (Sigma-Aldrich, South Africa) 20 g
    - Distilled water 1 L
    - The ingredients were mixed well, and the solution was autoclaved at 121°C for 30 min.

- **Normal Saline (1L)**
    - 8.5g Sodium chloride
    - Dissolve in 1L d.H2O

- **Bovine Serum Albumin (BSA) agar (for Proteinase test)**
    - 1.45 g MgSO
    - 1.45 g Yeast nitrogen base without amino acids
    - 4 g Glucose
    - 2 g BSA
    - 20 g Agar
    - 1000 ml distilled water
    - Dissolve the dry ingredients, except the BSA, into 1000 ml distilled water. Thereafter, autoclave and cooled to 45 °C. Filter sterilize the BSA and add to the mixture. Pour the plates.
-

- **Egg Yolk Media (For phospholipase test)**
    - Preparation of Egg York emulsion
    - Centrifuge 20ml egg York emulsion at 500 rpm for ten minutes at room temperature. Use supernatant for the plates.
    - 13 g Agar
    - 57,3 g Sodium Chloride
    - 0.55 g Calcium Chloride
    - 10 g Peptone
    - 30 g Glucose
    - 900 l Distilled Water
    - Dissolve all the ingredients into the distilled water, and autoclave. Cool to 45°C. At this temperature, add the egg York emulsion, mix gently and then pour the plates.

- **Gram's Crystal Violet Stain**
    - 10g 90% crystal violet dye
    - 500ml absolute Ethanol

- **Gram's Iodine**
    - 6g Iodine
    - 12g KI
    - 1800ml distilled water

- **Gram's Decolourizer**
    - 400ml acetone
    - 1200ml 95% Ethanol

- **Gram's Safranin**
    - 10g safranin dye
    - 1000ml distilled water
- **Phosphate buffered saline**
- 4.2 g sodium chloride
- 0.078 g sodium dihydrogen phosphate (NaH2PO4.2H2O)

- 0.64 g sodium hydrogen phosphate (NaHPO4)
- 500 ml distilled water

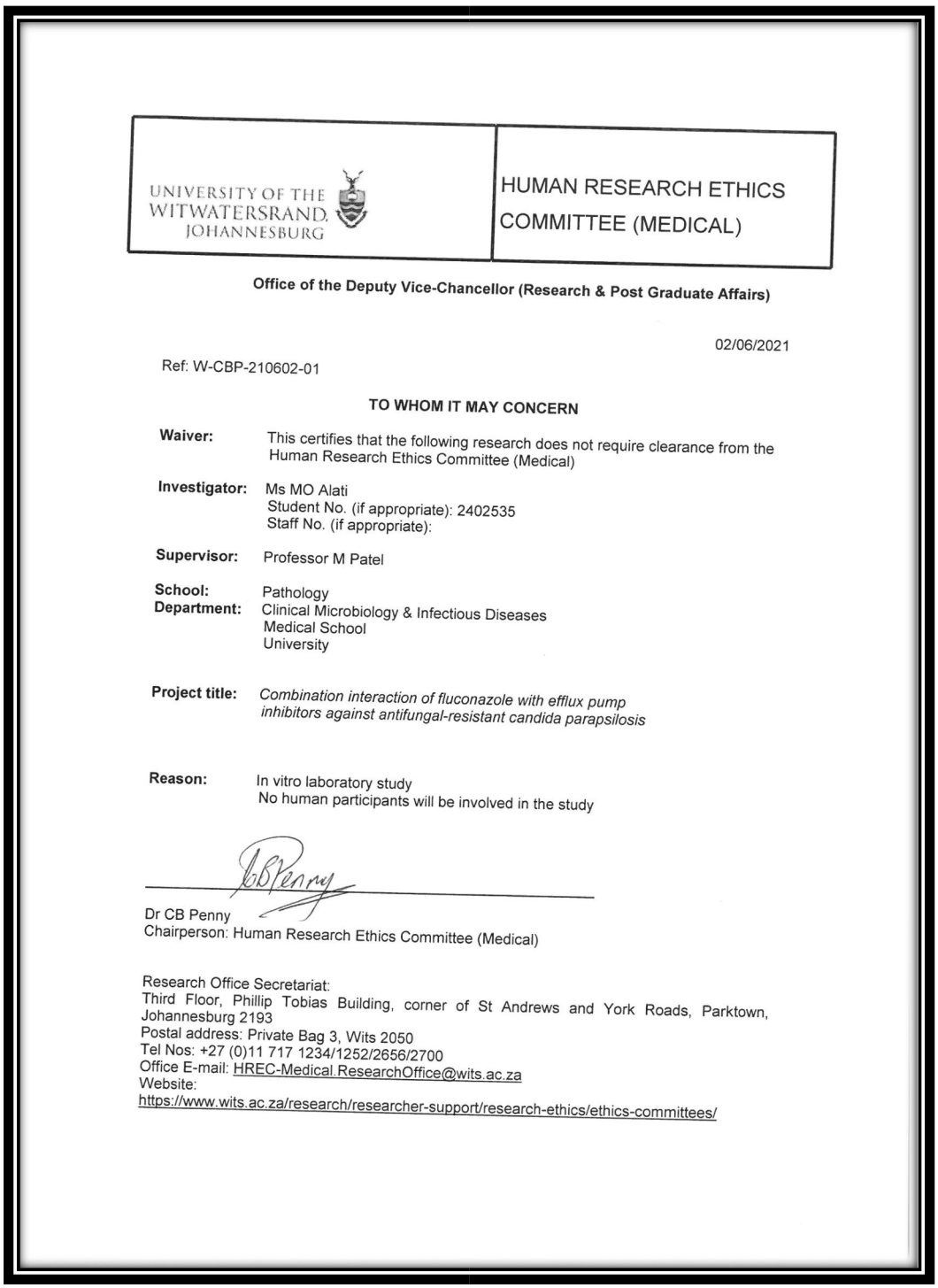
These were suspended in water and autoclaved at 151b and 121 °C for 15 minutes.

- **Rhodamine 6G**
- 0.05 g Rhodamine 6G (C 28H30N2O3HCl) (Sigma-Aldrich, South Africa)
- 10 ml Dimethyl sulfoxide 0.99%

- **Dinitrophenol**
- 0.92g Dinitrophenol $HOC_6H_3(NO_2)_2$
- 10 ml Dimethyl sulfoxide 0.99%

Appendix B

Ethical Clearance Certificate



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

02/06/2021

Ref: W-CBP-210602-01

TO WHOM IT MAY CONCERN

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

Investigator: Ms MO Alati
Student No. (if appropriate): 2402535
Staff No. (if appropriate):

Supervisor: Professor M Patel

School: Pathology
Department: Clinical Microbiology & Infectious Diseases
Medical School
University

Project title: *Combination interaction of fluconazole with efflux pump inhibitors against antifungal-resistant candida parapsilosis*

Reason: In vitro laboratory study
No human participants will be involved in the study

Dr CB Penny
Chairperson: Human Research Ethics Committee (Medical)

Research Office Secretariat:
Third Floor, Phillip Tobias Building, corner of St Andrews and York Roads, Parktown, Johannesburg 2193
Postal address: Private Bag 3, Wits 2050
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Website:
https://www.wits.ac.za/research/researcher-support/research-ethics/ethics-committees/

Appendix C

Statistical analysis of the results of Efflux inhibitory activity of pantoprazole and omeprazole

Table C.1: The effect of efflux pump inhibitors and its combination with fluconazole on resistant *C. parapsilosis* efflux pumps studied using extracellular accumulation of Rhodamine 6G assay

Fluconazole resistant *C. parapsilosis*		**Extracellular R6G concentration (µM)**			
Time (min)	Control	pantoprazole	Pnt +flu	omeprazole	Ome +flu
0	1.5	1.5	1.5	1.5	1.5
	1.5	1.4	1.5	1.52	1.5
	1.4	1,3	1.5	1.51	1.52
10	0.56	0.58	0.5	0.58	0.5
	0.56	0.576	0.51	0.56	0.5
	0.57	0.579	0.49	0.59	0.5
15	0.576	0.6	0.48	0.6	0.49
	0.576	0.6	0.48	0.6	0.491
	0.576	0.59	0.487	0.63	0.489
20	0.586	0.63	0.485	0.641	0.493
	0.589	0.631	0.482	0.644	0.492
	0.586	0.625	0.481	0.642	0.491
25	0.618	0.65	0.528	0.6	0.5026
	0.618	0.66	0.51	0.6	0.5
	0.617	0.65	0.512	0.6	0.503
30	0.840.84	0.68	0.56	0.642	0.56
	0.85	0.67	0.56	0.6	0.56
	0.83	0.68	0.562	0.64	0.562
35	0.89	0.7	0.569	0.648	0.568
	0.9	0.72	0.568	0.649	0.57
	0.88	0.71	0.568	0.65	0.57
40	0.91	0.72	0.59	0.67	0.576
	0.94	0.73	0.58	0.67	0.574
	0.93	0.74	0.5	0.68	0.573
50	1	0.73	0.6	0.7	0.586
	0.99	0.72	0.6	0.7	0.584
	1	0.74	0.61	0.69	0.589
60	1.041	0.73	0.6	0.7	0.6
	1.041	0.73	0.62	0.7	0.62
	1.043	0.74	0.6	0.72	0.63

Table C.2: The effect of Pantoprazole and its combination with fluconazole on efflux pump studied using extracellular accumulation of Rhodamine 6G assay, represents comparison of test results (60 min) to the control

Fluconazole resistant ***C. parapsilosis***	**Extracellular R6G concentration (µM)**				
Time (min)	Control	pantoprazole	Pnt +flu	omeprazole	Ome +flu
0	1.5	1.5	1.5	1.5	1.5
	1.5	1.4	1.5	1.52	1.5
	1.4	1.3	1.5	1.51	1.52
P values	0.09175171				
	0.211324865				
	0.164341443				
	0.211324865				

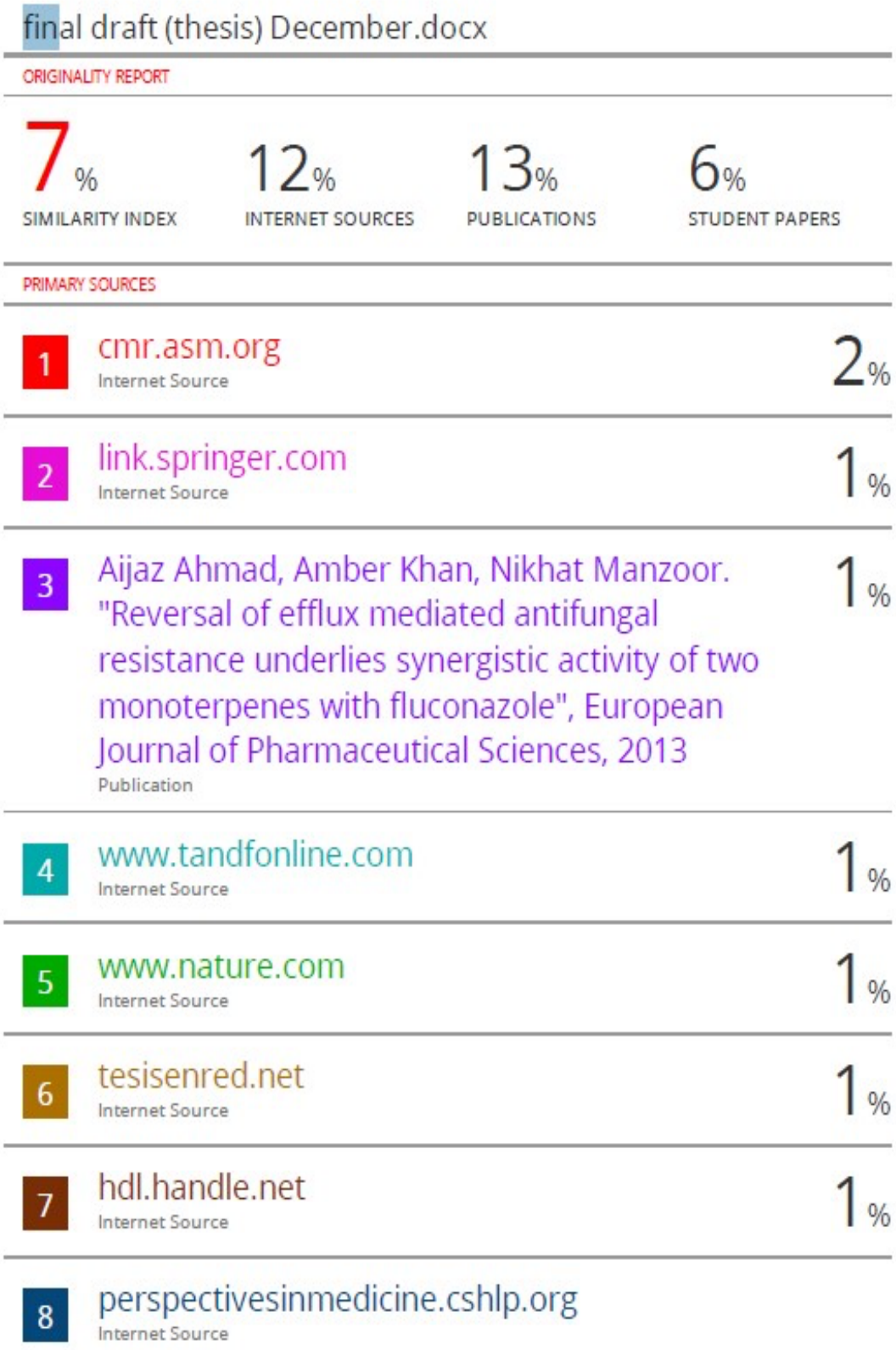
Table C.3: The effect of Omeprazole and its combination with fluconazole on efflux pump studied using extracellular accumulation of Rhodamine 6G assay, represents comparison of test results (60 min) to the control.

Fluconazole resistant ***C. parapsilosis***	**Extracellular R6G concentration (µM)**				
Time (min)	Control	pantoprazole	Pnt +flu	omeprazole	Ome +flu
60	1.041	0.73	0.6	0.7	0.6
	1.041	0.73	0.62	0.7	0.62
	1.043	0.74	0.6	0.72	0.63
P values	3.73954E-05				
	0.000130305				
	0.000160315				
	0.000191816				

Table C.4: The effect of proton pump inhibitors and their combination with fluconazole on efflux pump studied using intracellular accumulation of Hoechst 33342 assay, represents comparison of test results to the control

Intracellular Hoechst 33342 accumulation					
StrainS	**Control**	**Panthoprazole**	**Omeprazole**	**Flu+ Omp**	**Flu+Pnp**
	5100000	31000000	27000000	26000000	11000000
	5200000	34000000	22000000	28000000	24000000
C. p2	5400000	32000000	25000000	33000000	32000000
C. p resistant	5233333	32333333.33	24666666.7	29000000	22333333.33
SD	152752.5	1527525.232	2516611.48	3605551.28	10598742.06
P value	**0.000518883**				
	0.002854253				
	0.003481413				
	0.052637978				

Appendix E
Similarity index



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