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Combination interaction of proton pump inhibitors with fluconazole against multidrug resistant *Candida auris*

by

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Master of Science in Medicine by Research only

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South Africa

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DECLARATION

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



(Signature of candidate)

06th day of June 20 22 in WITS

DEDICATION

To my family, for their tremendous support throughout my education.

ABSTRACT

A significant challenge with controlling *C. auris* infections is its resistance to multiple antifungal agents, including azoles. Alternative strategies are required to combat the problem of antifungal resistance. Some of the suggested approaches include combination therapy and anti-virulence treatment strategies. There is growing interest in combining proton pump inhibitors (PPIs) with available antifungal drugs for better treatment outcomes. In this study, we investigated the antifungal activity of PPIs omeprazole and pantoprazole alone and in combination with fluconazole against resistant *C. auris* isolates. The anti-virulence activity of the synergistic combination against resistant *C. auris* isolates was determined. Lastly, the effect of the synergistic combination on *C. auris* energy-dependent efflux-activity was established.

The CLSI broth micro-dilution method was used to determine the antifungal susceptibility of 25 *C. auris* isolates against antifungal agents (fluconazole and amphotericin B) and PPIs (pantoprazole and omeprazole). The combination interaction of fluconazole and PPIs was established by determining the fractional inhibitory concentration index (FICI) of each combination. Time-kill curves were used to determine the antifungal activity of the synergistic combination over time and to confirm the combination interaction. All 25 *C. auris* isolates were screened for their ability to adhere to epithelial cells and proteinase secretion using microscopy and culture technique respectively. The effect of the synergistic combination on isolates with positive pathogenicity markers was determined.

Most of the *C. auris* isolates were resistant to fluconazole (92%) and all were sensitive to amphotericin B (100%). Omeprazole and pantoprazole had antifungal activity against *C. auris* at MIC values of 3125 µg/ml and 15625 µg/ml, respectively. The combination of fluconazole and pantoprazole reduced fluconazole MIC values by 4-fold, from 125 µg/ml to 31.2 µg/ml. The combination had synergistic effect against most *C. auris* isolates (56%), additive effect in 36% and indifference in 8%. There was no synergism in the fluconazole and omeprazole

combination. However, the combination had antagonist effect against most *C. auris* isolates (56%). All *C. auris* isolates displayed some degree of adherence to epithelial cells and 96% produced proteinases. Fluconazole and pantoprazole combination significantly reduced adherence (p values < 0.05) and proteinase secretion (p values< 0.001) at inhibitory and sub-inhibitory concentrations. Pantoprazole inhibited fluconazole efflux at inhibitory and sub-inhibitory concentrations.

The study showed that *C. auris* isolates express virulence factors such as adherence and proteinase production. The combination of pantoprazole and fluconazole has antifungal and anti-virulence activity against resistant *C. auris* isolates. In addition, it was shown that pantoprazole can attenuate fluconazole resistance by inhibiting the activity of energy-dependent efflux-pumps and it has synergistic effect with fluconazole. Therefore, pantoprazole has the potential to improve therapy outcomes when azoles are used.

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LIST OF ABBREVIATIONS AND ACRONYMS

ALS- Agglutinin-like sequence

ANOVA- Analysis of variance

API20- Analytical profile index 20

ATPase- Adenosine trisphosphate synthase

BSA- Bovine serum albumin

CDC - Centres for Disease Control and Prevention

CFU/ml- Colony forming units per millilitre

CHROMAgar- Chromogenic media agar

CLSI- Clinical and Laboratory Standards Institute

DNA- Deoxyribonucleic acid

DZ- Degradation zone

DMSO- Dimethyl sulfoxide

Erg- Ergosterol

FICI- Fractional inhibitory concentration index

FLC- Fluconazole

GPI- Glycosylphosphatidylinositol

g- gram

Hsp90- Heat shock protein 90

Hog1- High osmolarity glycerol 1

HIV- Human Immunodeficiency Virus

HWP- Hyphal wall protein

ITS- Internal transcribed region

Kb- Kilobase

MTL- Mating type locus

MALDI-TOF- Matrix-assisted laser desorption/ ionisation-time of flight mass spectrometry

MCF- McFarland standard

Mb- Megabase

µg/ml- Microgram per millilitre

µm- Micrometre

µM- Micro molar

ml- Millilitre

MIC- Minimum inhibitory concentration

MFC- Minimum fungicidal concentration

M- Molar

MDR- Multidrug resistant

NAC- non-ablicans *Candida*

PBS- Phosphate buffered saline

PPIs- Proton pump inhibitors

PTP-Pantoprazole

RCF- Relative centrifugal force

rpm- Revolutions per minute

R6G- Rhodamine 6G

RNA- Ribonucleic acid

SDA- Sabouraud dextrose agar

SDB- Sabouraud dextrose broth

SNPs- Short nucleotide polymorphisms

SAPK- Stress-activated protein kinase

NAC- Non-albicans

NaCl- Sodium chloride

OME- Omeprazole

wt/vol- Weight per volume

WOR1- White-opaque regulator 1

WGS- Whole genome sequencing

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Candida species are prevalent in human fungal infections causing morbidity and mortality, especially in immunocompromised individuals (Cortegiani et al. 2018). Members of the genus *Candida* have been implicated in a wide range of clinical infections associated with invasive and superficial candidiasis (Cortegiani et al. 2018). A gradual increase in infections caused by *Candida* pathogens has been observed over the previous decades (Cortegiani et al. 2018). This increase in *Candida* infections is mainly attributed to the extended use of broad-spectrum antimicrobial agents, an increase in invasive procedures and an upsurge in the number of immunocompromised patients. *C. albicans* is currently considered the predominant cause of *Candida* infections in health care settings. Nonetheless, it has been observed that non-albicans *Candida* (NAC) species such as *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* are also frequently associated with invasive fungal infections (Cortegiani et al. 2018). Recently, *Candia auris* has been identified as an emerging NAC species that is proving to be a serious threat to human health worldwide. This is primarily because it is associated with numerous hospital outbreaks globally. Furthermore, it has shown resistance to the three main classes of antifungal medicines, and it is difficult to identify with conventional laboratory techniques (CDC, 2016). As such, it is important to discover novel ways in which *C. auris* infections can be controlled. This includes a variety of strategies such as finding new antifungal drugs, using combination treatments to improve the efficiency of available antifungal agents and targeting virulence factors. Furthermore, the development of new therapeutic startegies can be used to combat infections caused by a wide range of fungal pathogens.

1.2 LITERATURE REVIEW

1.2.1 Fungal infections

The incidence of fungal infections has increased since the 1980s, especially in immunocompromised patients (Arendrup et al. 2005; Espinel-Ingroff et al. 2009; Sardi et al. 2013). Superficial infections account for most of the fungal diseases in humans (Brown et al. 2012; Havlickova et al. 2008). About 25% of the global population has fungal infections of the nails and skin (Ameen 2010; Brown et al. 2010; Havlickova et al. 2008). Dermatophytes are recognized as the causative agents of superficial infections, including common infections such as tenia pedis, tenia capitis and onychomycosis (Ameen 2010; Brown et al. 2012; Havlickova et al. 2008; Thomas et al. 2010). *Candida* species cause oral and genital mucosal infections, which are also common fungal infections. About 50-75% of women in their child-bearing years have vulvovaginal candidiasis (Brown et al. 2012; Sobel 2007). Young children, individuals with dentures, transplant patients, and HIV patients are more predisposed to oral fungal infections (Brown et al. 2012; Salerno et al. 2011; Samaranayake et al. 2009).

The frequency of invasive infections is substantially lower compared to superficial infections (Brown et al. 2012). However, the prevalence of invasive infections has increased over the years (Brown et al. 2012). The concern with invasive infections is that they are correlated with morbidity and mortality at significantly high rates (Brown et al. 2012; Enoch et al. 2006; Husain et al. 2003; Kibbler et al. 2003). *Candida* and *Aspergillus* species are frequently associated with invasive fungal infections. Between the two fungal species, *Candida* pathogens are the prevalent cause of invasive infections (Pfaller and Diekema, 2007).

Candida species form part of the normal human microbiota, colonizing several anatomically distinct sites such as the skin, oral cavity, gastrointestinal tract, vagina, esophagus and vascular system (Shao et al. 2007). Common manifestations caused by *Candida* species include

mucocutaneous overgrowth and bloodstream infections (Eggimann et al. 2003). A heterogeneous group of fungi form part of the *Candida* genus. More than 17 *Candida* species are known aetiological agents of fungal infections in humans, which include *Candia albicans*, *Candida glabarata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis* (Pfaller et al. 2007).

C. albicans has been identified as the leading cause of *Candida* infections. However, growing evidence indicates that several NAC species have emerged as opportunistic pathogens with noteworthy clinical significance (Pfaller et al. 2007; Pfaller et al. 2014). There has been a rise in the proportion of invasive infections caused by NAC species (Pfaller et al. 2007; Pfaller et al. 2014). This may be due to the extensive use of broad-spectrum antibiotics, an increase in the number of immunocompromised individuals, and the use of medical device implants (Pfaller et al. 2007; Pfaller et al. 2014). Furthermore, NAC species have shown decreased susceptibility to the main types of antifungal agents, especially fluconazole (Khan et al. 2007; Kim et al. 2009). Consequently, the rapid emergence of NAC fungal pathogens is a significant threat to human health. Amongst NAC species, *C. auris* has been recently described as a newly emerged pathogen with notable clinical significance. Some of the concerning *C. auris* features include its association with nosocomial outbreaks and multidrug-resistance.

1.2.2 Emergence of *C. auris*

The actual prevalence and incidence of *C. auris* is unknown, mainly because it is difficult to identify with conventional molecular techniques (Lockhart et al. 2017). *C.auris* was first reported in 2009 after it was isolated from the external ear canal of a patient in Japan (Satoh et al. 2009). *C. auris* was subsequently recovered from the ear canal of chronic otitis media patients in Korea (Kim et al. 2009). Two years later, Lee et al (2011) described *C. auris* as the cause of three bloodstream infections in South Korea (Lee et al. 2011). In the same study, *C. auris* was discovered in bloodstream isolates collected in 1996 (Lee et al. 2011). After the first

description of *C. auris*, the pathogen was reported in multiple countries worldwide, including India (Chakrabarti et al. 2015; Chowdhary et al. 2014; Chowdhary et al. 2013; Rudramurthy et al. 2017), Israel (Belkin et al. 2018), Canada (Schwartz and Hammond 2017), China (Wang et al. 2018), Colombia (Escandón et al. 2018; Morales-López et al. 2017; Parra-Giraldo et al. 2018), Europe (Gaitán et al. 2017; Kohlenberg et al. 2018; Riat et al. 2018; Ruiz-Gaitán et al. 2018), Kenya (Okinda et al. 2014), Kuwait (Emara et al. 2015), Malaysia (Tap et al. 2018), Oman (Al-Siyabi et al. 2017; Mohsin et al. 2017), Pakistan (Lockhart et al. 2017), Panama (Araúz et al. 2018; Ramos et al. 2018), Saudi Arabia (Abdalhamid et al. 2018), South Africa (Magobo et al. 2014), South Korea (Lee et al. 2011), United Arab Emirates (Alatoom et al. 2018), United Kingdom (Schelenz et al. 2016), United States of America (McCarthy 2016; Tsay et al. 2017; Vallabhaneni et al. 2016), Russia (Vasilyeva et al. 2018), and Venezuela (Calvo et al. 2016). Retrospective analysis from a SENTRY collection indicated that there were no misidentifications before 2009 (Lockhart et al. 2017). This suggested that *C. auris* has recently emerged as a human pathogen (Lockhart et al. 2017). The cause of the recent emergence of *C. auris* remains unclear but it is suggested that it may include health care practices, genetic changes, human activities and changes in environmental temperatures (Chybowska et al. 2020; Jackson et al. 2019).

1.2.2.1 Geographic clades of *C. auris*

Using Whole Genome Sequencing (WGS) analysis, Lockhart et al (2017) confirmed that *C. auris* emerged almost simultaneously in four different regions. 47 *C. auris* isolates from India, Japan, Pakistan, South Africa, and Venezuela were analysed using WGS (Lockhart et al. 2017). It was determined that the *C. auris* isolates shared about 119 000 Single Nucleotide Polymorphisms (SNPs). The generated phylogeographic structure indicated that the isolates formed four distinct clades, namely, East Asia, South Asia, South Africa, and South America (Lockhart et al. 2017). Tens of thousands of SNPs separated these geographic clades, however,

a smaller number of SNPs was found within each clade (Lockhart et al. 2017). Any 2 South American isolates were separated by less than 16 SNPs (Lockhart et al. 2017). Any 2 South African isolates were distinguished by less than 70 SNPs (Lockhart et al. 2017). 34 of 36 South Asian isolates were separated by less than 60 SNPs (Lockhart et al. 2017). Interestingly, 2 isolates from Pakistan were distinct from other South Asian isolates by 600 SNPs. It was also observed that there were 2 smaller clusters within the South Asian clade (Lockhart et al. 2017). There was a strong correlation between the small clusters and a single hospital (Lockhart et al. 2017). The large number of SNPs separating the clades and the low level of intra-region genetic diversity within each clades indicated near-simultaneous global emergence of the pathogen (Lockhart et al. 2017).

Recently, Chow et al (2019) has proposed a possible fifth clade. WGS analysis of the *C. auris* isolate demonstrated that it is separated from the other four clades by more than 200 000 SNPs (Chow et al. 2019). The representative of the probable clade is the first *C. auris* isolate to be reported in Iran (Abastabar et al. 2019). The isolate was obtained in 2018 from the ear canal of a 14 year old ontomycosis patient (Abastabar et al. 2019). It was further determined that the isolate was susceptibile to the major classes of antifungals (Abastabar et al. 2019). True *C. auris* incidence in Iran may be unknown due to the limitations of using conventional diagnostic tools (Abastabar et al. 2019). It was further suggested that *C. auris* may have emerged in Iran a while ago because the patient had never travelled outside Iran (Abastabar et al. 2019).

1.2.2.2 Genome features of *C. auris*

Clade I isolates were used to generate the first *C. auris* genome assemblies (Chybowska et al. 2020). Nanopore and Illumina-based technology was used to assemble the genomes of some of these Clade I isolates (Chatterjee et al. 2015; Rhodes et al. 2018). The genomes of the isolates from the other four geographic clades were assembled later by Muñoz et al. (2018). The generated optical maps of representative isolates indicated that *C. auris* has seven

chromosomes (Muñoz et al. 2018). This observation was consistent with previous findings that determined *C. auris* chromosome number with pulsed-field gel electrophoresis (Oh et al. 2011). The size of the assembled genomes was determined to range between 12.1 Mb and 12.7 Mb (Chybowska et al. 2020). Similar to other *Candida* species, *C. auris* protein-coding genes were predicted to be between 5288 and 5601 (Muñoz et al. 2018).

Amongst the geographic clades, chromosomal rearrangements have been observed in all *C. auris* chromosomes (Muñoz et al. 2019). Translocations and inversions have been shown to span the genome by hundreds of thousands of Kb (Muñoz et al. 2019). Telomere-telomere assemblies have indicated that chromosomal rearrangements are prevalent in Clade II genomes, relative to the other clades (Muñoz et al. 2019). Clade II genomes contain nine translocations and two inversions, hence they have a karyotype that is significantly distinct from that of the other clades (Muñoz et al. 2019). It was initially suggested that the chromosomal rearrangements could impede genetic exchange amongst the clades (Muñoz et al. 2018). However, the *C. auris* genome contains a highly conserved MTL (Mating type locus) which may indicate their capacity to mate (Muñoz et al. 2018). The *C. auris* MTLα isolates have the α1 gene whilst MTLa isolates contain a1 and a2 genes (Muñoz et al. 2018). Clade II and III contain the MTLα idiomorphs, which span 14.3 Kb of the genome (Muñoz et al. 2018). MTLa idiomorphs are found in clade I and IV isolates, spanning 14.9 Kb of the genomic region (Muñoz et al. 2018). MTL idiomorphs have not been described in clade V (Chybowska et al. 2020).

1.2.2.3 Identification and typing of *C. auris*

Rapid and accurate identification of *Candida* species during infections is important for the development of effective treatment solutions (Chowdhary et al. 2017). Technologies used to identify *Candida* species range from conventional biochemical techniques to nucleic-acid based methods (Sardi et al. 2013). Conventional identification techniques include the use of

commercial systems such as API20, BD Phoenix and Vitek® 2 (Kim et al. 2016). These identification systems contain growth tests that use nitrogen and carbon compounds (Chowdhary et al. 2017). The current challenge is that these commercial systems have been associated with *C. auris* misidentification (Kim et al. 2016). *C. auris* is difficult to identify because it is absent in the databases of most commercial systems (Chowdhary et al. 2017). For example, *C. auris* is frequently misidentified as *Rhodotora glutinis, Sachhromyces kluvyeri, C. famata* and *C. haemulonii* by API_20C AUX (bioMérieux), API ID32 (bioMérieux), Microscan (Beckman Coulter) and Vitek® 2 (bioMérieux), respectively (Jeffery-Smith et al. 2018). An updated version of Vitek® 2 (software version 8.01) demonstrates improved ability to distinguish between *C. auris* and *C. haemulonii* (Ambaraghassi et al. 2019). However, distinction between *C. auris* and *C. duobushaemulonii* remains suboptimal while using Vitek® 2 (Ambaraghassi et al. 2019). This suggests that commercial identification methods may not be suitable for the correct identification of *C. auris*. As such, molecular techniques and matrix associated laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) methods are currently used to identify *C. auris* isolates accurately (Girard et al. 2016; Lockhart et al. 2017). In addition to MALDI-TOF MS, it has been shown that *C. auris* can be correctly identified by sequencing regions in the ribosomal RNA gene such as the internal transcribed sequencer and D1/D2 region (Girard et al. 2016; Lockhart et al. 2017).

1.2.2.4 Biological characteristics of *C. auris*

Since *C. auris* has recently emerged as a human pathogen, very little information is available on its biology. Nonetheless, there are studies that describe some of its microbiological traits. It has been observed that *C. auris* grows as round, smooth, cream-white coloured colonies on Sabouraud agar. In contrast, *C. auris* forms light to dark pale-pink colonies on CHROMAgar. The optimal temperatures for *C. auris* growth are between 37 °C- 40 °C (Kathuria et al. 2015). However, unlike *C. haemulonii*, it can also grow at 42 °C, this characteristic is used to

distinguish the two (Kathuria et al. 2015). An analytical profile index indicates that carbon assimilation patterns of *C. auris* isolates differ (Chowdhary et al. 2013; Kathuria et al. 2015; Magobo et al. 2014; Satoh et al. 2009). South African and Indian isolates show assimilation of N-acetylglucosamine, in contrast, isolates from Japan and South Korea do not (Chowdhary et al. 2013; Kathuria et al. 2015; Magobo et al. 2014; Satoh et al. 2009). Microscopic view of *C. auris* morphology indicates that it assumes an oval-like shape, without the formation of hyphae (Borman et al. 2016). However, the formation of hyphae and other morphologies depends on certain physiological conditions (Borman et al. 2016).

1.2.2.5 *C. auris* and the CTG clade

C. auris belongs to the fungal CTG clade, which is a restricted group of ascomycete yeast with a specific genetic code (Papon et al. 2014). *Candida* species belonging to the CTG clade dominantly translate the universal leucine codon CUG as serine (Santos et al. 2011). About 75 *Candida* species display this reassignment of CUG codons, this includes well-known pathogenic yeast *C. albicans, C. parapsilosis, C. tropicalis* but not *C. glabarata* (Papon et al. 2013; Santos et al. 2011). *Candida* species belonging to the CTG clade produce proteins that contain about 3% leucine and 97% serine at CUG codon positions (Santos and Tuite 1995). There is evidence to suggest that CUG ambiguity plays a vital role in generating phenotypic diversity amongst the CTG clade (Miranda et al. 2013). Miranda et al (2013) indicated that the partial reversal of CUG codon identity from Serine to Leucine caused significant changes to the biology of the cells. The expression of *S. cerevisiae* $\mathrm{tRNA}_{\mathrm{CAG}}^{\mathrm{Leu}}$ in a *C. albicans* model caused significant cellular changes in gene expression, gene structure, morphogenesis, adhesion, and the secretion of hydrolytic enzymes (Miranda et al. 2013).Seeing as *C. auris* belongs to the CTG clade, it may be suggested that CUG ambiguity plays a role in *C. auris* adaptation and its ability to cause infections.

1.2.2.6 Adaptation to environmental stresses

C. auris has unique biological properties compared to many other *Candida* species. *C. auris* displays high thermo-tolerance and halo-tolerance relative to other *Candida* species. Most fungi cannot grow at the human physiological temperature range of 36.5°C-37.5 °C (Jackson et al. 2019; Wang et al. 2018; Welsh et al. 2017). This low tolerance for high temperatures inhibits fungal infections in humans (Jackson et al. 2019; Wang et al. 2018; Welsh et al. 2017). In contrast to other *Candida* species, *C. auris* can grow at temperatures greater than 40 °C (Jackson et al. 2019; Wang et al. 2018; Welsh et al. 2017). It has been observed that every 1% increase of temperature in the 30 °C-40 °C range resulted in a 6% decrease in the number of surviving *C. auris* isolates (Robert and Casadevall 2009). *C. auris* thermotolerance facilitates its ability to cause infections and survive fever temperatures in humans (Jackson et al. 2019). Another distinct *C. auris* trait is its ability to tolerate hypersaline environments. Relative to other *Candida* species, *C. auris* has been shown to survive in high salt concentrations (>10% NaCl, wt/vol) (Wang et al. 2018; Welsh et al. 2017). *C. auris* halotolerance and thermotolerance may be pivotal attributes to its ability to persistently survive harsh environments and host organisms (Jackson et al. 2019). For instance, *C. auris* is frequently isolated from the skin of the human groin and axilla (Jackson et al. 2019). These isolation sites are predisposed to increased temperature and salt concentration during strenuous physical activity (Jackson et al. 2019).

Adaptation to environmental stresses is facilitated by a wide range of signalling pathways in *Candida* species. It has also been established that the molecular pathways that control stress signalling are conserved throughout most fungal pathogens. Available studies indicate that the hog1-related stress-activated protein kinase (SAPK) pathway plays a key role in *C. auris* (Day et al. 2018). The calcineurin pathway is another important stress-response system observed in *C. auris* (Day et al. 2018). Calcineurin is a calcium and calmodulin dependent protein

phosphatase that interacts with the Hsp90 chaperone protein to regulate stress-response systems (Day et al. 2018).

1.2.3 Virulence factors of *C. auris*

Pathogenic *Candida* species cause disease in human hosts through three main stages: adhesion, invasion and damage (Polke et al. 2015). These steps are facilitated by a myriad of features called virulence factors (Casadevall and Pirofski 2001). Virulence factors may be described as microbial features that a pathogen expresses to establish an infection in a host (Casadevall and Pirofski 2001). Other eminent roles of virulence factors include mediating growth in a host, immune system evasion and intracellular survival (Casadevall and Pirofski 2001). Virulence factors are further characterised as components that can be weakened within a pathogen without altering its viability (Casadevall and Pirofski 2001). This suggests that antifungal activity against virulence factors may not induce resistance.

Extensive research has been done on *C. albicans* pathogenicity but limited data is available on NAC species, especially *C. auris*. Nonetheless, most *Candida* species express a similar set of virulence features which include adherence factors, morphogenesis, secretion of hydrolytic enzymes, biofilm formation, stress-response systems, host immune system evasion, and resistance to antifungals (Mba and Nweze 2020).

Furthermore, species-specific expression of virulence factors has been observed in *Candida* species. For example, hyphae and phospholipase are largely produced in *C. albicans* hence they are pivotal to its virulence. In contrast, South African *C. auris* isolates have been shown to not produce true hyphae and phospholipase (Shaban et al. 2020). This also suggests that *C. auris* virulence is attributed to other features such as adhesion, secretion of proteinases and biofilm formation.

1.2.3.1 Adhesion and invasion

Adhesion of *Candida* species to epithelial cells is essential for initial colonization and infection (Richardson et al. 2018). Adhesion to epithelial surfaces occurs through direct physical attachment, via interaction with co-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. 1990). The initial contact of *C. albicans* with the epithelium occurs through hydrophobic and electrostatic attractions (Ener and Douglas 1992; Hazen et al. 1990). Cell surface protein content and adhesion have been linked to hydrophobicity in *C. albicans* (Ener and Douglas 1992; Hazen et al. 1990). Hydrophobic fungi have been shown to be more virulent than their hydrophilic counterparts (Ener and Douglas 1992; Hazen et al. 1990). Essential to the adhesion of *Candida* species are adhesins, cell surface proteins responsible for facilitating the process (Richardson et al. 2018). Attachment to epithelial cells can activate the yeast to hyphae morphological transition in some *Candida* species (Richardson et al. 2018). Hence, two major adhesins in *C. albicans* are hypha-associated Agglutinin -like sequence proteins (Als) and hyphal wall protein 1(hwp1) (Polke et al. 2015; Richardson et al. 2018).

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). In *C. albicans*, the Als protein family consists of eight members, Als1-7 and Als9 (Butler et al. 2009; Hoyer 2001). The main epithelial adhesin for *C. albicans* adhesion is Als3p, homologues of this protein have been identified in NAC species (Butler et al. 2009; Hoyer 2001). It has been shown that these homologues may display similar roles in adhesion, for example, CPAR_404800 is a *C. parapsilosis* homologue of Als3p and its deletion results in reduced adhesion (Richardson et al. 2018).

Not enough studies characterize the adhesion of *C. auris* hence very little is known about the key molecular components involved in the process. It has been indicated that *C. auris* adhesins have repeated domains that are like those found in *C. albicans* Als proteins (Butler et al. 2009; Hoyer 2001). However, *C. auris* displays significantly less adhesion to epithelial cells, relative to *C. albicans*. This suggests that Als homologues may function differently because of slight structural differences. Hwp1 is another key adhesin in *C. albicans*. Hwp1 expression is increased during *Candida* oral infections and colonisation (Richardson et al. 2018). Since hwp1 is a substrate of transglutaminase in mammals, it mediates *C. albicans* adhesion to the oral mucosa through covalent links (Richardson et al. 2018). The absence of Als3 and hwp1 has resulted in a decrease of about 70% in *C. albicans* adhesion (Wächtler et al. 2011).

Following adhesion and colonisation, *Candida* species invade host cells through two distinct mechanisms, namely, induced endocytosis and active penetration (Wächtler et al. 2012). During induced endocytosis, fungal cells passively invade the epithelium of host cells. Fungal cells are endocytosed through the activity of microfilaments of host cells (Phan et al. 2007). Induced endocytosis is prompted when host cell receptors recognise invasins on the fungal cell surface. In *C. albicans*, Als3 interacts with mammalian cadherins during induced endocytosis (Phan et al. 2007). It has also been shown that Heat shock protein 1 (Ssa1) is another *C. albicans* invasin that mediates endocytosis (Sun et al. 2010). Ssa1 interacts with other host receptors such as HER2 and EGF (Phan et al. 2007). In contrast to induced endocytosis, active penetration is primarily facilitated by the fungal pathogen (Wächtler et al. 2012). During active penetration, the fungal cells may use the physical pressure of its hyphae or secrete hydrolytic enzymes to invade host cells (Wächtler et al. 2012).

1.2.3.2 Morphogenesis

A diverse set of microorganisms undergo changes in their morphology to become successful pathogens (Wang and Lin 2012). These transitions include a change in cell shape, size and

surface composition (Wang and Lin 2012). Morphogenesis has been observed in a number of pathogenic *Candida* species such as *C. glabrata*, *C. albicans* and *C. tropicalis* (Whiteway and Bachewich 2007). Morphological transitions may occur spontaneously or as a reaction to environmental challenges (Whiteway and Bachewich 2007). A wide range of internal and external environmental influences regulate morphological transitions (Biswas et al. 2007; Huang 2012; Whiteway and Bachewich 2007). Changes in temperature, serum concentration, oxygen and carbon dioxide levels may trigger morphogenesis in fungi during its life cycle or infection (Biswas et al. 2007; Huang 2012; Whiteway and Bachewich 2007).

In addition to adapting to environmental stresses, fungal species use morphological transitions to undergo microevolution which enhances phenotype variability (Jain et al. 2008). It is through rapid microevolution that the fungal pathogens become well-adapted to host microenvironments and even develop antifungal resistance (Jain et al. 2008). A substantial amount of studies have described the yeast-hyphal transition and white-opaque switch in *C. albicans* and *C. tropicalis* (Biswas et al. 2007; Huang 2012; Whiteway and Bachewich 2007). These morphological transitions play essential roles in mating and pathogenesis (Biswas et al. 2007; Huang 2012; Whiteway and Bachewich 2007). Like other members of the *Candida* genus, *C. auris* can assume a number of morphologies (Oh et al. 2011; Wang et al. 2018; Yue et al. 2018).

The morphological transition from yeast to filament is one of the key features of *C. albicans* pathogenicity (Polke *et al*, 2015). For instance, *C. albicans* can adhere firmly to epithelial cells in their hyphal form, relative to their yeast form (Richardson et al. 2018). Furthermore, hyphal formation promotes virulence by facilitating invasion of the epithelium and by helping the pathogen evade phagocytosis. A wide range of factors have been shown to stimulate filamentation in *C. albicans*, including serum, carbon dioxide levels and N-acetylglucosamine (Biswas et al. 2007). Conversely, it was shown that *C. auris* did not produce true hyphae under

these environmental conditions (Wang et al. 2018). Nonetheless, *C. auris* can form pseudohyphal-like structures under certain growth conditions (Wang et al. 2018). Pseudohyphal-like *C. auris* cells were found on Yeast Peptone Dextrose medium containing 10% NaCl at 37 ℃ and 42 ℃ (Wang et al. 2018). Therefore, *C. auris* filamentation occurs in hypersaline and high temperature conditions (Wang et al. 2018).

Hyphal formation in *C. albicans* is mediated by the expression of *HWP1* and *ECE1* genes, the latter encodes candidalysin (Muñoz et al. 2018). *C. auris* does not produce true hyphae but rather pseudohyphal-like structures in certain conditions. Consistent with this observation, it has been shown that *C. auris* does not express *HWP1* and *ECE1* genes (Muñoz et al. 2018). Recent studies indicate that other factors may induce filamentation in *C. auris* (Kim et al. 2019). For example, the depletion of Hsp90 is associated with the formation of pseudohyphal-like cells in *C. auris* (Kim et al. 2019). *C. auris* forms pseudohyphal filaments when exposed to genotoxins such as hydoxyurea, fluorocytosine, and methyl methanesulfate (Ruiz et al. 2020). A recent study has reported that passage of *C. auris* cells through a mouse infection model induces filamentation (Yue et al. 2018). The filamentous cells were morphologically similar to *C. albicans* true hyphae (Yue et al. 2018).

In a study that described *in vitro* filamentation of *C. auris* cells, orthologs of genes associated with *C. albicans* filamentation were found (Yue et al. 2018). Increased expression of *ALS4* and G1 cyclin –related gene *HGC1* orthologs was observed in filamentous *C. auris* cells (Yue et al. 2018). However, the expression of a key regulator of *C. albicans* filamentation *EFG1* was downregulated in filamentous *C. auris* (Yue et al. 2018). Differential expression of *C. albicans* orthologs *PGA34*, *PGA38* and *PGA58* was observed in *C. auris*. These cell-wall genes are involved in *C. albicans* filamentation. The lack of key filamentation genes suggests that hyphal formation may not play a significant role in *C. auris* virulence, compared to *C. albicans.*

1.2.3.3 Lytic enzyme secretion

The production of extracellular hydrolytic enzymes is recognized as an important feature of pathogenicity in *Candida* species. Lytic enzymes seem to play pivotal roles in adherence and invasion of host tissues (Polke et al. 2015). Furthermore, the secretion of hydrolytic enzymes perpetuates *Candida* survival by degrading host tissues for nutrient acquisition (Polke et al. 2015). Some of the eminent hydrolytic enzymes include hemolysins, lipases, phospholipases and proteinases (Polke et al. 2015).

Two of the major hydrolytic enzymes in *C.albicans* are phospholipases and secreted-aspartyl proteinases (SAPS). *C. albicans* has been shown to produce various phospholipases, which include lysophospholipase, lysophospholipase-transacylase and phospholipase A-D (Niewerth and Korting 2001). The function of phospholipases is to metabolise phospholipids, which are important constituents of cell membranes (Niewerth and Korting 2001). High phospholipase activity has been associated with increased pathogenicity in *C. albicans* (Niewerth and Korting 2001). For instance, invasive *C. albicans* strains display high phospholipase production, compared to non-invasive strains (Niewerth and Korting 2001). Significant phospholipase production seems to be limited to *C. albicans*, NAC species such as *C. auris*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata* rarely produce phospholipases (Niewerth and Korting 2001).

The conventional role given to SAPs is the degradation of host tissues as a source of nutrients for the pathogen. Recently, SAPs have been shown to have a plethora of roles in cell wall maintenance, formation of adhesins, production of biofilms, inactivation of host antimicrobial peptides, and evasion of host immune response (Larkin et al. 2017; Wang et al. 2018).Ten genes encoding SAPs have been identified in *C. albicans*, which are *SAP1* to *SAP10* (Naglik e al. 2004). These SAPs play different roles in *C. albicans* pathogenesis. *SAP1* to *SAP6* facilitate adherence, tissue degradation and immune system evasion in hosts (Naglik et al. 2004). *SAP9* and *SAP10* have a role in maintaining the integrity of the cell surface (Naglik et al. 2004). In

addition to SAPs, hemolysins have been shown to play a vital role in the colonization of host cells. These pore-forming toxins have been shown to have a high capacity for acquiring irons thereby promoting survival in mammalian cells by absorbing irons from the haemoglobin (Kumar et al. 2015; Tsang et al. 2007; Weissman and Kornitzer 2004).

The ability to produce hydrolytic enzymes has been observed in *C. auris*, and in a strain-dependent manner. Among the enzymes found in the *C. auris* genome, hydrolases are the most predominant (Chatterjee et al. 2015). Consistent with this observation, Larkin et al (2017) found that 64 % (9/14) of *C. auris* isolates from several geographic regions produced proteinases and 35% (6/16) displayed phospholipase activity. Similarly, Wang *et al* (2018) indicated that *C. auris* isolates produced SAPs at a significantly higher quantity than *C. albicans* isolates. Furthermore, *C. auris* SAPs maintained their pathogenicity when exposed to a temperature of 42 ℃ (Borman et al. 2016; Wang et al. 2018). This observation indicated that *C. auris* could sustain its pathogenicity under high temperature stress (Borman et al. 2016). Strain-dependent phospholipase production has also been observed in *C. auris* but it occurs at a much lower rate, relative to *C. albicans* (Kumar et al. 2015; Tsang et al. 2007). Comparative analyses have indicated that the quantity of lipases found in the *C. auris* genome is similar to that of *C. albicans* and *C. dubliniesies* (Kumar et al. 2015; Tsang et al. 2007). Production of hemolysins appears to be higher in *C. auris* isolates from hospitals compared to other environments (Kumar et al. 2015; Tsang et al. 2007).

1.2.3.4 Biofilm formation

Most microorganisms form biofilms to successfully survive in their natural environment instead of growing as planktonic organisms (Du et al. 2020; Hall-Stoodley et al. 2004). Microorganisms form biofilms by irreversibly attaching to surfaces, followed by the production of extra-polymeric polysaccharides that encase the structured microbial communities (Donlan 2001). The major components of the biofilm matrix are β-1.3 glucan, β-1.6 glucan and α-

mannan polysaccharides (Mitchell et al. 2015). Mature *Candida* biofilms appear as complex three-dimensional structures that have water-channels and micro-colonies enclosed in the extra cellular matrix (Ramage et al. 2001). This arrangement of the biofilm structure is optimal for the entry of nutrients and the removal of waste.

Limited information is available to characterise biofilm arrangement in *C. auris*. However, it has been shown that *C. auris* biofilm structure is similar to that of other *Candida* species (Dominguez et al. 2019). *C. auris* biofilms have a biomass that is smaller than that of *C. albicans* but greater than *C. glabrata* (Dominguez et al. 2019; Singh et al. 2019). *C. auris* biofilm-formation is linked to the ability to form aggregates (Borman et al. 2016; Singh et al. 2019). The capacity to form biofilms has been observed in both aggregating and non-aggregating *C. auris* isolates (Borman et al. 2016; Singh et al. 2019). However, non-aggregating cells display a higher capacity to form biofilms, relative to aggregating cells (Borman et al. 2016; Singh et al. 2019). Aggregating cells were mostly observed in colonising isolates which suggests that colonising isolates survive competitive ecological niches, such as the human skin, by forming aggregates and biofilms (Singh et al. 2019).

Biofilm formation in *Candida* species is associated with a substantial amount of infections in humans (Donlan 2001). This is primarily because biofilms facilitate persistence on medical devices and host cells (Kojic and Darouiche 2004; Ramage et al. 2006). A significant amount of *Candida* infections involve the colonization of pathogenic *Candida* on a wide range of medical devices including catheters, implants, pacemakers, prosthesis, dentures, and shunts (Kojic and Darouiche 2004; Ramage et al. 2006). Recent studies have clearly demonstrated strain-dependent biofilm-formation in *C. auris* (Dominguez et al. 2019; Kean et al. 2018; Larkin et al. 2017). *C. auris* is well-known for its ability to persist on multiple surfaces, which may be linked to biofilm formation (Biswal et al. 2017; Welsh et al. 2017). The observed ability

to survive on multiple surfaces supports the hypothesis that *C. auris* biofilms facilitate transmissibility and persistence in hospital settings (Horton et al. 2020).

1.2.3.5 *C. auris* virulence in infection models

Comparative studies have indicated that *C. auris* expresses decreased virulence, relative to *C. albicans*. Using a *G. mellonella* infection model, Borman et al (2016) showed that *C. auris* virulence was significantly reduced, in comparison to *C. albicans* strains. Furthermore, it was indicated that the expression of virulence factors in *C. auris* aggregating cells displayed less virulence than their non-aggregating counterparts (Borman et al. 2016). Similarly, Wang et al (2018) demonstrated that *C. auris* was less virulent than *C. albicans* in a mouse systemic infection model. Interestingly, *C. auris* displayed reduced virulence compared to *C. albicans* but greater than *C. glabrata* in a *G. mellonella* model (Wang et al. 2018). Consistent with these observations, it has also been shown that *C. auris, C. glabrata* and *C. haemullonii* are significantly less virulent when compared to *C. albicans* in a murine infection model (Fakhim et al. 2018; Rossato and Colombo 2018). The cause of reduced *C. auris* virulence remains unclear but is suggested that it may be due to its inability to produce true hyphae, which is important for host cell invasion (Yue et al. 2018). It also plausible that *C. auris* displays less virulence because it is haploid whilst *C. albicans* is diploid (Du et al. 2020). Supporting this theory, it has been demonstrated that *C. albicans* displayed reduced virulence and growth when their diploid state was changed to haploid (Hickman et al. 2013). Since filamentous *C. auris* cells produce fewer SAPs than yeast cells, it is possible that they do not cause systemic infections (Du et al. 2020).

1.2.4 Antifungal drug resistance

Antifungal agents interact with specific cellular targets to hinder fungal growth or survival. However, fungi can inhibit the action of antifungal agents using various mechanisms (Sanglard and Odds 2002). Antifungal resistance is demonstrated by an increase in the MIC value of a

particular antifungal agent, relative to the MIC of the same species (Sanglard and Odds 2002). Resistance may be defined as primary or secondary. Primary resistance is also referred to as intrinsic resistance; it is observed before exposure to treatment *in vivo* or *in vitro* (Sanglard and Odds 2002). In contrast, frequent exposure to antifungal agents may induce secondary resistance. Secondary resistance can be reversible when the organism adapts to the antifungal temporarily or it may be acquired due to genetic alterations (Sanglard and Odds 2002). Acquired resistance is rarely reported compared to intrinsic resistance. This is mainly because the resistance genes in fungi are not exchanged through plasmids. Hence, selection of resistance genes occurs in an individual during treatment with an antifungal (Arendrup and Patterson 2017). Acquired antifungal resistance has been reported in AIDS and bone-marrow transplant patients receiving azole treatment for *Candida* infections (Marr et al. 1997; White et al. 1998).

Resistance to the three main classes of antifungal drugs has been observed in *Candida* isolates. A substantial amount of studies report resistance to azole and echinocandins in *Candida* species, since resistance to polyenes (amphotericin B) is rare. Echinocandins are currently used as first-line therapy against invasive candidiasis. This is primarily because echinocandins are fungicidal against most *Candida* species including those that display resistance towards azole and polyene antifungals (Pfaller et al. 2019).

Candida species display differential resistance to antifungal agents. For example, a recent ARTEMIS DISK study indicated that *C. albicans* isolates displayed low resistance to fluconazole with very little change over the years, from 0.2% in 1997-2001 to 0.1% in 2015-2016 (Pfaller et al. 2019). In contrast, clinically relevant NAC species show increasing resistance to fluconazole (Pfaller et al. 2019). In the years 1994-2014, fluconazole resistance has generally increased in *C. glabrata* and *C. tropicalis*, 8.6% to 10% and 2.5% to 4.9%, respectively (Pfaller et al. 2019). Similarly, *C. parapsilosis* shows an increase in fluconazole resistance over time (Pfaller et al. 2019). This change in fluconazole resistance may be

attributed to the use of fluconazole as first-line therapy in the past (Pfaller et al. 2019). Recurrent use of fluconazole may have induced secondary resistance in these *Candida* species (Pfaller et al. 2019). Resistance to echinocandins is rarely observed in *Candida* species such as *C. albicans* (0.0%-0.1%), *C. krusei* (0.0%-0.7%) *C. parapsilosis* (0.0%-0.1%), and *C. tropicalis* (0.5%-0.7%) isolates (Pfaller et al. 2019). A general increase in echinocandin resistance was not observed in any of the species overtime (Pfaller et al. 2019).

Another major concern in antifungal resistance is that multidrug resistance is developing in *Candida* species (Arendrup and Patterson 2017). Multidrug resistance is uncommon in species that lack intrinsic resistance because acquiring several resistance mechanisms would compromise the fitness of the fungi (Arendrup and Patterson 2017; Ben-Ami and Kontoyiannis 2012; Vincent et al. 2013). Amongst *Candida* species, multidrug resistance has been reported in *C. glabrata*, *C. guillermondii*, *C. krusei* and *C. auris* (Arendrup and Patterson 2017). Early reports of *C. auris* infections in South Korea demonstrated that the isolates were resistant to fluconazole and amphotericin B (Kim et al. 2009). Subsequently, multi-drug resistant (MDR) *C. auris* strains were associated with cases of invasive infections (Lee et al. 2011). The MDR isolates displayed decreased susceptibility to caspofungin, 5-flucytosine, fluconazole, and voriconazole (Lee et al. 2011). In a study conducted by the CDC, antifungal resistance of 54 *C. auris* isolates collected from India, Pakistan, South Africa and Venezuela was determined (Lee et al. 2011). It was found that 7% of the isolates were resistant to echinocandins, 35% to amphotericin B, and 93% to fluconazole (Lee et al. 2011). Moreover, resistance to two 2 antifungal drugs was observed in 43% of the isolates and 4% to three antifungal drugs (Lee et al. 2011).

1.2.5 Mechanisms of antifungal resistance

The pyrimidine analogue, flucytosine, acts on fungi by inhibiting DNA and protein synthesis (Kanafani and Perfect 2008). Genetic alterations in cytosine permease can impair drug uptake

thereby inducing intrinsic resistance (Kanafani and Perfect 2008). Cytosine deaminase and uracil phosphoribosyl-transferase genes have been linked to acquired flucytosine resistance (Kanafani and Perfect 2008). Flucytosine usually combined with other antifungals, especially amphotericin B due to the observed resistance (Holmes et al. 2016).

Polyene antifungal agents act by inserting into the fungal cell membrane through binding to ergosterol (Kanafani and Perfect 2008). The polyenes subsequently form porin channels which results to leakage of cytoplasmic contents, loss of transmembrane potential and cell death (Georgiev 2000; Kanafani and Perfect 2008; McCarthy et al. 2017) . Erg3 gene mutations confer resistance to polyene antifungals (McCarthy et al. 2017). Typical features of polyene resistance include increased sterol content and a decrease in the amount of ergosterol (McCarthy et al. 2017). It is also suggested that increased catalase activity protects the fungal cell from oxidative damage, thereby facilitating amphotericin B resistance (Sokol-Anderson et al. 1986).

Echinocandins inhibit the synthesis of β-1,3-D glucan which is a vital part of the fungal cell wall (Pfaller 2012). Resistance to echinocandins is predominantly related to point mutations in the FKS genes of the β-1,3-D glucan synthase complex (Perlin 2007). Mutations in the FKS genes cluster around two highly conserved hotspot regions (Perlin 2007). *FKS1* mutations in hotspot regions have been described in *C. dubliniesis, C. glabrata, C.krusei,* and *C. tropicalis* (Garcia-Effron et al. 2008; Park et al. 2005). Another probable mechanism of resistance to echinocandins comprises the adaptive stress response system (Stevens et al. 2006; Walker et al. 2008; Wiederhold et al. 2005). Inhibition of β-1,3-D glucan synthesis induces increased chitin synthesis *in vitro* (Stevens et al. 2006; Walker et al. 2008; Wiederhold et al. 2005). Chitin synthesis impedes echinocandin antifungal activity by inducing the calcium-calcineurin signalling pathway, protein kinase C, and high-osmolarity glycerol response (Stevens et al. 2006; Walker et al. 2008; Wiederhold et al. 2005).

Azole antifungal agents act on fungi by inhibiting an Erg11 encoded enzyme, lanosterol 14-α sterol demethylase (Kanafani and Perfect 2008). 14-α sterol demethylase facilitates the production of ergosterol from lanosterol (Kanafani and Perfect 2008). Azole resistance occurs through various mechanisms, including mutations in the target protein, genetic changes in ergosterol biosynthesis, upregulation of the target protein, and upregulation of multidrug efflux transporters (Prasad et al. 2016). Mutations in Erg11 induce resistance by decreasing the optimal binding affinity to azoles (Orozco et al. 1998). Alterations in Erg3 prevent the accumulation of toxic by-products resulting from 14-α sterol demethylase inhibition (Kelly et al. 1997). Increased intracellular concentration of the target protein overwhelms the azole antifungal, thereby inhibiting its antifungal activity (Sanglard and Odds 2002). The difference between the upregulation of Erg11 in azole resistant strains and the relative expression in susceptible strains is a factor of three to five (Sanglard and Odds 2002). This suggests that this mechanism contributes little to the overall resistance of *Candida* species (Sanglard and Odds 2002). Azole resistance is mostly caused by the increased activity of multidrug efflux pump (Liu et al. 2015; Mane et al. 2016). Efflux pumps transport drugs outside the fungal cell thereby decreasing drug concentration at the target site. Studies indicate that multidrug resistance in *Candida* species is caused by multidrug-efflux systems (Arendup and Patterson 2017).

1.2.5.1 Efflux pump activity

Azole resistance is mostly caused by the increased activity of multidrug efflux pumps (Liu et al. 2015; Mane et al. 2016). Efflux pumps transport drugs outside the fungal cell thereby decreasing drug concentration at the target site (Prasad et al. 2016). Studies indicate that multidrug resistance in *Candida* species is caused by multidrug-efflux systems (Arendup and Patterson 2017). Genes encoding *Candida* efflux-pumps proteins belong to the ABC (ATP-binding Cassette) and MFS (Major Facilitator) superfamily of proteins (Prasad et al. 2016). ABC and MFS utilize different energy sources to translocate compounds (Cannon et al. 2009).

ABC proteins use ATP hydrolysis to transport compounds whilst MFS efflux pumps use the proton-motive force generated across the cell membrane (Cannon et al. 2009). Usually, azole resistant species overexpress *MDR* genes of MFS transporters and *CDR* genes of ABC transporters (Prasad et al. 2016). *CDR* and *MDR* encoded efflux pumps interact with a distinct spectra of azole substrates (Kanafani and Perfect 2008). *MDR* specificity is limited to fluconazole whilst *CDR* transporters confer resistance to most azoles (Kanafani and Perfect 2008).

In *C. albicans*, azole resistant clinical isolates have been shown to express Cdr1p, Cdrp2 and CaMdr1p efflux pumps (Sanglard et al. 1995). Azole resistance caused by increased efflux activity has been observed in NAC species. For example, the expression of CgCDR1 and CgCDR2 from *C. glabrata*, and CdCDR1 from *C. dubliniensis* has been associated with azole resistance (Sanglard et al. 1995). Similarly, it has been shown that efflux activity plays a significant role in *C. auris* resistance to azole antifungals. Analysis of the *C. auris* genome demonstrated that this pathogen has a variety of efflux pump proteins belonging to the ABC and MFS superfamily (Chatterjee at al. 2015).

Kean et al. (2018) indicated that susceptibility to fluconazole was increased by the overexpression of genes encoding 2 ABC and 3 MFS efflux pumps in resistant *C. auris* isolates. There is evidence to suggest that fluconazole resistance is mostly caused by ABC type efflux activity, relative to MFS in *C. auris*. Rybak et al (2019) demonstrated that *CDR1* was highly overexpressed, relative to *MDR1* in the majority of fluconazole resistant *C. auris* isolates. Furthermore, it was found that fluconazole MIC values decreased substantially when *CDR1* was deleted (Rybak et al. 2019). In contrast, deleting *MDR1* did not affect fluconazole resistance in *C. auris* (Rybak et al. 2019). Two other studies have shown that fluconazole resistant *C. auris* isolates display increased ABC efflux pump activity. These observations

suggest that fluconazole resistance may be attenuated by targeting ABC efflux pumps in *C. auris*.

1.2.5.2 Biofilm mediated resistance

Candida biofilms have been shown to facilitate resistance by sequestering antifungal drugs and upregulating efflux pump activity. It has been suggested that biofilm-mediated resistance is mostly attributed to antifungal sequestering by the biofilm matrix (Mitchell et al. 2013). β-1.3 glucan is frequently associated with this type of biofilm resistance mechanism (Mitchell et al. 2013). Antifungal drug sequestering has been observed in *C. auris* biofilms (Dominguez et al. 2019). Dominquez et al (2019) indicated that *C. auris* matrix reduced susceptibility to azoles by sequestering about 70% of triazoles. It was also observed that hydrolysing matrix polysaccharides increased *C. auris* susceptibility to fluconazole (Dominguez et al. 2019). In addition to drug sequestering, biofilm-forming *Candida* species resist the effect of antifungal drugs by upregulating a variety of resistance genes. Transcriptome assembly by Kean et al (2018) indicated that *C. auris* biofilm development upregulated a variety of virulence genes including, adhesins, cell wall proteins, and efflux pumps.

1.2.6 Strategies to combat antifungal resistance

With the observation that there is an increase in resistance to antifungal drugs, there are numerous developments made to improve therapeutic strategies. This includes improving the efficiency of available antifungal drugs and finding new drug targets. However, the rapid emergence of antifungal resistance continues to decrease treatment options. Consequently, it is important to develop novel therapeutic strategies that will effectively tackle *Candida* infections whilst inducing minimal antifungal resistance. A wide range of potential treatment strategies are currently explored, which include combining antifungal agents and targeting virulence factors.

1.2.6.1 Combination treatments

Combination therapy is being used to treat multiple infectious diseases such as HIV/AIDS, tuberculosis (Spitzer et al. 2017). Furthermore, there is evidence to suggest that combination treatments may be used to control invasive fungal infections (Spitzer et al. 2017) . There are multiple ways in which combination therapy is used to potentiate antifungal drugs, which include increasing the range of drug targets or improving bioavailability (Spitzer et al. 2017). For example, there was synergistic antifungal activity against *C. albicans* when azoles and amphotericin B were combined with flucytosine (Yamamoto et al. 1997). The synergism between these drugs may have resulted from an increase in bioavailability (Spitzer et al. 2017). In other cases, an additive increase in therapeutic efficiency could occur because the combined drugs have different targets in the same protein or cellular pathway (Spitzer et al. 2017). For instance, terbinafine and azoles both target the ergosterol biosynthesis pathway and their combination has resulted in increased antifungal activity against *C. albicans* (Perea et al. 2001). Lastly, drugs with parallel targets may have converging antifungal activity (Spitzer et al. 2017). For example, synergistic combinations of echinocandins with azoles and polyenes target the membrane and cell wall of the fungal pathogen at the same time (Shalit et al. 2003).

Most importantly, combination treatments can be used to decrease or prevent the evolution of drug resistance (Ghannoum et al, 1995). For example, when drugs with distinct intracellular targets are used in combination therapy, development of antifungal resistance is reduced (Spitzer et al. 2017). This is because it will be hard for the pathogen to develop mutations in multiple genes (Spitzer et al. 2017). Furthermore, combination therapy may increase the fungicidal activity of generally fungistatic drugs like azoles (Spitzer et al. 2017). In that case, the likelihood of the pathogen developing resistance would be decreased because the population size has rapidly decreased (Spitzer et al. 2017). Although rare, antifungal resistance can be completely reversed in a process called selection inversion (Baym et al. 2016). This

occurs when the selective advantage of the pathogen is deactivated and there is a fitness cost to developing resistance (Baym et al. 2016). This highlights that combination therapy has great potential in successfully treating infections caused by resistant *Candida* species.

There is growing interest in the use of combination treatments that target factors that cause antifungal resistance. As mentioned previously, one of the major drivers of antifungal resistance in *Candida* species such as *C. auris* is the upregulation of efflux pump activity. As a result, targeting efflux pumps can significantly increase the susceptibility of resistant strains towards azoles. A small molecule named iKIXI has been shown to impede gene activation by Pdr-1 in, thereby sensitizing resistant *C. glabrata* isolates to azoles (Nishikawa et al. 2016). Moreover, the antifungal activity of fluconazole was improved when combined with iKIXI *in vivo* (Nishikawa et al. 2016). These results indicate that targeting efflux activity is an efficient treatment strategy against azole-resistant pathogens (Lee et al. 2021).

1.2.6.2 Targeting virulence factors

Treatment plans that target virulence factors seek to inhibit the ability of the fungal pathogen to cause disease in its host (Lee et al. 2021). There are multiple advantages to targeting virulence factors, including the increase in the range of drug targets and decreasing the development of drug resistance by minimizing the selection pressure (Lee et al. 2021). Studies have indicated that antivirulence drugs and their combinations can substantially inhibit virulence activity in *Candida* species. For instance, the activity of SAPs can be inhibited by HIV proteinase inhibitors indinivar, saquinavor and pepstatin A in *C. albicans* (Gauwerky et al. 2009). Tacrolimus and cyclosporin A target calcineurin in the stress-response pathway of *Candida* species (Gauwerky et al. 2009). In most cases, antivirulence drugs attenuate the activity of multiple virulence features. For example, a small molecule called filastatin impedes *in vivo* filamentation and biofilm formation in *C. albicans* (Fazly et al. 2013). Targeting Hsp90

activity has also decreased *C. albicans* virulence by inhibiting morphogenesis and biofilm formation (Robbins et al. 2011).

Above all, combination treatments have the potential to simultaneously target antifungal drug efficiency and the emergence of drug resistance. For instance, beauvericin potentiates the antifungal activity of azoles by repressing TOR1 kinase and Hsp90 activity, which are important for morphogenesis (Shekhar-Guturja et al. 2016). Furthermore, beauvericin impedes drug resistance by blocking multidrug efflux activity (Shekhar-Guturja et al. 2016). Indeed, combination therapy is an effective approach when addressing drug resistance in currently available antifungal drugs. For this reason, there is a need to expand the repertoire of drugs that can be used in combination therapy. There is a growing interest in the use of proton pump inhibitors in combination treatment of *Candida* infections.

1.2.6.2 Using proton pumps to target virulence and antifungal resistance

Proton pump inhibitors are a class of drugs that decrease the production of acids in the stomach. Hence, they are currently used as first-line treatment for conditions that are associated with acid production such as gastroesophageal reflux disease, and ulcers of the stomach, esophagus and duodenum (Malfertheiner et al. 2017; Scarpignato et al. 2016). It is suggested that they impede acid production by reducing H^+ /K^+ -ATPase activity in the cell membrane. Some of the commonly used proton pump inhibitors in clinical settings include esomeprazole, ilaprazole, lansoprazole, pantoprazole, omeprazole and rabeprazole (Lu et al. 2020).

Several studies have demonstrated that PPIs have antifungal activity against *Candida* species. For example, growth of susceptible *C. albicans* isolates was inhibited by omeprazole at a dose of 10 µM (Liu and Köhler 2016.). It was also observed that lansoprazole could inhibit hyphal formation of sensitive *C. albicans* at a concentration of ≥ 200 µM (Biswas et al. 2001). There is growing interest in using PPIs to reverse antifungal resistance. However, it has been reported

that there is no synergistic antifungal activity when PPIs were combined with fluconazole *in vitro*. For example, Liu and Köhler (2016) demonstrated that omeprazole antagonized fluconazole activity in *C. albicans*. It was also observed that omeprazole and pantoprazole attenuated growth inhibition by fluconazole (Kaneko et al. 2013).

Nonetheless, other studies found that proton pump inhibitors improved the antifungal activity of azoles against *Candida* species. Monk and colleagues (2005) indicated that BM2, a plasma membrane PPI, enhanced the antifungal activity of fluconazole against resistant *C. dubliniesis* and *C. albicans* isolates at sub-inhibitory MICs (<5 µM). Likewise, synergistic activity of common PPIs and antifungal drugs against *Candida* species has been observed. Lu et al (2020) indicated that omeprazole and lansoprazole act synergistically with fluconazole against resistant *C. albicans*, with the minimum inhibitory concentration decreased to 1-4 µg/mL from >512 µg/Ml. It was further observed *in vivo* that the combination treatment prolonged the survival rate of infected *Galleria mellonella* larvae (Lu et al. 2020). It was also shown that treatment with PPIs significantly inhibited efflux pump activity. Similarly, phospholipase production and phenotypic switching decreased in the *C. albicans* isolates (Lu et al. 2020). This observation indicated that PPIs and fluconazole combination treatment may be used to reverse fluconazole resistance in *C. auris* by inhibiting virulence factors.

1.3 Justification for this study

C. auris has been associated with a wide range of nosocomial outbreaks of invasive and superficial infections. *C. auris* has notable clinical significance because of its increasing resistance to multiple antifungal drugs, causing high mortality and morbidity. Increased tolerance by *C. auris* to azoles, especially fluconazole, is reported in most isolates. Decreased susceptibility to fluconazole is largely associated with an increase in efflux pump activity. This is a global health concern because azoles such as fluconazole are commonly used since they are inexpensive and have low toxicity. As such, more studies are aimed at finding novel

strategies to reverse resistance to antifungal drugs. Some of the proposed approaches include combining antifungal agents and targeting virulence factors. The rationale behind targeting virulence factors instead of pathogen survival is that it will not induce acquired drug resistance. Very little data is available to demonstrate synergistic activity of combination therapy against *C. auris*. Furthermore, very few studies elucidate the mechanisms in which this approach decreases resistance to antifungal drugs. As such, studying the effect of combination treatment against *C. auris* will provide data that may support the use of this approach in treating infections.

1.4 Aims

The aim of this study is to determine the combination interaction of proton pump inhibitors with fluconazole on *C. auris* growth, virulence factors, adhesion and proteinase production, and reversal of drug resistance.

1.4.1 Objectives

- To determine minimum inhibitory concentrations of fluconazole and proton pump inhibitors against *C. auris*.
- To determine the combination interaction between fluconazole and proton pump inhibitors against *C. auris* by determining the FICI.
- To study the combination of fluconazole and proton pump inhibitors on the reversal of fluconazole drug resistance in *C. auris*.
- To evaluate the effect of fluconazole and proton pump inhibitors on *C. auris* virulence factors, adhesion and proteinase production.

CHAPTER 2: METHODS AND MATERIALS

2.1 Reagents and media

Analytical grade reagents were used to conduct the experiments. Appendix B contains a list of the reagents, composition of the media and buffers.

2.2 Antifungal agents and proton pump inhibitors

The antifungal agents used in this study were fluconazole (Sigma-Aldrich, South Africa) and amphotericin B (Sigma-Aldrich). Stock solutions of 1000 µg/ml fluconazole and 250 µg/ml amphotericinB were prepared in 1% DMSO and stored at -20 °C until needed. The proton pump inhibitors used in this study were pantoprazole (Sigma-Aldrich, South Africa) and omeprazole (Sigma-Aldrich, South Africa). Stock solutions of 12500 µg/ml pantoprazole and 250000 µg/ml omeprazole were prepared in 1% DMSO and stored at -20 °C until required.

2.3 *Candida* strains

C. auris isolates (25) and *C. albicans* SC5314 were used to conduct this study. *C. albicans* SC5314 was used as a positive control strain since it has been used extensively in virulence studies. The isolates were obtained from the National Institute of Communicable Diseases (Johannesburg, South Africa). Table 2.1 contains a list of the fungal strains used. The *Candida* isolates were stored at -80 °C at the Department of Clinical Microbiology and Infectious Diseases. An ethical clearance waiver to use the isolates was obtained from the University of the Witwatersrand's Human Research Ethics Committee (Appendix A, reference number W-CBP-200827-03). The glycerol stocks of the stored *Candida* isolates were revived by culturing on Sabouraud dextrose agar (SDA) plates at 37 °C for 24 hours.

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

***Candida* species**	**Number**	***Candida* species**	**Number**
C. albicans	SC5314	*C. auris*	MRL6057
C. auris	MRL2397	*C. auris*	MRL6059
C. auris	MRL2921	*C. auris*	MRL6065
C. auris	MRL3499	*C. auris*	MRL6125
C. auris	MRL3758	*C. auris*	MRL6173
C. auris	MRL4000	*C. auris*	MRL6183
C. auris	MRL4587	*C. auris*	MRL6194
C. auris	MRL4888	*C. auris*	MRL6277
C. auris	MRL5418	*C. auris*	MRL6326
C. auris	MRL5762	*C. auris*	MRL6333
C. auris	MRL5765	*C. auris*	MRL6334
C. auris	MRL6005	*C. auris*	MRL6338
C. auris	MRL6015	*C. auris*	MRL6339

2.4 Antifungal susceptibility test

The Minimum inhibitory concentrations (MIC) of fluconazole and proton pump inhibitors against all 25 *C. auris* isolates was determined using the broth-microdilution method, following the recommended guidelines described by the Clinical and Laboratory Institute (CLSI) M27Ed4 document (CLSI, 2017). *Candida* colonies were suspended in Sabouraud dextrose broth (SDB) and incubated at 37 °C for 24 hours. A 0.5 McFarland (MCF) standard of the 24 hour cultures was prepared by adding the inoculum (1.5×10^8 cfu/ml) to 0.085 % saline. The 96 well microtitre plates were prepared by adding 100 µl SDB to all wells and 100 µl of antifungal agents to the first well of each row. A 2-fold serial dilution of the antifungal agents was performed at their respective rows. Following the dilutions, 100 µl of the standardised inoculum was added to the wells. The range of the resulting concentrations of the antifungal agents was 1.95-250 µg/ml for fluconazole, 0.48-62.5 µg/ml for omeprazole, and 0.024-3.125 µg/ml for pantoprazole.

Every experimental set up included SDB alone as a sterility control, culture and SDB media as a growth control, 1% DMSO as a negative control, and 2 µg/ml Amphotericin B as a positive control. The 96 well plates were incubated at 37 °C for 24 hours and the MIC was determined afterwards. The MIC was described as the lowest concentration of the antifungal agent that completely inhibited visible fungal growth, in comparison to the growth control. Using the MICs, the isolates were described as resistant or sensitive. Currently, there are no concrete CLSI guidelines for the MIC breakpoints to distinguish resistant and susceptible *C. auris* isolates. As such, drug resistance against antifungal agents was based on tentative breakpoints established by the CDC using MIC breakpoints of closely related species. The tentative susceptibility breakpoints are as follows; isolates that are resistant to fluconazole have MICs ≥ 32 µg/ml, isolates that are susceptible to fluconazole have MICs ≤ 32 µg/ml, isolates that are resistant to amphotericin B have MICs ≥ 2µg/ml, isolates that are susceptible to amphotericin B have MICs ≤ 2µg/ml. The experiments were done in triplicates.

The minimum fungicidal concentrations (MFCs) were defined as the lowest concentration of the antifungal agent to decrease the colony forming units (CFUs). To determine the MFCs, a volume of 10 µl was collected from the wells that did not produce visible growth on the 96 well microtitre plates. The collected volumes were spread on SDA and incubated at 37 °C. The SDA plates were inspected for growth after 24 hours of incubation. Based on the MICs and MFCs, three fluconazole resistant isolates with the highest MIC were selected for further studies, which were *C. auris* 6015, 6057 and 5762. The experiments were performed in three replicates.

2.5 Combination interaction

The combination interaction of fluconazole and proton pump inhibitors was determined using a method a previously described method (Ahmad et al. 2015). The MICs of the combinations were determined with all 25 *C. auris* strains and *C. albicans* SC5314. At their MIC values,

fluconazole and proton pump inhibitors were combined in 1:1 volume ratios. The 96 well microtitre plates were prepared by adding 100 µl SDB to all wells and 100 µl of the combinations at the first well of their respective rows. A 2-fold serial dilution of the drug combinations was performed. Subsequently, 100µl of the standardised inoculum (1.5×10^8 cfu/ml) was added to the wells. The 96 well microtitre plates were then incubated at 37 ℃ for 24 hours and the MICs were established after incubation. The experiments were done in triplicates.

The degree of synergy between the drug combinations was determined by the Fractional Inhibitory Concentration Index (FICI) based on the zero-interaction theory of Loewe additivity. The FICI is the sum of the FICs (Fractional Concentration Index) of the combined test agents. The FICI was calculated using the following:

$$FICI = FICIa + FICIb = \frac{MICa}{A} + \frac{MICb}{B}$$

Where MICa and MICb were the MICs of the test compounds (fluconazole and pump inhibitors) individually, A and B were the MIC of the test compounds in combination.

The value of the FICI was interpreted as follows:

- 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 the combined drugs produce an effect that is greater than the sum of their individual effect. An additive effect occurs when the combination effect is consistent with the potency of the drugs individually. Indifference denotes that the effect of the combination is

equivalent to the potency of the most effective drug. Antagonism signifies that the combination is less efficient compared to the potency of the most effective drug.

2.6 Time-kill kinetics

Time-kill kinetics assay was used to study the antimicrobial activity of fluconazole and proton pump inhibitors against *C. auris* over time. Three resistant *C. auris* strains (6015, 5762, 6057) were subcultured twice on SDA and grown overnight in SDB at 37 °C. The inoculum of the isolates was diluted in SDB at a 1:100 volume ratio. Each isolate was treated with ½ MIC fluconazole and pantoprazole in combination. 10 µl aliquots were collected at 0, 2, 4, 8, 12 and 24 hour time points. The aliquots were diluted in 90 µl distilled water. The 100 µl suspensions were diluted as required and subsequently spread on SDA plates. The SDA plates were then incubated at 37 °C for 48 hours. Thereafter, the plates were evaluated for growth and the surviving 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 antifungal activity of fluconazole and pantoprazole in combination.

Time-kill curves were used to confirm the combination interaction of fluconazole and pantoprazole. Relative to the most active drug, a ≥ 2log10 decrease in cfu/ml represented synergy and a <2log10 increase in cfu/ml indicated indifference. A ≥2 log10 increase in cfu/ml indicated antagonism, when compared to the most active drug. An increase between 1log10 cfu/ml to 2log10 cfu/ml indicated additivity, relative to the least active drug (Li et al. 2008).

2.7 Efflux activity

ABC-type efflux activity was determined by measuring the glucose-induced efflux of Rhodamine 6G (R6G), using a method described by Ahmad et al (2013). *C. auris* isolates were

sub-cultured on SDA for 24 hours at 37 °C. 1×10^6 cells (A_{595}=0.1) were grown aerobically in 50 ml SDB for 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). 5 mM deoxyglucose and 5mM dinitrophenol were added to de-energise the cells for 45 minutes in PBS. After de-energizing, the cells were centrifuged, washed twice and re-suspended in PBS. Fluconazole, pantoprazole and their combinations were added to the cells at their ½ MIC values. 10 µM R6G was added to the cells and the suspensions were incubated at 37 °C for 60 minutes. Aliquots of the samples were removed after 0, 10, 20, 30, 40, 50, and 60 minutes of incubation. The cells were then collected by centrifugation for 2 minutes at 9000 RCF. The absorbance of the resulting supernatants was measured at 527 nm. Energy dependent R6G efflux was estimated by adding 0.1 M glucose after 25 minutes of incubation, and the readings were recorded until 60 minutes. Controls were prepared with glucose-free PBS. A standard concentration curve of R6G was prepared to determine the actual concentration of R6G effluxed (Figure 2.1). The experiments were done in triplicates.

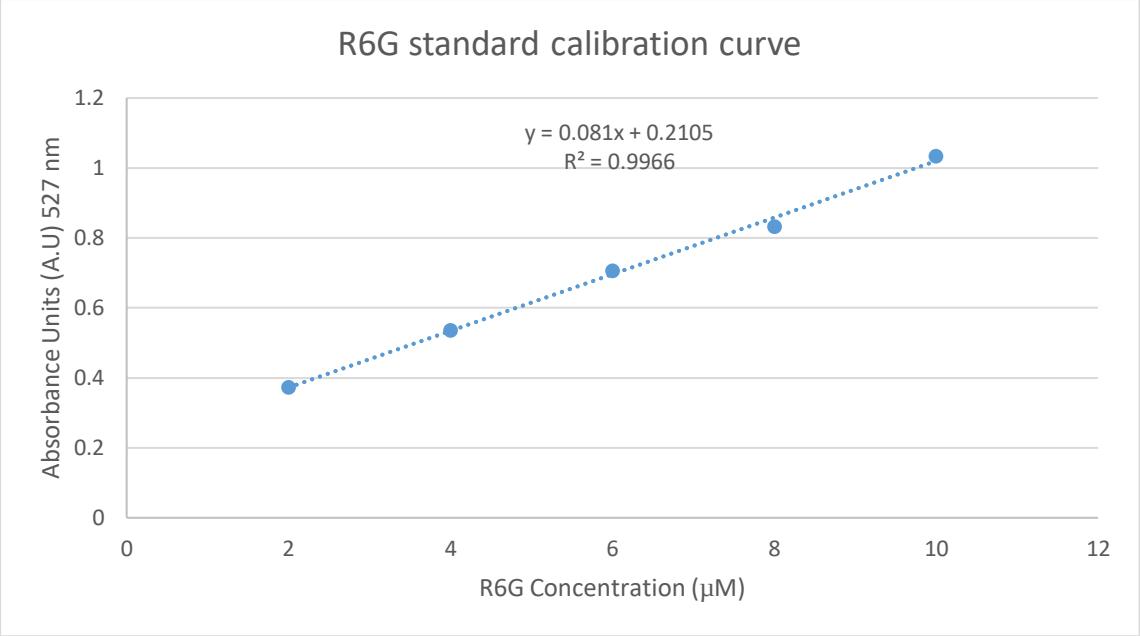


Figure 2.1: R6G assay standard curve of concentration versus absorbance units. The equation y= 0.081+0.2105, with a R^2 value of 0.9966, was used to determine the concentration of R6G effluxed, where x is the concentration and y is the absorbance. To construct the curve, standard solutions of R6G at concentrations of 2 µM, 4 µM, 6 µM, 8 µM and 10 µM were prepared. The absorbance of each R6G solution was measured at 527 nm.

2.8 Pathogenicity markers

The effect of fluconazole and pantoprazole in combination on pathogenicity markers was determined. This was done by exposing *C. auris* isolates showing these markers with MIC, ½ MIC, and ¼ MIC of the drug combination. The experiments were done in triplicates.

2.8.1 Adhesion assay

25 *C. auris* isolates were screened for their ability to adhere to epithelial cells using a method described by Patel et al (2009) with modifications. *C. albicans* SC5314 was used as a positive control strain. Oral epithelial cells were collected from the investigator (myself), washed three times and suspended in sterile distilled water. The number of oral epithelial cells was adjusted to 1×10^7 cells/ml using a haemocytometer. *Candida* cells were inoculated in 5 ml SDB and incubated in a 200 rpm shaker at 37 °C for 18 hours. The cells were collected by centrifugation, washed three times and re-suspended in 2 ml broth. The number of *Candida* cells was adjusted

to 1×10^7 cells/ml. 2ml of yeast cells and 2 ml of epithelial cells were mixed and incubated for 3 hours at 37 °C. Using a 20 µm pore nylon filter, adherent and non-adherent cells were separated from each other. Adherent cells were then washed and re-suspended in distilled water. The cells were viewed under a light microscope after gram staining. Adherence was measured as the number of adherent cells per 100 epithelial cells. The experiment was performed in triplicates by preparing three slides for each isolate.

2.8.1.1 The effect of drug combinations on adherence

Subsequent to screening, the effect of exposing adherent *C.auris* isolates to the fluconazole and pantoprazole combination was investigated. As described in 2.8.1, oral epithelial cells and *Candida* cells were collected and standardized to 1×10^7 cells/ml. *C.auris* cells were exposed to the fluconazole and pantoprazole combination at their ¼ MIC, ½ MIC and MIC values for 2 hours in a 200 rpm shaker at 37 °C. After exposure, the *C.auris* cells were washed three times in distilled water and re-suspended in 2 ml SDB. 2ml of oral epithelial cells were mixed with 2 ml treated *C.auris* cells. The mixture was incubated for 2 hours in a 200 rpm shaker at 37 °C. Afterwards, adherent and non-adherent cells were separated with the 20 µm pore nylon filter and observed under a light microscope after gram staining. Three slides were prepared for each isolate.

2.8.2 Proteinase assay

25 *C. auris* isolates were first screened for extracellular proteinase activity using a previously described method (Shaban et al. 2020). *C. auris* isolates were transferred into flasks containing 5 ml SDB and incubated at 37 °C for 18 hours. The cells were centrifuged for 5 minutes at 3000 RCF and re-suspended into fresh media. The fresh culture was adjusted to 10^6 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. *C. albicans* SC5314 was used as a positive

control strain. The experiment was done in triplicates for each strain. Proteinase activity was determined by measuring the 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

2.8.2.1 The effect of drug combinations on proteinase activity

After screening, proteinase production of three *C. auris* isolates with very strong enzyme activity was evaluated after exposure to the fluconazole and pantoprazole combination. Using the method described above (section 2.8.2), a fresh culture of 10^6 cfu/ml was prepared for all isolates. The cells were then exposed to the drug combinations at concentrations of MIC, ½ MIC, and ¼ MIC for 2-3 hours. Aliquots of 2 µl were placed at equidistant points on BSA agar plates. The cells without any treatment functioned as a control. The plates were then incubated for 3-4 days at 37 °C. Afterwards, the P_Z value of the isolates was determined and interpreted. The experiment was done three times.

2.9 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software version 5. Since all experiments were performed in three replicates, the results were reported as the mean standard deviation. A student's t-test was used to compare the controls to the tests to determine the effect

of drug combinations on *C. auris* across the experiments. Statistical significance was denoted by p values of ≤ 0.05.

Experimental plan

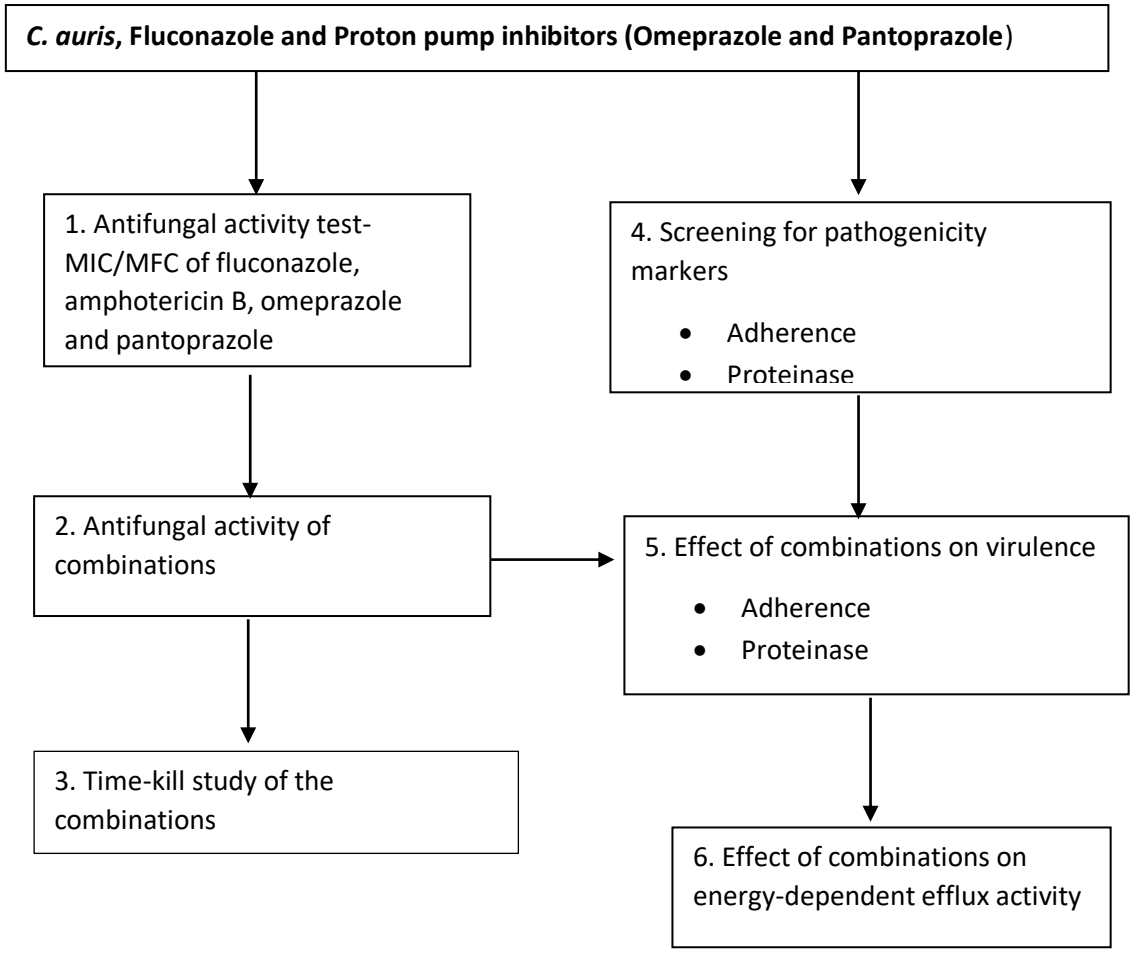


Figure 2.2: Flow diagram demonstrating the experimental plan. The antifungal activity test was performed on 25 *C. auris* isolates with fluconazole, amphotericin B, pantoprazole and omeprazole. The combination interaction of the antifungal agents was performed on all *C. auris* isolates. The effect of the synergistic combination on time-kill kinetics was determined using a *C. auris* representative strain (MRL 6015). All 25 *C. auris* strains were screened for adherence to oral epithelial cells and proteinase production. A *C. auris* representative strain (MRL 6339) was used to investigate the effect of the combination on adherence and proteinase activity. The effect of the combination on energy-dependent efflux activity was determined with a *C. auris* representative strain (MRL 6339).

CHAPTER 3: RESULTS

3.1 MIC and MFC of amphotericin B, fluconazole, pantoprazole and omeprazole

MIC and MFC were performed on 25 strains of *C. auris* and one *C. albicans* against two antifungal agents and two proton pump inhibitors. The results are shown in table 3.1 (summary) and table C1 (raw data). For *C. auris*, median MIC of amphotericin B and fluconazole were 1.25 µg/ml (range 0.312-1.25 µg/ml) and 125 µg/ml (range 15.6-250 µg/ml), respectively. Whereas for *C. auris*, median MIC of pantoprazole and omeprazole were 3125 µg/ml (range 781-3125 µg/ml) and 15625 µg/ml (range 7812-31250 µg/ml) respectively. Based on CLSI guidelines, the results of fluconazole and amphotericin B were interpreted as resistant and sensitive strains (Table 3.2). It was found that 92% (23/25) of *C. auris* strains were resistant and *C. albicans* was sensitive to fluconazole. It was found that all *C. auris* strains (25/25) and *C. albicans* were sensitive to amphotericin B.

The MFC values of fluconazole against all tested 25 *C. auris* strains and *C. albicans* were reported as N/A (not applicable) since the drug did not show 99-99.5% killing activity (Table 3.1). In contrast, amphotericin B displayed fungicidal activity against *C. auris* strains, with a median MFC value of 1.25 µg/ml (range 0.625-2.5 µg/ml). Pantoprazole and omeprazole also had fungicidal activity against *C. auris*, with median MFC values of 6250 µg/ml (range 1560-6250 µg/ml) and 31250 µg/ml (range 15625-62500 µg/ml), respectively. The MFC values of amphotericin B, pantoprazole, and omeprazole against *C. albicans* were 0.625 µg/ml, 6250 µg/ml, and 31250 µg/ml, respectively.

Table 3.1: Summary of antifungal susceptibility test results for *C. auris* and *C. albicans*.

Candida species	**Antifungal agent**	**Median MIC (range)**	**Median MFC (range)**
C. auris (25) n=75	Amphotericin B	1.25 µg/ml (0.312-1.25)	1.25 µg/ml (0.625-2.5)
	Fluconazole	125 µg/ml (15.6-250)	N/A
	Pantoprazole	3125 µg/ml (781-3125)	6250 µg/ml (1560-6250)
	Omeprazole	15625 µg/ml (7812-31250)	31250 µg/ml (15625-62500)
C. albicans (1) n=3	Amphotericin B	0.156 µg/ml (0.156-1.25)	0.625 ug/ml (0.625-2.5)
	Fluconazole	7.8 µg/ml	N/A
	Pantoprazole	3125 µg/ml	6250 µg/ml
	Omeprazole	15625 µg/ml	31250 µg/ml

- N/A - not applicable

Table 3.2: Interpretation of antifungal susceptibility results.

***Candida* species**	**Antifungal agent**	**Number of isolates (percentage)**	
		Resistant (R)	**Sensitive (S)**
C. auris (25)	Amphotericin B	0(0)	25(100)
	Fluconazole	23 (92)	2(8)
	Pantoprazole	N/A	N/A
	Omeprazole	N/A	N/A
C. albicans (1)	Amphotericin B	0(0)	1(100)
	Fluconazole	0(0)	1(100)
	Pantoprazole	N/A	N/A
	Omeprazole	N/A	N/A

- N/A - not applicable

3.2 Susceptibility of *C. auris* and *C. albicans* to combination of fluconazole and pantoprazole

Based on these MIC results, at their MIC values, fluconazole and pantoprazole were combined in 1:1 volume ratios and MIC values were obtained to study the combination effect on *C. auris*. Table 3.3 shows the results of MIC values alone and in combination, calculated FICI values and the interpretation of the results. A summary of the results is shown in table 3.4.

The median MIC value for fluconazole against all *C. auris* strains was reduced from 125 µg/ml to 31.2 µg/ml when combined with pantoprazole (Table 3.4). Similarly, the median MIC value for pantoprazole against all tested *C. auris* isolates was decreased to 391 µg/ml from 3125 µg/ml, in combination with fluconazole. For *C. albicans*, the median MIC value for fluconazole did not change when combined with pantoprazole. However, the median MIC value for pantoprazole decreased from 3125 µg/ml to 195 µg/ml.

For *C. auris*, the FICI values varied depending on the test strain with synergistic, indifference and additive effects. None of the strain showed antagonistic effect. For *C. albicans*, the combination effect was indifferent. It was found that most of the *C.auris* strains (56%) had synergistic effect and all of these strains were resistant to fluconazole. The two sensitive strains of *C. auris* showed an additive effect.

Table 3.3: Susceptibility of *C. auris* and *C. albicans* to fluconazole in combination with pantoprazole.

Candida strains	Median MIC values (n=3)				FICI	Interpretation
	Alone		In combination			
	FLC (µg/ml)	PTP (µg/ml)	FLC (µg/ml)	PTP (µg/ml)		
SC5314	15.6	3125	15.6	195	1.1	IND
MRL2397	31.25	3125	15.6	195	0.6	ADD
MRL2921	125	3125	31.25	391	0.4	SYN
MRL3499	62.5	3125	31.25	391	0.6	ADD
MRL3758	31.25	3125	15.6	195	0.6	ADD
MRL4000	125	781	62.5	781	1.5	IND
MRL4587	250	3125	62.5	781	0.5	SYN
MRL4888	250	3125	31.25	391	0.25	SYN
MRL5418	62.5	3125	31.25	781	0.75	ADD
MRL5762	125	781	15.6	195	0.368	SYN
MRL5765	125	3125	31.25	391	0.375	SYN
MRL6005	125	3125	62.5	781	0.75	ADD
MRL6015	250	3125	62.5	781	0.5	SYN
MRL6057	250	3125	62.5	781	0.5	SYN
MRL6059	125	3125	31.25	391	0.375	SYN
MRL6065	250	3125	62.5	781	0.5	SYN
MRL6125	62.5	3125	31.25	391	0.625	ADD
MRL6173	62.5	3125	31.25	391	0.625	ADD
MRL6183	62.5	3125	62.5	781	1.25	IND
MRL6194	125	3125	31.25	391	0.4	SYN
MRL6277	125	3125	31.25	391	0.375	SYN
MRL6326	125	3125	31.25	391	0.375	SYN
MRL6333	125	3125	62.5	781	0.75	ADD
MRL6334	125	3125	31.25	391	0.375	SYN
MRL6338	125	3125	62.5	781	0.75	ADD
MRL6339	125	3125	31.25	0.39	0.375	SYN

- FLC- fluconazole; PTP – pantoprazole; FICI - fractional inhibitory concentration index; SYN - synergy; IND - indifference; ADD - additive; ANT – antagonistic, SC 5314 – C. *albicans,* All MRL – *C. auris.*

Table 3.4: Summary of results for the susceptibility of *C. auris and C. albicans* to fluconazole combined with pantoprazole

***Candida* species**	**No. of strains**	**Median MIC**				**FICI Mean±SD (Range)**	**Interpretation No. of isolates (%)**			
		FLC A	**PTP A**	**FLC B**	**PTP B**		**SYN**	**IND**	**ADD**	**ANT**
C.auris	Total (25)	125	3125	31.2	391	0.58±0.28 (0.25-1.5)	14 (56)	2 (8)	9 (36)	0 (0)
	R (23)	125	3125	31.2	391	0.58±0.29 (0.25-1.5)	14 (56)	2 (8)	7 (28)	0 (0)
	S (2)	31.2	3125	15.6	195	0.56±0 (0.56)	0 (0)	0 (0)	2 (8)	0 (0)
C. albicans	Total (1)	15.6	3125	15.6	195	1.06±0 (1.06)	0 (0)	1 (100)	0 (0)	0 (0)

- FLC - fluconazole; PTP - pantoprazole; R - resistant; S – Sensitive.
- Median MIC for FLC (µg/ml); Median MIC for PTP (µg/ml).
- A - median MIC of antifungal alone; B - median MIC of antifungal in combination.
- FICI - fractional inhibitory concentration index; SYN - synergy; IND - indifference; ADD - additive; ANT - antagonistic.

3.3 Susceptibility of *C. auris* and *C. albicans* to combination of fluconazole and omeprazole

Based on these MIC results, at their MIC values, fluconazole and omeprazole were combined in 1:1 volume ratios and MIC values were obtained to study the combination effect on *C. auris*. Table 3.5 shows the results of MIC values alone and in combination, calculated FICI values and the interpretation of the results. A summary of the results is shown in Table 3.6.

The median MIC value for fluconazole against *C. auris* strains decreased from 125 µg/ml to 31.2 µg/ml when combined with omeprazole. In *C. albicans*, the median MIC value of fluconazole did not change when combined with omeprazole. For *C. auris*, the FICI values varied depending on the test strain with indifference, additive and antagonistic effects. None of the strains showed synergistic effect. For *C. albicans*, the combination effect was indifferent. When the results were summarised (Table 3.6), it was found that most of the *C.auris* strains (56%) had an antagonistic effect and majority of these strains were resistant to fluconazole.

The two sensitive strains of *C. auris* showed an indifferent effect. Omeprazole was excluded from the rest of the study since it did not display synergism with fluconazole.

Table 3.5: Susceptibility of *C. auris* and *C. albicans* to fluconazole in combination with omeprazole.

***Candida* strains**	**Median MIC values (n=3)**				**FICI**	**Interpretation**
	Alone		**In combination**			
	FLC (µg/ml)	**OME (µg/ml)**	**FLC (µg/ml)**	**OME (µg/ml)**		
SC5314	15.6	15625	15.6	3906	1.25	IND
MRL2397	31.25	15625	62.5	15625	4	IND
MRL2921	125	7812	62.5	15625	2.5	IND
MRL3499	62.5	7812	125	31250	6	ANT
MRL3758	31.25	15625	31.25	7812	1.5	IND
MRL4000	125	7812	62.5	15625	2.5	IND
MRL4587	250	7812	125	31250	4.5	ANT
MRL4888	250	15625	250	62500	5	ANT
MRL5418	62.5	15625	125	31250	4	IND
MRL5762	125	15625	250	62500	6	ANT
MRL5765	125	15625	250	62500	6	ANT
MRL6005	125	15625	62.5	15625	1.5	IND
MRL6015	250	15625	125	31250	2.5	IND
MRL6057	250	15625	62.5	15625	1.25	IND
MRL6059	125	7812	125	31250	5	ANT
MRL6065	250	7812	250	62500	9	ANT
MRL6125	62.5	7812	125	31250	6	ANT
MRL6173	62.5	7812	125	31250	6	ANT
MRL6183	62.5	15625	250	62500	8	ANT
MRL6194	125	15625	62.5	15625	1.5	IND
MRL6277	125	15625	250	62500	6	ANT
MRL6326	125	15625	62.5	15625	1.5	IND
MRL6333	125	15625	62.5	15625	1.5	IND
MRL6334	125	31250	125	31250	2	IND
MRL6338	125	15625	250	62500	6	ANT
MRL6339	125	31250	125	31250	2	IND

- FLC- fluconazole; OME– omeprazole; FICI - fractional inhibitory concentration index; IND - indifference; ADD - additive; ANT – antagonistic, SC 5314 – C. *albicans*, All MRL – *C. auris*.

Table 3.6: Summary of results for the susceptibility of *C. auris and C. albicans* to fluconazole combined with omeprazole.

***Candida* species**	**No. of strains**	**Median MIC**				**FICI Mean±SD (Range)**	**Interpretation No. of isolates (%)**			
		FLC A	**OME A**	**FLC B**	**OME B**		**SYN**	**IND**	**ADD**	**ANT**
C. auris	Total (25)	125	15625	31.2	3125	3.96±2.3 (1.25-9)	0 (0)	11 (44)	0	14 (56)
	R (23)	125	15625	31.2	3125	4.18±2.3 (1.25-9)	0 (0)	11 (44)	0 (0)	11 (44)
	S (2)	31.2	15625	15.6	11700	2.75±0.7 (1.5-4)	0 (0)	2 (8)	0 (0)	0 (0)
C. albicans	Total (1)	15.6	15625	15.6	3950	1.25±0 (1.25)	0 (0)	1 (100)	0 (0)	0 (0)

- FLC - fluconazole; OME - omeprazole; R - resistant; S – Sensitive.
- Median MIC for FLC (µg/ml); Median MIC for OME (µg/ml).
- A - median MIC of antifungal alone; B - median MIC of antifungal in combination.
- FICI - fractional inhibitory concentration index; SYN - synergy; IND - indifference; ADD - additive; ANT – antagonistic.

3.4 Effect of combination of fluconazole and pantoprazole on time-kill kinetics

One resistant strain of *Candida auris* (MRL6015) was selected and the effect of combination of fluconazole and pantoprazole on time-kill kinetics was studied. Figure 3.1 displays time-kill curves for fluconazole and pantoprazole alone and in combination against MRL6015 at their ½ MIC values. Given the starting inoculum of $\sim 10^{6.5}$ cfu/ml, fluconazole alone yielded a 2 fold increase in $\log_{10}$ cfu/ml after 24 hours. A slight increase of 0.76 $\log_{10}$ cfu/ml was found in pantoprazole alone. In contrast, the fluconazole and pantoprazole combination yielded a 2.07 $\log_{10}$ cfu/ml decrease after 24 hours. Relative to fluconazole alone, pantoprazole was the most active test agent. Therefore, pantoprazole results were compared to the combination to determine synergism or antagonism against *C. auris*. Relative to pantoprazole alone, it was found that the combination resulted in a 2.8 $\log_{10}$ cfu/ml decrease after 24 hours, which indicated a synergistic interaction.

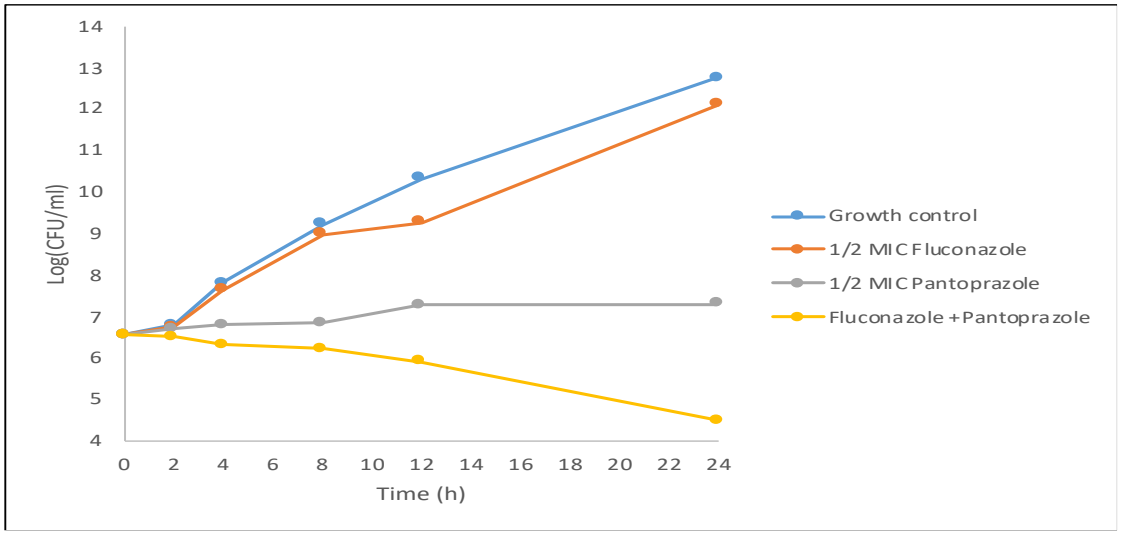


Figure 3.1: Time-kill plots of fluconazole and pantoprazole at ½ MIC values alone and in combination against fluconazole-resistant *C. auris* strain (MRL 6015). The starting inoculum of $\sim 10^{6.5}$ CFU/ml was exposed to 62.5 µg/ml fluconazole and 15625 µg/ml pantoprazole alone and in combination for 24 hours. The untreated culture of the same strain functioned as the growth control. The number of cfu/ml was determined by removing aliquots at 0, 2, 4, 8, 12, 24 h.

3.5 C. *auris* screening for the adherence ability and proteinase production

In order to select proteinase producing strain and the best with the adherence ability, all the strains were screened.

The adherence ability was studied in the oral epithelial cells (Figure 3.2). The results showed that all the test strains of *C. auris* were capable of some level of adherence to the epithelial cells (Table 3.7, raw data in table C2). Summary of the results (Table 3.8) showed that for *C. auris*, the mean number of cells adherent to 100 epithelial cells was 143 whereas 209 cells (mean) of *C. albicans* were adherent to 100 oral epithelial cells.

Proteinase activity was measured from selective media (Figure 3.3). The results showed that 96% (24/25 strains) *C. auris* strains produced some degree of proteinase with Pz ranging from 0.5 to 0.86 (Table 3.9, raw data in table C3). Very strong proteinase activity was noted in 60%

of these *C. auris* strains (Table 3.10). Similarly *C. albicans* also showed a very strong proteinase activity (Pz 0.6).

Based on these adherence and proteinase assay results, it was found that MRL6339 strain of *C. auris* produced the highest quantities of proteinase and it had the highest adherence ability. In the combination study this strain also showed a synergistic effect. Therefore, this strain was selected for further assays to study the effect of combination of fluconazole and pantoprazole on the adherence ability and proteinase production.

Table 3.7: Adherence of *C. auris* and *C. albicans* to oral epithelial cells.

***Candida* strains**	**No of adherent cells to 100 epithelial cells Mean±SD**
SC5314	209±19
MRL2397	195±10
MRL2921	126±12
MRL3499	138±17
MRL3758	121±7
MRL4000	105±10
MRL4587	196±22
MRL4888	136±8
MRL5418	120±7
MRL5762	125±7
MRL5765	113±9
MRL6005	118±16
MRL6015	123±13
MRL6057	134±21
MRL6059	179±13
MRL6065	131±18
MRL6125	169±9
MRL6173	121±18
MRL6183	121±18
MRL6194	99±12
MRL6277	140±11
MRL6326	123±13
MRL6333	156±7
MRL6334	143±6
MRL6338	163±6
MRL6339	224±9

- SC5314- *C. albicans*; All MRL- *C. auris*.

Table 3.8: Summary results of the *C. auris* and *C. albicans* adherence assay.

***Candida* species**	**No. of strains (%)**	**No. of *Candida* cells/100 oral epithelial cells Mean ± SD (range)**
C. auris	25(100)	144 ±34 (98-234) n=75
C. albicans	1(100)	209 ±19 (190-228) n=3

Table 3.9: Proteinase activity of *C. auris* isolates and *C. albicans.*

Candida strains	**Proteinase activity (Pz value) Mean±SD**	**Proteinase activity level**
SC5314	0.6±0	++++
MRL2397	0.61±0.1	++++
MRL2921	0.65±0.4	++++
MRL3499	0.75±0	+++
MRL3758	0.72±0.05	+++
MRL4000	1±0	-
MRL4587	0.6±0	++++
MRL4888	0.56±0.03	+++
MRL5418	0.6±0	++++
MRL5762	0.7±0.05	+++
MRL5765	0.67±0	++++
MRL6005	0.7±0.05	+++
MRL6015	0.67±0	++++
MRL6057	0.75±0	+++
MRL6059	0.5±0.02	++++
MRL6065	0.55±0.02	++++
MRL6125	0.59±0.02	++++
MRL6173	0.65±0.04	++++
MRL6183	0.82±06	++
MRL6194	0.58±0.03	++++
MRL6277	0.6±0	++++
MRL6326	0.72±0.05	+++
MRL6333	0.6±0	++++
MRL6334	0.8±0.06	++
MRL6338	0.67±0	++++
MRL6339	0.5±0	++++

- Proteinase activity level: Pz = 1 (- no proteinase activity); +, Pz = 0.9-0.99 (weak activity); ++, Pz = 0.80-0.89 (medium activity); +++, Pz = 0.70-0.79 (strong activity); ++++, Pz <0.69 (very strong activity), SC 5314 – C. *albicans,* All MRL – *C. auris*.

Table 3.10: Summary results of the proteinase activity of *C. auris* and *C. albicans.*

***Candida* species**	**Proteinase activity level**	**No. of strains (%)**
C. auris (25)	++++	15(60)
	+++	7(28)
	++	2(8)
	+	0(0)
	-	1(4)
C. albicans (1)	++++	1(100)

- Proteinase activity level: no activity (-) weak activity (+); medium activity (++); strong activity (+++); very strong activity (++++).

3.6 Effect of combination of fluconazole and pantoprazole on the adherence ability and proteinase production

The effect of combination of fluconazole and pantoprazole at MIC and ½ MIC concentrations on the adherence ability of *C. auris* representative strain (MRL6339) to epithelial cells was studied. The results showed that for the control, the mean number of cells adherent to 100 epithelial cells were 204 whereas for ½ MIC and for MIC there were 185 and 86 respectively (Table 3.11, raw data in table C5). When the control counts were compared to the test counts at both the concentrations using a student t test, it was found that both combination concentrations reduced the adherence of *C. auris* significantly with p values <0.05 (Figure 3.4).

The effect of combination of fluconazole and pantoprazole at MIC and ½ MIC concentrations on the proteinase production in *C. auris* was also studied. The results showed that for the control, the mean Pz value was 0.51 whereas for the ½ MIC and MIC Pz was 0.67 and 0.8 respectively (Table 3.12, raw data in table C5). When the control Pz was compared to the test Pz values at both the concentrations using a student t test, it was found that both combination concentrations reduced the proteinase production in *C. auris* significantly with p values of <0.001 (Figure 3.5).

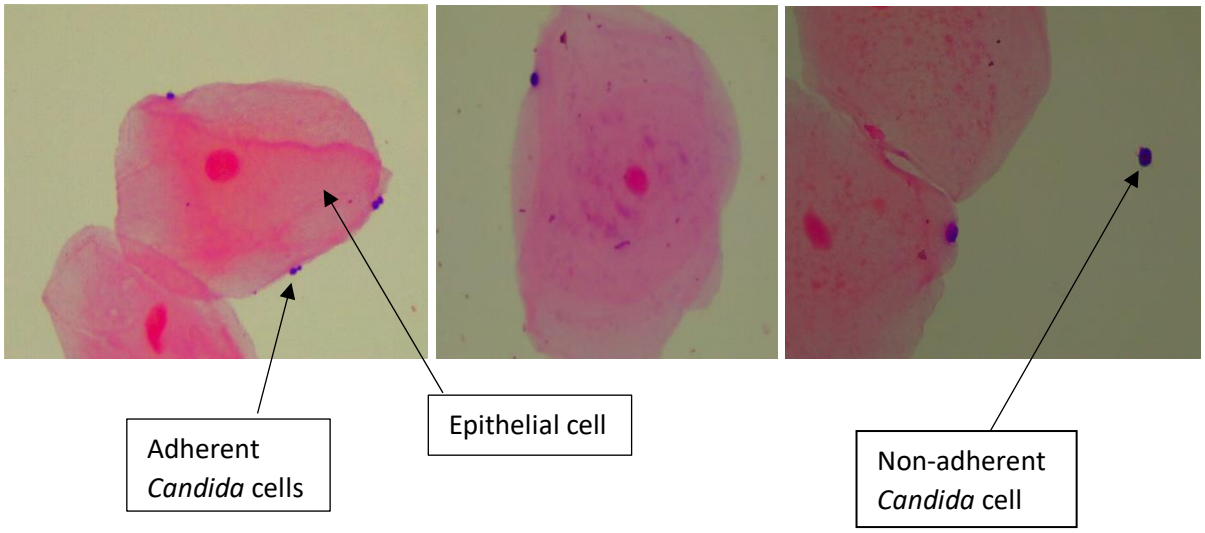


Figure 3.2: Epithelial cell (pink) with adherent and non-adherent *Candida* cells (violet). The cells were viewed under a 100X objective.

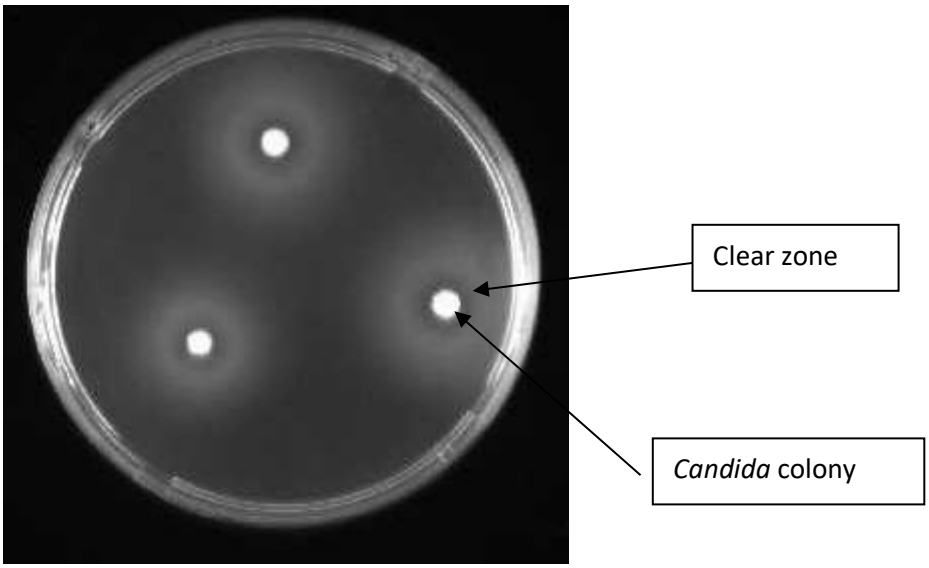


Figure 3.3: The proteinase activity of *Candida* strains on BSA agar. Proteinase production is marked by clear zones around the colonies.

Table 3.11: The effect of various concentrations of fluconazole and pantoprazole combinations on the adherence activity of *C. auris* representative strain (MRL6339).

***Candida* auris** MRL 6339	**FLC-PTP concentrations**		
	0	**1/2 MIC**	**MIC**
No of adherent *Candida* cells Mean±SD	204±18	185±22	86±24

- MIC for FLC - fluconazole (125 µg/ml) and PTP - pantoprazole (3125 µg/ml).

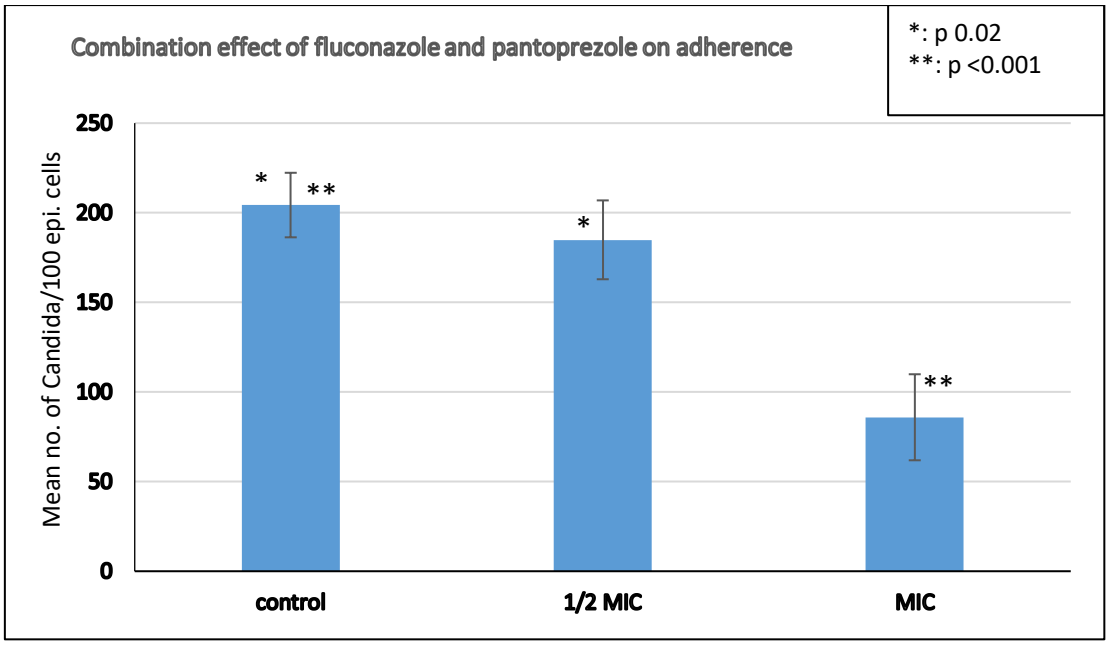


Figure 3.4: The effect of various concentrations of fluconazole and pantoprazole combinations on the adherence activity of *C. auris* representative strain (MRL6339). *: comparison of control to ½ MIC combination, **: comparison of control to MIC combination.

Table 3.12: The effect of various concentrations of fluconazole and pantoprazole combinations on the proteinase activity of *C. auris* representative strain (MRL6339).

***Candida* auris** MRL 6339	**FLC-PTP concentrations**		
	0	**1/2 MIC**	**MIC**
Proteinase activity (Pz value) Mean±SD	0.51±0.02	0.67±0.06	0.8±0.04

- MIC for FLC- fluconazole (125 µg/ml) and PTP- pantoprazole (3125 µg/ml).

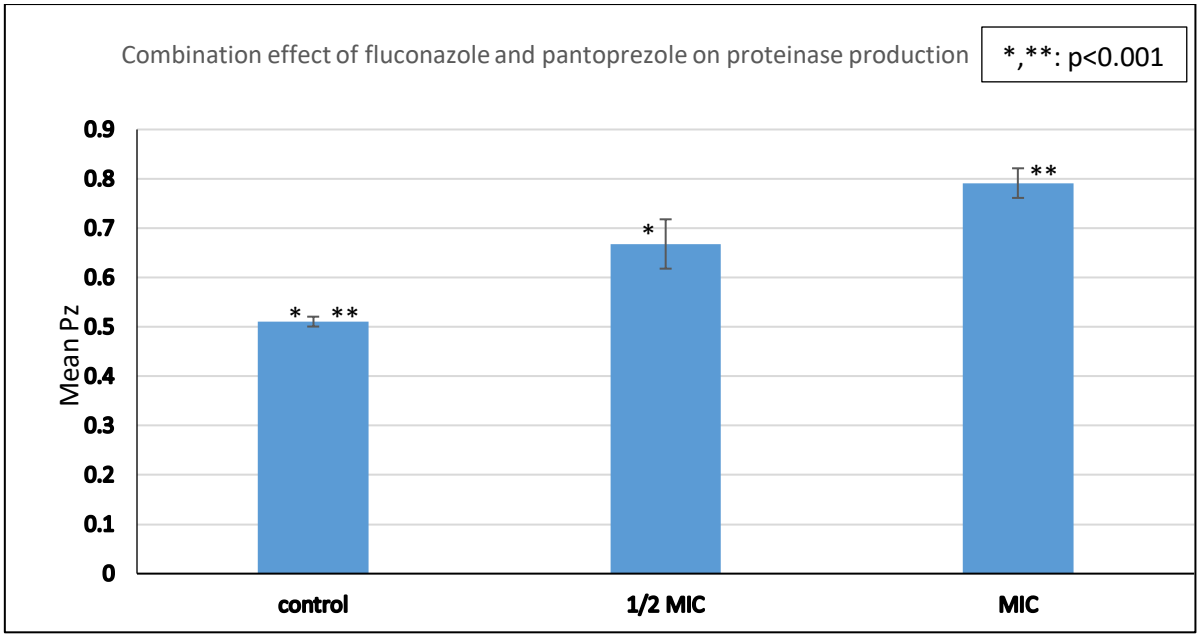


Figure 3.5: The effect of various concentrations of fluconazole and pantoprazole combinations on the proteinase activity of *C. auris* representative strain (MRL6339). *: comparison of control to ½ MIC combination, **: comparison of control to MIC combination.

3.7 Effect of combination of fluconazole and pantoprazole on efflux pump

To investigate the effect of fluconazole and pantoprazole on energy-dependent efflux activity, the uptake and efflux of R6G by fluconazole-resistant *C. auris* cells (MRL6015) was studied (Figure 3.6). For comparison, R6G activity in the absence of the test agents was observed in *C. auris* and fluconazole-sensitive *C. albicans* SC5314.

R6G uptake increased shortly after *C. auris* and *C. albicans* cells were incubated in glucose-free PBS. This is indicated by a sharp drop in the extracellular concentration of R6G after 10 minutes of incubation. Intracellular uptake of R6G stabilised after 20 minutes of incubation. The addition of 0.1 M glucose induced a sharp increase in R6G efflux in control *C. auris* cells and cells exposed to FLC at MIC. This is displayed by an increase in extracellular R6G concentrations after 30 minutes of incubation. *C. auris* cells with PTP only at MIC values displayed a slight increase in R6G efflux. In contrast, *C. auris* cells exposed to the combination

(FLC+PTP) do not show immediate efflux of R6G after 30 minutes of incubation. A similar observation was made in *C. albicans* cells.

A delay in R6G efflux was detected in *C. auris* cells exposed to the combination (FLC+PTP) at ¼ MIC, ½ MIC and MIC values, relative to the *C. auris* control. This is shown by a sharp increase in extracellular R6G concentration after 50 minutes of incubation, instead of 30 minutes. The red dashes represent the length of time R6G spent in the cells exposed to FLC+PTP. Within the 10-50 min time window, the combination yielded a wider stretch of the curves at ½ MIC and MIC, relative to ¼ MIC. Which indicated that RG6 spent more time in *C. auris* cells exposed to FLC+PTP at ½ MIC and MIC values. The combination at all tested concentrations yielded trend-lines similar to *C. albicans*.

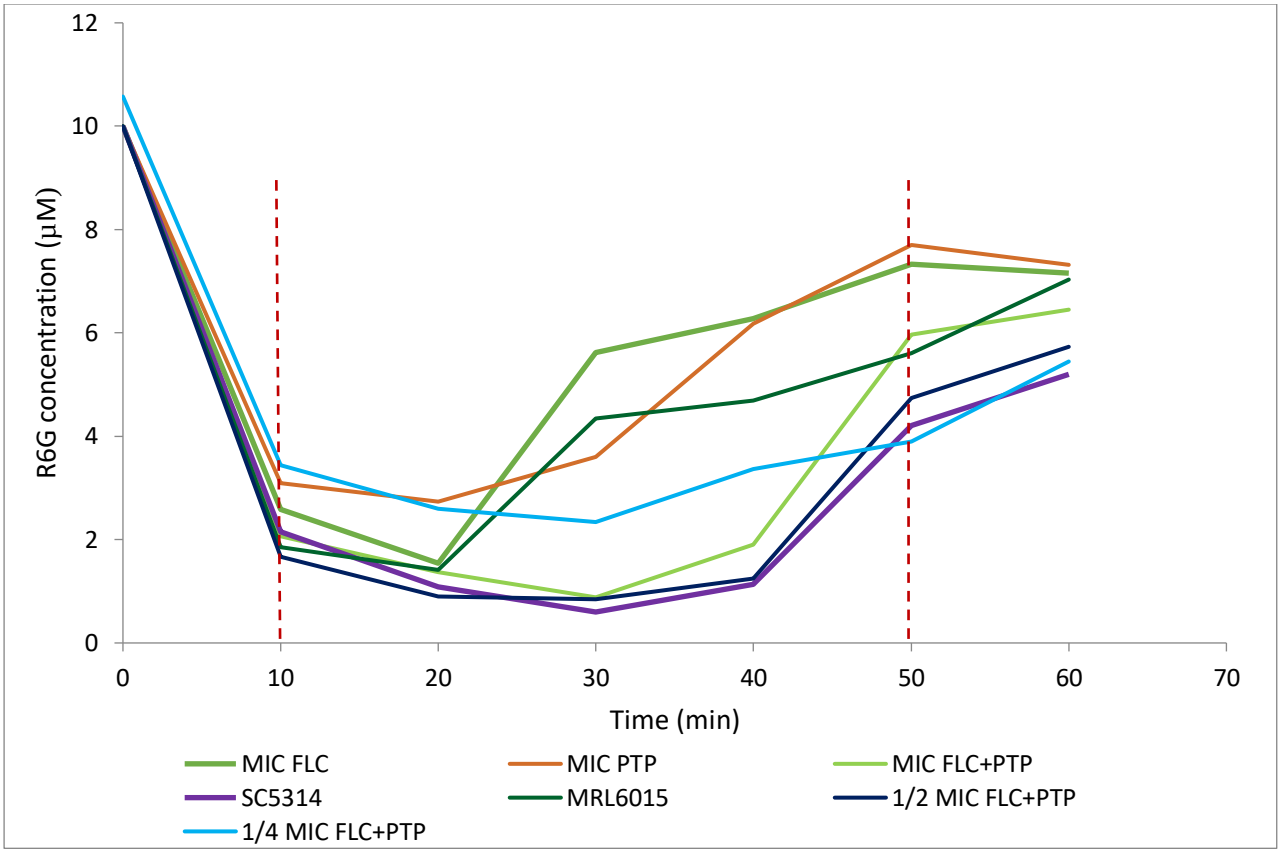


Figure 3.6: The extracellular uptake and efflux of R6G in fluconazole-resistant *C. auris* MRL6015 and sensitive *C. albicans* SC5314. R6G efflux activity was studied in *C. auris* cells exposed to FLC and PTP alone and in combination at their ¼ MIC, ½ MIC and MIC values

(orange, red, light green, dark blue, light blue). R6G efflux in the absence of FLC and PTP functioned as controls (dark green and purple). Energy-dependent R6G efflux was activated by adding 0.1 M glucose to the cells after 25 min. Absorbance of the aliquots collected at 0, 10, 20, 30, 40, 50 and 60 min time points was measured at 527 nm. Extracellular R6G concentration was determined with a standard curve.

CHAPTER 4: DISCUSSION

Most recently identified *Candida auris* has become a unique species for a number of reasons. It causes outbreaks in hospitals and long term institute facilities due to its ability to survive on surfaces increasing transmissibility (Chowdhary et al. 2017). It also has intrinsic resistance to many conventional antifungal drugs making treatment difficult (Lockhart et al. 2017). In addition, the identification of *C. auris* is difficult, delaying diagnosis and treatment (Chowdhary et al. 2017). Therefore, development of new drugs and treatment strategies are necessary. Proton pump inhibitors are known to have antifungal activities (Liu and Köhler 2016). In addition, combination therapy is known to have good therapeutic effectiveness (Spitzer et al. 2017). Therefore the use of proton pump inhibitors in conjunction with conventional drugs was studied.

4.1 Antifungal susceptibility testing in *C. auris*

The *in vitro* antifungal susceptibility testing has become routine in a number of clinical laboratories. This is because of increasing reports of antifungal resistance in *Candida* species such as *C. auris*. The CLSI broth-microdilution method is extensively used to determine resistant and sensitive *Candida* isolates against antifungal drugs. Other frequently used methods include Etest, Vitek® 2, and Sensititre™ YeastOne™ (Lone and Ahmad 2019). The MIC values for fluconazole are consistently high across these methods (Lone and Ahmad 2019). In contrast, there are differences in the MIC values for amphotericin B amongst these methods (Lone and Ahmad 2019). For example, MIC values for amphotericin B are elevated with Vitek® 2, relative to Etest and broth-microdilution (Lockhart 2019). Furthermore, there is evidence to suggest that the broth-microdilution method is not robust in determining amphotericin B MIC values in *C. auris*, relative to automated methods like Etest (Lockhart 2019). Therefore, MIC values obtained in this study may not be the best representation of amphotericin B susceptibility in these isolates. It has been reported that Etest and

Sensititre™ YeastOne™ commercial systems display significant agreement with CLSI data for amphotericin B susceptibility tests (Ruiz-Gaitán at al. 2019). Therefore, the use of Etest and Sensititre™ YeastOne™ instead of CLSI broth-microdilution should be considered when determining susceptibility to amphotericin B.

4.1.1 MIC and MFC of antifungal agents (amphotericin B and fluconazole)

Results from this study showed that 92% of the *C. auris* isolates were resistant to fluconazole (MIC ≥ 32 µg/ml) and 100% sensitive to amphotericin B (MIC≤ 2 µg/ml). These findings are consistent with the results from other studies on South African *C. auris* isolates. Magobo et al (2020) found that 97% of 85 *C. auris* isolates were resistant to fluconazole and all were sensitive to amphotericin B (Magobo et al. 2020). In a more comprehensive study that included 400 *C. auris* isolates, 90% were resistant to fluconazole and only 0.3% displayed resistance to amphotericin B (Maphanga et al. 2021). Similarly, high resistance to fluconazole has been recorded in many other regions in the USA, East Asia and South Asia (Calvo et al. 2016; Chowdhary et al. 2013; Lockhart et al. 2017). Although a significant number of sensitive isolates have been found in some regions, global reports of tested isolates indicate that *C. auris* is highly resistant to fluconazole. These findings confirm that fluconazole cannot be used alone to treat *C. auris* infections.

Currently available susceptibility data implies that *C. auris* is often characterized by high MICs for fluconazole whereas there is variable susceptibility to other antifungal agents, including amphotericin B (Arendrup et al. 2017). *In vitro* susceptibility of amphotericin B against *C. auris* is inconsistent across multiple studies. Like this study, multiple reports show that *C. auris* is sensitive to amphotericin B but others have reported substantial resistance. For example, a wide range of MIC values were found in a study of 104 *C. auris* isolates, with some having a high MIC of 8 µg/ml (Prakash et al. 2016). Similarly, another study has found high MIC values of >1 µg/ml for amphotericin B in 50% *C. auris* isolates (Calvo et al. 2016). In general,

resistance to amphotericin B is uncommon but most *C. auris* populations display 0-30% resistance. Nonetheless, the inconsistent MIC results suggest that an antifungal susceptibility test should be performed before defining therapy that requires amphotericin B (Calvo et al. 2016).

C. auris is globally recognized as a multidrug resistant (resistant to 2 or more classes of antifungals) pathogen. This is because there is evidence to show that about 40-50% of reported *C. auris* isolates are multidrug resistant (Kathuria et al. 2015; Lockhart 2019; Sarma and Upadhyay 2017). The *C. auris* isolates used in this study could not be classified as multidrug resistant since they showed resistance to fluconazole only. In addition to the issue of using CLSI methods for amphotericin B MIC values, it is possible that these isolates could be classified as MDR if more antifungal drugs were tested.

4.1.2 MIC and MFC of proton pump inhibitors (pantoprazole and omeprazole)

It has been shown that proton pump inhibitors have antifungal activity against *Candida* species (Liu and Köhler 2016). Currently, there are no provisional MIC breakpoints established for proton pump inhibitors against *C.auris*. Therefore, the MIC values for pantoprazole and omeprazole could not be interpreted as resistant and sensitive. In this study, it was found that the MIC values for both proton pump inhibitors (pantoprazole and omeprazole) were much higher than those of the antifungal drugs (amphotericin B and fluconazole). This indicated that the proton pump inhibitors had very weak antifungal activity against all tested *C. auris* isolates. Although observed in *C. albicans*, other studies also show that high concentrations of proton pump inhibitors are required to inhibit yeast growth. Respectively, MIC values as high as 256 µg/ml and > 600 µg/ml for pantoprazole and omeprazole were recorded (Biswas et al. 2001; El-Ganiny et al. 2022). The MIC values for omeprazole and pantoprazole obtained in this study are higher than those previously recorded in *C. albicans* (Biswas et al. 2001; El-Ganiny et al. 2022). These findings suggest that the tested *C. auris* isolates are more tolerant to PPIs than *C.*

albicans. However, these observations indicate that omeprazole and pantoprazole alone may not be suitable for treating *Candida* infections. This is because the PPI concentrations required to inhibit pathogen growth are too high for therapeutic applications.

4.2 Combination interaction of PPIs and FLC

Proton Pump Inhibitors (PPIs) are the most effective drugs for suppressing gastric acids hence they are used to treat acid-related illnesses. PPIs have a wide range of clinical applications, including combination therapy with fluconazole against *Candida* infections.

In the current study, it was found that the fluconazole and pantoprazole combination had a synergistic effect against the majority of the *C. auris* isolates (56%) and they were all resistant to fluconazole. The MIC values of fluconazole were reduced by 4 folds when combined with pantoprazole. These findings indicate that pantoprazole increased the sensitivity of resistant *C. auris* isolates to fluconazole. Consistent with these results, El-Ganiny (2022) found that pantoprazole reduced fluconazole resistance in *C. albicans* and NAC-species, *C. dubliniensis, C. glabrata,* C. *krusei*, *C. parapsilosis,* and *C. tropicalis*. Similarly, Lu et al (2020) was the first to indicate that PPIs can act synergistically with fluconazole against resistant *C. albicans* isolates. It was shown that the MICs of fluconazole decreased from > 512 µg/ml to 1µg/ml in combination with pantoprazole (Lu et al. 2020). The results show that there is evidence to support the use of pantoprazole to reduce fluconazole resistance against clinically relevant *Candida* species, including *C. auris*. Azole resistance occurs through various mechanisms including increased efflux activity and target gene erg11 overexpression. In the present study, the high reduction in fluconazole MIC values in combination could have resulted from the blockage of *C. auris* efflux pump activity by pantoprazole. Decreased efflux pump activity would result in an increase in the concentration of fluconazole in *C. auris* cells. This suggestion is based on the previous findings that synergistic drug combinations that reduced fluconazole resistance also decreased efflux pump activity (El-Ganiny et al. 2022; Lu et al 2020; Vega-

Chacón et al. 2021). Gene expression analysis of azole resistance genes in *C. auris* could give a clearer explanation for the susceptibility results of the combination.

The results of the present study showed that the drug combination of fluconazole and omeprazole has an antagonistic effect against most of the tested *C. auris* isolates (56%). Important to note, this antagonistic interaction was observed in all resistant *C. auris* isolates. There was a 2 fold increase in the MIC values of omeprazole when combined with fluconazole. Similarly, it has been reported that omeprazole antagonises the antifungal activity of fluconazole against resistant *C. albicans* isolates (Urai et al. 2014). Urai et al (2014) has shown that omeprazole increased the MIC values of fluconazole by 4 fold in *C. albicans*. In the same study, it was proposed that omeprazole increased *C. albicans* sensitive to fluconazole by inducing the activity of an efflux pump (Urai et al. 2014). Other studies have also found that omeprazole antagonizes fluconazole in susceptible *C. albicans* isolates (Kaneko et al. 2013; Liu and Köhler 2016). Rabeprazole, an analogue of omeprazole, also decreased the antifungal activity of fluconazole against *C. albicans* (Liu and Köhler 2016). In contrast to these findings, it has been reported that there is synergism between omeprazole and fluconazole against resistant *C. albicans* (Lu et al. 2020). This indicates that more combination interaction studies should be performed for *Candida* isolates for the discovery of novel treatment strategies.

4.3 Time-kill kinetics

Time-kill curves are used to determine the effect of antimicrobial compounds over time by providing information on the growth rate of pathogens (Mukherjee et al. 2005). Furthermore, time-kill curves can be used to establish the effect of drug combinations on cell viability (Mukherjee et al. 2005). In the current study, it was found that fluconazole at its ½ MIC values did not have an effect on the viability (cfu/ml) of a *C. auris* representative strain (MRL6015). In contrast, cell viability decreased when the cells were exposed to pantoprazole at ½ MIC values. The fluconazole and pantoprazole combination yielded the highest decrease in cell

viability. It was further demonstrated that the combination was synergistic against the resistant *C. auris* isolate. This is consistent with the interpretation of FICI values obtained in the current study. Therefore, the time-kill assay was successfully used to confirm the interaction effect between fluconazole and pantoprazole against resistant *C. auris*.

Unlike the results of the current study, disagreements between time-kill curves and FICI interpretations have been observed. For example, Li et al (2008) found inconsistent results between FICI values and time-kill curves for the combination interaction of azoles with cyclosporine A against sensitive *C. albicans*. Interestingly, the same study found that there was an agreement in the interpretation of results between the two methods in resistant *C. albicans* isolates (Li et al. 2008). These observations show that multiple methods should be used to determine the combination interaction of drugs in *Candida* species to improve the accuracy of the results.

4.4 Virulence assays

Pathogenic *Candida* species use virulence factors to establish an infection in human hosts. As such, it is important to identify and characterise virulence features in clinically relevant *Candida* species. Since *C. auris* is a recently emerged pathogen, very few studies are available to describe its virulence factor. Nonetheless, *C. auris* has been shown to express a myriad of virulence factors including adhesion factors and hydrolytic enzymes. In the present study, 25 *C. auris* isolates were screened for adhesion and the production of proteinases.

4.4.1 *C. auris* adhesion to oral epithelial cells

Adhesion of *Candida* species is a vital step during the initial colonization and infection of epithelial cells. Whilst most studies discuss the adhesion of *C. auris* to abiotic surfaces, there is very limited data on *C. auris* adhesion to epithelial cells. In our study, we found that all tested *C. auris* isolates were capable of attaching to oral epithelial cells. However, the number of *C. albicans* cells adhered to epithelial cells was greater than *C. auris* cells. This is consistent

with previous reports that *C. auris* adherence to buccal epithelial cells is reduced, relative to *C. albicans* (Shaban et al. 2020). *C. albicans* adhesion to epithelial cells is mostly facilitated by adhesins of the Als protein family. Gene expression analysis by Muñoz et al (2018) showed that *C. auris* expressed less Als proteins, compared to *C. albicans*. Therefore, is possible that *C. auris* displays reduced adherence to buccal epithelial cells because it expresses less Als adhesins.

Although Als proteins play a vital role in adherence to epithelial cells, other molecular interactions facilitate this process (Nikou et al. 2019). For example, *C. albicans* has receptors that can bind to carbohydrates, fibronectin and salivary proteins found on the surface of human cells (Nikou et al. 2019). Similarly, the expression of hydrophobins is positively associated with adherence in *C. albicans* (Nikou et al. 2019). Therefore, it is plausible that decreased adherence in *C. auris* is attributed to other features such as cell surface hydrophobicity and the composition of the cell-surface. The results of our study indicate that more studies are required to characterise the molecular mechanisms that facilitate adherence of *C. auris* to epithelial cells.

4.4.2 Proteinase production in *C. auris*

The extracellular secretion of hydrolytic enzymes is recognized as a relevant feature of *Candida* pathogenicity in humans. Hydrolytic enzymes have several functions in *Candida* cells, which include nutrient acquisition, adherence and invasion of host cells. Analysis of the *C. auris* genome show that hydrolases are the prominent type of enzymes (Chatterjee et al. 2015). Furthermore, strain-dependent production of *C. auris* has been reported by several studies (Kumar et al. 2016; Larkin et al. 2017). Nonetheless, few studies are available to describe the production of lytic enzymes in *C. auris*.

In the current study, we showed that 96% of all tested *C. auris* strains (24/25) can produce proteinase. Amongst the strains that tested positive for proteinase activity, most of them displayed very strong proteinase activity. These findings are in agreement with results from

previous studies, where proteinase activity was found in the majority of the tested isolates (Larkin et al. 2017; Shaban et al. 2020). Furthermore, the same studies found very strong proteinase production in the majority of the tested isolates (Larkin et al. 2017; Shaban et al. 2020). Combined with our study, these results indicate that proteinase production is a vital virulence factor in *C. auris*. Our study also shows that the series of *C. auris* strains tested had different levels of proteinase production. This supports the previous observations that the expression of virulence factors, including hydrolytic enzymes is strain-dependent (Kumar et al. 2015; Larkin et al. 2017).

4.5 Effect of the combination on virulence factors

Combination therapy is used to improve the efficiency of available drugs through multiple mechanisms, which include increasing the number of drug targets, enhancing bioavailability or reducing toxicity (Ghannoum et al. 1995). For example, fungistatic drugs like azoles can be fungicidal in combination with other drugs (Spitzer et al. 2017). Therefore, this treatment strategy can be used to re-sensitize resistant pathogens to antifungal-drugs.

There is growing interest in the use of combination treatments to target virulence factors in resistant *Candida* species. Virulence factors appeal as potential drug targets because decreasing their activity reduces the development of drug resistance. PPIs alone have anti-virulence activity against *Candida* species. For example, lansoprazole can decrease the formation of hyphae in *C. albicans* (Biswas et al. 2001). Very few studies indicate the anti-virulence activity of PPIs in combination with azoles.

In the current study, we found that the fluconazole and pantoprazole combination has anti-virulence activity against the tested *C. auris* isolates. The combination had an effect on the adherence ability and proteinase secretion in resistant *C. auris* isolates. It was found that the combination at both MIC and ½ MIC values significantly decreased adherence in the tested *C. auris* isolate (p values < 0.05). These results suggest that the combination can impede the

adherence of *C. auris* cells to epithelial cells. In doing so, the combination can prevent the initial step of infection.

We also found that the pantoprazole and fluconazole combination at MIC and ½ MIC values reduced proteinase activity in the representative *C. auris* isolate significantly (p values < 0.01). The combination at ½ MIC values lessened the level of proteinase secretion from "very strong activity" to "strong activity". At MIC values, the combination reduced the proteinase activity level to "medium activity". These results indicate that the combination can inhibit the hydrolytic activity of SAPs in *C. auris*. Therefore, it may be concluded that this combination can impede active penetration of epithelial cells. Since SAPs have multiple functions, inhibition of proteinase activity by the combination may also impede other molecular processes that support *C. auris* virulence. For instance, the degradation of host tissues and the evasion of immune response systems in host cells may be impeded by the combination. Overall, these findings indicate that the combination of fluconazole and pantoprazole has notable anti-virulence activity against *C. auris*. Consistent with our results, Lu et al (2020) found that phospholipase secretion and phenotypic switching was reduced in *C. albicans* when fluconazole was combined with PPIs.

4.6 Effect of combination on R6G efflux activity

Antifungal resistance to azoles is predominantly caused by increased activity of multidrug efflux pumps (Liu et al. 2015; Mane et al. 2016). R6G and Hoechst 33342 fluorescent dyes can be used to trace energy-dependent efflux activity. This is because these dyes are substrates of multiple MDR efflux pumps (Maesaki et al. 1999).

In our assay, we used the R6G dye to track efflux pump activity. Without exposing the cells to test agents, we found that both resistant *C. auris* cells and sensitive *C. albicans* cells could accumulate R6G in the absence of glucose. This is consistent with previous reports, where it was shown that the extracellular concentration of R6G decreased rapidly in glucose-free

conditions (Ahmad et al. 2013; Maesaki et al. 1999). R6G efflux was increased by the addition of glucose in resistant *C. auris* cells. These findings suggest that the *C. auris* cells were capable of decreasing the intracellular accumulation of fluconazole by inducing energy-dependent efflux activity. Therefore, it was concluded that efflux pump activity was one of the mechanisms of fluconazole-resistance in the tested *C. auris* isolate.

It has been reported that sensitivity to azoles can be restored by inhibiting efflux pump activity in resistant *Candida* isolates (Liu et al. 2015; Mane et al. 2016). In the current study, we found that the efflux activity of a resistant *C. auris* isolate was impeded by the combination of fluconazole and pantoprazole. Sub-inhibitory and inhibitory concentrations of the combination delayed energy-dependent efflux activity. The intracellular concentration of fluconazole was not reduced rapidly in *C. auris* cells that were exposed to the combination. This suggests that the combination extended the time for the antifungal agents to interact with drug targets in *C. auris* cells. Overall, these results show that pantoprazole attenuated fluconazole resistance by decreasing ABC-type efflux activity.

Consistent with our results, it was shown that a PPI verampil can inhibit efflux activity in *C. albicans*. Furthermore, verampil and fluconazole had synergistic antifungal activity against *C. albicans* (Vega-Chacón et al. 2021). Similarly, El-Ganiny et al (2022) recently showed that pantoprazole significantly decreased the expression of CDR1 and MDR1 efflux pumps in *C. albicans*. In the same study, the results were confirmed by molecular docking of pantoprazole into the efflux pump proteins of *C. albicans* (El-Ganiny et al. 2022). It was found that pantoprazole binds to the cavity of the active site in *C. albicans* CDR1 and MDR1 efflux pumps (El-Ganiny et al. 2022). Furthermore, it was observed that pantoprazole has good affinity for CDR pumps (El-Ganiny et al. 2022). These results confirmed that pantoprazole can block the function of ABC efflux pumps.

4.7 Clinical implications

The study has shown that *C. auris* isolates are highly resistant to fluconazole and it should not be used to treat *C. auris* infections alone. Proton pump inhibitors have antifungal activity against *C. auris*. However, it may not be realistic to use them for treating *C. auris* infections because very high doses may be required to inhibit this pathogen. In contrast, fluconazole and pantoprazole in combination can inhibit *C. auris* growth at lower concentrations. During combination treatment, lower doses of each drug will be required to impede *C. auris* growth. The anti-virulence activity of the combination will prevent surviving *C. auris* cells from causing an infection due to its reduced virulence. Furthermore, the combination has therapeutic potential because it can re-sensitize resistant *C. auris* cells to fluconazole.

4.8 Limitation

The limitation of this study is that it is done *in vitro*. An *in vivo* study should be done to investigate the effect of the combination treatment in a living organism. In doing so, we can observe other important factors such as the pharmacokinetics of the drug combination.

4.9 Future research

- Performing the same study on a larger number of *C. auris* isolates from different geographic clades would make the findings more inclusive.
- Using a larger set of proton pump inhibitors may assist in finding more synergistic interactions with fluconazole.
- A study on the effect of the fluconazole and pantoprazole combination *in vivo* would provide information regarding the clinical implications of the treatment strategy.
- The effect of the combination on the expression of resistance and virulence genes in *C. auris* can be determined to support the use of this therapy further.

CHAPTER 5: CONCLUSION

The present study showed that *C. auris* isolates are highly resistant to fluconazole. The virulence of the *C. auris* isolates may be attributed to their ability to adhere to human epithelial cells and to produce proteinases. The tested PPIs displayed antifungal activity against resistant *C. auris* alone, with pantoprazole being the most active. However, very high concentrations of both PPIs alone are required to inhibit *C. auris* growth. Pantoprazole displayed synergism with fluconazole against resistant *C. auris* isolates. The pantoprazole and fluconazole combination can significantly reduce virulence activity of resistant *C. auris* isolates at sub-inhibitory and inhibitory concentrations. This suggests that the combination can prevent the initial step of infection by inhibiting the adherence of *C. auris* to epithelial cells. The cells that manage to adhere will be prevented from invading host cells via active penetration because proteinase production is impeded. In that way, the combination reduces *C. auris* virulence through multiple mechanisms. It was also shown that fluconazole resistance was caused by efflux-pump activity. The combination delayed fluconazole efflux from the *C. auris* cells. Therefore, the combination can attenuate fluconazole resistance by blocking ABC type efflux activity. Overall, these findings indicate that pantoprazole has the potential to enhance the efficiency of fluconazole to improve treatment outcomes.

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APPENDICES

Appendix A

Figure A1: Ethical clearance waiver

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

27/09/2020

Ref: W-CBP-200827-03

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/Professor K/M Galane/Patel Student No. (if appropriate): 1149444 Staff No. (if appropriate):
Supervisor:	Dr A Ahmad
School: **Department:**	Pathology Clinical Microbiology & Infectious Diseases Medical School University
Project title:	*Combination interaction of proton pump inhibitors with fluconazole against multidrug-resistant Candida auris*
Reason:	In vitro laboratory study. No human participants will be involved in the study.

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

Research Office Secretariat:
Physical address: Phillip Tobias Building, 3rd Floor, Office 302, Corner York Road and Princess of Wales Terrace, Parktown, Johannesburg 2193.
Postal address: Private Bag 3, Wits 2050
Tel Nos. +27 (0)11-717-1234/2656/2700/1252
Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za.
Website:http://www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Appendix B: Preparation and composition of media and buffers

Bovine Serum Albumin Agar

- 1.45 g Ammonium Sulphate
- 1.45 g Yeast nitrogen base without amino acids
- 2 g BSA
- 4 g Glucose
- 20 g Agar
- 1L Distilled water
- BSA was dissolved in distilled water and filter sterilized
- The rest of the dry ingredients were dissolved in distilled water, autoclaved for 30 minutes at 121°C and cooled to 45 °C.
- BSA was added to the cooled mixture and poured onto petri dishes.

Sabouraud Dextrose Agar

- 60g Sabouraud Dextrose Agar (Sigma-Aldrich, South Africa)
- 1L Distilled water
- The SDA was completely dissolved in distilled water and autoclaved at 121 °C for 30 minutes. Thereafter, the solution was cooled to 40-45 °C and then poured onto sterile petri plates.

Sabouraud Dextrose Broth

- 30g Sabouraud Dextrose Broth (Sigma-Aldrich, South Africa)
- 1L Distilled water
- SDB was thoroughly mixed with distilled water and autoclaved at 121 ° C for 30 minutes.

Normal saline

- 0.85 g Sodium Chloride
- 1L Distilled water
- Sodium Chloride was dissolved in distilled water

Appendix C: Raw data for findings

Table C1: MIC and MFC of amphotericin B, fluconazole, pantoprazole and omeprazole against *C. auris* and *C. albicans.*

***Candida* strains**	**Replicates**	**AMP (µg/ml)**		**FLC (µg/ml)**		**PTP (µg/ml)**		**OME (µg/ml)**	
		MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
SC 5314	1	0.156	0.625	15.6	N/A	3125	6250	15625	31250
	2	0.156	0.625	7.8	N/A	3125	6250	15625	31250
	3	0.156	0.312	7.8	N/A	3125	6250	15625	31250
MRL 2397	1	0.625	1.25	31.25	N/A	3125	6250	15625	31250
	2	0.625	1.25	31.25	N/A	3125	6250	15625	31250
	3	0.625	1.25	31.25	N/A	3125	6250	15625	31250
MRL 2921	1	1.25	2.5	125	N/A	3125	6250	15625	31250
	2	1.25	2.5	125	N/A	3125	6250	15625	31250
	3	1.25	2.5	125	N/A	3125	6250	15625	31250
MRL 3499	1	0.625	1.25	62.5	N/A	3125	6250	15625	31250
	2	0.625	1.25	62.5	N/A	3125	6250	15625	31250
	3	0.625	1.25	62.5	N/A	3125	6250	15625	31250
MRL 3758	1	0.312	0.625	31.25	N/A	3125	6250	7812	15625
	2	0.312	0.625	31.25	N/A	3125	6250	7812	15625
	3	0.312	0.625	31.25	N/A	3125	6250	7812	15625
MRL 4000	1	0.625	1.25	125	N/A	781	1562	15625	31250
	2	0.625	1.25	125	N/A	781	1562	15625	31250
	3	0.312	0.625	125	N/A	781	1562	15625	31250
MRL 4587	1	0.625	1.25	250	N/A	3125	6250	15625	31250
	2	0.312	0.625	250	N/A	3125	6250	15625	31250
	3	0.312	0.625	250	N/A	3125	6250	15625	31250
MRL 4888	1	0.625	1.25	125	N/A	3125	6250	15625	31250
	2	0.625	1.25	125	N/A	3125	6250	15625	31250
	3	0.625	1.25	125	N/A	3125	6250	15625	31250
MRL 5418	1	1.25	2.5	31.25	N/A	3125	6250	7812	15625
	2	1.25	2.5	62.5	N/A	3125	6250	7812	15625
	3	1.25	2.5	62.5	N/A	3125	6250	7812	15625
MRL 5762	1	1.25	2.5	125	N/A	781	1562	15625	31250
	2	1.25	2.5	125	N/A	781	1562	15625	31250
	3	0.625	1.25	125	N/A	781	1562	15625	31250
MRL 5765	1	1.25	2.5	125	N/A	3125	6250	15625	31250
	2	1.25	2.5	125	N/A	3125	6250	15625	31250
	3	1.25	2.5	125	N/A	3125	6250	15625	31250

- AMP- amphotericin B; FLC- fluconazole; PTP- pantoprazole; OME- omeprazole; N/A - not applicable, SC 5314 – C. *albicans,* All MRL – *C. auris.*

Table C1: MIC and MFC of amphotericin B, fluconazole, pantoprazole and omeprazole against *C. auris* and *C. albicans* – continued.

***Candida* strains**	**Replicates**	**AMP (µg/ml)**		**FLC (µg/ml)**		**PTP (µg/ml)**		**OME (µg/ml)**	
		MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
MRL 6005	1	1.25	2.5	125	N/A	3125	6250	15625	31250
	2	1.25	2.5	125	N/A	3125	6250	15625	31250
	3	1.25	2.5	125	N/A	3125	6250	15625	31250
MRL 6015	1	0.312	0.625	250	N/A	3125	6250	15625	31250
	2	0.312	0.625	250	N/A	3125	6250	15625	31250
	3	0.312	0.625	250	N/A	3125	6250	15625	31250
MRL 6057	1	1.25	2.5	250	N/A	3125	6250	15625	31250
	2	1.25	2.5	250	N/A	3125	6250	15625	31250
	3	1.25	2.5	250	N/A	3125	6250	15625	31250
MRL 6059	1	0.625	1.25	125	N/A	3125	6250	7812	15625
	2	0.625	1.25	125	N/A	3125	6250	7812	15625
	3	0.312	0.625	125	N/A	3125	6250	7812	15625
MRL 6065	1	1.25	2.5	125	N/A	3125	6250	7812	15625
	2	0.625	1.25	62.5	N/A	3125	6250	7812	15625
	3	0.625	1.25	62.5	N/A	3125	6250	7812	15625
MRL 6125	1	0.625	1.25	62.5	N/A	3125	6250	15625	31250
	2	0.625	1.25	62.5	N/A	3125	6250	15625	31250
	3	0.625	1.25	62.5	N/A	3125	6250	15625	31250
MRL 6173	1	0.312	0.625	62.5	N/A	3125	6250	7812	15625
	2	0.312	0.625	62.5	N/A	3125	6250	7812	15625
	3	0.312	0.625	62.5	N/A	3125	6250	7812	15625
MRL 6183	1	0.312	0.625	62.5	N/A	3125	6250	7812	15625
	2	0.312	0.625	62.5	N/A	3125	6250	7812	15625
	3	0.312	0.625	62.5	N/A	3125	6250	7812	15625
MRL 6194	1	0.312	0.625	125	N/A	3125	6250	15625	31250
	2	0.312	0.625	125	N/A	3125	6250	15625	31250
	3	0.625	1.25	125	N/A	3125	6250	15625	31250
MRL 6277	1	0.625	1.25	125	N/A	3125	6250	15625	31250
	2	0.625	1.25	125	N/A	3125	6250	15625	31250
	3	0.625	1.25	125	N/A	3125	6250	15625	31250
MRL 6326	1	0.312	0.625	125	N/A	3125	6250	15625	31250
	2	0.312	0.625	125	N/A	3125	6250	15625	31250
	3	0.312	0.625	125	N/A	3125	6250	15625	31250
MRL 6333	1	0.625	1.25	125	N/A	3125	6250	15625	31250
	2	0.625	1.25	125	N/A	3125	6250	15625	31250
	3	0.312	0.625	125	N/A	3125	6250	15625	31250

- AMP- amphotericin B; FLC- fluconazole; PTP- pantoprazole; OME- omeprazole; N/A - not applicable, SC 5314 – C. *albicans*, All MRL – *C. auris*.

Table C1: MIC and MFC of amphotericin B, fluconazole, pantoprazole and omeprazole against *C. auris* and *C. albicans* – continued.

***Candida* strains**	**Replicates**	**AMP (µg/ml)**		**FLC (µg/ml)**		**PTP (µg/ml)**		**OME (µg/ml)**	
		MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
MRL 6334	1	0.625	1.25	125	N/A	3125	6250	31250	6250
	2	0.625	1.25	125	N/A	3125	6250	31250	6250
	3	0.312	0.625	125	N/A	3125	6250	31250	6250
MRL 6338	1	1.25	2.5	125	N/A	3125	6250	15625	31250
	2	1.25	2.5	125	N/A	3125	6250	15625	31250
	3	1.25	2.5	125	N/A	3125	6250	15625	31250
MRL 6339	1	1.25	2.5	125	N/A	3125	6250	31250	6250
	2	1.25	2.5	125	N/A	3125	6250	31250	6250
	3	0.625	1.25	125	N/A	3125	6250	31250	6250

- AMP- amphotericin B; FLC- fluconazole; PTP- pantoprazole; OME- omeprazole; N/A - not applicable, SC 5314 – C. *albicans*, All MRL – *C. auris*.

Table C2: Raw data for adherence in *C. auris* and *C. albicans.*

***Candida* strains**	**No. of *Candida* cells adherent to 100 epithelial cells**		
	1	**2**	**3**
SC 5314	190	228	209
MRL2397	192	206	187
MRL2921	112	137	128
MRL3499	122	156	135
MRL3758	129	114	120
MRL4000	98	101	117
MRL4587	221	180	188
MRL4888	138	142	127
MRL5418	123	112	125
MRL5762	132	143	129
MRL5765	120	102	116
MRL6005	136	113	105
MRL6015	134	127	109
MRL6057	158	121	119
MRL6059	179	166	192
MRL6065	146	135	111
MRL6125	169	177	160
MRL6173	124	102	137
MRL6183	101	135	127
MRL6194	108	85	103
MRL6277	128	150	143

- SC 5314- *C. albicans*, All MRL- *C. auris.*

Table C2: Raw data for adherence in *C. auris* and *C. albicans*- continued.

MRL6326	110	122	136
MRL6333	148	160	159
MRL6334	137	142	150
MRL6338	162	158	170
MRL6339	216	222	234

- SC 5314- *C. albicans,* All MRL- *C. auris.*

Table C3: Raw data for proteinase activity in *C. auris* and *C. albicans*.

***Candida* strains**	**Proteinase activity (Pz value)**		
	1	**2**	**3**
SC5314	0.6	0.6	0.6
MRL2397	0.67	0.67	0.5
MRL2921	0.67	0.67	0.6
MRL3499	0.75	0.75	0.75
MRL3758	0.75	0.75	0.67
MRL4000	1	1	1
MRL4587	0.6	0.6	0.6
MRL4888	0.6	0.54	0.54
MRL5418	0.6	0.6	0.6
MRL5762	0.67	0.75	0.67
MRL5765	0.67	0.67	0.67
MRL6005	0.75	0.75	0.67
MRL6015	0.67	0.67	0.67
MRL6057	0.75	0.75	0.75
MRL6059	0.5	0.5	0.54
MRL6065	0.54	0.54	0.57
MRL6125	0.6	0.57	0.6
MRL6173	0.67	0.67	0.6
MRL6183	0.86	0.86	0.75
MRL6194	0.54	0.6	0.6
MRL6277	0.6	0.6	0.6
MRL6326	0.75	0.75	0.67
MRL6333	0.6	0.6	0.6
MRL6334	0.86	0.75	0.86
MRL6338	0.67	0.67	0.67
MRL6339	0.5	0.5	0.5

- SC 5314 – C. *albicans,* All MRL – *C. auris.*

Table C4: Raw data for the effect of various concentrations of fluconazole and pantoprazole combinations on the adherence activity of *C. auris* representative strain (MRL6339).

***Candida* strain**	**Replicates**	**No. of adherent *Candida* cells to 100 epithelial cells at various FLC-PTP concentrations**		
MRL6339		**0**	**1/2 MIC**	**MIC**
	1	216	186	81
	2	222	172	54
	3	234	148	98
	4	201	190	76
	5	198	188	68
	6	215	203	53
	7	184	204	90
	8	175	168	110
	9	208	166	99
	10	190	224	130

- MIC for FLC - fluconazole (125 µg/ml) and PTP - pantoprazole (3125 µg/ml).

Table C5 : Raw data for the effect of various concentrations of fluconazole and pantoprazole combinations on the proteinase activity of *C.auris* representative strain (MRL6339).

***Candida* strain**	**Replicates**	**Proteinase activity (Pz value)**		
		0	**1/2 MIC**	**MIC**
MRL6339	1	0.5	0.6	0.8
	2	0.5	0.67	0.8
	3	0.5	0.67	0.75
	4	0.54	0.75	0.86
	5	0.5	0.67	0.75
	6	0.54	0.6	0.8
	7	0.5	0.67	0.75
	8	0.5	0.6	0.75
	9	0.5	0.67	0.8
	10	0.5	0.67	0.75
	11	0.5	0.75	0.8
	12	0.54	0.6	0.8
	13	0.5	0.75	0.8
	14	0.5	0.75	0.8
	15	0.54	0.6	0.86

- MIC for FLC - fluconazole (125 µg/ml) and PTP - pantoprazole (3125 µg/ml).

Appendix D: Similarity index report

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