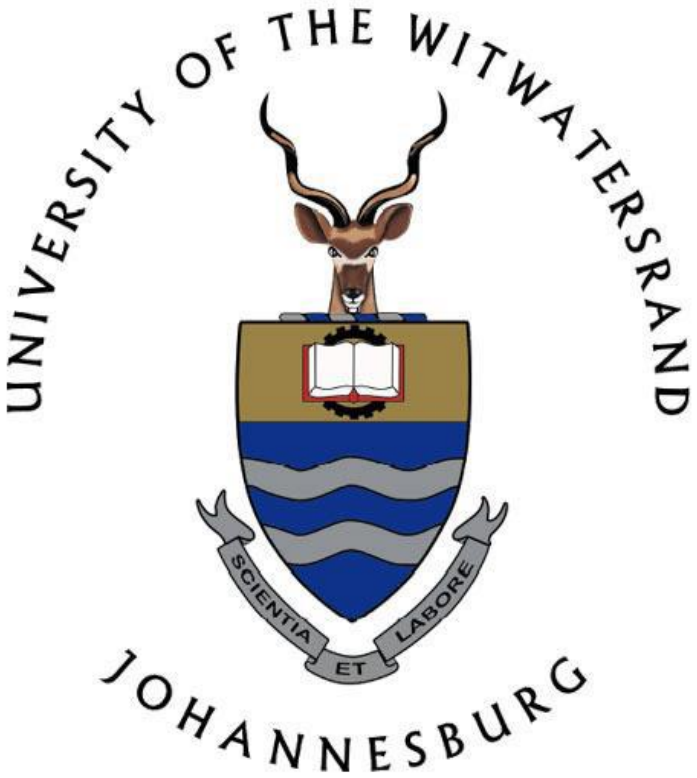
Expression and activity of oxidative stress enzymes in mediating fluconazole resistance in *Candida albicans* and their regulation by berberine

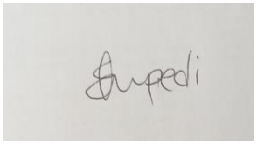
Evida Poopedi



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

Declaration

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



08 April 2019

Evida Poopedi

Date

350208

Student Number

Dedication

This dissertation is dedicated to my mother, Maphuthi Johannah Poopedi.

Publications and Presentations

Publication

Poopedi, E., Marimani, M.D. and Ahmad, A. Role of antioxidant defense in fluconazole susceptibility and its modulation by berberine in *Candida albicans*. Submitted to journal *PloS One*. (Refer to **Appendix 7.1** for abstract).

Presentations

Poster presentation: Poopedi, E., Marimani, M.D. and Ahmad, A. Upregulation of oxidative stress enzymes confers fluconazole resistance in *Candida albicans* clinical isolates. Faculty of Health Science Biennial Research Day and Postgraduate Expo. University of the Witwatersrand, Johannesburg, South Africa, 6 September 2018. (Refer to **Appendix 7.2** for abstract).

Poster presentation and flash talk: Poopedi, E., Marimani, M.D. and Ahmad, A. The effect of berberine on gene expression of antioxidant enzymes in *Candida albicans* clinical isolates. 9th Wits Cross Faculty Postgraduate Symposium. University of the Witwatersrand, Johannesburg, South Africa, 29th to 30th October 2018. (Refer to **Appendix 7.3** for abstract).

Abstract

Introduction

Despite the availability of several antifungal drugs, *Candida* infections remain a major health threat worldwide. The *Candida* infections problem has been amplified by the emergence of multidrug resistant *Candida* species towards the conventional antifungal drugs. In addition, activation of antioxidant defense system by *Candida* species has been known to be forefront mechanism to escape drug toxicity.This indicates an urgent need for the development of new therapeutic strategies and antifungal drugs. Natural products have served for centuries for the treatment of infectious diseases and are among the major sources for finding new antifungal drugs. Berberine (BER), an isoquinoline alkaloid found in a variety of plant species, has been shown to possess multiple biological and pharmacological properties including antimicrobial activity against *C. albicans* and other *Candida* species. However, the mechanism of action exerted by BER and its effect on *Candida* cells is not yet fully elucidated. Therefore, this study was conducted to evaluate the role of antioxidant enzymes in the susceptibility to fluconazole (FLC) in *C. albicans*. Another aspect was to determine the effect of BER on growth, antioxidant enzymes and their gene expression in *C. albicans*.

Materials and methods

Candida albicans clinical isolates (10 FLC susceptible and 10 FLC resistant) and one ATCC strain were obtained from the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand. Species identification was confirmed using API 20C AUX. Antifungal susceptibility was determined following CLSI M27-A3 guidelines. Gene expression of *SOD1*, *SOD2*, *GPx2*, *GLR1*, *GTT11,* and *CAT1* in untreated and BER treated *C. albicans* cells was measured by RT-qPCR. The activity level of the corresponding enzymes in the presence of BER was determined using a spectrophotometer.

Results

Gene expression analysis showed an increase in mRNA expression level of *SOD1*, *SOD2*, *GPx2*, *GLR1* and *GTT11* genes in FLC resistant isolates than in the susceptible group. The most significantly expressed gene was *SOD1* with 50.69-fold increase. The other genes showed moderate increase in the expression with fold change ranging from 1.2 to 4.2. The susceptibility test showed MICs ranging from 125 to 500 μg/ml with a significant difference in the activity of BER between FLC susceptible and resistant *C. albicans*. BER treatment

induced upregulation in the mRNA expression and enzymatic activities of major antioxidants. In FLC resistant *C. albicans*, treatment with ½ MIC value of BER caused downregulation of the targeted antioxidant genes indicating that BER at this concentration induced an intense oxidative stress, therefore, surpassing the antioxidant capacity of the cells.

Conclusion

The findings in this study showed that drug resistance is not only caused by mutations in a particular gene but could also arise from proteomic modulations. The study also demonstrated that *C. albicans* activates several antioxidant enzymes that form an integral component of the cell's response against oxidative stress. *Candida albicans* showed efficient antioxidant response at lower concentrations of BER. However, BER at ½ MIC value induced robust oxidative stress, especially in FLC resistant *C. albicans,* surpassing the antioxidant capacity of the cells. This demonstrates that BER at sub-inhibitory concentrations is able to render *C. albicans* avirulent by suppressing its antioxidant defense response without compromising cell viability of the fungi. Therefore, BER has a potential to be developed into a therapeutic agent for the treatment of *C. albicans* infections and other pathogenic fungi to overcome drug resistance.

Acknowledgements

I would like to first acknowledge God for granting me the strength, understanding and wisdom throughout my research work. May His name be praised, now and forever.

I would like to thank Dr Aijaz Ahmad for the opportunity given to pursue this degree under his supervision. Thank you for your support, guidance and motivation that have led to successful completion of this study.

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To all members and staff from the department of Oral Biological Sciences and Clinical Microbiology and Infectiuos Diseases, University of the Witwatersrand, thank you for providing the facilities to carry out the experiments for this study.

Finally, I would like to express my deepest gratitude towards my family and friends for their unconditional love and support throughout the course of my life.

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%	Percentage
<	Less than
±	Plus-minus
≤	Less than or equal
≥	Greater than or equal
µg	Microgram
µl	Microlitre
µM	Micromolar
µmol	Micromole
5-FC	5-flucytosine
5-FU	5-fluorouracil
ALS	Agglutinin-like sequence
API	Analytical profile index
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BER	Berberine
bp	Base pair
BSA	Bovine serum albumin
C. albicans	*Candida albicans*
C. glabrata	*Candida glabrata*
C. krusei	*Candida krusei*
C. parapsilosis	*Candida parapsilosis*

C. tropicalis	*Candida tropicalis*
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CDNB	1-chloro-2.4-dinitrobenzene
cfu	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
Cm	Centimeter
Cu/Zn	Copper-zinc
DDC	N, N′-diethyldithiocarbamate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ETC	Mitochondrial electron transport chain
FLC	Fluconazole
FMN	Flavin mononucleotide
g	Gram
GPI	Glycosylphosphatidylinositol
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced form of glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide

HCl	Hydrochloric acid
HDC	Hematogenously disseminated candidiasis
HIV	Human immunodeficiency virus
hrs	Hours
kb	Kilobase
kg	Kilogram
KOH	Potassium hydroxide
L	Litre
L. monocytogenes	*Listeria monocytogenes*
MAPK	Mitogen-activated protein kinase
mb	Megabase
MFC	Minimum fungicidal concentration
mg	Milligram
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MMLV	Moloney murine leukemia virus
mmol	Millimole
Mn	Manganese
Mol	Mole

mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NCAC	Non-*C.albicans Candida*
ng	Nanogram
NHLS	National Health Laboratory Service
nm	Nanometre
O_2^-	Superoxide anion
ºC	Degree celsius
OC	Oral candidiasis
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pH	Potential hydrogen
PMSF	Phenylmethane sulfonyl fluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT-qPCR	Quantitative reverse transcription PCR
S. cerevisiae	*Saccharomyces cerevisiae*

SAP	Secreted aspartyl proteinase
SD broth	Sabouraud dextrose
SD	Standard deviation
sec	Seconds
SOD	Superoxide dismutase
TAE	Tris acetate-EDTA buffer
TCA	Tricarboxylic acid
Tris-HCl	Tris hydrochloride
U	Units
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VVC	Vulvovaginal candidiasis
w/v	Weight per volume
WHO	World Health Organisation
×g	Gravity at the earth's surface
α	Alpha
β	Beta
γ	Gamma

Chapter 1: Introduction

1.1 Background to the study

Candida albicans is an opportunistic pathogen generally known for causing infections in immunocompromised individuals. *Candida albicans* forms part of the human microbiome colonizing mostly the skin, oral cavity, gastrointestinal tract, and urogenital tract. This pathogenic fungus causes two major types of infections: superficial mucosal infections and life-threatening systemic infections (Brown *et al.*, 2014). *Candida* species have been reported as the fifth most common pathogen responsible for nosocomial fungal infections (Yapar, 2014). Among all the *Candida* species, *C. albicans* represent the most predominant species. Recent studies have revealed an increasing trend of invasive fungal infections in hospitalized patients due to *C. albicans* and it is highly associated with the thriving Human Immunodeficiency Virus (HIV) pandemic. The ever-increasing incidences of *C. albicans* infections have led to an increased morbidity and mortality rates (da Silva *et al.*, 2016).

In healthy individuals, cells from the innate immune defense system recognize *C. albicans* and engulf the fungus through phagocytosis. Subsequently, the macrophages stimulate immediate production of reactive oxygen species (ROS) exposing the fungus to toxic chemicals (Kaloriti *et al.*, 2014). The ROS then interacts with cellular components causing irreversible cell damage to this pathogenic fungus. This detoxification by the ROS is the primary defense mechanism convenient for protection of the host from *C. albicans* infections in immunocompetent individuals (Miramón *et al.*, 2014). However, humans with suppressed immunity remain highly susceptible to *C. albicans* infections, indicating the critical importance of oxidative stress induction for effective killing of the pathogen (Kaloriti *et al.*, 2014).

Oxidative stress poses a great challenge for pathogens to persist and proliferate within the host. To overcome these destructive effects, *C. albicans* has evolved to acquire robust mechanisms to nullify the oxidative attack. The response of *C. albicans* to mount resistance to detoxify and repair of damage caused by ROS is mediated by activation of the AP-1 transcription factor Cap1 protein. *CAP1* gene regulates gene expression of vital enzymes or antioxidants involved in repairing damages caused by oxidative stress. These antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione or glutaredoxin and thioredoxin (Abegg *et al.*, 2012; Kaloriti *et al.*, 2014). Upregulation of these

intracellular antioxidant enzymes is essential for stress adaptation or rather intrinsically oxidative resistance in *C. albicans*. Notably, studies have shown that attenuation of *CAP1* modulates genes encoding antioxidant enzymes, therefore, rendering *C. albicans* susceptible to killing by ROS (Brown *et al.*, 2014).

There is a limited number of effective antifungal drugs mainly because fungal cells are eukaryotic thus closely related to humans. Therefore, it is much more challenging to develop antifungal drugs with selective toxicity. The current treatment option for invasive fungal infections is restricted to only four classes of antifungal agents namely azoles, polyenes, flucytosine and echinocandins (Roemer and Krysan, 2014). The first-line of antifungal agent for the treatment of *C. albicans* infections is fluconazole (FLC) because it has high oral bioavailability and relatively low toxic effects to the host. However, the high prevalence of indiscriminate use of the conventional antifungal drugs has led to rapid acquisition of resistance especially to FLC (Ahmad *et al.*, 2016). This indicates an urgent need for the development of novel effective antifungal therapeutics with no or minimal side effects.

The four classes of antifungal agents target mainly the biosynthesis and integrity of the fungal cell envelop. The paucity of selective antifungal drugs necessitates the urgency of a new approach in the development of new drugs (Roemer and Krysan, 2014). Researchers have identified new drug targets to overcome the challenge of few available effective antifungal drugs. Among these emerging drug targets, oxidative stress response is a potential drug target for the development of new and safe antifungal drugs. Therefore, comprehensive studies focusing on the enzymatic activities which neutralize the ROS of the host are of great importance in the new antifungal drug discovery. This is merely because of the role of this oxidative stress response in inducing *C. albicans* infections in the host (Ahmad *et al.*, 2016).

Natural products have already gained a lot of attention in the development of novel drugs. Berberine (BER) is a well-known isoquinoline alkaloid found in a variety of medicinal plant species belonging to the *Berberidaceae* family (Dhamgaye *et al.*, 2014; da Silva *et al.*, 2016). Berberine extract has been extensively studied and shown to possess multiple biological and pharmacological properties including anti-inflammatory, antitumor, and antimicrobial activities. Dhamgaye and co-workers exposed *C. albicans* cells to BER and measured ROS production after treatment. A significant increase in the endogenous ROS generation was observed demonstrating a possible antifungal effect exerted by the compound against FLC resistant *C. albicans* (Dhamgaye *et al.*, 2014). Another study conducted by da Silva and

colleagues to assess the effect of BER in FLC resistant *C. albicans* has indicated that this compound is able to induce a loss of cell viability and permanent plasma membrane damage (da Silva *et al.*, 2016). Moreover, BER has demonstrated to have low toxicity to mammalian cells when administered at moderate dosages (Gu *et al.*, 2015). However, the direct effect of BER compound on oxidative stress enzymes produced by *C. albicans* is not yet revealed.

1.2 Problem statement

Candidiasis is becoming a major public health threat worldwide, particularly in the immunodeficient population leading to increased mortality rates. The progress in the development of effective antifungal drug has been mostly slow with the last antifungal drug introduced in the past 30 years. In antifungal drug development, it is a challenge to selectively identify pathogen-specific drug targets. In addition, there has been an increasing trend of emerging *C. albicans* resistant isolates to the first-line antifungal drugs.

Recent studies have focused on virulence properties and their markers as new therapeutic drug targets because of their role in the advancement of fungal infections. This new approach aims to weaken the defense armoury of the pathogen rather than eradicating it from the host's system and eventually slowing down the progression of the disease. Oxidative stress response in *C. albicans* is a key defense mechanism and is critically required for the expression of virulence and pathogenesis to survive in stressful conditions and most importantly to mediate oxidative resistance. Assessment of oxidative stress enzymes in *C. albicans* is of paramount importance and can give a better understanding of the impact of these intracellular antioxidant enzymes on antifungal susceptibility and fungal pathogenicity. Moreover, BER extract has been reported to possess antifungal properties against *C. albicans*. However, the effect of this natural compound particularly on oxidative stress enzymes as an emerging drug target has not been addressed yet.

1.3 Aim of the study

To evaluate gene expression of oxidative stress enzymes mediating FLC resistance and to study the regulation of these enzymes by BER in FLC susceptible and resistant *C. albicans* isolates.

1.4 Objectives

i. To study gene expression profile of major antioxidant enzymes in the clinical FLC susceptible and resistant *C. albicans* isolates.

ii. To determine the minimum inhibitory concentration of BER against FLC susceptible and resistant *C. albicans* isolates.

iii. To study the effect of BER on the activity and gene expression of identified antioxidant enzymes in FLC susceptible and resistant *C. albicans* isolates.

Chapter 2: Literature review

2.1 Introduction to fungi

Fungi are eukaryotic organisms characterized by a rigid cell wall typically made up of chitin and glucans (Bowman and Free, 2006; Lowman *et al*., 2011). The majority of fungi have a filamentous body composed of microscopic tubular cells known as hyphae, while only a few species grow as single cellsand are classified as yeasts (Wang *et al*., 2009). Up to 99000 fungal species have been described so far, however, recent phylogenetic studies revealed a new estimate of the total number of fungi that exist on earth to be 5.1 million species (Mueller and Schmit, 2007; Carris *et al*., 2012). The major phyla of true fungi have been divided into seven groups: *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Microsporidia*, *Glomeromycota*, *Neocallimastigomycota* and *Blastocladiomycota* (Hibbett *et al*., 2007; Carris *et al*., 2012).

Fungi are ubiquitous and mostly found in soil, plant material, and organic substrates. Fungi have also been found to colonize human and other animal bodies (Carris *et al*., 2012; Badiee and Hashemizadeh, 2014). Fungi play an essential role in influencing the overall well-being of humans in both beneficial and detrimental ways. Some fungi are used in industries such as textiles, pulp, and paper to replace chemical processing that often has negative impacts on the environment. They are also used in the food industry for production of several food products including bread, wine, and juices. Edible fungi such as mushrooms, morels, and truffles are known to contain high levels of dietary minerals. Some fungi are used in scientific research studies in the discovery of new therapeutic agents for medicinal use (Mueller and Schmit, 2007).

2.2 Human fungal pathogens

Although fungi infect billions of people throughout the world, fungal infections are largely unappreciated due to lack of accurate epidemiological data. Recently, it has been estimated that fungal infections kill as many people as malaria and tuberculosis (Brown *et al*., 2012). There are about 600 different fungal species known to cause infections in humans, ranging from harmless to fatal diseases (Brown *et al*., 2012; Badiee and Hashemizadeh, 2014). Superficial or cutaneous infections such as *Tinea versicolor*, athlete's foot, and ringworm are caused by dermatophytes and *Malassezia* species (Badiee and Hashemizadeh, 2014; Underhill and Pearlman, 2015). It is believed that cutaneous infections affect nearly 25% of the global population each year, and the number of incidences has been rising in recent years.

In addition, humans constantly inhale fungal spores of moulds such as *Fusarium* and *Aspergillus*, which can become pathogenic especially in immunocompromised individuals (Hardison and Brown, 2012). The common fungi causing life-threatening systemic diseases include *Candida* species, *Aspergillus* species, *Histoplasma capsulatum*, *Cryptococcus neoformans* and *Pneumocystis jirovecii*. These fungal pathogens can invade a local site and disseminate to several viscera such as the liver, kidney, central nervous system and many other vital organs. The frequency of invasive infections due to these opportunistic fungi often correlates with the degree of immunosuppression (Mayer *et al*., 2013). Individuals at highest risk of invasive fungal infections are those with leukopenia, solid organ transplant recipients, long-term use of corticosteroids, HIV infection, cancer patients and neonates. Other predisposing factors include malnutrition, severe burns, prolonged stay in intensive care and advanced age. Even though the incidences of fungal infections are generally underestimated, they pose a great concern especially because invasive fungal infections affect over a million people annually with mortality rates of up to 50% (Spampinato and Leonardi, 2013; Roemer and Krysan, 2014).

2.3 *Candida* species

The genus *Candida* is comprised of more than 150 species, however, only few species are capable of causing infections in humans. These include *C. albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida auris*, *Candida krusei*, *Candida parapsilosis*, *Candida kefyr*, *Candida lipolytica*, *Candida famata*, *Candida rugose*, *Candida guilliermondii*, *Candida lusitaniae*, *Candida dublieniensis*, *Candida norvegensis*, *Candida pelliculosa* and *Candida incospicua*. The isolation frequencies of all these species have been reported to vary in the past years. However, up to 95% of invasive candidiasis in humans is caused by *C. albicans*, *C. krusei*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and recently identified *C. auris*. Among all these opportunistic *Candida* species, *C. albicans* remain the leading fungal pathogen (Abegg *et al*., 2012; Sardi *et al*., 2013; Yapar, 2014).

2.4 *Candida albicans*

Candida albicans is a polymorphic fungus that primarily exists as a commensal organism. The fungus asymptomatically colonizes various parts of the human body, especially the mucosal sites such as the oral cavity, vagina and gastrointestinal tract of the majority of healthy people (Kaloriti *et al*., 2014; Yapar, 2014). Carriage of *C. albicans* does not necessarily indicate an infection or disease. As a matter of fact, the oral cavity in the majority of healthy individuals is dominated by *Candida* species with carriage ranging from 17 to 75%

worldwide (Williams and Lewis, 2011). Furthermore, approximately 75% of women who are of childbearing age experience vulvovaginal candidiasis (VVC) at least once in their lifetime (Mayer *et al*., 2013; Peters *et al*., 2014). Epidemiological surveillance studies from different parts of the world have indicated that *C. albicans* is among the prevalent etiologic fungal species responsible for invasive fungal infections in hospitalized patients. With the ever-increasing number of *C. albicans* infections and several other factors, this has become a serious public health challenge (Perlroth *et al*., 2007; Karacaer *et al*., 2014).

Because of its clinical importance, *C. albicans* is the first fungal pathogen to be selected for whole genome sequencing. *Candida albicans* SC5314 was chosen for sequencing owing to its widespread and increasing use in genetic manipulation studies, virulence in animal experiments, and possession of standard chromosomal patterns in the progeny cells. The genome of *C. albicans* SC5314 strain has eight different chromosomes ranging from 1030 to 3200 kb. The total size of a complete genome is estimated to be 14.3 Mb and contains more than 6000 open reading frames (ORFs) (Jones *et al*., 2004; Kabir *et al*., 2012). Amongst the characterized genes, about 774 ORFs are specific to *C. albicans* and are not present in any of the closely related organisms such as *Schizosaccharomyces pombe, Saccharomyces cerevisiae* or even in humans. The number of these ORFs is constantly changing as new information arises regarding the functions of other genes (Kabir and Hussain, 2009; Lim *et al*., 2012).

2.5 Disease spectrum

The versatility of *C. albicans* to thrive in different environments within the host is key to its ability to cause such a wide spectrum of diseases. However, common infections caused by *C. albicans* are generally restricted to the oral cavity, vaginal tract and in the bloodstream (Dabas, 2013; Jabra-Rizk *et al*., 2016).

2.5.1 Oral candidiasis

Oral candidiasis (OC) is common in the elderly people, neonates and those with a debilitated immune system, particularly in HIV patients. Oral candidiasis is often one of the initial clinical manifestations of an underlying HIV infection and has been estimated that approximately 80% of HIV patients experience recurrent episodes of OC during the course of HIV progression. However, these numbers have been significantly reduced post the introduction of antiretroviral therapy (Fidel, 2011; Garcia-Cuesta *et al*., 2014). Oral candidiasis may appear in different lesion forms based on clinical presentation: 1)

Pseudomembranous candidiasis (**Figure 2.1**) is characterized by the presence of white curd-like lesions that can easily be removed by wiping, often leaving a red painful and bleeding surface. The most frequent sites of infection include buccal mucosa, tongue, palate, and oropharynx. Pseudomembranous candidiasis is commonly seen in neonates and immunocompromised individuals such as those infected with HIV, cancer patients, and those on corticosteroids or broad-spectrum antibiotic therapy; 2) Erythematous candidiasis (**Figure 2.2**) present as localized erythema usually on the dorsal surface of the tongue, the palate, and sometimes on the buccal mucosa. Erythematous candidiasis is the only form of *Candida* infection associated with consistent pain. Moreover, HIV patients often suffer from a chronic form of this infection; 3) Chronic hyperplastic candidiasis (**Figure 2.3**) also referred to as *Candida* leukoplakia may present as homogeneous white plaque-like lesion or speckled white lesion on an erythematous background. These lesions are commonly found on the commissural region of the buccal mucosa, and sometimes on the palate and tongue. A defining characteristic of hyperplastic lesions of the oral cavity is that they cannot be stripped away like those of pseudomembranous candidiasis. Hyperplastic lesions appear to have a possible association with smoking and malignancy formation in the oral cavity. However, a direct role of *Candida* species in the development of oral cancer is still not clear; 4) *Candida*-associated denture stomatitis presents as erythema confined to the denture-bearing tissues. This type of oral infection is seen in up to 65% of denture wearers. Although generally, these lesions are asymptomatic, patients may experience slight pain and burning sensations on the affected area. A major predisposing factor for *Candida*-associated denture stomatitis is poor oral hygiene (Farah *et al*., 2010; Williams and Lewis, 2011; Patil *et al*., 2015; Jabra-Rizk *et al*., 2016).

Even though *C. albicans* is by far the most prevalent opportunistic fungi associated with oral infections, incidences of non-*C. albicans Candida* (NCAC) species have been significantly increasing in recent years. These include *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* (Williams and Lewis, 2011; Patil *et al*., 2015).

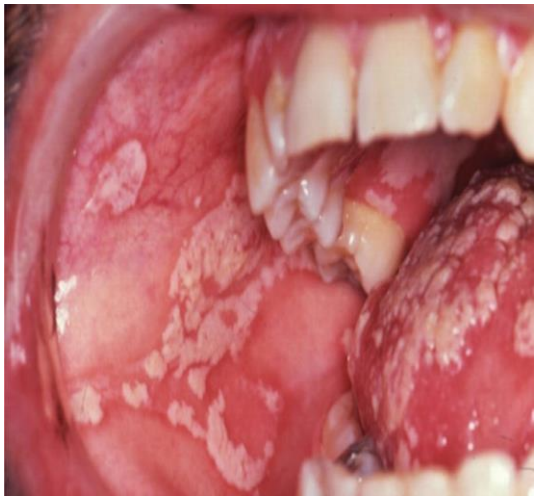

Figure 2.1: Pseudomembranous candidiasis (Jabra-Rizk *et al*., 2016).

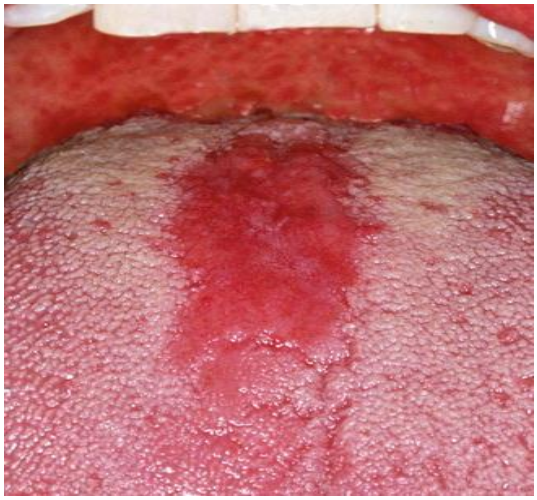

Figure 2.2: Erythematous candidiasis (Jabra-Rizk *et al*., 2016).

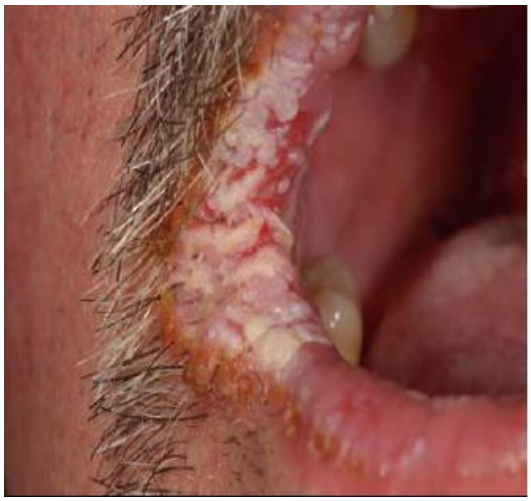

Figure 2.3: Hyperplastic candidiasis (Williams and Lewis, 2011).

2.5.2 Vaginal candidiasis

Vaginal candidiasis remains one of the most frequent reasons for gynaecology consultation in healthy women of childbearing age. In fact, gynaecology reports have indicated that VVC is the second most common cause of vaginal infections. Typical clinical symptoms include vulvar erythema, intense itching, dyspareunia, and vaginal discharge. The use of oral contraceptives, broad-spectrum antibiotics, pregnancy, diabetes mellitus and high levels of estrogen have been identified as predisposing factors for women to develop this infection (Del-Cura González *et al*., 2011; Peters *et al*., 2014; Goulart *et al*., 2016).

Statistical reports have indicated that up to 138 million women worldwide suffer from multiple episodes of VVC each year due to treatment failure. This is an alarming public health issue especially because this infection can lead to many health complications in women and it is also associated with morbidity, increased healthcare costs, and susceptibility to HIV infection (Gonçalves *et al*., 2016; Sherry *et al*., 2017). Despite the prevalence of several other *Candida* species, *C. albicans* accounts for majority of VVC infections (**Figure 2.4**). Over the past decades, scientific evidence has demonstrated an increased frequency of NCAC species with *C. glabrata* being the consistent second most prevalent fungus isolated from patients with VVC (Dabas, 2013; Gonçalves *et al*., 2016; Sherry *et al*., 2017).

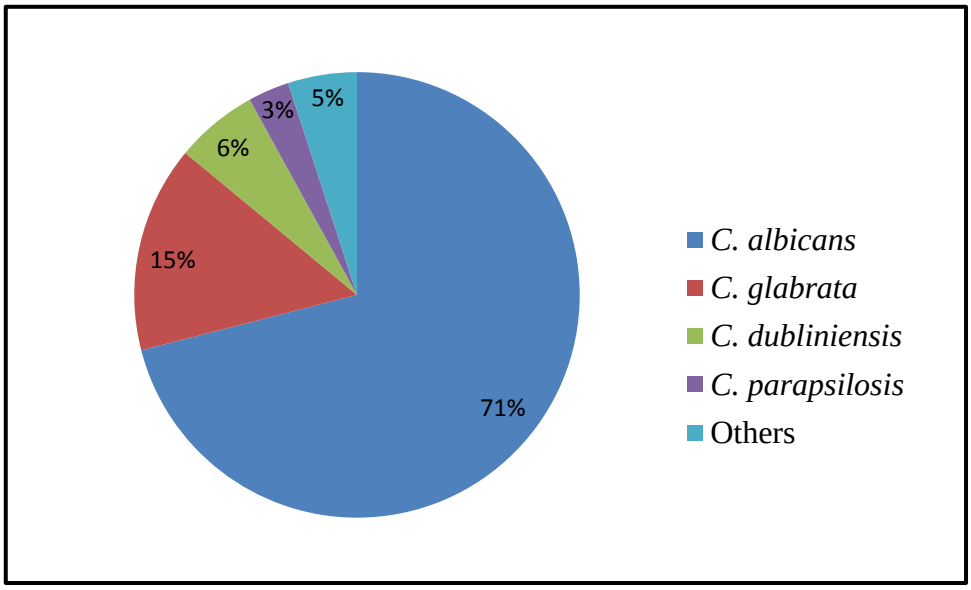

Figure 2.4: Distribution of *Candida* species in VVC patients, data obtained from Sherry *et al*., 2017.

2.5.3 Hematogenously disseminated candidiasis

Hematogenously disseminated candidiasis (HDC) is a serious life-threatening type of infection caused by *Candida* species in severely immunocompromised patients. This infection arises when *Candida* cells breach the mucosal barriers and enter the bloodstream of the host. Once the fungus is in the bloodstream, it can disseminate to virtually every important organ including the kidneys, heart, lungs, liver, spleen, and even brain. It is believed that in most cases the source of HDC is the host's endogenous flora or contaminated medical devices (Dabas, 2013; Jabra-Rizk *et al*., 2016).

Candida species have been identified as the leading cause of nosocomial bloodstream infections in most regions of the world. The number of populations at risk of HDC is rapidly expanding with mortality rates exceeding 50% (Mathé and Van Dijck, 2013; Calandra *et al*., 2016; Bassetti *et al*., 2018). One of the major contributing factors to this outcome is the lack of timely diagnosis tools of which delays initiation of antifungal therapy (Colombo *et al*., 2014; Jabra-Rizk *et al*., 2016).

In countries such as Switzerland and United States, *Candida* is at present ranked as the third most common infectious fungus responsible for up to 8 to 10% of bloodstream infections. However, this situation is quite different in European countries since *Candida* species only accounts for 3% of bloodstream infections and are ranked between the sixth and tenth most frequent organism isolated from blood cultures (Delaloye and Calandra, 2014; Calandra *et al*., 2016). Approximately 35% of these incidences occur in severely ill patients admitted in the intensive care units (Colombo *et al*., 2014; Yapar, 2014; Kullberg and Arendrup, 2015). In South Africa, hospital-based studies have reported *C. albicans*, *C. parapsilosis* and *C. glabrata* to be the most frequent pathogens causing fungal bloodstream infections. National surveillance representing both public and private hospitals for systemic fungal infections revealed that 98% of these cases were caused by *C. albicans* (Naicker *et al*., 2016). More recently, *C. albicans* and *C. krusei* were identified as the most predominant organisms present in the sterile sites of neonates admitted at Dr. George Mukhari Academic Hospital (Makhado *et al*., 2014). Recent studies have revealed a global shift in the epidemiology of *Candida* species. However, even today, *C. albicans* accounts for 50% of the cases of HDC (Yapar, 2014; Kullberg and Arendrup, 2015).

2.6 Laboratory diagnosis

For appropriate treatment of any infectious disease, rapid and correct identification of the causitive pathogen is important. Timely and correct diagnosis of *C. albicans* can lead to appropriate antifungal treatment and thereby can help in preventing severe infections and reduce the associated morbidity and mortality rates. The most common methods of *C. albicans* diagnosis are as follows:

2.6.1 Microscopy

A wet mount test is usually performed by experts for rapid detection and identification of *Candida* cells. This technique allows direct visualization of *Candida* yeast cells isolated from different anatomical sites. Commonly tested clinical specimens include those collected from mucosal membranes, tissue biopsies, scrapings, and cerebrospinal fluid. In addition, germ tube test can be used for presumptive identification of *C. albicans*. Microscopic examination is considered a reliable tool for diagnosis of fungal infections in resource-limited settings because it is rapid and cost-effective (Madhavan *et al*., 2011; Badiee and Hashemizadeh, 2014; Calandra *et al*., 2016).

2.6.2 Fungal culturing

Culturing is the current gold standard for diagnosis of *Candida* infections with sensitivity of up to 71% (Kullberg and Arendrup, 2015; Jabra-Rizk *et al*., 2016). The most frequently used media for primary isolation of clinical *Candida* species include Potato Dextrose agar, Sabouraud brain heart infusion, Yeast Potato Dextrose, Sabouraud Dextrose agar, and Blood agar. Lee's synthetic medium is used to show different morphologies during phenotypic switching in *C. albicans*. The other medium used for selective identification of *C. albicans* is CHROMagar *Candida*. The typical growth of *C. albicans* on this medium appears as light to medium green colonies (Nadeem *et al*., 2010; Madhavan *et al*., 2011).

Currently, culture method is the only diagnostic approach that detects viable fungi for susceptibility testing (Clansy and Nguyen, 2013; Kullberg and Arendrup, 2015). Even though culturing recovers *C. albicans* from clinical specimens, it suffers from serious limitations. On average, it takes about 4 days to reveal a positive detection of *C. albicans*. In addition, specimens collected after treatment often produce false negative results. A negative result in patients with HDC is because of the fact that *Candida* cells are continuously eliminated from the bloodstream. Given all these limitations, it is clear that there is a need for the development of rapid diagnostic tools to ensure prompt administration of therapy and also

improve patient's outcome (Pfeiffer *et al*., 2011; Schell*et al*., 2012; Badiee and Hashemizadeh, 2014).

2.6.3 Biochemical tests

Several commercial kits are available today for phenotypic identification of yeast. These include API 32C, API 20C, and RapID Yeast Plus System. Carbohydrate assimilation test is based on the ability of *Candida* yeast cultures to utilize carbohydrates for their metabolic processes. Some of the carbon or nitrogen sources that *Candida* assimilates include sucrose, maltose, raffinose, trehalose, N-Acetyl-Glucosamine, and xylose. This test is currently the only rapid and reliable commercial method for the identification of *Candida* species in resource-limited diagnostic laboratories. Carbohydrate assimilation is very useful in differentiating *C. albicans* from other *Candida* species with the same phenotypic characteristics (Marinho *et al*., 2010; Madhavan *et al*., 2011).

2.6.4 Serology

Conventional diagnostic methods exhibit a limited sensitivity to detect *Candida* species especially in patients with invasive candidiasis. Consequently, serological tests are recommended for timely diagnosis of *Candida* infections. However, antibody detection may be limited by two major drawbacks: 1) *C. albicans* colonization may elicit a temporal antibody response in healthy individuals. 2) Immunocompromised patients may fail to produce detectable antibodies even when they have active *Candida* infection (Yeo and Wong, 2002; Cuenca-Estrella *et al*., 2012).

The detection of *Candida* mannan antigens, anti-mannan antibodies, and β-D-glucan has been used as a surrogate marker for specific detection of *Candida* species. A meta-analysis study on the use of mannan and anti-mannan antibodies has revealed varying sensitivity in *Candida* species. However, the best performance of this serological test was observed in *C. albicans* with 58 to 70% sensitivity. The overall specificity of this test ranges between 46 and 89% as opposed to culture. Moreover, several studies have indicated that *Candida* mannan test performs well in patients with cell-mediated immune defects (Kurita *et al*., 2009; Clansy and Nguyen, 2013; Kullberg and Arendrup, 2015).

2.6.5 Nucleic acid detection

Polymerase chain reaction has a potential to overcome some of the challenges faced by conventional culture methods. This technique is based on the detection of *C. albicans* DNA from clinical specimens without relying on the viability of the organism. Some of the advantages of PCR assays over culture-based methods include reduced turnaround time without sacrificing accuracy, detection of low levels of DNA in blood and tissue samples and high sensitivity (Lucignano *et al*., 2011; Dabas, 2013). A number of PCR tests have been evaluated for the detection of invasive candidiasis, however, their accuracy differs depending on the type of the test. In a recent study, Avni and colleagues revealed a pooled sensitivity and specificity to be over 85% (Avni *et al*., 2011). One of the extensively studied commercial PCR tests is the LightCycler SeptiFast. This diagnostic test showed about 94% sensitivity when evaluated in patients suspected of having candidemia (Lucignano *et al*., 2011; Cuenca-Estrella *et al*., 2012; Badiee and Hashemizadeh, 2014; Kullberg and Arendrup, 2015).

It is worth mentioning that the use of molecular methods for routine identification in diagnostic laboratories remains a challenge, especially in developing countries as some of these tests are expensive and require well-trained personnel. A major pitfall in using PCR could be the interference of DNA contamination from sources other than the targeted organism (Lucignano *et al*., 2011; Madhavan *et al*., 2011; Pfeiffer *et al*., 2011).

2.7 Treatment of *Candida* infections

Compared to the antibacterial agents, the progress in the development of effective antifungal drugs has been slow. This is mainly complicated by the fact that the fungal cells have the same phylogenetic origin with the human cells. Therefore, it is a challenge to selectively identify pathogen-specific drug targets without causing any harm to the host (Tsui *et al*., 2016). Most of the antifungal drugs available in the market for clinical use target the fungal cell envelop, such as biosynthesis of fungal cell membrane by focusing on ergosterol or the cell wall linker molecule 1,3-β-glucan (Pianalto and Alspaugh, 2016). The current therapeutic agents for the treatment of *Candida* infections belong to the following antifungal classes: azoles, polyenes, pyrimidine analogs, allylamines, and echinocandins (Denning and Hope, 2010; Gulati and Nobile, 2016). Although most of the drugs from these antifungal classes are effective in their appropriate contexts, their use for clinical purposes has limitations because of several reasons such as high toxicity, drug resistance, narrow drug spectrum, and many others. All the major drug classes are briefly discussed below and summarized in **Table 2.1**:

2.7.1 Azoles

Azoles are the most important class of antifungal drugs which target ergosterol biosynthesis pathway of fungal cells. Azoles are synthetic compounds which have been classified into two groups based on the number of nitrogen atoms in the five-membered azole ring. The imidazoles such as miconazole and ketoconazole contain two nitrogen atoms, whereas the triazoles such as fluconazole (FLC), itraconazole, and miconazole have three nitrogen atoms (Maertens, 2004; Vandeputte *et al*., 2011).

Azoles interfere with ergosterol biosynthetic pathway through inhibition of lanosterol 14-α-demethylase, an enzyme dependent on cytochrome P-450. Azoles act by binding directly in the catalytic site of the target enzyme lanosterol 14-α-demethylase. Consequently, the enzyme will produce less ergosterol end products (Shapiro *et al*., 2011; Prasad *et al*., 2016). The depletion of ergosterol and accumulation of 14-α-methylated sterol precursors alters the normal functionality of the fungal membrane, affecting mainly the fluidity of the membrane and the activity of other several membrane-associated enzymes. The net effect of this mechanism is increased permeability of the fungal cell membrane, inhibition of cell growth and replication (Nigam, 2015).

Table 2.1: Drug target and mode of action of currently used antifungal agents

Antifungal class	Mode of action	Drugs
Azoles	Inhibitors of lanosterol 14-α-demethylase	Miconazole Clotrimazole Ketoconazole Voriconazole Posaconazole Fluconazole Itraconazole
Polyenes	Binding ergosterol and membrane disintegration	Amphotericin B Nystatin
Echinocandins	Inhibitors of 1,3-β-glucan synthase	Caspofungin Micafungin Anidulafungin
Nucleoside analogues	Inhibitor of DNA/RNA synthesis	Flucytosine
Allylamines	Inhibitors of squalene epoxidase	Terbinafine Naftifine Amorolfine

Of the azole class, FLC is the most widely used azole and possesses good pharmacokinetic properties. It has good absorption through the gastrointestinal tract and can easily penetrate through the cerebrospinal fluid or cerebrospinal barrier. Consequently, FLC is recommended as initial therapy for the treatment of superficial, cutaneous and disseminated infections caused by *Candida* species(Spampinato and Leonardi, 2013; Whaley *et al*., 2017).

With the frequent use and abuse of azole drugs especially FLC, drug resistance to azoles has drastically increased in the last few decades. Resistance to azole derivatives is mostly due to the occurrence of mutations in the *ERG11* gene encoding lanosterol leading to a decreased affinity for the drug. In addition, overexpression of efflux pump activity is a major mechanism of resistance in azole resistant clinical *Candida* isolates. There are two principal families of efflux proteins in *C. albicans*, the major facilitator superfamily and the ATP-binding cassette transporters encoded by the *MDR1* gene and *Candida* drug resistance 1 and 2 (*CDR1*/*CDR2*) genes, respectively. In addition, the formation of biofilms by *Candida* species

and their role in drug resistance has been extensively investigated by numerous researchers (Pierce and Lopez-Ribot, 2013).

2.7.2 Polyenes

Polyenes are cyclic amphiphilic organic molecules which are synthesized from *Streptomyces* bacteria and preferentially bind to ergosterol consisting membranes. There are more than 200 chemical molecules belonging to this class. However, only three are used for clinical purposes: amphotericin B, nystatin and natamycin (Vandeputte *et al*., 2011). Polyene class acts by binding to the ergosterol, inducing pores within the membrane lipid bilayer of the fungal cell. This allows intracellular electrolytes and other cytoplasmic contents to leak out through the transmembrane channels. The altered cellular permeability leads to cell death. In addition, the use of polyene agents especially amphotericin B has shown to induce intracellular accumulation of toxic reactive oxygen species responsible for cellular death (Mesa-Arango *et al*., 2014). Of note, resistance to the polyene class in *C. albicans* is mostly rare and species dependent within the *Candida* genus (Spampinato and Leonardi, 2013). Some *Candida* species are either intrinsically resistant to amphotericin B or require higher doses of the drug (Bondaryk *et al*., 2013).

Amphotericin B remains the cornerstone of therapy for the treatment of systemic fungal infections (Anderson *et al*., 2014). Despite having been available for clinical use for more than five decades, amphotericin B continues to exhibit the broadest spectrum of antifungal activity with a very gradual development of antifungal resistance. Amphotericin B, despite having a broad spectrum antifungal activity, its clinical use is severely hampered as it is often associated with severe side effects such as nephrotoxicity and infusion-related side effects. For these reasons, amphotericin B is only administered intravenously while nystatin and natamycin are used for oral and topical applications (Hoehamer *et al*., 2010). Lipid-based amphotericin B formulations have been developed in an attempt to attenuate the toxicity of this conventional drug. Despite the fact that liposomal amphotericin B formulations demonstrate improved safety profile, these formulations are generally costly and not readily available in some regions (Hamill, 2013).

2.7.3 Echinocandins

This class is a recent addition to the antifungal arsenal and has been approved for clinical use to combat invasive fungal infections (Denning, 2002; Roemer and Krysan, 2014). Due to impressive safety and efficacy profiles, echinocandin drugs have become the recommended alternative to the conventional treatment in patients with invasive fungal infections (Arendrup and Perlin, 2014). Echinocandins derivatives are semisynthetic lipopeptide compounds which have been naturally synthesized from several fungi species such as *Zalerion arboricola*, *Aspergillus rugulovalvus* and *Papularia sphaerosperma* (Vandeputte *et al*., 2011; Bondaryk *et al*., 2013). There are currently three antifungal drugs of this class licensed for the treatment of fungal infections: micafungin, caspofungin, and anidulafungin. The echinocandins class is novel in such that its derivatives selectively target a specific component on the fungal cell wall which is not present in the host cells (Pierce and Lopez-Ribot, 2013). Antifungal drugs of the echinocandinsclass impair the biosynthesis of 1,3-β-glucan synthase. This enzyme is crucial in the synthesis of 1,3-β-glucan, a key component essential for the integrity of the fungal cell wall of *Candida* species (Bondaryk *et al*., 2013). Lack of 1,3-β-glucan in the fungal cell wall induces osmotic instability and ultimately cell death for most fungal species (Spampinato and Leonardi, 2013).

Despite prolonged exposure and increasing use of echinocandin drugs, the incidence of resistance in *Candida* species remains relatively uncommon. However, *C. glabrata* is an exception as several studies have revealed a significant increase in the prevalence of emerging echinocandin resistant isolates. In addition, resistance to echinocandin drugs in *C. glabrata* is often associated with cross-resistance to azole agents yielding multidrug resistant isolates (Perlin, 2015).The mechanism of resistance to echinocandins drugs is attributed to point mutations in 1,3-β-glucan synthase subunits Rho1p and FKsp. These mutations often result in clinical treatment failures and have been linked with elevated minimum inhibitory concentration values (Beyda *et al*., 2012).

2.7.4 Nucleoside analogues

5-flucytosine (5-FC) is a fluorinated pyrimidine analogue with no intrinsic antifungal activity. However, the compound has to be enzymatically converted to metabolites that inhibit fungal DNA and protein synthesis (Vandeputte *et al*., 2011; Fang *et al*., 2017). 5-FC is transported through the fungal cell membrane by cytosine permease. Once 5-FC penetrates the fungal cell, cytosine deaminase rapidly converts 5-FC into 5-fluorouracil (5-FU). The selective toxicity of 5-FC relies on cytosine deaminase, a crucial enzyme within the fungal cell and

absent in mammalian cells. However, the use of this drug has been associated with negligible side effects in humans such as diarrhoea, vomiting, and skin rash. Severe adverse effects include hepatotoxicity and bone marrow depression. These toxic effects are presumed to be caused by the conversion of 5-FC to 5-FU by the intestinal microflora (Vermes *et al*., 2000; Costa *et al*., 2015). Even with these side effects, 5-FC in combination with either FLC or amphotericin B remains useful in the treatment of invasive fungal infections (Morace *et al*., 2014). By far, *in vitro* susceptibility testing has revealed about less than 10% of *C. albicans* with intrinsic resistance to this antifungal class. Up to 30% of clinical isolates rapidly develop resistance during the treatment (Espinel-Ingroff, 2008; Bondaryk *et al*., 2013). The use of 5-FC as monotherapy is problematic as it has been linked to increased development of acquired resistance (Morace *et al*., 2014; Fang *et al*., 2017).

Inactivation of key enzymes of the pyrimidine pathway due to mutations is the reason for resistance (Spampinato and Leonardi, 2013). Innate resistance to 5-FC is primarily caused by loss of activity of the *FCY2* gene encoding cytosine permease. Deficiency in this enzyme leads to impaired uptake of the drug, decreasing accumulation of the drug within fungal cells (Vermes *et al*., 2000; Morace *et al*., 2014). The second mechanism of resistance is related to inactivation of cytosine deaminase and uracil phosphoribosyltransferase of the pyrimidine pathway. These enzymes are encoded by *FCY1* and *FUR1* genes, respectively. Both *FCY1* and *FUR1* genes interfere with the conversion of 5-FC to 5-FU. The most frequently occurring type of acquired 5-FC resistance is induced by point mutations in the *FUR1* gene of the fungal isolates (Edlind and Katiyar, 2010; Spampinato and Leonardi, 2013).

2.7.5 Allylamines

This class of antifungal agents was discovered by accident from research intended to chemically synthesize new treatment options for the central nervous system. Naftifine and terbinafine are synthetic analogs of this class and have shown to possess antifungal activity in clinical studies (Birnbaum, 1990). Allylamines target a membrane-bound enzyme, squalene epoxidase involved in the conversion of squalene into squalene 2,3-epoxide. The latter product is subsequently converted into ergosterol and lanosterol (Denning and Hope, 2010). The inhibition of squalene epoxidase leads to accumulation of high concentrations of the intracellular squalene. This is believed to interfere with the functionality of the fungal membrane and cell wall synthesis due to ergosterol deficiency (Ryder, 1992; Campoy and Adrio, 2017).

Although allylamines have a broad spectrum activity against fungal pathogens, they are commonly used as topical agents for the management of dermatophyte infections (Sanglard *et al*., 2009).

2.8 Antifungal drug resistance

Despite the availability of several classes of antifungal drugs for clinical use, the incidence of fungal infections coupled with increasing number of death rates cannot be neglected. This outcome is mostly attributed to the emergence of resistance to the available therapeutic drugs (Srinivasan *et al*., 2014). Antifungal drug resistance has been traditionally classified into three major groups: 1) intrinsic resistance, which is present in clinical isolates independent of prior exposure to the drug, 2) acquired resistance develops after drug exposure in previously susceptible isolates either by horizontal gene transfer or due to genetic alterations within the gene of interest, 3) clinical resistance is when the fungi are fully susceptible to the antifungal drug *in vitro* but patients show poor response to clinical treatment. The latter is often displayed by progression or relapse of the infection particularly in patients with immune defects (Kontoyiannis and Lewis, 2002).

Antimicrobial drug resistance is an inevitable evolutionary process and is not exclusive to fungi only, but also bacterial pathogens have shown resilience towards the toxic effects of the drugs (Cort *et al*., 2016). One of the major factors exacerbating the development of antifungal drug resistance is the fact that most of the antifungal drugs available in clinical medicine today have been in use for several decades. This pressurizes the fungi to develop resistance to withstand the toxic effects of the drugs (Sanglard *et al*., 2009; Srinivasan *et al*., 2014). Most of the current antifungal drugs target ergosterol synthesis, β-1,3 glucan synthesis or bind directly to the ergosterol interfering with the functionality of the membrane (Pierce and Lopez-Ribot, 2013). With the increasing antifungal drug resistance development, there is a need for the development of new antifungal drugs which can slow down the development of antifungal drug resistance and have minimal undesirable side effects (Parente-Rocha *et al*., 2017).

2.9 Antifungal therapeutic drug targets in *Candida* species

2.9.1 Current drug targets

As described in the treatment section, most of the current antifungal agents used in clinic settings, directly or indirectly, focus on the cell envelop and particularly on membrane sterols, cell wall and their biosynthesis. The established targets of the current antifungal drugs used are summarised in **Figure 2.5**.

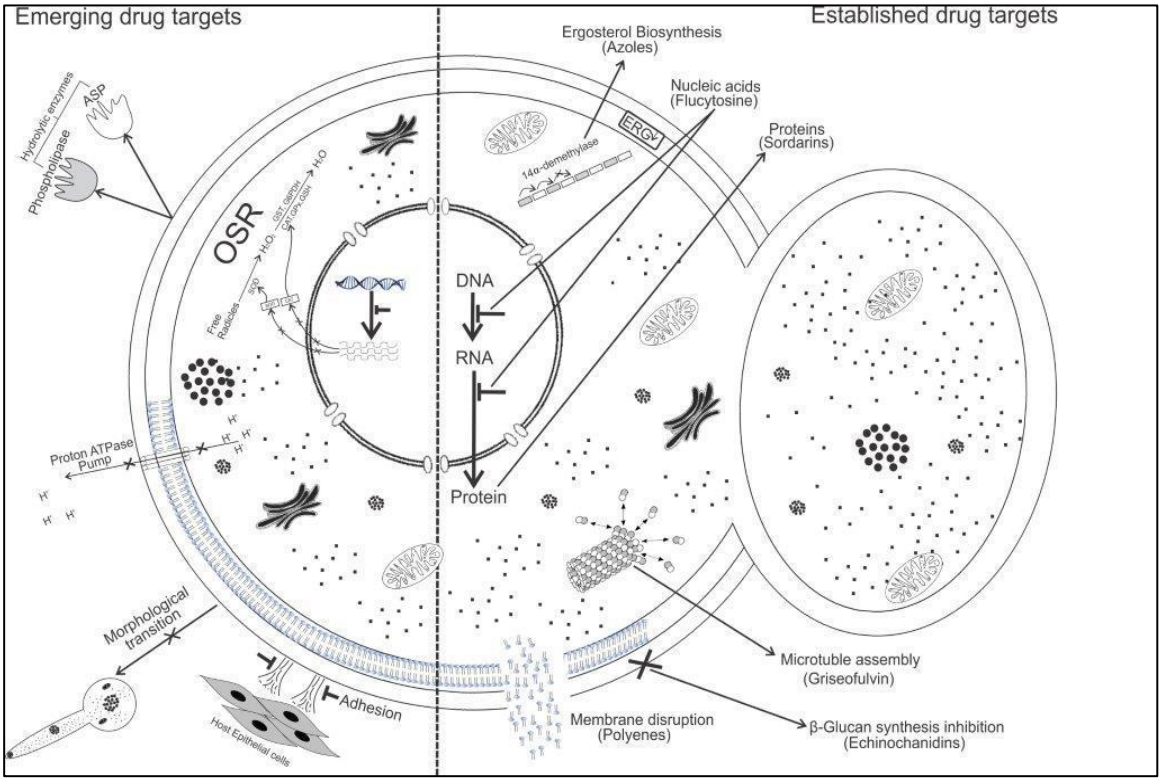

Figure 2.5: Established and emerging antifungal drug targets in *C. albicans* (Ahmad *et al.*, 2016)

2.10 Pathogenicity markers as an emerging drug target

All opportunistic pathogens have developed specialized mechanisms for colonization or even to cause infections to the host under suitable predisposing conditions. Unlike bacteria, which are simple microorganisms, *C. albicans* is a eukaryotic organism and has a larger genome which can utilize even more advanced mechanisms to cause infectious diseases and also resist host defense mechanisms (Hube and Naglik, 2001). *Candida albicans* exist primarily in humans as a commensal organism but has developed subtle strategies required to colonize and inflict damage to host tissue cells. The transition from harmless commensal to a disease-causing pathogen is facilitated by several virulence determinants (Weindl *et al*., 2010; Naglik

et al., 2011). Some of the important virulence determinants essential for the pathogenicity of *C. albicans* include expression of adhesins on the cell surface, biofilm formation, secretion of hydrolytic enzymes, morphology switching from unicellular yeast cells to its filamentous form, secretion of antioxidant enzymes to cope with oxidative stress, proton ATPases pump and many others (Mayer *et al*., 2013). *Candida albicans* rely on these virulence determinants for successful colonization, invasion of host tissues and evasion of host immune system (Gow and Hube, 2012).

Anti-virulence therapeutics represents an attractive approach to the development of novel classes of antifungal drugs. This approach intends to weaken specific virulence properties that promote or may cause *C. albicans* infections in the host without compromising cell viability of the pathogen. The advantages of targeting the pathogenicity markers or virulence factors include: 1) expansion of the number of potential drug targets which can be exploited for the development of novel antifungal classes, 2) preservation of the natural microbiota of the host, 3) exerting a reduced selection pressure for the development of antifungal drug resistance (Cegelski*et al*., 2008; Karkowska-Kuleta *et al*., 2009; Pierce and Lopez-Ribot, 2013).

2.10.1 Phenotypic switching

The ability of *C. albicans* to switch spontaneously between several colony morphologies was first discovered in 1985. Different *C. albicans* strains exhibit distinct variants of colony phenotypes including smooth, rough, star, ring, irregular wrinkle, and fuzzy (Slutsky *et al*., 1985; Kabir *et al*., 2012). This distinctive attribute is the reason for increased virulence of *C. albicans* during infection because it allows the organism to react flexibly and thrive in continuously changing extracellular environments within the host (Khan *et al*., 2010; Tao *et al*., 2014). Of all these mentioned phenotypic variants, white-opaque transition has received significant attention from researchers because of its role in the pathogenesis of *C. albicans* (Calderone and Fonzi, 2001; Bommanavar *et al*., 2017). The white cells give rise to smooth and shiny, dome-shaped colonies whereas opaque cells are large and rough, with elongated bean-shaped colonies on solid media (Slutsky *et al*., 1985; Bommanavar *et al*., 2017). White and opaque cells have shown a number of different features in terms of gene expression profiles, susceptibility to the host immune system, mating competency and virulence characteristics (Soll, 2009; Tao *et al*., 2014; Ene *et al*., 2016). Experimental murine models investigating the role of phenotypic switching in pathogenesis have shown that opaque cells cause cutaneous infections whereas the white cells are mainly responsible for HDC (Kvaal *et*

al., 1997; Bommanavar *et al*., 2017). A study by Tao and colleagues discovered novel colony morphology in *C. albicans*, grey cell type. Their finding suggests that *C. albicans* is capable of undergoing at least three stable phenotypic transitions under favourable conditions. The grey cells exhibit the highest virulence ability to cause cutaneous infections when compared with white and opaque cells *in vitro* studies (Tao *et al*., 2014).

2.10.2 Morphological dimorphism

Unlike many other pathogenic fungi capable of infecting humans, *C. albicans* exists in a number of different morphological cell types (**Figure 2.6)**. This includes yeast, hyphae, pseudohyphae, and chlamydospores (Kabir *et al*., 2012; Noble *et al*., 2017). However, morphological switching from single-celled budding yeast to filamentous form (pseudohyphae and true hyphae) has been extensively reviewed due to its correlation with virulence during *Candida* infections and also in the identification and development of novel antifungal drug targets (Khan *et al*., 2010; Lim *et al*., 2012; Tyc *et al*., 2014). The role of morphological transition in virulence has been studied in detail and it has been reported that mutant yeast cells in murine experimental studies are often avirulent and incapable of causing systemic *Candida* infections in comparison to the cells that grow exclusively as hyphae (Shapiro *et al*., 2011). A study by Saville and colleagues has pointed out roles for yeast and hyphae during *Candida* infections (Saville *et al*., 2003). The hyphal form of *C. albicans* is required for active penetration of the host barriers to cause tissue damage and invasion whereas the yeast form is more suitable for systemic dissemination and can promote multiple organ infections (Saville *et al*., 2003; Shapiro *et al*., 2011). Moreover, expression of other virulence determinants such as adhesins, hydrolytic enzymes, and superoxide dismutase enhance virulence potential of the hyphae forms in comparison with yeast forms of *C. albicans* (Khan *et al*., 2010; Noble *et al*., 2017). Of all the virulence properties in *C. albicans*, yeast to hyphae transition has been extensively studied in the development of new drug targets. For example, a study by Watanabe and colleagues showed that the use of their newly synthesized compound targeting yeast to hyphae transition and biofilm formation in *C. albicans* suppressed its virulence (Watanabe *et al*., 2012). In addition, a plant-derived compound, the gymnemic acids inhibited the transition of yeast to hyphae under different hyphal inducing environments in *C. albicans* without compromising its viability (Vediyappan *et al*., 2013).

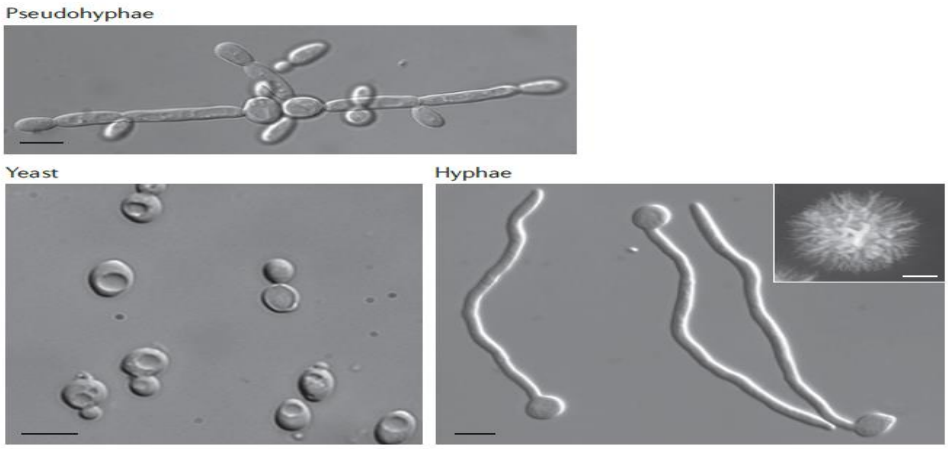

Figure 2.6: Different morphological forms of *C. albicans* (Sudberry, 2011).

2.10.3 Adhesion molecules

Adhesion to host cells is the most critical step for colonization and establishment of *C. albicans* infections. The cell wall surface of *C. albicans* is equipped with specialized proteins, known as adhesins responsible for adhesion to different substrates such as epithelial and endothelial cells, implanted medical devices, extracellular matrix proteins, and many others (Kabir *et al*., 2012; Lim *et al*., 2012). Among the cell surface adhesins, agglutinin-like sequence (ALS) protein family is one of the best studied (Tronchin *et al*., 2008; Mayer *et al*., 2013). ALS protein family consists of at least eight members encoded by distinct genetic loci *ALS1-7* and *ALS9* (Murciano *et al*., 2012; Hoyer and Cota, 2016). These genes encode for glycosylphosphatidylinositol (GPI)-linked cell surface proteins which are highly homologous in their molecular structure. Even though all the eight ALS proteins have shown to be implicated in the adherence of *C. albicans* cells to host constituents, adhesion assay studies have revealed that ALS3 protein is upregulated during the infection of oral epithelial cells and vaginal infections (Liu and Filler, 2011; Naglik *et al*., 2011; Mayer *et al*., 2013). It is believed that the adhesive properties of the ALS3 protein are enhanced by a strong affinity binding of N-terminal region to either N- or E-cadherin of the host cells (Phan *et al*., 2007). Another key adhesin molecule involved in the attachment of *C. albicans* cells is GPI-anchored hyphal wall protein (Hwp1). This adhesin is able to interact with host proteins, thereby causing a strong covalent attachment between *C. albicans* and host epithelial cells (Sundstrom *et al*., 2002). However, researchers have focused on the initial step of adherence to specific host tissues for the development of new antifungal drugs (Ahmad *et al*., 2016).

2.10.4 Hydrolytic enzymes

Candida albicans is able to produce a range of various extracellular hydrolytic enzymes to adapt to different niches within the host (Khan *et al*., 2010). Among these enzymes, secreted aspartyl proteinases (SAPs), phospholipases and lipases have received the most attention from researchers because of their role in fungal pathogenicity (Sachin *et al*., 2012; Mayer *et al*., 2013; Pawar *et al*., 2014). These enzymes facilitate penetration of the fungus to cause tissue damage, iron acquisition, and dissemination within the host. In addition, the secreted hydrolytic enzymes help the fungal pathogen to overcome and render the host immune system ineffective (Karkowska-Kuleta *et al*., 2009; Staniszewska *et al*., 2012; Tsai *et al*., 2013). Furthermore, hemolysin production by *C. albicans* followed by iron acquisition mediates hyphal invasion in HDC (Sachin *et al*., 2012). *Candida albicans* mutants lacking specific genes responsible for encoding hydrolytic enzymes have shown attenuated pathogenicity and are easily phagocytosed (Aoki *et al*., 2011).

i. Secretion of aspartyl proteinases (SAPs)

SAPs by *C. albicans* are important because of their role in the virulence activity of *C. albicans* during adhesion, tissue damage and evasion of antimicrobial attack (Deepa *et al*., 2015). SAPs isoenzymes hydrolyze important immunological and structural defense proteins such as mucin, immunoglobulin A, complement component 3, and others to cause defects in the host innate immune system (Silva *et al*., 2011; Aoki *et al*., 2012; Pawar *et al*., 2014). To date, 10 different members of the *SAP* gene family *SAP1* to *SAP10* have been identified in *C. albicans* for the expression of aspartyl proteinases (Feng *et al*., 2015; Dutton *et al*., 2016). *SAP* genes are known for encoding enzymes with similar functions and characters but have distinct molecular properties such as molecular weight, pH and isoelectric point (Karkowska-Kuleta *et al*., 2009; Pawar *et al*., 2014). *SAP* genes are regulated differentially depending on the morphological form of the fungus, stage or type of infection and the surrounding environment. *In vitro* studies provide evidence that *SAP1-3* and *SAP4-6* are highly expressed in the yeast and hyphal phases, respectively. As both yeast and hyphae forms produce *SAP9* and *SAP10,* which encodes GPI anchoring domains, indicating their association with the integrity of the fungal cell wall (Khan *et al*., 2010; Aoki *et al*., 2012; Staniszewska *et al*., 2012).

The exact contribution of individual Sap isoenzymes secretion and enzymatic activities in *C. albicans* pathogenicity is not conclusive because of different expression levels during the course of infection (Naglik *et al*., 2008; Mayer *et al*., 2013; Kumar *et al*., 2015). However, majority of murine experiments have demonstrated that expression of Sap1-3 proteins is necessary for early stages of mucocutaneous infections and for virulence in HDC (Correia *et al*., 2010; Staniszewska *et al*., 2012; Pawar *et al*., 2014). Sap4-6 proteins are involved in both mucosal and systemic infections and have been found to play a role in organ invasion (Staniszewska *et al*., 2012; Kumar *et al*., 2015).

ii. Phospholipases

The production of phospholipases is important for the establishment of *C. albicans* infections. This enzyme can lead to impairment or even rupture of the host cell membrane which is necessary for adherence and invasion (De Luca *et al*., 2012). It has been found that phospholipases activity is usually elevated during tissue damage because these enzymes are responsible for hydrolysis of one or more ester linkages of glycophospholipids of the host cell membranes (Karkowska-Kuleta *et al*., 2009; Sardi *et al*., 2013). The family of the secreted phospholipases is classified into four different classes (PLA to PLD) based on the ability of the enzyme to cleave a specific ester bond in the phospholipid molecule. This action affects the stability of the host cell membranes and eventually causes cell lysis (Ghannoum, 2000; Khan *et al*., 2010; Pereira *et al*., 2015).

In *C. albicans*, five members of class B (PLB1-5) have been implicated in the pathogenicity of the organism and may contribute to the disruption of host cell membranes. Inactivation of *PLB1* and *PLB5* genes in *C. albicans* have shown weakened virulence coupled by reduced ability to penetrate cell membranes in a murine intravenous infection model (Theiss *et al*., 2006). Furthermore, other studies have revealed increased level of extracellular phospholipases production in *C. albicans* clinical isolates compared to commensal isolates (Pinto *et al*., 2008; Mahmoudabadi *et al*., 2010).

iii. Lipases

In vitro studies focusing on the role of extracellular lipases in *C. albicans* as virulence factor have revealed that this enzyme contributes to host invasion in diverse ways (Hube *et al*., 2000; Nguyen *et al*., 2011). Some of the important attributes of extracellular lipase in the pathogenicity of *C. albicans* include adhesion to host tissues, triggering of local inflammatory

disorders which in turn enhance host cell damage (Hube *et al*., 2000; Nguyen *et al*., 2011). Moreover, this enzyme has shown to possess cytotoxic characteristics causing damage to host cells (Park *et al*., 2013). The lipase gene family is made up of at least ten members, *LIP1* to *LIP10* (Paraje *et al*., 2008). A study conducted by Stehr and colleagues to better understand expression profile of lipases during *C. albicans* infections revealed the involvement of extracellular lipases in both cutaneous and systemic infections. Their study showed that the expression level of *LIP* genes depend on the progress of infection and type of infection (Stehr *et al*., 2004). In comparison with other hydrolytic enzymes such as proteinases and phospholipases, extracellular lipases have been widely neglected. As a result, very limited information is available in the literature (Bhat *et al*., 2011).

2.11 Biofilm formation

A biofilm is a population of microbial cells that are adherent to each other or to surfaces, enclosed by a self-produced slimy extracellular polymeric matrix (Mathé and Van Dijck, 2013; Gulati and Nobile, 2016). *Candida* biofilms can develop in a set of diverse environments. This is a great concern especially in public health since dispersed cells from biofilms can colonize implanted medical devices such as the urinary and central venous catheters, dentures, or orthopaedic implants and potentiate invasive candidiasis in humans. It is not surprising that biofilm formation on indwelling medical devices is a major risk factor for the unacceptable increased mortality rates in hospitalized patients (Römling and Balsalobre, 2012; Nobile and Johnson, 2015). Nosocomial device-related infections caused by *Candida* biofilms are inherently difficult to eradicate or control with common antifungal drug therapies, making these infections challenging to combat. One of the key defining characteristics of *C. albicans* biofilms is their resilience to broad spectrum of antifungal drugs with minimum inhibitory concentrations of up to one thousand-fold higher than those necessary to inhibit planktonic cells (Taff *et al*., 2012; Silva-Dias *et al*., 2015; Sandai *et al*., 2016). Increased resistance of *C. albicans* biofilms is caused by several factors, but it has been mostly linked to upregulation of drug efflux pumps, an impermeable extracellular matrix and existence of recalcitrant persister cells (Nobile and Johnson, 2015).

2.12 Oxidative stress

Oxygen free radicals also known as reactive oxygen species (ROS) are the by-products of normal cellular metabolism in living organisms. ROS can be beneficial in living systems at low to moderate concentrations, however, excess production of ROS can cause adverse modifications to cellular components such as lipids, proteins, and nucleic acids. These modifications can induce cell apoptosis (Rahman, 2007; Lone *et al*., 2013; Wang *et al*., 2017). The detrimental effect of ROS to cause cellular damage is often termed as oxidative stress and the host's defense against this stress is known as antioxidant defense system. Oxidative stress is often caused by a shift in the balance between formation of ROS and antioxidant defense response (Birben *et al*., 2012; Fisher *et al*., 2012). Oxidative stress has been implicated in the pathophysiology of several human diseases such as cancer, cardiovascular diseases, neurodegenerative disorders and other chronic diseases associated with aging (Ďuračková, 2010; Fisher *et al*., 2012; Rahman *et al*., 2012).

Free radicals are inherently unstable chemical molecules containing one or more unpaired electrons. The presence of unpaired electrons increases the chemical reactivity of the free radical towards other substances (Betteridge, 2000; Lenaz, 2012). Some of the most studied radical species that are of physiological significance in living systems include hydroxyl radical (•OH), O_2^- (superoxide anion) and H_2O_2 (hydrogen peroxide) (**Figure 2.7**) (Rahman *et al*., 2012; Wang *et al*., 2017). During normal cellular respiration, molecular oxygen is continuously reduced to water by the mitochondrial electron transport chain (ETC). This process is mediated by different respiratory burst enzymes such as NADPH oxidase and xanthine oxidase. However, the majority of the O_2^- is generated within the inner mitochondrial membrane in living organisms via the respiratory chain. It has been identified that a small proportion of about 1 to 3% of electrons leak to oxygen prematurely during oxidative phosphorylation by healthy mitochondria, leading to production of mitochondrial O_2^- instead of reducing molecular oxygen to water (Birben *et al*., 2012; Lenaz, 2012; Lone *et al*., 2013; Kim *et al*., 2015). In addition, there are several external stimuli which can potentially trigger the production of free radicals. These include ultraviolet radiation, gamma rays, air pollutants, heavy metal ions and chemotherapy drugs (Betteridge, 2000; Gu *et al*., 2015).

Several signaling pathways have been shown to be directly involved and activated in response to a diverse range of environmental stresses. Of these, high osmolarity protein (Hog1p) MAPK and activating protein-1 (Cap1p) are the most contributing pathways to

oxidative stress tolerance in *C. albicans*. This transcriptional response promotes osmotic adaptation and glycerol accumulation (Enjalbert *et al*., 2006; Mayer *et al*., 2013; Kaloroti *et al*., 2014). *In vitro* studies have shown that inactivation of these regulators impair stress resistance and attenuate virulence in *C. albicans* (Cheetham *et al*., 2011; Jain *et al*., 2013; Tillmann *et al*., 2015).

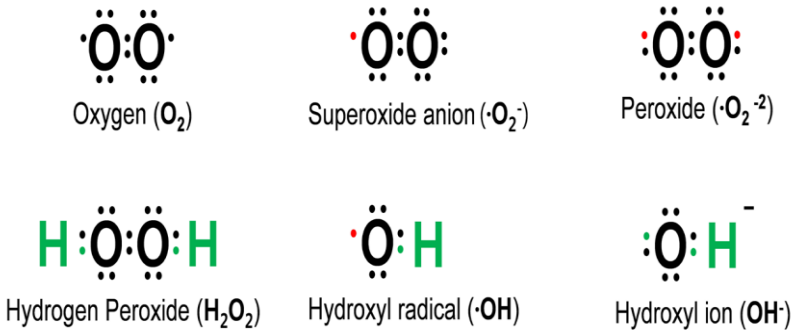

Figure 2.7: Reactive oxygen species generated as natural by-product of normal cellular respiration. The unpaired electrons are shown in red (Kim *et al*., 2015).

The mitochondrial ETC is composed of five major multi-subunit complexes including NADH-coenzyme Q (CoQ) reductase (Complex I), succinate dehydrogenase (Complex II), ubiquinol-cytochrome c reductase (Complex III), cytochromeCoxidase (Complex IV), and ATP synthase (Complex V). The respiratory chain complexes I and III are recognized for their maximum capacities to produce significant amounts of ROS, particularlyO_2^- (Brand, 2010; Gupta *et al*., 2014). In Complex I, NADH: ubiquinone oxidoreductase catalyzes the transfer of electrons from NADH to ubiquinone. This complex serves as an entry point of electrons from NADH into the mitochondrial ETC. The reduced form of flavin mononucleotide (FMN) accepts electrons from NADH and transfers them via a series of seven conserved iron-sulfur clusters to the reduction site of ubiquinone (Murphy, 2009; Lone *et al*., 2013). There is evidence for at least two sites involved in the production of O_2^- in complex I, the FMN group or iron-sulfur clusters binding site and the ubiquinone binding site. The latter is commonly associated with the copious production of ROS levels. This is a consequence of the reduction of CoQ pool coupled with a high proton gradient via reverse electron flow (Murphy, 2009). In contrast, forward electron transfer favors the reduction of FMN leading to an increase of NADH/NAD^+ ratio. The FMN site generates relatively low amounts of O_2^- (Murphy, 2009; Andreyev *et al*., 2015).

Complex III is an enzyme complex that funnels electrons from coenzyme Q (QH_2) to cytochrome C, an electron acceptor. This complex produces significantly high amounts of O_2^- in the presence of a respiratory inhibitor such as antimycin. Antimycin interferes with the transfer of electrons from cytochrome C to the quinone at centrei (Q_i) site, leading to accumulation of semiquinone at centre o (Q_o) site. This is the reason for complex III to leak electrons to molecular oxygen, thereby enhancing O_2^- formation (Kowaltowski *et al*., 2009; Brand, 2010). Complex III is distinguished from other ROS producing sites by the ability to release electrons to both the matrix and the inner membrane of the mitochondria (Lone *et al*., 2013; Orr *et al*., 2013).

2.13 Resistance to oxidative stress by production of antioxidant enzymes

In order to thrive and establish infections within the host, *C. albicans* has developed robust antioxidant defense mechanisms to evade or neutralize ROS produced either by the host's innate immune system or antifungal drugs (Belenky and Collins, 2011; Bink *et al*., 2011; Miramo´n *et al*., 2012; Youseff *et al*., 2012). The endogenous antioxidant compounds have been classified into two major categories: enzymatic and non-enzymatic antioxidants. The major enzymatic antioxidants known to directly counterbalance the harmful effects of ROS and repair cellular damage include SOD, catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) (Rahman *et al*., 2012; Gupta *et al*., 2014). Upon oxidative stress, fungal cells are exposed to significant levels of O_2^- that will eventually lead to the killing of the organism if not neutralized by the activity of antioxidant enzymes. Antioxidant enzyme SOD is able to convert O_2^- to a more stable oxidant H_2O_2 and molecular oxygen. The spontaneous dismutation of O_2^- by SOD is considered the primary and vital step for regulating intracellular ROS production in living organisms (Gupta *et al*., 2014; Wang *et al*., 2017). Subsequently, the produced end product H_2O_2 is reduced to water by either catalase or GPx. The GPx reaction for detoxification of H_2O_2 oxidizes the reduced form of glutathione (GSH) into glutathione disulfide (GSSG). This is followed by the regeneration of GSH from GSSG, with the help of a reducing agent NADPH through the enzymatic reaction of GR. The GSH recycling process is of fundamental importance in scavenging free radicals in living cells (Gupta *et al*., 2014; Miramo´n *et al*., 2014). One more important antioxidant enzyme involved in detoxifying xenobiotic substrates or rather reactive metabolites generated from oxidative stress is glutathione S-transferase (GST). This enzyme catalyzes the conjugation of GSH to xenobiotics, thereby preventing their interaction with cellular proteins and biological membranes (Birben *et al*., 2012; Wang *et al*., 2017).

The nonenzymatic antioxidants include low molecular weight compounds such as GSH, vitamin E and C, carotenoids and many others (Birben *et al*., 2012).

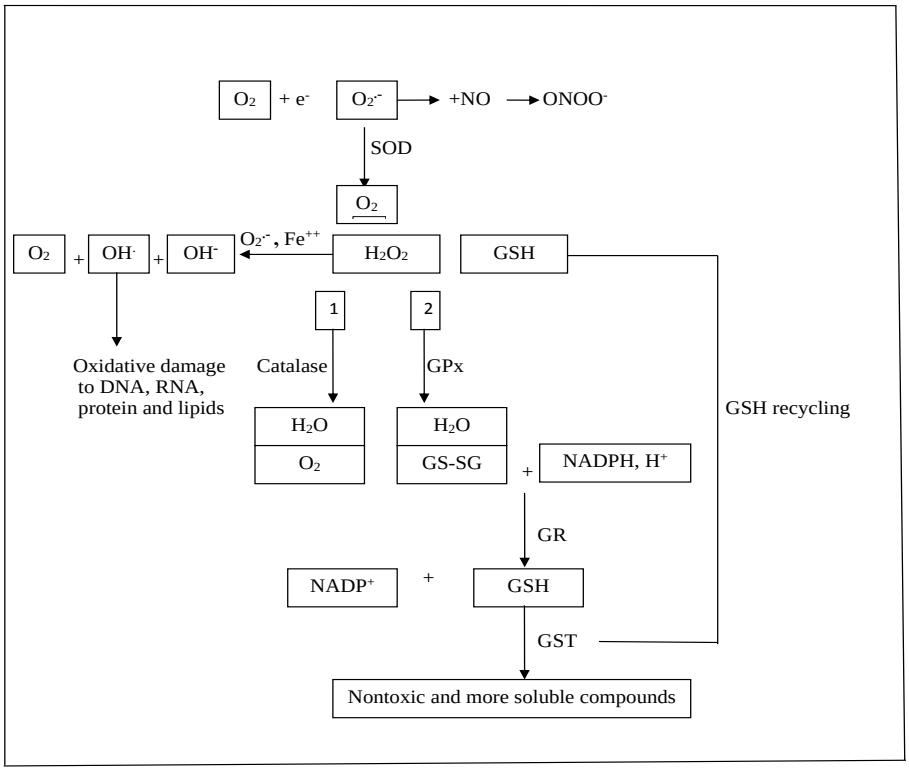

Figure 2.8: A schematic illustration of O_2^- detoxification via enzymatic antioxidant pathways.

2.13.1 Superoxide dismutase

A typical eukaryotic organism solely expresses two SODs, copper-zinc (Cu/Zn) dependent SOD1 that resides mainly in the cytosol and manganese (Mn) dependent SOD2 found within the mitochondrial matrix. *Candida albicans* possesses four additional SOD isoforms: MnSOD3 localized in the cytosol and copper only GPI- anchored SOD4 to SOD6 (Bink *et al*., 2011; Peterson *et al*., 2016). According to Li and colleagues, *C. albicans* expresses the cytosolic SOD1 and SOD3 as a mechanism of survival regardless of copper availability in the environment. In the presence of copper, Cu/Zn SOD1 expression level remain significantly elevated. Conversely, depletion of intracellular copper induces switching of expression from Cu/Zn SOD1 to MnSOD3. This switch is mediated by the sensing regulator MAC1 in response to copper status. Moreover, this phenomenon was not only observed in murine model studies but also confirmed in *C.albicans* isolates from human blood samples (Li *et al*., 2015[a]; Broxton and Culotta, 2016).

The expression of SOD enzymes in *C. albicans* is dependent on the morphology of the organism. In the hyphal state of *C. albicans*, SOD5 is highly expressed whereas SOD4 increase is only observed in the yeast phase. Upregulation of SOD5 during the infection is typically due to the fact that hyphal form of *C. albicans* is necessary for interactions with the host phagocytic cells (Heilmann *et al*., 2011; Dantas *et al*., 2015). *In vitro* studies have demonstrated the virulence role of SOD enzymes especially SOD1 and SOD5 (Frohner *et al*., 2009; Miramo´n *et al*., 2012).

A number of studies investigating the mechanism of action used in natural and synthetic compounds known to exhibit antifungal activity have shown that some of these compounds potentiate fungicidal action against *C. albicans* by modifying the expression level of SOD genes following exposure to the compounds (Ojha *et al*., 2010; Yousuf *et al*., 2010; Linares *et al*., 2013; De Cremer *et al*., 2016). Furthermore, inactivation of genes encoding for SOD enzymes in *C. albicans* renders the mutant strains hypersensitive to killing (Frohner *et al*., 2009; Chaves and da Silva, 2012; Miramo´n *et al*., 2012; Gleason *et al*., 2014). It has been reported that miconazole treatment induced a significant upregulation in all SOD genes except for SOD3 when compared to the untreated control group in *C. albicans* biofilms. These findings demonstrate the important role of the antioxidant response in drug resistance. To further confirm the specific action of the compound in treated cells, *C. albicans* deletion mutants (ΔΔΔ*sod4sod5sod6 C. albicans* cells) or SOD inhibitor N,N′-diethyldithiocarbamate (DDC) were used. These evaluations led to an increased antifungal activity of miconazole, which is directly linked with the rise of endogenous ROS production (Bink *et al*., 2011; De Cremer, 2016; Sun *et al*., 2017).

2.13.2 Catalase

The catalase enzyme is a tetrameric protein with a single iron-containing heme group embedded in the catalytic site of each monomer. This enzyme is present in the peroxisome of almost all aerobic organisms, thereby preventing accumulation of toxic compounds more specifically H_2O_2. Catalase efficiently converts H_2O_2 to water and molecular oxygen using iron as a cofactor. One striking characteristic of catalase enzyme is the highest turnover numbers withone molecule of catalase able to convert approximately six million of H_2O_2 molecules to water and oxygen in a second (Rahman, 2007).

Unlike with many other yeast organisms such as *Saccharomyces cerevisiae* that encode two different catalase genes, *C. albicans* expresses only one catalase gene (Wysong *et al*., 1998;

Nishimoto *et al*., 2016). Catalase is considered to be one of the pathogenic factors that significantly contribute to the establishment of *C. albicans* infections. This enzyme is required for resistance to peroxide stress, as well as for protection against PMNs killing (Wysong *et al*., 1998; Nakagawa *et al*., 2003; Kaloroti *et al*., 2014). Several studies have rigorously evaluated the role of catalase enzyme through inactivation or attenuation of the *CAT1* gene. It was revealed that disruption of the *CAT1* gene in *C. albicans* increases the susceptibility of the organism to killing and also causes defects in virulence in an experimental model of HDC (Wysong *et al*., 1998; Komalapriya *et al*., 2015).

2.13.3 Glutathione

Glutathione (GSH) is the most abundant low molecular weight thiol present in living organisms including plants, animals, and microorganisms. GSH is involved in several metabolic processes and the imbalance of its redox homeostasis is implicated in the etiology and progression of many diseases. It is estimated that the intracellular concentration of GSH in yeast cells is approximately 10 mM (Yadav *et al*., 2011).

GSH is a tripeptide molecule containing three amino acids: cysteine, glycine and glutamic acid with a free thiol group. The synthesis process of GSH is catalyzed by ATP-requiring cytosolic enzymes, GSH synthetase and γ-glutamylcysteine synthetase (Lushchak, 2012; Maras *et al*., 2014). GSH that exist in cells is mostly in its reduced form. Upon oxidation, the reduced form of GSH serves as an electron donor that reduces disulphide bonds and in turn, becomes converted to its oxidized form GSSG. GSSG is then reverted to the reduced form GSH through an enzymatic reaction regulated by glutathione reductase (GLR), using NADPH as a cofactor. Disturbance of the GSH/GSSG ratio in *C. albicans* cells affect the redox status and induce elevated ROS levels, auxotrophy and low virulence (Baek *et al*., 2004; Yadav *et al*., 2011; Zhu *et al*., 2011; Guedouari *et al*., 2014).

In *C. albicans*, GSH plays a key role in defense against oxidative damage and detoxification of free radicals and many other toxic compounds. Exposure of *Candida* cells to antifungal drugs is known to induce ATP driven transporters. These antifungal drugs conjugate with GSH and are exported out of the cell (Zhu *et al*., 2011). Studies investigating the mechanism of action in selected natural and synthetic compounds known to exert antifungal activity have reported a dose-dependent depletion of intracellular GSH levels coupled with elevated ROS production in *Candida* cells (Abegg *et al*., 2012; Bertóti *et al*., 2016; Thangamani *et al*., 2017). Furthermore, assessment of the influence of FLC and micafungin in *C. albicans* cells

have shown a decrease in the overall GSH/GSSG ratio demonstrating that these drugs interfere with the normal regulation of redox state of the cells. Interestingly, FLC resistant *C. albicans* showed a significant increase in GSH content as opposed to the FLC sensitive isolate. The increment of GSH content enhanced fungal protection against oxidative stress. The study also speculated that high levels of GSH in FLC resistant *C. albicans* may have a role in the removal of the drug from the cell (Maras *et al*., 2014).

2.14 Berberine

2.14.1 Berberine history and use

Chemical compounds derived from natural sources especially plants are used throughout the world for medicinal purposes and commonly as the major source of antimicrobial agents. The use of herbal medicine for primary health care is very popular in developing countries. The World Health Organisation (WHO) has reported that approximately 80% of the populations, particularly in the African and Asian countries continue to widely use herbal medicine because it is often affordable and accessible as opposed to modern medicine. Phytomedicine has received increasing attention from researchers in recent years in an attempt to obtain scientific evidence on the safety and effectiveness of these medicinal plants and eventually integrate herbal medicine into the national health care systems (Ahmad *et al*., 2016).

Berberine (**Figure 2.9**) is an isoquinoline alkaloid that is isolated from different Chinese medicinal plants such as *Coptis chinensis* (goldenthread), *Hydrastis Canadensis* (goldenseal), *Berberis aquifolium* (Oregon grape), *Berberis aristata* (tree turmeric) and many other natural products. BER is present in the roots, rhizomes, and stem bark of these medicinal plant species (Kumar *et al*., 2015; Elsheikh *et al*., 2018). This compound is bright yellow in colour and is characterized by its odourless and acidic nature. BER is slightly soluble in water and alcohol, but sparingly soluble in methanol. In general, the salt forms of BER are often more soluble (Battu *et al*., 2010; Kumar *et al*., 2015). Traditionally, BER had been used for many years in Chinese medicine for the treatment of gastroenteritis. However, clinical investigations in recent years have focused on unraveling the biological properties of BER and it has been revealed that this compound possesses a wide spectrum of pharmacological effects. These include properties such as antitumor, antimicrobial, anti-inflammatory, anti-hypertensive, anti-hyperglycaemic, anti-depressant, neuroprotective and many others (Tan *et al*., 2011; Nguyen *et al*., 2014).

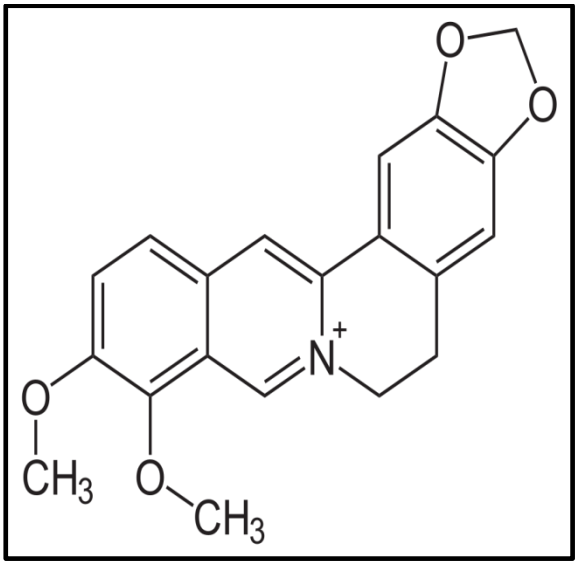

Figure 2.9: A chemical structure of berberine (Pang *et al*., 2015).

2.14.2 Bioavailability

Despite the fact that BER and its derived compounds have shown to exhibit various pharmacological properties, the use of BER is limited due to its poor bioavailability after oral administration. *In vivo* studies in murine animal experiments have reported oral bioavailability of BER to be less than 1% (Liu *et al*., 2010[b]; Zhang *et al*., 2014). This is mostly attributed to its hydrophilic nature, low intestinal absorption and poor aqueous solubility (Zhang *et al*., 2014; Kumar *et al*., 2015; Elsheikh *et al*., 2018). It was revealed by Pan and colleagues that P-glycoprotein pumping activity is responsible for the poor bioavailability of BER (Pan *et al*., 2002). The intestinal P-glycoprotein is a cell membrane ATP-dependent efflux protein that is found in the human intestinal epithelia. This protein interacts with BER and excretes it from the enteric cells back into the intestinal lumen. P-glycoprotein efflux activity highly contributes to the reduced efficacy of BER and many other drugs that are known as P-glycoprotein substrates (Pan *et al*., 2002; Zhang *et al*., 2013). To overcome this challenge of poor bioavailability, novel mechanisms have been tried in the recent works of literature that promise to enhance the drug delivery system as well as intestinal absorption of BER. The research focuses mainly on the use of nanoparticle-based carriers and P-glycoprotein inhibitors to suppress the efflux process (Zhang *et al*., 2013; Nguyen *et al*., 2014; Elsheikh *et al*., 2018). In addition, the use of BER in combination with other clinically approved drugs often produces a synergistic effect in experimental studies (Zhou *et al*., 2012; Imenshahidi and Hosseinzadeh, 2016).

2.14.3 Toxicity

In general, BER shows very low toxicity with minimal side effects (Pang *et al*., 2015; Mahmoudi *et al*., 2016). However, it is believed that toxicity in mammalian cells is concentration-dependent with safety levels ranging from 0.5 g to an excessive dosage of more than 4g (Gu *et al*., 2015). A study by Kheir and colleagues has shown that not only does excessive concentration of BER contribute to its acute toxicity but also the route of drug administration. They injected BER in mice through three different administration routes and found a median lethal dosage of BER to be 9.03 and 57.61 mg/kg in intravenous and intraperitoneal groups respectively, but no significant median lethal dosage was reported for the intragastric group (Kheir *et al*., 2010). Side effects that have been reported in humans due to BER overdose include mild gastrointestinal discomfort and allergies (Zhang *et al*., 2008; Gu *et al*., 2015). However, some clinical studies have reported unexpected adverse effects such as suppression of cellular and humoral immune functions, elevation of bilirubin levels in the blood serum and modification of the sex hormone synthesis pathway (Linn *et al*., 2012; Lao-Ong *et al*., 2013; Mahmoudi *et al*., 2016).

2.14.4 Distribution

Although BER has shown to be present at very low concentrations in the blood, *in vivo* study evaluating the pharmacokinetic profile of BER has revealed a much higher concentration of BER and its metabolites in the body tissues than in the blood following oral administration (Tan *et al*., 2013). BER was administered orally to rats and showed a rapid distribution to the organs including the liver, lungs, kidneys, body muscle, brain, heart, and fat. The concentration of BER and its major metabolites was high in the studied tissues than in the blood 4 hours after administration. The study also suggested that BER was able to reach the target organs within 15 minutes after dosing (Tan *et al*., 2013). Moreover, it has been reported that BER is able to permeate the blood-brain barrier which is a prerequisite for neuroprotective drugs (Wang *et al*., 2005; Kumar *et al*., 2015).

2.14.5 Combination treatment in *C. albicans*

According to studies that have investigated the biological properties of natural compounds, BER exerts potent synergistic antifungal activity when used in combination with known antifungal drugs recommended for the treatment of *Candida* infections including FLC (Iwazaki *et al*., 2010; Wei *et al*., 2011; Li *et al*., 2015[b]). The use of combination therapy has gained momentum in clinical practice, with many advantages over monotherapy. These

include improved therapeutic efficacy, lower side effects and reduced development of drug resistance (Zhu *et al*., 2014).

Typically, BER exhibits weak antifungal activity with higher MIC values compared to the available drugs. However, the concomitant use of BER and FLC in *C. albicans* shows fractional inhibitory concentration index value of less than 0.5 (Li *et al*., 2015[b]; Xu *et al*., 2017; Yang *et al*., 2018). Interestingly, the synergistic antifungal efficacy of BER when combined with other antifungal drugs is most evident in FLC resistant *C. albicans* than in FLC susceptible *C. albicans* isolates. This interaction represents a promising and safer approach for the development of novel antifungal drugs against FLC resistant *C. albicans* (Quan *et al*., 2006; Li *et al*., 2015[b]).

Based on these promising results, researchers have focused on understanding the underlying mechanisms of action involved in BER alone or with other antifungal drugs against *Candida* species (Dhamgaye *et al*., 2014; da Silva *et al*., 2016; Zorić *et al*., 2017). It has been observed in numerous studies that the combination of BER and FLC induce plasma membrane damage in FLC resistant *C. albicans* (Shao *et al*., 2016; Zorić *et al*., 2017). It is with no doubt that FLC inhibits ergosterol synthesis of the cell membrane which results in loss of membrane integrity and increased permeability. Li and colleagues performed transmission electron microscopy and it was found that FLC treatment caused damage to the cell membrane, leading to an increase of intracellular accumulation of BER in *C. albicans* cells. This effect of FLC on the cell membrane is believed to enhance the antifungal activity of BER. The same study revealed that the use of other antifungal drugs that target the cell membrane such as ketoconazole, itraconazole and amphotericin B were able to enhance the susceptibility of resistant *C. albicans* to BER. Conversely, 5-FC and caspofungin were not able to synergize with BER (Li *et al*., 2013; Yang *et al*., 2018).

2.15 Anti-virulence properties of BER in *Candida* species

2.15.1 Efflux pumps

Studies investigating the possible involvement of efflux pump in the antifungal activity of BER have revealed that BER reduce expression of efflux pumps in *Candida* species (Li *et al*., 2013; Shao *et al*., 2016). However, it has been observed that the use of BER and FLC cause significant inhibition of *CDR1* and *CDR2* genes especially in FLC resistant isolate (Li *et al*., 2013; Shao *et al*., 2016; Xu *et al*., 2017). In a study conducted by Zhu and co-workers, BER

inhibited upregulation of *CDR1* and *CDR2* genes and their transport function in *C. albicans* (Zhu *et al*., 2014).

2.15.2 Morphological transition

Morphological transition from non-pathogenic yeast form to pathogenic hyphae form is one of the intial virulence factors of *C. albicans.* BER has been reported to hault this morphological transition in *C. albicans.*A study done by Zorić and colleagues tested the effect of BER on morphological transition in different hyphal inducing media. Their findings showed that BER inhibited the transition of *C. albicans* from yeast to its filamentous form in all media used (Zorić *et al*., 2017).

2.15.3 ROS

It has been reported that intracellular ROS augmentation is an important mediator for antifungal activity of azole drugs. Interestingly, the induction of low levels of endogenous ROS in FLC resistant *Candida* species correlates highly with drug resistance (Kobayashi *et al*., 2002; Bink *et al*., 2011). However, exposure of FLC resistant *Candida* to BER and FLC increase endogenous ROS. This is an indication of a positive mechanism exerted by BER to overcome FLC resistance (Xu *et al*., 2009; Shao *et al*., 2016). A study done in *C. albicans* showed enhanced levels of ROS in the presence of BER (Dhamgaye *et al.*, 2014). In another study, Zorić and colleagues also showed a dose-dependent generation of ROS in BER treated *C. albicans* cells (Zorić *et al*., 2017).

To further characterize and understand the mechanism of action used by BER and its effect on *Candida* cells, assessment of primary antioxidant enzymes as a response and defense mechanism at enhanced levels of ROS is important. This study was conducted to generate new information on the antioxidant defense system of *C. albicans* under BER induced oxidative stress.

Chapter 3: Materials and Methods

3.1 Study population

This study was conducted in the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand. A total of twenty clinical isolates of *C. albicans* stored in glycerol stocks at -80 ºC in the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand, were used. In addition, a control laboratory strain *C. albicans* ATCC MYA2876, also known as *C. albicans* SC5314, was used in all the experiments.

3.2 Ethical approval

The clinical isolates of *C. albicans* were collected from HIV positive (Group A), cancer (Group B) and other immunocompromised patients (Group C) attending different clinics at Charlotte Maxeke Johannesburg Academic Hospital. All these strains were isolated from patients under the ethical clearance number M000402, obtained from the Human Research Ethics Committee, University of the Witwatersrand. To use these isolates in this study, approval was granted by the Human Research Ethics Committee of University of the Witwatersrand under ethical waiver reference number: W-CJ-170524-1 (**Appendix 7.4**).

3.3 *Candida albicans* identification

3.3.1 API 20 C AUX

An API 20 C AUX was performed using BioM`erieux API kit (BioM`erieux, France) according to the manufacturer's instructions. The isolates were cultured onto Sabouraud 4% glucose agar (Sigma-Aldrich, MO, USA) and incubated at 37 ºC for 18-24 hours. An incubation box was prepared by distributing approximately 5 ml of sterile distilled water in the tray to create a moist environment. A fresh yeast colony was inoculated into 5 ml of 0.85% NaCl, mixed well in preparation of approximately 3 x 10^8 cfu/ml by adjusting cell density to 0.2 at 600 nm using Siemens Microscan Turbidity Meter. A 100 µl of the suspension was transferred to API C medium and gently mixed. About 140 µl of the suspension obtained from the API C medium was carefully transferred to each cupule. The tray was closed with the provided lid and incubated at 30 ºC for 72 hours.

The reading and interpretation of the results were done using the BioM`erieux API web software. A cupule more turbid than the 0 cupule was recorded as a positive reaction.

3.3.2 Germ tube test

A fresh yeast colony was emulsified in 0.5 ml of fetal bovine serum (Roche Diagnostics, GmbH, Germany) and was incubated at 37 ºC for 2 hours. Subsequently, a drop of the suspension was placed at the centre of the microscope slide (Lasec, South Africa) using sterile glass capillary tubes (Lasec, South Africa). The suspension drop was carefully covered with a coverslip (Lasec, South Africa) kept in a slanting position to minimize the formation of air bubbles. The wet mount slide was examined using a light microscope to visualize production of germ tubes. *Candida albicans* SC5314 (germ tube positive) and *C. parapsilosis* ATCC (germ tube negative) were included in this experiment as controls.

3.4 Antifungal susceptibility testing

3.4.1 Minimum inhibitory concentration (MIC)

To determine the antifungal activity of berberine (BER) against 21 test isolates, MIC was determined using broth microdilution method following the guidelines as recommended by the CLSI M27-A3, with modifications (CLSI, 2008). A working solution of 8000 µg/ml of BER (**Appendix 7.5.1**) and 500 µg/ml of FLC (**Appendix 7.5.2**) were prepared for this experiment. Separately, a 100 µl of Sabouraud dextrose (SD) broth (Sigma-Aldrich, MO, USA) was dispensed into a 96 well microtiter plate (Thermo Fischer Scientific, MA, USA). From the working solution, 100 µl of BER was added in the first column and mixed properly by pipetting up and down three times. A serial twofold dilution was done by transferring 100 µl of the mixture from the first well to the second. This process was successively repeated until the last well. Aliquots of 100 µl exponentially growing *C. albicans* cells were carefully dispersed into all the wells except for the media control wells. In every set of the experiment, positive (FLC), negative (20% methanol), media and drug free culture controls were included. The final concentrations in the plate ranged from 125 to 0.06 µg/ml for FLC and those of BER were from 2000 to 0.98 µg/ml. All the microtiter plates were sealed with lids and were incubated at 37 ºC for 18-24 hours. The results were read visually and the lowest concentration with no visible growth was considered as the MIC value. The isolates were further categorized as either susceptible or resistant to FLC following the CLSI breakpoints (CLSI, 2012). *Candida albicans* isolates with MIC $\leq$ 8 µg/ml were declared susceptible and resistant isolates were those with MIC $\geq$ 8 µg/ml. All the experiments were done in triplicate and all the results were expressed in µg/ml.

3.4.2 Minimum fungicidal concentration (MFC)

MFC values were determined as described previously by Borman *et al.*, 2017, with modifications. A 10 µl of the sample was removed from MIC wells, two concentrations below and above MIC concentration. The sample was spread onto Sabouraud 4% glucose agar plates and incubated at 37 ºC for 48 hours. MFC was determined as the lowest concentration of compound in which no viable cells of *C. albicans* were recovered.

3.5 Determination of viable cells

The survival rate of *C. albicans* cells was estimated as previously described by Lee *et al*., 1995, with modifications. A single colony of FLC susceptible *C. albicans* SC5314 and FLC resistant *C. albicans* 4122 were inoculated in SD broth and incubated at 37 ºC with shaking at 100 ×g for 16 hours. The cultures were harvested by centrifugation at 2200 ×g for 15 min. The pelleted cells were resuspended in a fresh SD broth and adjusted cell density to OD_{600} = 0.5, equivalent to 6×10^8cfu/ml. The suspension was exposed to 0 (control), 63, 125, 250 and 500 µg/ml BER for 2 hours. An aliquot of 10 µl of the test sample was taken from each concentration at 0, 1 and 2 hours and was ten-fold diluted using sterile distilled water. A 10 µl of the diluted sample was spread onto Sabouraud 4% glucose agar plates and incubated for 48 hours at 37 ºC. After incubation, colonies were counted in all the plates and survival percentage was calculated as follows:

$$\text{Survival } \% = \frac{\text{cfu at each point}}{\text{cfu of untreated cells}} \times 100$$

3.6 Gene expression

To study the role of antioxidant defense genes in the susceptibility to FLC in *C. albicans* isolates and to determine the effect of BER on these genes, gene expression of *SOD1*, *SOD2*, *GPx2*, *GLR1*, *GTT11*, and *CAT1* in untreated and BER treated *C. albicans* cells was measured by Quantitative reverse transcription PCR (RT-qPCR).

3.6.1 RNA extraction

Candida albicans RNA was extracted using the Zymo Research Quick-RNA Fungal/Bacterial Miniprep Kit (Zymo Research Corp, USA), according to the manufacturer's instructions with slight modifications. All the fungal cultures were grown as described above in section 3.5. After centrifugation at 2200 ×g for 15 min, pelleted cells were resuspended in 800 µl of RNA lysis buffer and transferred to ZR BashingBead lysis tube. The samples were vortexed for 2 min and centrifuged at 12 000 ×g. After centrifugation, 400 µl of the

supernatant was transferred to a Zymo-Spin IIICG column in a 2 ml collection tube and centrifuged at the same conditions. This was followed by addition of 400 µl absolute ethanol (Diagnostic Media Products-NHLS, SA) to the flow-through. The mixture was transferred to a Zymo-Spin IIC column and centrifuged at 12 000 ×g for 1 min. A 400 µl RNA prep buffer was added to the column and centrifuged at 12 000 ×g for 1 min. A 700 µl RNA wash buffer was added to the column and centrifuged at 12 000 ×g for 1 min. A 400 µl RNA wash buffer was added to the column and centrifuged at 12 000 ×g for 2 min. Lastly, 30 µl DNase/RNase-free water was directly added to the column matrix and centrifuged at 12 000 ×g for 60 seconds. The eluted RNA was aliquoted and stored at -70 ºC for further use.

3.6.2 DNase treatment

Aliquots of 10 µg RNA samples were suspended in 1X DNase buffer (**Appendix 7.5.3**) to make a final volume of 100 µl. 1 µl of 250 U DNase (Zymo Research Corp, USA) was added to the mixture and briefly vortexed to spin down the sample. The sample was incubated for 30 min at 37 ºC. Inactivation of the reaction was done by adding 1 µl of 0.5 mol/L EDTA and incubated at 75 ºC for 10 min. Following the incubation period, the sample was placed on ice for 10 min before use.

3.6.3 RNA quality assessment

A 10 µl of RNA sample was gently mixed with 5 µl of the loading dye (Thermo Fischer Scientific, MA, USA). The sample was loaded into the wells of 1.8% agarose gel stained with ethidium bromide (Merck KGaA, Darmstadt, Germany). The gel was placed in the tank filled with 1X TAE buffer (**Appendix 7.5.4**) and ran for 1 hour at 75 V. A 50 bp DNA ladder (Thermo Fischer Scientific, MA, USA) was included in this experiment. Imaging was done using ChemiDocXRS gel documentation system (Bio-Rad, CA, USA) under ultraviolet illumination to visualize the separated *C. albicans* fragments 25S, 18S, and 5.8S rRNA.

3.6.4 cDNA synthesis

Reverse transcription of the RNA was performed using iScript cDNA synthesis kit (Bio-Rad, CA, USA) following the manufacturer's instructions. A reaction mixture of 20 µl volume consisting of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase, 5X iScript reaction mix containing oligo (dT) and random hexamer primers, nuclease-free water and 1 µg of RNA sample was prepared in an Eppendorf tube (Thermo Fischer Scientific, MA, USA). The mixture was incubated in an Eppendorf Thermomixer using the following conditions: priming for 5 min at 25 ºC, reverse transcription for 20 min at 46 ºC, and

reversetranscription inactivation for 1 min at 95 ºC. cDNA samples were stored at -20 ºC for further use.

3.6.5 Quantitative reverse transcription PCR (RT-qPCR)

RT-qPCR was performed with PowerUp SYBR Green Master Mix (Thermo Fischer Scientific, MA, USA) using the LightCycler Nano Real-Time PCR System (Roche, Basel, Switzerland). A reaction mixture of 20 µl consisting of 10 µl SYBR Green master mix, 0.5 µl of 10 µM each primer (Integrated DNA Technologies, IA, USA), 6 µl nuclease-free water and 100 ng RNA template, was prepared. Primer sequences for different genes are shown in **Table 3.1**. The cycling conditions were as follows: uracil-DNA glycosylase activation at 50 ºC for 2 min, initial denaturation at 95 ºC for 2 min, followed by 40 cycles of denaturation at 95 ºC for 15 sec, annealing at 50 ºC for 15 sec, elongation at 72 ºC for a minute.

A blank or no template control reaction was included in every run to identify PCR contamination. Melting curve analysis was performed to ensure amplification of specific PCR products. Gene expression level was calculated using the formula:

$$2^{-\Delta\Delta Cq}$$

Where ΔCq was the mean Cq value of the target gene minus the mean of housekeeping genes, and ΔΔCq was the ΔCq of the tested cells minus ΔCq of the control cells.

Table 3.1: List of primer sequences used in this study

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Source
Genes of interest			
CAT1	ACACAGGAA ATACCCAAT GAG	GCA TCA GCC AAG TCT TGA GAG	Westwater *et al*., 2005
SOD1	TTG AAC AAG AAT CCG AAT CC	AGC CAA TGA CAC CAC AAG CAG	Westwater *et al*., 2005
SOD2	ACC ACC CGT GCT ACT TTG AAC	GCC CAT CCA GAA CCT TGA AT	Westwater *et al*., 2005
GPx2	TGT GTG GGT TCA CAC CTC AA	ATG GGG AAA CTG ACA CCA AA	Zhu *et al*., 2011
GLR1	GCT CAT CTA AGT CAT TGT GAC C	GCT GGA CCA GAA GAA AAA GTT G	Dai *et al*., 2013
GTT11	TGC TAG ACA TTC CTC CCT GT	TGG CCA GTT TCA GCA ATC AC	This study
Housekeeping genes			
RIP	TGT CAC GGT TCC CAT TAT GAT ATT T	TGG AAT TTC CAA GTT CAA TGG A	Nailis *et al*., 2006
PMA1	TTG CTT ATG ATA ATG CTC CAT ACG A	TAC CCC ACA ATC TTG GCA AGT	Nailis *et al*., 2006

CAT1 = catalase, *SOD1* = copper-zinc superoxide dismutase, *SOD2* = manganese superoxide dismutase, *GPx2* = glutathione peroxidase, *GLR1*= glutathione reductase, *GTT11* = glutathione S-transferase, *RIP* = ubiquinol cytochrome-c reductase complex component, *PMA1* = plasma membrane ATPase.

3.7 Enzymatic activity

Activities of different enzymes, such as catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione S-transferase; which play an important role as an antioxidant defense system in *C. albicans* was measured spectrophotometrically.

3.7.1 Preparation of cell-free extract

Cell-free extract of *C. albicans* cells were prepared according to the method described by Jethwaney *et al*., 1997, with modifications. Exponentially growing cells of FLC susceptible *C. albicans* SC5314 and FLC resistant *C. albicans* 4122 were exposed to 0 (control), 63, 125 and 250 µg/ml BER for 2 hours. Approximately 1 g wet weight cells were suspended in 5 ml grinding medium consisting of 250 mmol/L sucrose (**Appendix 7.5.5**), 10 mmol/L Tris-HCl buffer at pH 7.5 (**Appendix 7.5.6**), 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) (**Appendix 7.5.7**), and 2 g of 0.5 mm glass beads (Lasec, South Africa). The suspension was disrupted using cell homogenizer (Sigma-Aldrich, MO, USA) and vigorously agitated for 3 min. The homogenate was centrifuged at 4000 ×g for 10 min at 4 ºC to remove the cell debris and glass beads. The supernatant obtained was transferred to a sterile Eppendorf tube and centrifuged for 45 min at 12 000 × g at 4 ºC. The supernatant was used as cell-free extract and stored at -20 ºC for further analysis.

3.7.2 Total protein estimation

A series of bovine serum albumin (BSA) protein (Roche Diagnostics, GmbH, Germany) standards with concentrations ranging from 0.1 to 1.5 mg/ml were prepared to make a final volume of 100 µl as shown in **Table 3.2**. A 50 µl of the unknown samples and standards were added to 1.95 ml Bradford reagent (Sigma-Aldrich, MO, USA). The mixture was incubated at room temperature in the dark for 10 to 25 min until a blue colour or protein-dye complex was formed. Protein concentration was measured at 595 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was plotted to determine the amount of protein in the unknown samples (Bradford, 1976).

Table 3.2: Protein dilutions for preparation of a standard curve

Final concentration (mg/ml)	2 mg/ml BSA (µl)	10 mmol/L Tris-HCl buffer, pH 7.5 (µl)
0	0	100
0.1	5	95
0.2	10	90
0.4	20	80
0.6	30	70
0.8	40	60
1.0	50	50
1.5	75	25

3.7.3 Catalase

Catalase activity was performed following Khan *et al*., 2011, with modifications. The assay mixture consisted of 2.8 ml potassium phosphate buffer (50 mmol/L, pH 7.0) (**Appendix 7.5.8**), 100 µl H_2O_2 30% w/v (Merck Chemicals (Pty) Ltd, Darmstadt, Germany), and 100 µl cell-free extract. The decrease in absorbance due to the removal of H_2O_2 by catalase was measured at every 30 seconds interval for up to 3 min at 240 nm using a spectrophotometer. Catalase activity was expressed as µmol H_2O_2 consumed per minute per mg protein using an extinction coefficient of 43.6 mM^{-1} cm^{-1}. The following equation was used for calculations:

$$\text{Unit activity (units/ min/g)} = \frac{\Delta\text{A test/ min} \times \text{total volume(ml)}}{\text{Ext. coefficient} \times \text{sample volume (ml)}}$$

$$\text{Specific activity (mol UA/mg protein)} = \frac{\text{unit activity (units/min/g)}}{\text{total protein content (mg/g)}}$$

3.7.4 Superoxide dismutase (SOD)

SOD activity was performed following Khan *et al*., 2011, with modifications. A fresh solution of 50 mmol/L Tris buffer, pH 8.5 (**Appendix 7.5.9**) and 20 mmol/L pyrogallol (**Appendix 7.5.10**) were prepared at the time of the assay. The test sample contained 2.8 ml Tris buffer, 100 µl pyrogallol, and 100 µl cell-free extract. A 90 seconds induction period was allowed. SOD activity was determined by monitoring inhibition of pyrogallol

autoxidation at every 30 seconds for 3 min at 420 nm. Pyrogallol autoxidation was calculated as:

$$\%\ \text{Inhibition of pyrogallol autoxidation} = \frac{\Delta A\ \text{test}}{\Delta A\ \text{control}} \times 100$$

$$\text{Therefore, SOD activity (U/ml)} = \text{inhibition of pyrogallol autoxidation (\%)/50\%}$$

3.7.5 Glutathione peroxidase (GPx)

GPx activity was measured as previously described by Yousuf *et al*., 2010, with modifications. The assay mixture contained 2 ml potassium phosphate buffer (50 mmol/L, pH 7.0) (**Appendix 7.5.8**), 100 µl of 1 mmol/L sodium azide (**Appendix 7.5.11**), 100 µl of 1 mmol/L EDTA (**Appendix 7.5.12**), 100 µl of freshly prepared 1 mmol/L tripeptide glutathione (**Appendix 7.5.13**), 100 µl of 0.2 mmol/L NADPH (**Appendix 7.5.14**), 100 µl H_2O_2 30% w/v and 100 µl cell-free extract. GPx activity was determined by measuring decrease in absorbance due to oxidation of NADPH to $NADP^+$ at every 30 seconds for 3 min at 340 nm. The following equation was used for calculations:

$$\text{Unit activity (units/ min/g)} = \frac{\Delta A\ \text{test/ min} \times \text{total volume(ml)}}{\text{Ext. coefficient} \times \text{sample volume (ml)}}$$

Extinction coefficient = 6.22 mM^{-1} cm^{-1}

$$\text{Specific activity (mol UA/mg protein)} = \frac{\text{unit activity (units/min/g)}}{\text{total protein content (mg/g)}}$$

3.7.6 Glutathione reductase (GLR)

The activity of GLR was measured as previously described by Yousuf *et al*., 2010, with modifications. The assay mixture consisted of 2 ml potassium phosphate buffer (50 mmol/L, pH 7.4) (**Appendix 7.5.8**), 100 µl of 0.1 mmol/L NADPH (**Appendix 7.5.15**), 100 µl of 0.5 mmol/L EDTA (**Appendix 7.5.16**), 100 µl of freshly prepared 1 mmol/L oxidized glutathione disulphide (GSSG) (**Appendix 7.5.17**) and 100 µl cell-free extract. The decrease in absorbance due to reduction of GSSG in the presence of NADPH was monitored at an interval of 30 seconds for 3 min at 340 nm. The following equation was used for calculations:

$$\text{Unit activity (units/ min/g)} = \frac{\Delta A\ \text{test/ min} \times \text{total volume(ml)}}{\text{Ext. coefficient} \times \text{sample volume (ml)}}$$

Extinction coefficient = 6.22 mM^{-1} cm^{-1}

$$\text{Specific activity (mol UA/mg protein)} = \frac{\text{unit activity (units/min/g)}}{\text{total protein content (mg/g)}}$$

3.7.7 Glutathione S-transferase (GST)

GST activity was determined following Maras *et al*., 2014, with modifications. The assay mixture consisted of 2 ml potassium phosphate buffer (50 mmol/L, pH 6.5) (**Appendix 7.5.8**), 100 µl of 100 mmol/L tripeptide glutathione (GSH) (**Appendix 7.5.18**), 100 µl of 100 mmol/L 1-chloro-2.4-dinitrobenzene (CDNB) (**Appendix 7.5.19**) and 100 µl cell-free extract. The increase in absorbance corresponding to conjugation of GSH to CDNB was recorded at every of 30 seconds for 3 min at 340 nm. The following equation was used for calculations:

$$\text{Unit activity (units/ min/g)} = \frac{\Delta\text{A test/ min} \times \text{total volume(ml)}}{\text{Ext. coefficient} \times \text{sample volume (ml)}}$$

Extinction coefficient = 9.6 mM^{-1} cm^{-1}

$$\text{Specific activity (mol UA/mg protein)} = \frac{\text{unit activity (units/min/g)}}{\text{total protein content (mg/g)}}$$

3.8 Statistical analysis

All the experiments in this study were performed in triplicates and reported as mean ± SD. Unpaired student's t-test was used to analyze statistical significance between two independent experimental groups using GraphPad PRISM Software Version 7.04 (GraphPad Software Inc., San Diego, CA, USA). P-value of <0.05 was considered statistically significant.

Chapter 4: Results

4.1 Identification of *C. albicans* clinical isolates

Twenty-one strains of *C. albicans* (10 FLC susceptible, 10 FLC resistant and one ATCC strain) were obtained from the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand and the identification was confirmed using an API 20 C AUX kit (**Figure 4.1**). It generates a 7-digit numerical profile with percentage similarity to *C. albicans*. The test strains similarity percentages ranged from 97 to 99.5 %.

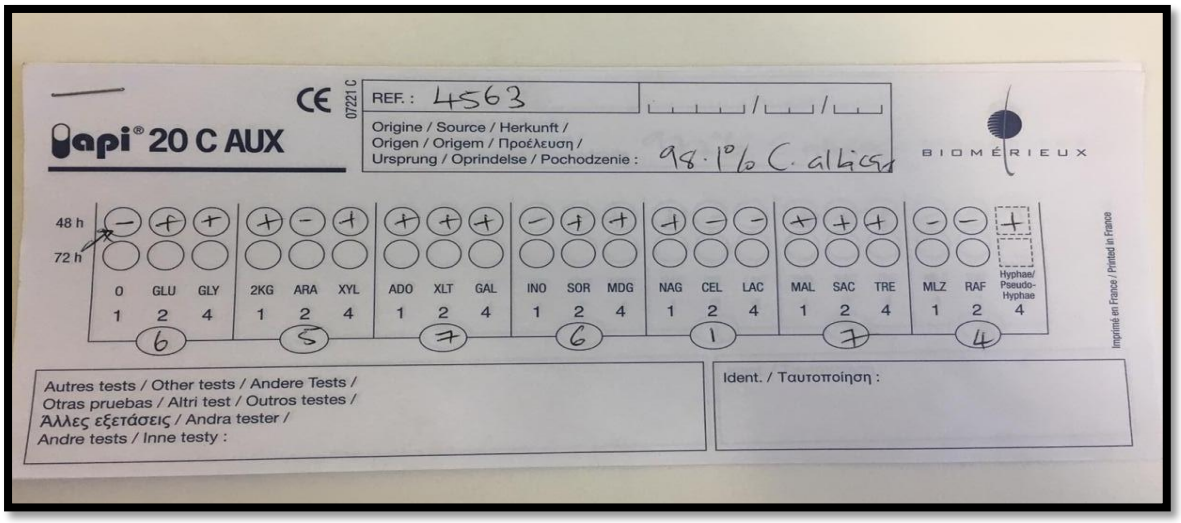

Figure 4.1: API 20 C AUX results sheet for *C. albicans*

4.2 Upregulation of oxidative stress responsive genes in *C. albicans*

4.2.1 FLC susceptibility

To understand the relationship between antioxidant enzymes and the level of susceptibility to FLC in *C. albicans*, expression of genes involved in the detoxification of ROS in FLC susceptible and resistant *C. albicans* was analyzed. In this experiment, three isolates in each group were considered depending on their phenotypic susceptibility profile to FLC as shown in **Table 4.1**. Each group consisted of one FLC susceptible and one FLC resistant *C. albicans* which were determined following the CLSI breakpoints.

Table 4.1: Isolates used in RT-qPCR

Group ID	FLC susceptible *C. albicans*	FLC (MIC) µg/ml	FLC resistant *C. albicans*	FLC (MIC) µg/ml
HIV patients	4180	0.49	4122	63
Cancer patients	4554	0.49	A71	63
Other immunocompromised conditions	4563	0.49	4135	63

4.2.2 RNA quantification

RNA integrity and purity were assessed using agarose gel electrophoresis. Total RNA extraction from exponentially growing *C. albicans* yielded high-quality RNA samples for downstream application. The gel picture in **Figure 4.2** shows intact fragments of ribosomal RNA (rRNA) 25S, 18S, and 5.8S under UV light.

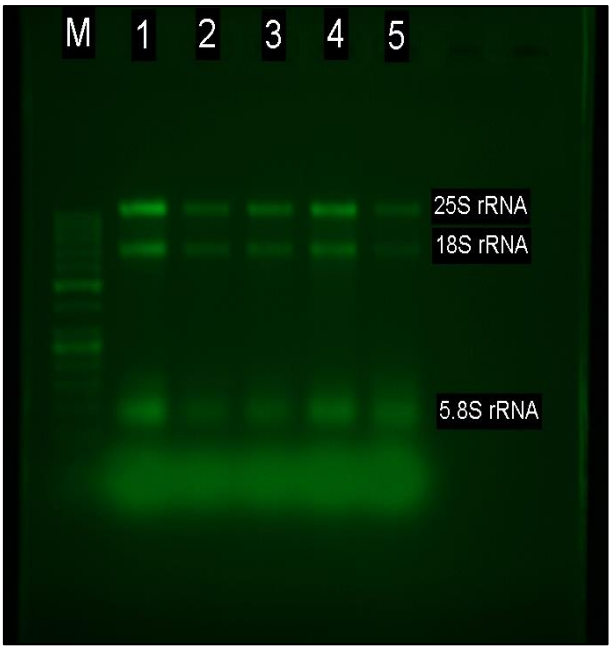

Figure 4.2: Total RNA run on 1.8 % agarose gel. Lane M = 50 bp DNA ladder, Lane 1 to 5 show intact total RNA of different *C. albicans* isolates with clear bands.

4.2.3 Expression stability of reference genes in FLC susceptible and resistant *C. albicans*

In RT-qPCR, accurate normalization is important, and it requires reference genes whose expression would not change under the investigated conditions. Two reference genes, *PMA1* and *RIP* were selected in this study based on the stability of their expression in planktonic cells of *C. albicans* (Nailis *et al*., 2006). **Figure 4.3** represents mRNA expression level of *PMA1* and *RIP* genes. The results show that both *PMA1* and *RIP* were constitutively expressed in both FLC susceptible and resistant *C. albicans*. Statistical analysis revealed no significant difference in the expression of reference genes.

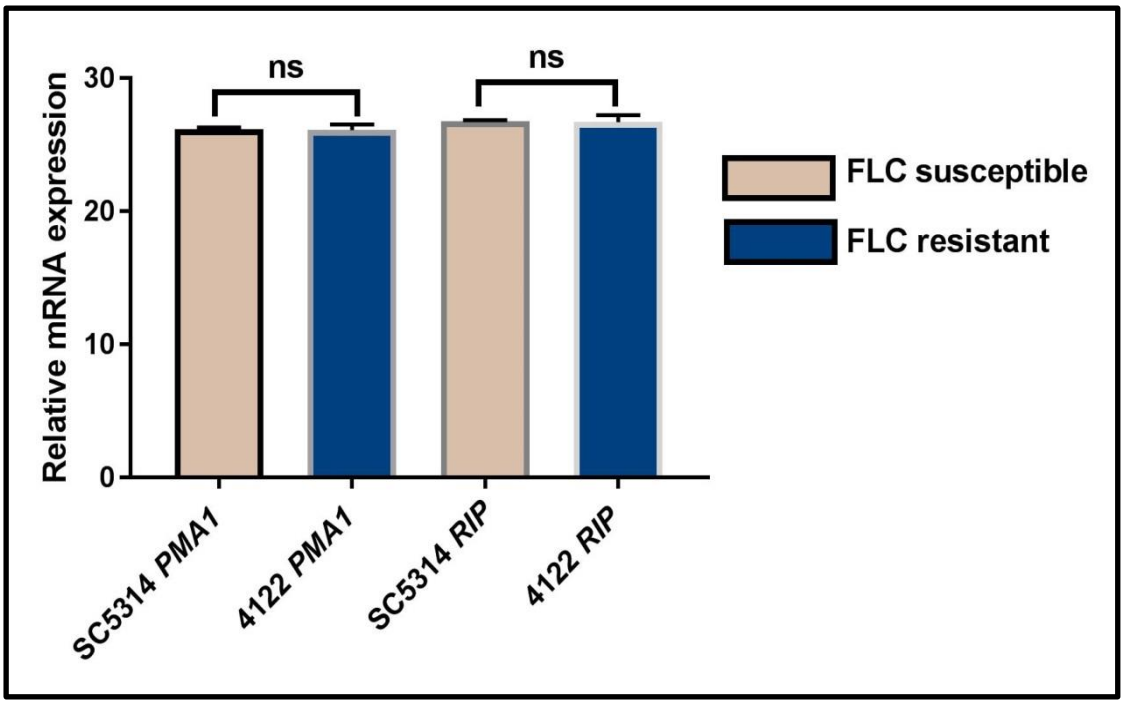

Figure 4.3: Stability of *PMA1* and *RIP* genes in FLC susceptible and resistant *C. albicans*. Mean of three independent repeats in each group were considered. Unpaired student's t-test was used to analyze statistical significance in FLC susceptible and resistant *C. albicans.*

4.2.4 mRNA expression level of antioxidant enzymes in FLC susceptible and resistant *C. albicans*

Transcription profiling of genes encoding oxidative stress enzymes namely: *SOD1*, *SOD2*, *GPx2*, *GLR1*, *GTT11*, and *CAT1* were done using RT-qPCR. The results are shown in **Figure 4.4** to **Figure 4.8**. There was an increase in mRNA expression level of *SOD1* (**Figure 4.4**), *SOD2* (**Figure 4.5**), and *GTT11* (**Figure 4.8**) genes in FLC resistant isolates compared to findings for the susceptible group. However, a different trend was observed in the expression of *GPx2* (**Figure 4.6**) and *GLR1* (**Figure 4.7**). HIV patients and cancer patients showed low expression of the aforementioned genes in the resistant *C. albicans* relative to FLC

susceptible isolates, while as in other immunocompromised conditions mRNA expression level for these genes was increased in FLC resistant isolate.

In general, *SOD* and *GLR1* showed markedly high transcription levels regardless of their antifungal susceptibility profile. These data indicate that *SOD* and *GLR1* could have a positive involvement in the antioxidant defense system in *C. albicans*.

On the contrary, *CAT1* expression seemed to follow a different pattern. Lower level or no expression of the *CAT1* gene was found in both FLC susceptible and resistant *C. albicans*. Statistical analysis for this particular gene revealed no significant difference in the tested groups ($p = 0.05$). The results for *CAT1* were omitted because of insufficient data to plot graphs.

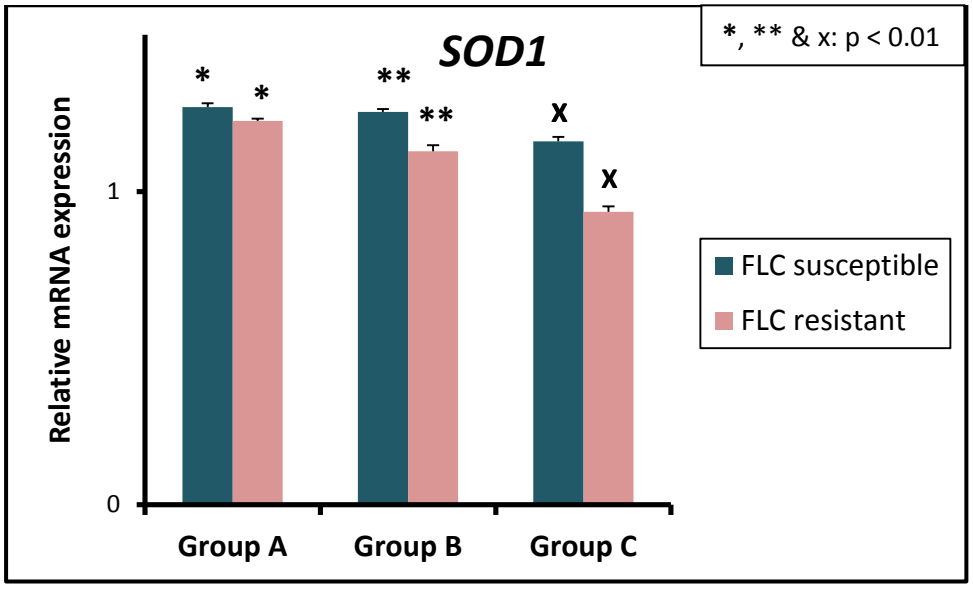

Figure 4.4: mRNA expression of *SOD1* in FLC susceptible and resistant *C. albicans*. Group A = HIV patients, Group B = cancer patients and Group C = other immunocompromised conditions. The graphs were plotted using the mean of three independent repeats ± SD after normalization to reference genes. Unpaired student's t-test was used to analyze statistical significance.

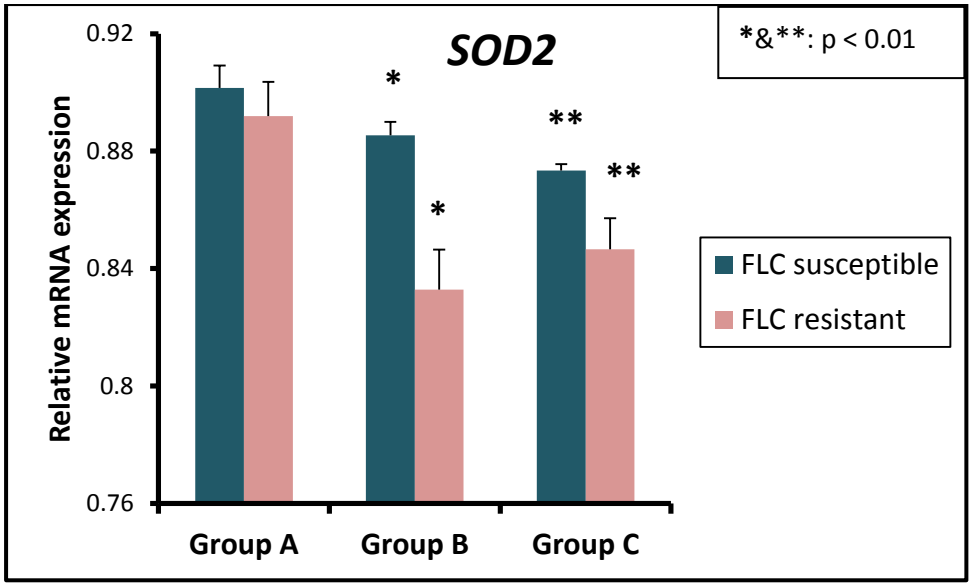

Figure 4.5: mRNA expression of *SOD2* in FLC susceptible and resistant *C. albicans*. Group A = HIV patients, Group B = cancer patients and Group C = other immunocompromised conditions.The graphs were plotted using the mean of three independent repeats ± SD after normalization to reference genes. Unpaired student's t-test was used to analyze statistical significance.

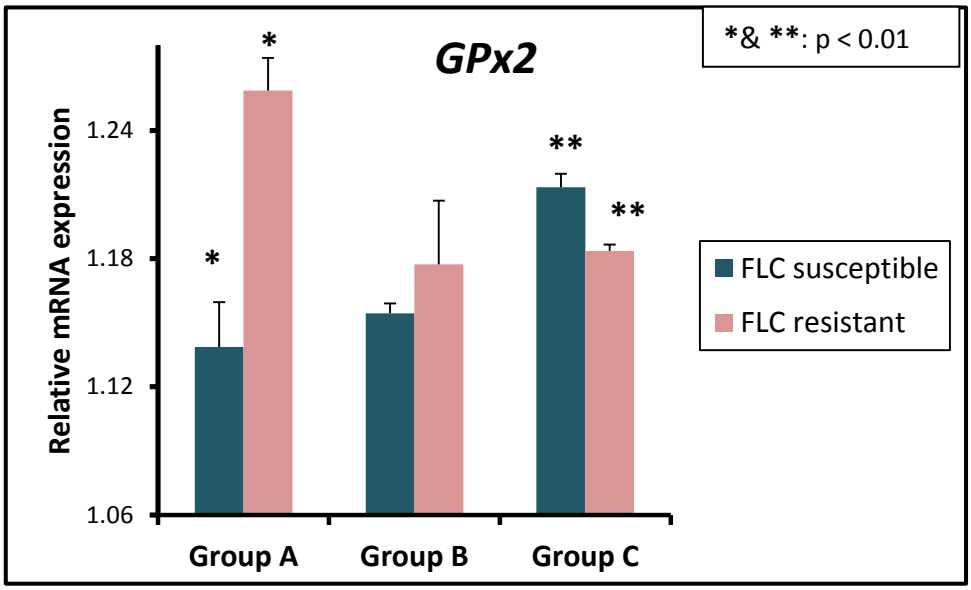

Figure 4.6: mRNA expression of *GPx2* in FLC susceptible and resistant *C. albicans*. Group A = HIV patients, Group B = cancer patients and Group C = other immunocompromised conditions.The graphs were plotted using the mean of three independent repeats ± SD after normalization to reference genes. Unpaired student's t-test was used to analyze statistical significance.

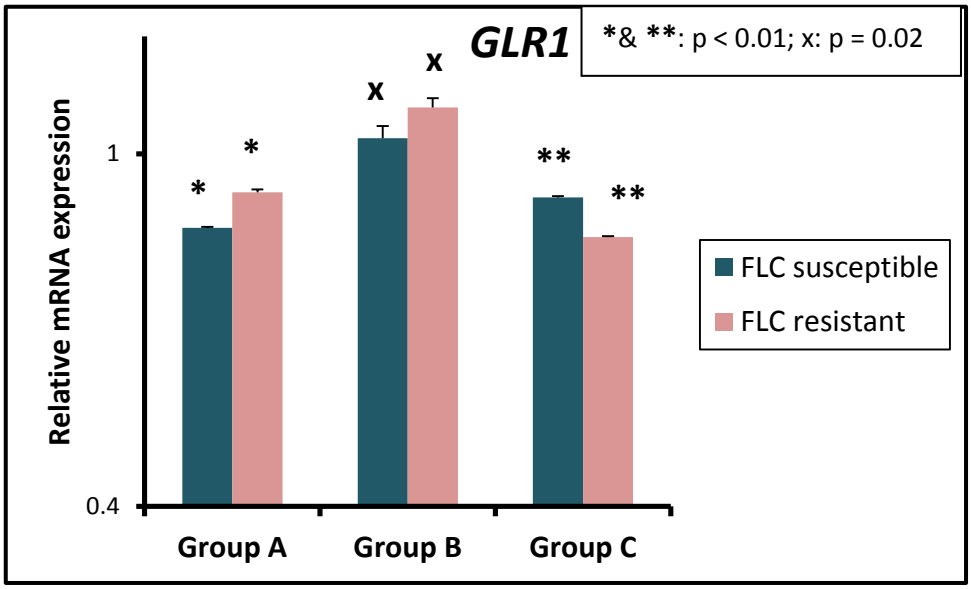

Figure 4.7: mRNA expression of *GLR1* in FLC susceptible and resistant *C. albicans*. Group A = HIV patients, Group B = cancer patients and Group C = other immunocompromised conditions.The graphs were plotted using the mean of three independent repeats ± SD after normalization to reference genes. Unpaired student's t-test was used to analyze statistical significance.

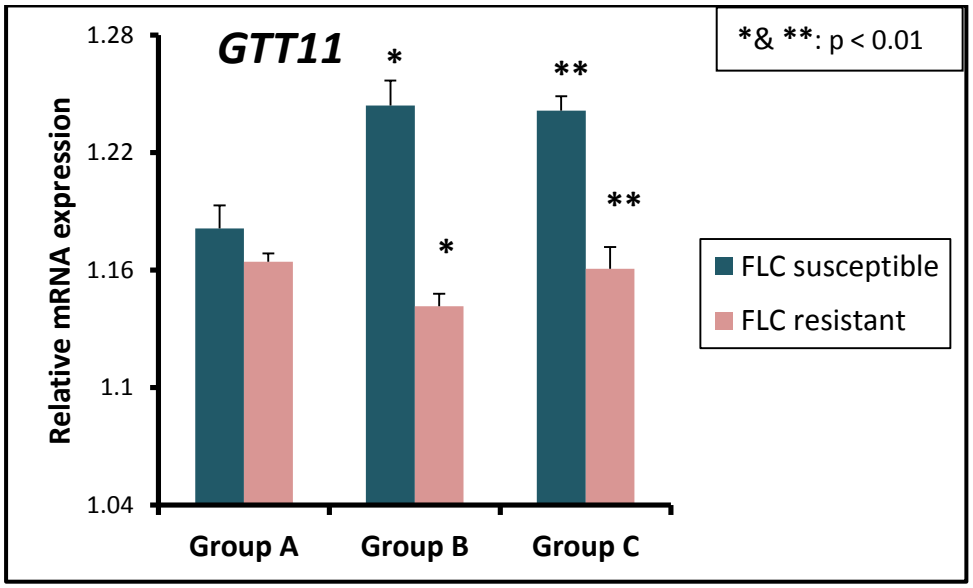

Figure 4.8: mRNA expression of *GTT11* in FLC susceptible and resistant *C. albicans*. Group A = HIV patients, Group B = cancer patients and Group C = other immunocompromised conditions.The graphs were plotted using the mean of three independent repeats ± SD after normalization to reference genes. Unpaired student's t-test was used to analyze statistical significance.

4.2.5 Fold change in FLC resistant relative to FLC susceptible *C. albicans*

The results of a fold change difference ($2^{-\Delta\Delta Cq}$) are summarized in **Table 4.2**. The most significantly expressed genes in all the three groups were *SOD1, SOD2,* and *GTT11* producing greater fold difference in FLC resistant *C. albicans*. The highest was *SOD1* with a 50.69-fold greater than FLC susceptible *C. albicans* in other immunocompromised conditions. The other genes showed a moderate increase in the expression with fold change ranging from 1.20 to 6.98.

Table 4.2: Summary of fold change difference in FLC resistant *C. albicans*

Genes	**Fold change ($2^{-\Delta\Delta Cq}$)**		
	HIV patients	**Cancer patients**	**Other immunocompromised conditions**
SOD1	2.30	10.94	50.69
SOD2	1.20	2.71	1.57
GPx2	0.10	0.65	1.67
GLR1	0.32	0.37	3.22
GTT11	1.38	6.98	4.03

4.3 The antifungal activity of BER against *C. albicans* isolates

The activity of BER against *C. albicans* clinical isolates and one ATCC strain was determined. The results are shown in **Table 4.3**. Out of the 21 tested *C. albicans* isolates, 48% (10/21) showed MIC of 500 µg/ml, 43% (9/21) showed MIC of 250 µg/ml, and 9% (2/21) showed MIC of 125 µg/ml. The overall MIC values ranged from 125 to 500 µg/ml for BER.

MFC values in the tested isolates were ranging from 500 to 2000 µg/ml. There was no significant difference between the tested groups.

Table 4.3: Antifungal activity of BER against *C.albicans*

Isolates		MIC (µg/ml) n=3	
		FLC	**BER**
ATCC	SC5314	0.49	250
FLC susceptible			
Group A	4180	0.49	500
	CR34	1.95	500
	C87	1.95	500
	CR 03	0.1	500
Group B	4554	0.49	500
	027 GRN	1.95	500
	167-1	0.49	500
Group C	4563	0.49	250
	4085	0.49	500
	5112	0.1	250
FLC resistant			
Group A	4122	63	500
	4175	31	250
	4179	8	500
	N27	31	125
Group B	180-1	31	250
	C89	31	250
	A71	63	250
Group C	004	31	125
	CR-02	8	250
	4135	63	250

Group A = HIV patients

Group B = Cancer patients

Group C = Other immunocompromised conditions

Figure 4.9 shows a summary of BER antifungal activity against twenty-one *C. albicans* isolates (10 FLC susceptible, 10 FLC resistant and one ATCC strain). The mean MIC values were 275 and 450 µg/ml BER in FLC resistant and susceptible *C. albicans* respectively. The overall difference in the antifungal activity of BER between FLC susceptible and resistant *C. albicans* was statistically significant (p= 0.004).

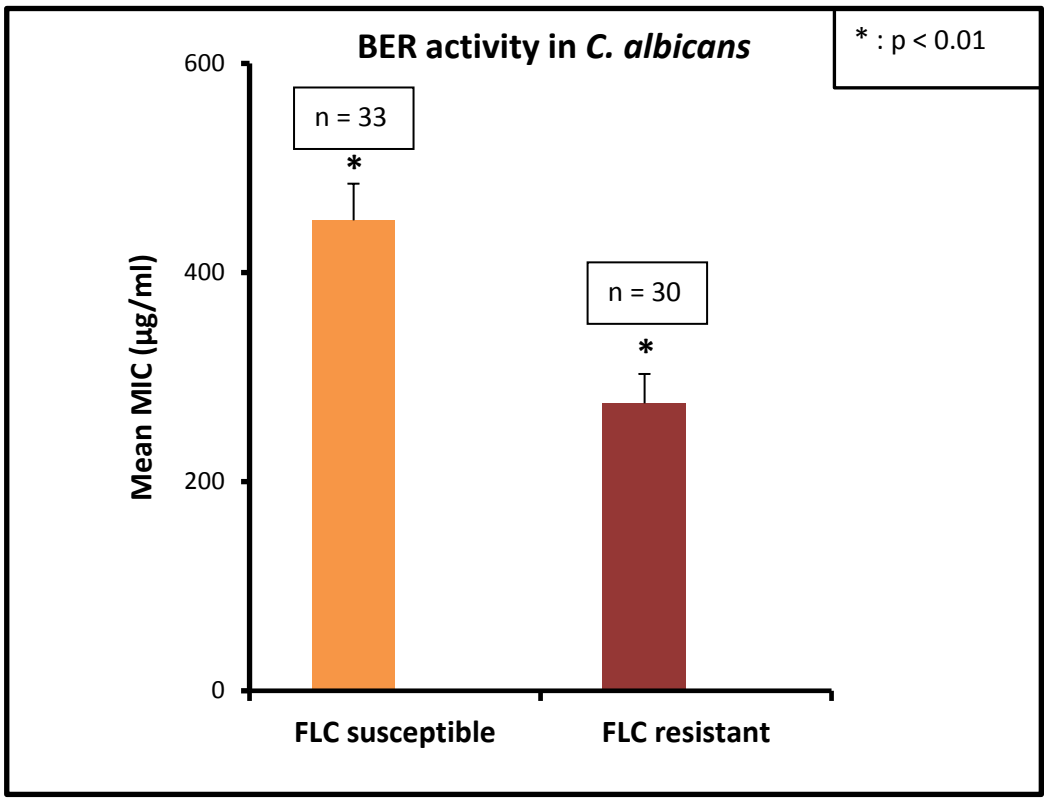

Figure 4.9: Summary of the antifungal activity of BER against *C. albicans* isolates. Mean of three independent repeats in each group were considered. Unpaired student's t-test was used to analyze statistical significance between FLC susceptible and resistant *C. albicans*.

4.4 The effect of BER on the expression of antioxidant enzymes

4.4.1 *C. albicans* survival rate in the presence of BER

Exponentially growing isolates of *C. albicans* SC5314 (FLC susceptible) and *C. albicans* 4122 (FLC resistant) were treated with MIC and sub-inhibitory concentrations of BER for 2 hours to determine cell viability. The number of colonies was recorded at 0, 1 and 2 hours to monitor growth and estimate survival rate in treated cells relative to the control. The results are shown in **Table 4.4**, **Figure 4.10**, and **Figure 4.11**. The two isolates showed similar results. *Candida albicans* cells were able to continue growing exponentially even in the presence of BER after 1 hour, with up to 75% rate in comparison with untreated cells. In contrast, BER caused a dose-dependent reduction in *C. albicans* after 2 hours. Inhibition of

growth was most evident in cells treated with 500 µg/ml BER resulting in 41% and 57% survival rate in *C. albicans* SC5314 and *C. albicans* 4122 respectively.

Table 4.4: Cell viability of *C. albicans* in the presence of BER

BER concentration(µg/ml)	Survival rate (%) n=3			
	***C. albicans* SC5314**		***C. albicans* 4122**	
	1 hr	**2 hrs**	**1 hr**	**2 hrs**
0	100	100	100	100
63	98	95	92	91
125	92	89	75	79
250	84	71	75	70
500	72	41	75	57

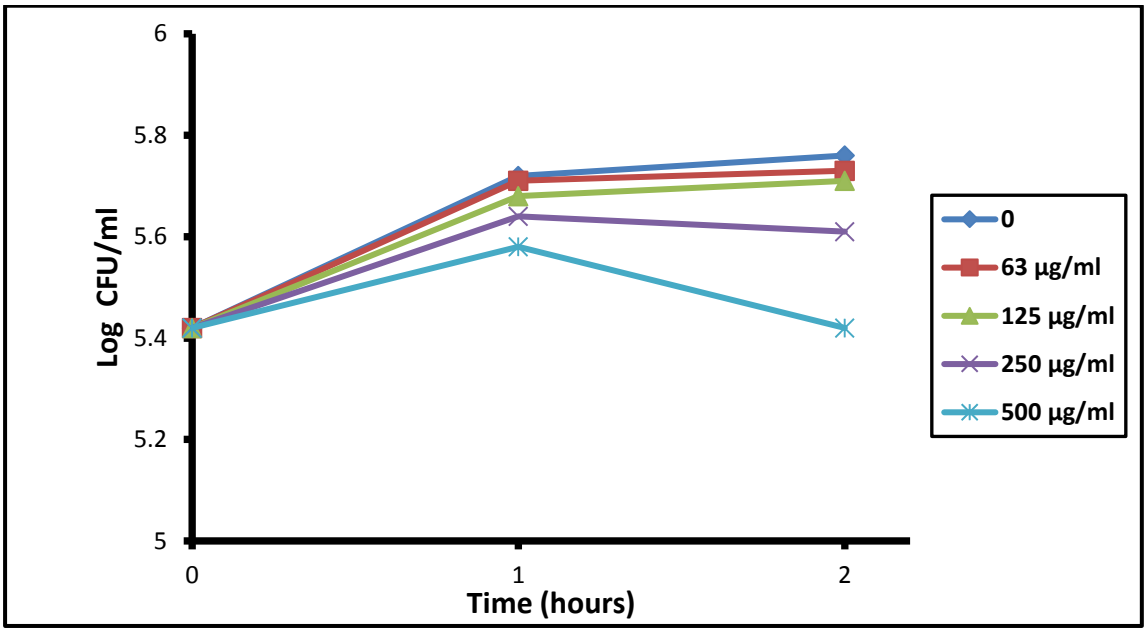

Figure 4.10: Growth curve of exponentially growing cells of *C. albicans* SC5314 in the presence of 0, 63, 125, 250 and 500 µg/ml BER. Data represent means of three independent experiments.

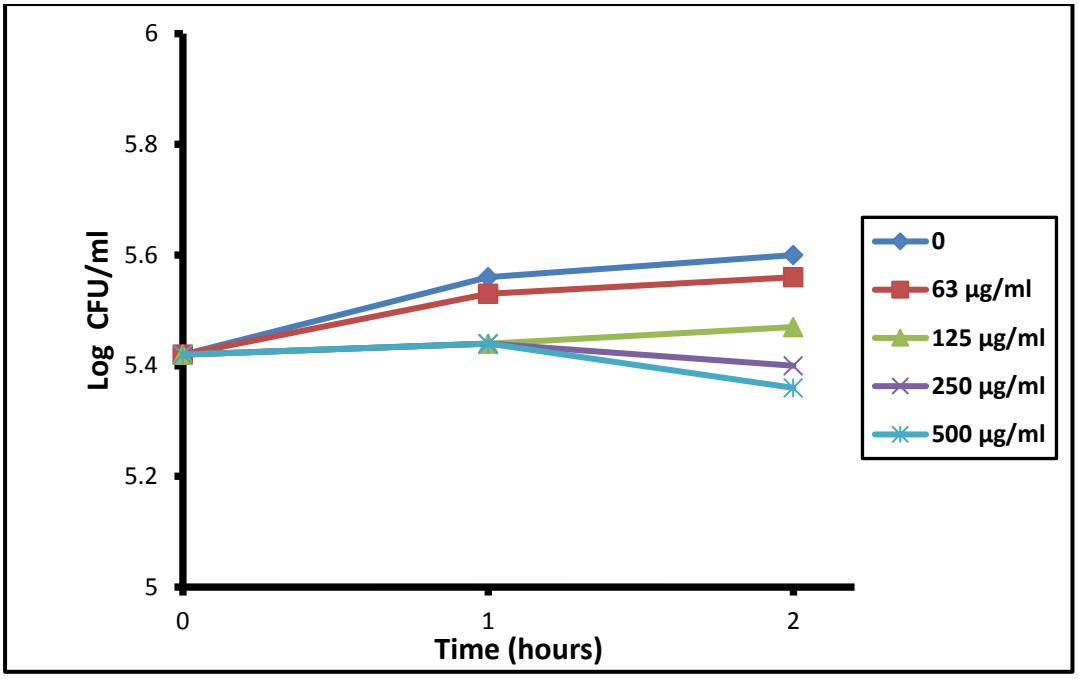

Figure 4.11: Growth curve of exponentially growing cells of *C. albicans* 4122 in the presence of 0, 63, 125, 250 and 500 µg/ml BER. Data represent means of three independent experiments.

Depending on the results of viability test as shown above in **Table 4.4**, three concentrations (63, 125 and 250 µg/ml BER) showing more than 70% survival of *C. albicans* cells after 2 hours exposure were selected in subsequent analysis investigating the effect of BER on antioxidant enzymes to eliminate the antioxidant effect of other processes such as apoptosis or necrosis.

4.4.2 Expression stability of reference genes in BER treated and untreated *C. albicans*

The stability of reference genes was measured in the presence of BER using RT-qPCR. *C. albicans* SC5314 was exposed to 0 and 250 µg/ml BER for 2 hours. The results are shown in **Figure 4.12**. The two reference genes, *PMA1* and *RIP* were stably expressed in both BER treated and untreated *C. albicans* cells. Statistical analysis revealed no significant difference in the tested groups.

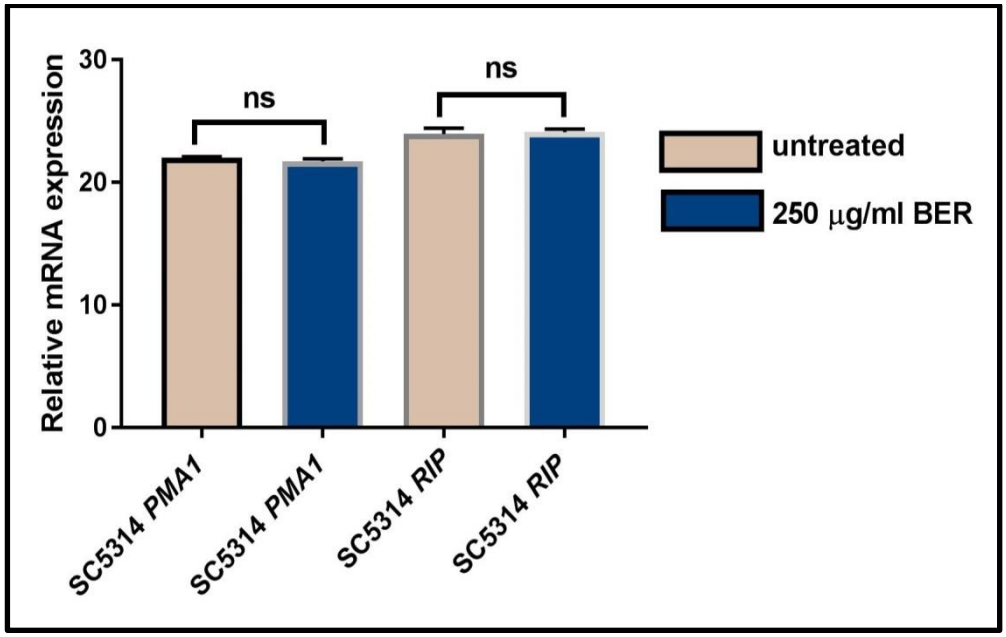

Figure 4.12: Stability of *PMA1* and *RIP* genes in BER treated and untreated *C. albicans* SC5314. Mean of three independent repeats in each group were considered. Unpaired student's t-test was used to analyze statistical significance in BER treated cells relative to the control.

4.4.3 Gene expression of antioxidant enzymes in the presence of BER

The effect of BER on mRNA expression level of antioxidant genes *SOD1*, *SOD2*, *GPx2*, *GLR1*, *GTT11*, and *CAT1* in the presence of 0, 63,125 and 250 BER µg/ml following 2 hours of exposure was investigated using RT-qPCR. The results of the effect of BER on expression of these genes are shown in **Figure 4.13** and **Figure 4.14** for *C. albicans* SC5314 (FLC susceptible) and *C. albicans* 4122 (FLC resistant) respectively. BER induced oxidative stress in *C. albicans* cells of which is characterized by an increase in the expression of antioxidant genes particularly *SOD1*, *SOD2*, *GPx2*, *GLR1* and *GTT11* after exposure to increasing concentrations of BER.

Even though BER treatment caused an increase in the expression level of these antioxidant genes, *C. albicans*'s antioxidant response to BER varied depending on the degree of susceptibility to FLC. For instance, only 250 µg/ml of BER was able to induce an intense expression of antioxidant genes in FLC susceptible *C. albicans* SC5314 (**Figure 4.13**). In contrast, low to moderate concentrations (63 and 125 µg/ml) were sufficient to induce internal oxidative stress in FLC resistant *C. albicans* 4122. However, expression level of these genes was more pronounced at 125 µg/ml of BER as shown in **Figure 4.14**. Interestingly, treatment with high concentrations of BER (250 µg/ml) in FLC resistant *C. albicans* 4122 showed a decrease in the expression of *SOD1*, *SOD2*, *GLR1* and *GTT11* in

comparison to lower levels or less than 250 µg/ml of BER. This effect might be caused by the robust induction of oxidative stress which in turn overwhelms the antioxidant capacity of the organism.

According to these data, only moderate to high concentrations of BER (125 to 250 µg/ml) were able to induce significant upregulation of *GLR1* expression in both tested isolates.

The results for *CAT1* were omitted because no significant effects were observed on expression even after exposure to all three different concentrations of BER.

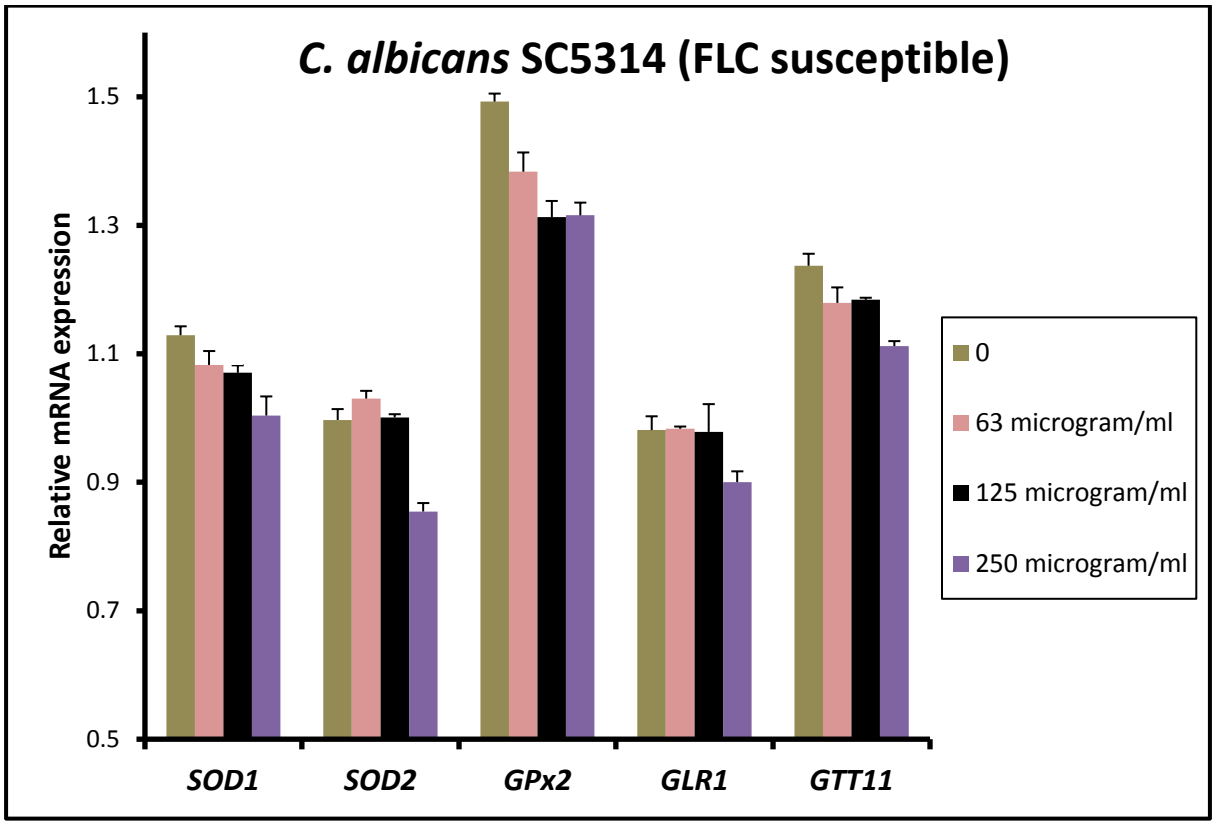

Figure 4.13: Expression levels of *SOD1*, *SOD2*, *GPx2*, *GLR1* and *GTT11* in *C. albicans* SC5314 in response to BER exposure. The graphs were plotted using the mean of three independentrepeats ± SD after normalization to reference genes.

Table 4.5: Statistical values of the data obtained in gene expression analysis in *C. albicans* SC5314

Genes	Comparison (n=3)	P value
SOD1	Control to 63 µg/ml	0.035
	Control to 125 µg/ml	0.005
	Control to 250 µg/ml	0.003
SOD2	Control to 63 µg/ml	
	Control to 125 µg/ml	
	Control to 250 µg/ml	<0.001
GPx2	Control to 63 µg/ml	0.017
	Control to 125 µg/ml	0.001
	Control to 250 µg/ml	<0.001
GLR1	Control to 63 µg/ml	
	Control to 125 µg/ml	
	Control to 250 µg/ml	0.007
GTT11	Control to 63 µg/ml	0.004
	Control to 125 µg/ml	0.006
	Control to 250 µg/ml	<0.001

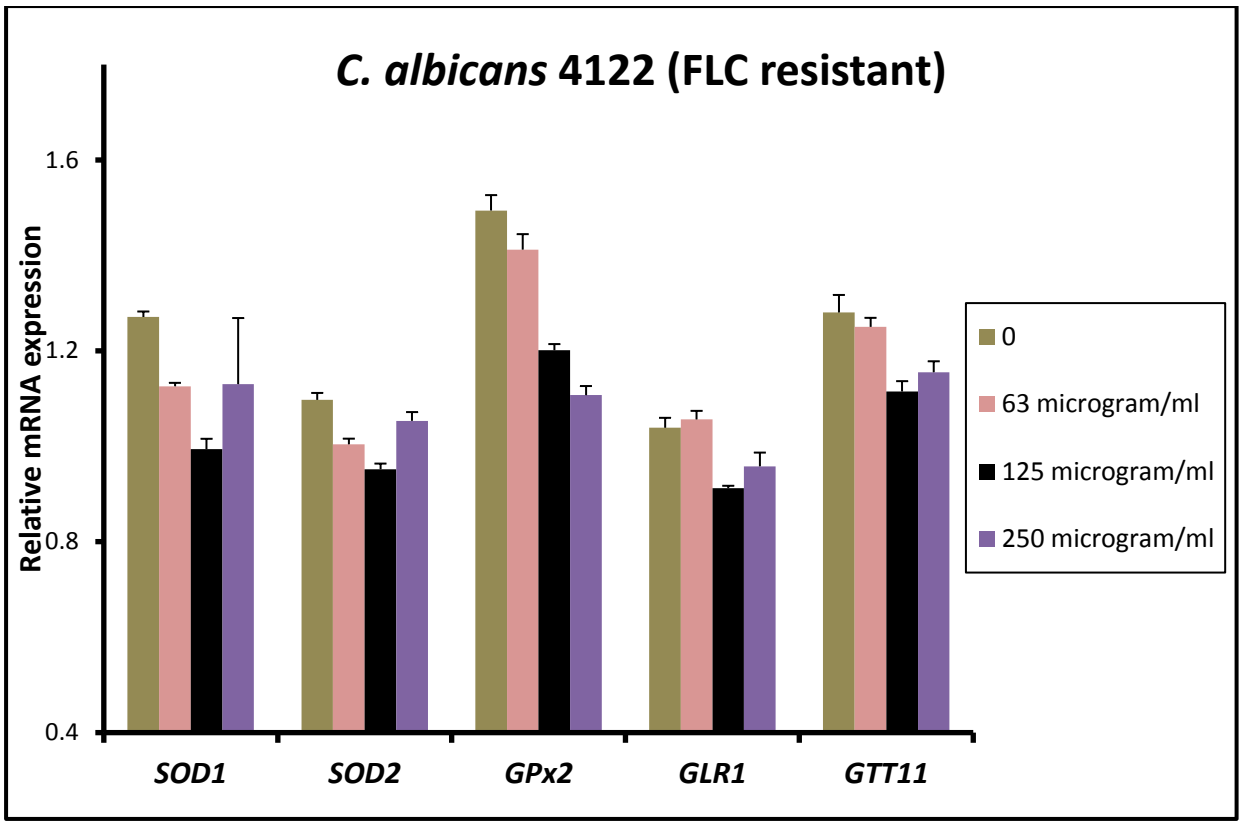

Figure 4.14: Expression levels of *SOD1, SOD2, GPx2, GLR1* and *GTT11* in *C. albicans* 4122 in response to BER exposure. The graphs were plotted using the mean of three independent repeats ± SD after normalization to reference genes.

Table 4.6: Statistical values of the data obtained in gene expression analysis in *C. albicans* 4122

Genes	Comparison (n=3)	P value
SOD1	Control to 63 µg/ml	<0.001
	Control to 125 µg/ml	<0.001
	Control to 250 µg/ml	<0.001
SOD2	Control to 63 µg/ml	0.001
	Control to 125 µg/ml	<0.001
	Control to 250 µg/ml	0.032
GPx2	Control to 63 µg/ml	0.002
	Control to 125 µg/ml	<0.001
	Control to 250 µg/ml	<0.001
GLR1	Control to 63 µg/ml	
	Control to 125 µg/ml	<0.001
	Control to 250 µg/ml	0.019
GTT11	Control to 63 µg/ml	
	Control to 125 µg/ml	<0.001
	Control to 250 µg/ml	0.003

4.4.4 Expression fold change in BER treated relative to untreated cells of *C. albicans*

Table 4.7 and **Table 4.8** show the results of fold change difference ($2^{-\Delta\Delta Cq}$) in BER treated *C. albicans* SC5314 (FLC susceptible) and *C. albicans* 4122 (FLC resistant) relative to untreated cells. In *C. albicans* SC5314 (**Table 4.7**), expression of *SOD1*, *SOD2*, *GLR1* and *GTT11* showed the same trend. All these genes increased expression level with increasing concentrations of BER. However, fold change difference was most pronounced at 250 µg/ml BER. In contrast, *GPx2* fold change in *C. albicans* SC5314 remained the same in the presence of all the three tested concentrations of BER.

A different pattern was observed in *C. albicans* 4122 as shown in **Table 4.8**. Fold difference in the expression of *SOD1*, *SOD2*, *GLR1*, and *GTT11* was highest at 125 µg/ml BER but decreased sharply at 250 µg/ml BER. However, this was not the case with *GPx2*. This gene showed a dose-dependent fold change.

The overall results revealed higher fold change in BER treated *C. albicans* 4122 cells than observed in *C. albicans* SC5314.

Table 4.7: Expression fold change in response to BER exposure in *C. albicans* SC5314

Genes	**Fold change ($2^{-\Delta\Delta Cq}$)**		
	63 µg/ml	**125 µg/ml**	**250 µg/ml**
SOD1	2.10	2.55	7.41
SOD2	0.59	0.94	9.86
GPx2	5.47	6.03	5.97
GLR1	0.97	1.05	3.74
GTT11	2.32	2.51	7.33

Table 4.8: Expression fold change in response to BER exposure in *C. albicans* 4122

Genes	Fold change ($2^{-\Delta\Delta Cq}$)		
	63 µg/ml	**125 µg/ml**	**250 µg/ml**
SOD1	10.10	82.40	16.78
SOD2	4.30	9.79	1.99
GPx2	3.89	106.90	483.18
GLR1	0.76	7.38	3.58
GTT11	1.61	13.80	7.26

4.5 Antioxidant enzymatic activities

4.5.1 Estimation of total protein concentration

The Bradford assay was performed to determine total protein concentration in the cell-free extract. Absorbance values of BSA protein standards with concentrations ranging from 0.1 to 1.5 mg/ml were used to plot the standard curve shown in **Figure 4.15**. The absorbance values ranged from 0.010 to 0.363 at 595 nm.

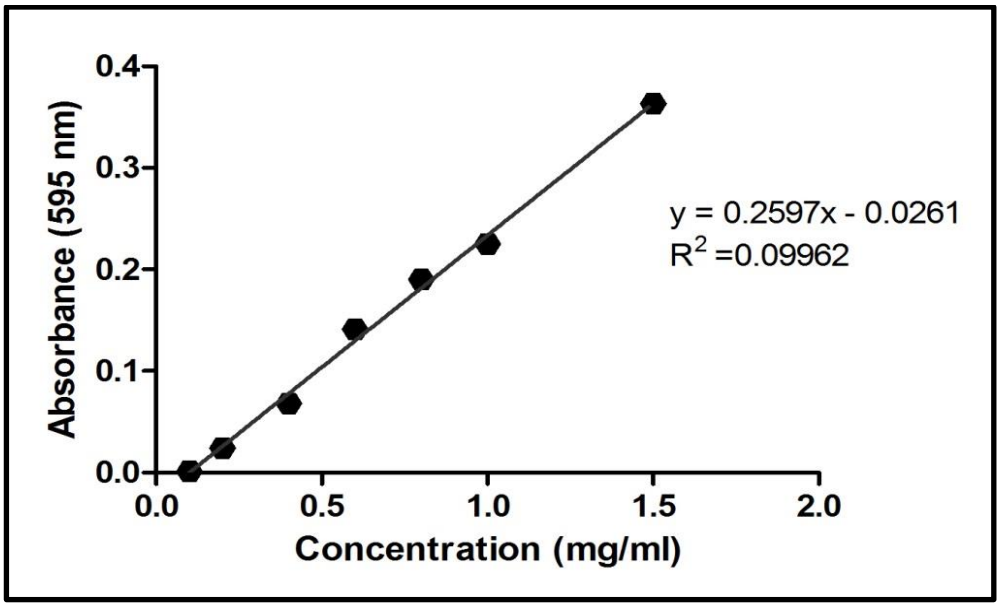

Figure 4.15: Bradford assay standard curve with BSA protein concentrations ranging from 0.1 to 1.5 mg/ml.

4.5.2 Protein concentrations in the presence of BER

Total protein concentrations extrapolated from the Bradford standard curve are shown in **Table 4.9**. The results for *C. albicans* SC5314 were ranging from 0.8 to 1.2 mg/ml whereas those of *C. albicans* 4122 ranged from 0.6 to 0.7 mg/ml. These protein concentrations were used to calculate specific activity of antioxidant enzymes.

Table 4.9: Cell-free extract protein concentration extrapolated from the Bradford assay standard curve

Strain	BER conc. (µg/ml)	Absorbance (595 nm)	Mean protein conc. in mg/ml (n=3)
***C. albicans* SC5314**	0	0.243	1.0
	63	0.284	1.2
	125	0.203	0.9
	250	0.185	0.8
***C. albicans* 4122**	0	0.136	0.6
	63	0.143	0.7
	125	0.139	0.6
	250	0.12	0.6

4.5.3 The effect of BER on antioxidant enzymes at protein level

The results of the effect of BER on antioxidant enzymes at the protein level are represented in **Table 4.10** and **Table 4.12** for *C. albicans* SC5314 (FLC susceptible) and *C. albicans* 4122 (FLC resistant) respectively. Specific enzymatic activities of total SOD, glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), and catalase were assessed at 2 hours after BER treatment. Compared with untreated *C. albicans* cells, enzymatic activities gradually increased along with BER concentrations from 63 to 250 µg/ml. *C. albicans* SC5314 specific enzyme activities remained at basal levels following 63 µg/ml BER treatmentbut showed a striking increase after 125 and 250 µg/ml exposures (**Table 4.10**). These findings are in accordance with the results obtained earlier in antifungal activity testing (**Figure 4.9**) and gene expression (**Table 4.7** and **Table 4.8**) indicating once again that BER has a weaker activity or effect against FLC susceptible *C. albicans*.

Although BER treatment in *C. albicans* SC5314 caused a dose-dependent increase in specific enzyme activities, these results showed significant change in activities of total SOD, GPx, GR and catalase at 125 and 250 µg/ml BER. Statistical values are shown in **Table 4.11**. The activity of GST was not statistically significant at all three tested concentrations in comparison with the untreated cells ($p= 0.05$).

Despite the fact that BER treatment in *C. albicans* 4122 (**Table 4.12**) generally showed higher levels of enzymatic activities, there was no significant effect in cells exposed to sub-inhibitory concentrations except for GR activity at 125 µg/ml BER. Total SOD, GPx, GST and catalase activities reached statistical significance after 250 µg/ml BER treatment (**Table 4.13**).

Of note is the fact that SOD activity levels were the highest in both FLC susceptible and resistant *C. albicans*. These findings demonstrate the significant role of SOD in regulating intracellular levels of ROS in *C. albicans*.

Table 4.10: The effect of BER on specific enzymatic activities in *C. albicans* SC5314

Specific enzyme	BER (µg/ml)	Calculated specific enzymatic activity from absorbance (n=3)	
		control	treated
SOD (U/ml)	63	0.93 ± 0.004	1.16 ± 0.022
	125	0.93 ± 0.004	1.41 ± 0.003
	250	0.93 ± 0.004	1.80 ± 0.003
GPx (µmolUA/mg)	63	36.23 ± 0.003	33.67 ± 0.005
	125	36.23 ± 0.003	140.88 ± 0.003
	250	36.23 ± 0.003	193.33± 0.041
GR (µmol UA/mg)	63	23.15 ± 0.002	20.36 ± 0.003
	125	23.15 ± 0.002	51.45 ± 0.003
	250	23.15 ± 0.002	112.54 ± 0.020
GST (µmol UA/mg)	63	45.52 ± 0.004	41.93 ± 0.002
	125	45.52 ± 0.004	66.55 ± 0.004
	250	45.52 ± 0.004	118.8 ± 0.032
Catalase (µmol UA/mg)	63	11.47 ± 0.003	12.04 ± 0.002
	125	11.47 ± 0.003	23.45 ± 0.009
	250	11.47 ± 0.003	35.55 ± 0.003

Table 4.11: Statistical analysis of experimental data used to calculate enzyme activity in *C. albicans* SC5314

Specific enzyme	Comparison of absorbance readings (n=3)	P value
SOD	Control to 63 µg/ml	
	Control to 125 µg/ml	0.031
	Control to 250 µg/ml	0.004
GPx	Control to 63 µg/ml	
	Control to 125 µg/ml	0.005
	Control to 250 µg/ml	
GR	Control to 63 µg/ml	
	Control to 125 µg/ml	0.030
	Control to 250 µg/ml	0.005
GST	Control to 63 µg/ml	
	Control to 125 µg/ml	
	Control to 250 µg/ml	
Catalase	Control to 63 µg/ml	
	Control to 125 µg/ml	
	Control to 250 µg/ml	<0.001

Table 4.12: The effect of BER on specific enzymatic activities in *C. albicans* 4122

Specific enzyme	BER (µg/ml)	Calculated specific enzymatic activity from absorbance (n=3)	
		untreated	treated
SOD (U/ml)	63	1.83 ± 0.001	1.74 ± 0.001
	125	1.83 ± 0.001	2.13 ± 0.049
	250	1.83 ± 0.001	3.03 ± 0.011
GPx (µmolUA/mg)	63	51.09 ± 0.005	93.56 ± 0.007
	125	51.09 ± 0.005	146.3 ± 0.019
	250	51.09 ± 0.005	348.34 ± 0.022
GR (µmol UA/mg)	63	27.85 ± 0.002	40.42 ± 0.004
	125	27.85 ± 0.002	72.88 ± 0.001
	250	27.85 ± 0.002	96.46 ± 0.005
GST (µmol UA/mg)	63	83.85 ± 0.004	94.7 ± 0.013
	125	83.85 ± 0.004	145.07 ± 0.024
	250	83.85 ± 0.004	169.03 ± 0.008
Catalase (µmol UA/mg)	63	17.58 ± 0.006	57.67 ± 0.055
	125	17.58 ± 0.006	72.25 ± 0.062
	250	17.58 ± 0.006	81.8 ± 0.016

Table 4.13: Statistical analysis of experimental data used to calculate enzyme activity in *C. albicans* 4122

Specific enzyme	Comparison of absorbance readings (n=3)	P value
SOD	Control to 63 µg/ml	
	Control to 125 µg/ml	
	Control to 250 µg/ml	0.013
GPx	Control to 63 µg/ml	
	Control to 125 µg/ml	
	Control to 250 µg/ml	0.029
GR	Control to 63 µg/ml	
	Control to 125 µg/ml	0.007
	Control to 250 µg/ml	0.021
GST	Control to 63 µg/ml	
	Control to 125 µg/ml	
	Control to 250 µg/ml	0.011
Catalase	Control to 63 µg/ml	
	Control to 125 µg/ml	
	Control to 250 µg/ml	0.005

Chapter 5: Discussion

There have been an ever-increasing number of *C. albicans* infections in recent years. Despite the high prevalence and mortality rates of *C. albicans* infections worldwide, treatment options are still a challenge and only a few antifungal drugs are available for clinical use. As a result, the search for new therapeutic drugs has gained popularity in an attempt to combat the high burden of *Candida* infections. Thus far, natural products are considered a promising source of potent antifungal drugs. However, it is important to understand the mechanism of action employed by these natural compounds in order to circumvent the development of fungal resistance and also identify relevant cellular targets (Neto *et al*., 2014; Parente-Rocha *et al*., 2017).

5.1 Differential gene expression of major antioxidant enzymes in *Candida albicans*

Research evidence indicates that some conventional antimicrobial drugs induce apoptosis or cellular death by stimulating the production of endogenous ROS (Delattin *et al*., 2014; Mesa-Arango *et al*., 2014). As a result, microorganisms have adopted well-defined mechanisms in response to ROS for tolerance, survival, and virulence. Several free radical scavenging enzymes such as SOD and catalase are involved in ROS detoxification. Upregulation of these primary protective enzymes has been found to promote drug tolerance and biofilm-associated persistence in microorganisms (Linares *et al*., 2006; Belenky and Collins, 2011; Bink *et al*., 2011; Van Acker *et al*., 2013). Some classes of drugs, such as azoles have been found to induce accumulation of ROS in fungal cells (Bink *et al*., 2011; Ferreira *et al*., 2013), which could be correlated to the development of drug resistance against this class of antifungal drugs. The current study sought to evaluate expression patterns in the mRNA expression level of genes encoding antioxidant enzymes in FLC susceptible and resistant *C.albicans* clinical isolates. The results showed clear differences in the expression level of oxidative stress enzymes between these *C. albicans* isolates (**Figure 4.4** to **Figure 4.8**). Higher expression levels were observed in *C. albicans* resistant isolates in comparison with the susceptible group.

SOD is considered a key antioxidant enzyme in regulating the accumulation of ROS in living organisms. SOD acts as a primary antioxidant enzyme that alleviates the effects of ROS by converting O_2^- into less toxic H_2O_2 and water (Khan *et al*., 2011). The results of this study showed a significant increase in the mRNA expression level of *SOD* genes in FLC resistant isolates compared to the susceptible isolates. Moreover, *SOD1* was the most significantly expressed antioxidant gene in this study (**Table 4.2**). Upregulation of *SOD* in FLC resistant

C. albicans correlates with previous findings where SOD activity in FLC resistant *C. albicans* was 1.93-fold greater than that of the sensitive isolates (Linares *et al*., 2013). In contrast, a study conducted in *Listeria monocytogenes* found no direct influence between *SOD* expression and antibiotic sensitivity. This is most probably due to the fact that *L. monocytogenes* possess an incomplete tricarboxylic acid (TCA) cycle resulting in no or low production of ROS during antibiotic exposure (Kohanski *et al*., 2007; Feld *et al*., 2012). However, it is worth mentioning that inactivation of *SOD* gene in several pathogenic bacterial species confers increased susceptibility to antimicrobial agents (Wang and Zhao, 2009; Mosel *et al*., 2013; Heindorf *et al*., 2014). This again demonstrates the importance of SOD in protecting microorganisms from antibiotic-mediated oxidative stress.

Catalase is a key antioxidant enzyme in the detoxification of H_2O_2 to prevent excess accumulation of ROS. In this study, we could not differentiate the mRNA expression of catalase in FLC susceptible and resistant *C. albicans*, due to extremely low expression. Similar results of low expression were observed for *GPx2*. A possible explanation for low mRNA expression could be i) oxidative inactivation as antioxidant defense system is induced in the presence of oxidant species, ii) the results of this study showed overexpression of *SOD*, a prominent ROS scavenger. It is, therefore, logical to expect minimum production of H_2O_2 within the cells. We, therefore, postulate that low expression of *CAT1* and *GPx2* may be correlated to the increased SOD expression.

In addition to SOD and catalase, several other enzymes such as intracellular glutathione (GSH), glutathione S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR) also play an important role as antioxidant defense system in *C. albicans.* Disruption of the GSH redox state has been associated with high production of ROS, GSH auxotrophy and reduced virulence (Yadav *et al*., 2011; Guedouari *et al*., 2014). GSH dependent enzymes regulate the level of GSH in order to maintain cellular homeostasis. The results obtained in this study showed upregulation of the gene encoding GST in the resistant isolates with fold change ranging from 1.38 to 6.98. One of the major properties of GST is the ability to export xenobiotics out of the cells. As a consequence of their involvement in detoxification of stress factors, an increase in the *GST* gene has been implicated in antimicrobial resistance (Veal *et al*., 2002; Pugazhendhi *et al*., 2017). With regard to GPx and GR, varying patterns at mRNA transcription level were identified. For instance, expression of *GPx2* and *GLR1* were elevated in FLC susceptible *C. albicans* isolates in group A (HIV patients) and group B (cancer patients); whereas group C (other immunocompromised

condition) showed a different pattern where gene expression of the aforementioned genes was higher in FLC resistant compared to that of the susceptible isolates. Previous studies revealed that *GPx* in *C. albicans* is differentially expressed depending on the oxidizing agents (Miramón *et al*., 2012).

The results of this study revealed a clear correlation between genes encoding ROS scavenging enzymes and the level of susceptibility to FLC in *C. albicans*. These findings showed that drug resistance is not only caused by mutations in a particular gene but could also arise from proteomic modulations. Therefore, co-application of conventional antifungal agents with redox-potent agents targeting the antioxidant system could serve as a new therapeutic strategy to overcome drug resistance. Furthermore, this approach could greatly reduce the cost and risks of severe side effects.

5.2 Berberine activity against *Candida albicans* cells

The use of BER for clinical purposes has been intensively explored. BER has been found to possess broad spectrum activity against microorganisms including pathogenic fungi (Wei *et al.*, 2011; Dhamgaye *et al.*, 2014; Shao *et al.*, 2016). *In vitro* experiments show variations in the susceptibility profiles of BER treatment against *C. albicans*. It has been revealed that as little as 8 to 32 µg/ml BER can exert relatively good antimicrobial activity (da Silva *et al.*, 2016). Conversely, some studies have shown quite high MIC values of up to 400 µg/ml BER, which is consistent with the findings of the current study (Wei *et al.*, 2011; Zhou *et al.*, 2015; Liu *et al.*, 2017). The present study found MICs ranging from 125 to 500 µg/ml. A study by Li and colleagues has shown that BER treatment alone is not sufficient to weaken the fungal cell membrane for intracellular accumulation (Li *et al.*, 2013). Therefore, it is no surprise that the current study found MICs ranging from 125 to 500 µg/ml. Due to concerns about this limitation, strategies focusing on enhancing the efficacy of BER are needed.

The overall data obtained in the activity of BER showed a significant difference ($p = 0.004$) between FLC resistant and susceptible *C. albicans* (**Figure 4.9**). These results revealed that BER is more active against FLC resistant isolates than FLC susceptible *C. albicans*. FLC resistance in *C. albicans* is well known for causing changes in the organization or functionality of the fungal membrane (Mishra *et al*., 2008). We, therefore, speculate that the differences in BER activity between FLC susceptible and resistant *C. albicans* might be due to alterations of membrane structure leading to increased cell permeability for BER in FLC resistant *C. albicans*. However, further studies are required to test this hypothesis.

5.3 The effect of BER on major antioxidant enzymes in *Candida albicans*

It is known that stress adaptation can alter the antioxidant defense system in many living organisms. This often involves activation of antioxidant enzymes in response to specific stress conditions. However, these antioxidant enzymes may decrease as cells enter the apoptotic pathway (Beach *et al*., 2016). To determine the effect of BER on the enzymes and their genes expression and to exclude the effect on cell viability, appropriate concentrations and treatment time was determined. The results showed cell viability with up to 71% surviving *C. albicans* cells in the presence of sub-inhibitory of BER (**Table 4.4**). A significant reduction in the number of viable cells in samples treated with 500 µg/ml BER was observed. Hence, the latter concentration was excluded in our investigations to ensure that the antioxidant effect was caused by the tested compound and not due to any other biological process.

In *C. albicans*, activation of antioxidant machinery is a direct reflection of internal oxidative stress. In fact, *C. albicans* use this strategy as a defensive mechanism to survive in hostile environments (Kaloriti *et al*., 2014). The results of this study revealed an increase in gene expression and activity of key enzymes essential in the antioxidant defense system. BER treatment enhanced the production capacity of antioxidant enzymes in *C. albicans* to cope with oxidative stress. In all living organisms including fungi, SOD is one of the main antioxidant enzymes involved in the detoxification of intracellular ROS generated either within the mitochondrion during normal cellular respiration or under stress conditions (Liu *et al*., 2010[a]). *Candida albicans* has six SOD isoforms that differ depending on their location and metal co-factors (Bink *et al*., 2011). Several studies have shown overexpression of SOD in *C. albicans* in response to superoxide anion generators such as phenylpropanoids, diallyl disulphide, honokiol and many others (Yousuf *et al*., 2010; Khan *et al*., 2011; Sun *et al*., 2017). In this study, overexpression of genes encoding SOD and increased enzyme activity was observed when *C. albicans* cells were treated with BER. Among all the other antioxidant enzymes, enzymatic level of SOD was the highest. These results demonstrate the importance of SOD in the detoxification of ROS under oxidative stress conditions faced by *C. albicans*.

Even though SOD plays a crucial role in preventing cellular damage caused by ROS, it does not provide complete protection against the deleterious effects of oxidative stress. In order to effectively protect cellular components that are susceptible to oxidative stress, aerobic organisms utilize other antioxidant enzymes for fine modulation of ROS. Among these enzymes, catalase and GPx are considered the major H_2O_2 scavenging enzymes in most

fungal cells (de Olieveira *et al*., 2013). As expected, there was a significant increase in the expression of *GPx2* at all the three concentrations of BER. Enzymatic activity levels of GPx revealed that this enzyme was among the most produced antioxidants in *C. albicans* treated cells. GPx is predominantly responsible for reducing H_2O_2, a detrimental oxidizing agent to water and oxygen by using glutathione as an electron donor. Therefore, overexpression of *GPx* in the current study is not a surprising finding but an important phenomenon in maintaining H_2O_2 content at physiological concentration. Despite the fact that both GPx and catalase are capable of breaking down the reaction of H_2O_2, the results obtained in this study indicated that only increased GPx took part in the antioxidant defense response against BER induced stress. This was particularly supported by low enzymatic activity and gene expression level of catalase in this study. In fact, RT-qPCR failed to reveal *CAT1* amplification in the presence of BER. However, we identified an increasing pattern of catalase at the translation level. Previous studies have reported poor correlation between mRNA and protein expression in response to stress in yeast cells (Lackner *et al*., 2012; Cheng *et al*., 2016). The discrepancy is mainly due to the fact that mRNA and protein have different regulatory mechanisms occurring at different levels. For instance, mRNA is generally unstable with short half-life times.On the other hand, protein stability and half-life could be increased because of post-translational modifications. Hence, the difference in the amount of specific protein and expression levels of the corresponding mRNA (Lackner *et al*., 2012; Cheng *et al*., 2016). The low level of catalase in the present study is in stark contrast to previous research. Khan and co-workers reported significantly increased levels of catalase in response to eugenol and estragole (Khan *et al*., 2011). Similarly, Román and colleagues reported increased *CAT1* expression following treatment with different oxidants and antifungal drugs (Román *et al*., 2016). The differences may be caused by 1) longer exposure period in their experiments, 2) lower numbers of viable cells, 3) or the use of different oxidizing agents. Based on the results of this study, it can be concluded that catalase is not a main H_2O_2 scavenger under BER induced oxidative stress.

In addition, glutathione reductase (GR) is an important enzyme required for survival of yeast cells under oxidative stress conditions, while playing an important role in maintaining the intracellular glutathione redox ratio of GSH/GSSG (Grant *et al*., 1996; Gostimskaya and Grant, 2016). In this study, sub-lethal concentrations of BER caused an increase in GR activity. However, higher concentrations (125 and 250 µg/ml) triggered a much more significant activation of GR. The results of this study are in agreement with the previous

findings reporting a marked induction of GR in *C. albicans* following exposure to severe oxidative stress conditions (50 mM H_2O_2) (Gonz´alez-P´arraga *et al*., 2003). Furthermore, their study reported higher survival percentage in cells with elevated GR under stress conditions, emphasizing the importance of GR for survival in toxic environments. It is therefore not surprising that the current study found significant activation of GR activity and gene expression only in cells treated with 125 and 250 µg/ml of BER.

Another key antioxidant enzyme important in maintaining glutathione content and GSH/GSSG ratio in cells is GST. An alteration of glutathione levels is known to cause severe cell damage. GST is a key determinant in protecting cells against oxidative stress products, as well as in the detoxification of foreign compounds. This detoxification reaction involves spontaneous conjugation of reduced glutathione and xenobiotics via GST activity. The conjugates formed during this reaction can be readily excreted from cells (Gostimskaya and Grant, 2016). In this study, upregulation of GST encoding gene was observed which is congruent with previous findings where *GST* gene expression increased upon alkaline and oxidative stresses (Garcerá *et al*., 2010). These results also revealed that among the specific enzymatic levels, GST was the second most expressed antioxidant in response to BER treatment (**Table 4.10** and **Table 4.12**). This finding suggests a positive contribution of GST in resisting the effects of oxidative stress in *C. albicans*.

Although BER caused an increase of these targeted antioxidant enzymes, the overall results indicated that *C. albicans* exhibited efficient antioxidant response at lower concentrations. However, *C. albicans* could not sufficiently alleviate oxidative stress occurring at higher concentrations, especially in FLC resistant *C. albicans*. One plausible explanation could be that BER at ½ MIC values of BER induced robust oxidative stress in *C. albicans* surpassing the antioxidant capacity of the cells, thereby resulting in reduced production of antioxidant enzymes.This demonstrates that BER at sub-inhibitory concentrations is able to render *C. albicans* avirulent by suppressing its antioxidant defense response without compromising cell viability of the fungi. Therefore, BER could serve as a potent ROS-inducing agent, disrupting the antioxidant system especially in *C. albicans* resistant isolates to overcome antifungal drug resistance. To the best of our knowledge, this study is the first to assess the antioxidant response system in *C. albicans* as a potential antifungal target for BER.

5.4 Conclusion

The findings in this study showed that drug resistance is not only caused by mutations in a particular gene but could also arise from proteomic modulations. The study also demonstrated that *C. albicans* activates several antioxidant enzymes that form an integral component of the cell's response against oxidative stress. Our findings clearly revealed that BER modulates the activity and gene expression of major antioxidant enzymes in *C. albicans*. These findings provide further insight into the mechanism of antifungal action employed by BER. This information has revealed that BER has a potential to be developed into a therapeutic agent for the treatment of *C. albicans* infections and other pathogenic fungi.

5.5 Future research

1. Sequencing of oxidative stress responsive genes to assess any variations in the genetic information between FLC susceptible and resistant *C. albicans*.
2. *Candida albicans* cells comprise of six different SOD isoforms which are involved in the detoxification of stress. This study focused on the expression of *SOD1* and *SOD2* which does not provide a complete representation of the antioxidant capacity of SOD. The other four *SOD* genes could be studied to understand the role of each *SOD* gene in oxidative stress.
3. In this study, FLC resistant *C. albicans* cells were most susceptible to BER. To better understand this finding, ergosterol content and membrane permeability in FLC susceptible and resistant *C. albicans* in the presence of BER would be an important study.
4. The effect of BER on oxidative stress parameters such as lipid peroxidation, superoxide levels, and H_2O_2 could be assessed.
5. The effect of BER on key virulence factors in *C. albicans* such as biofilm formation and hydrolytic enzymes could be studied.
6. The enzyme activities were done in the external reaction mixtures and the results may vary from *in situ* studies. Future testing using animal models is recommended.

Chapter 6: References

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Chapter 7: Appendices

Appendix 7.1: Abstract submitted to journal plos one

PLOS ONE

Role of antioxidant defense system in fluconazole susceptibility and its modulation by berberine in Candida albicans

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Full Title:	Role of antioxidant defense system in fluconazole susceptibility and its modulation by berberine in Candida albicans
Short Title:	Antioxidant defense system as novel antifungal drug target in C. albicans
Corresponding Author:	Aijaz Ahmad, Ph.D. University of the Witwatersrand Johannesburg, Gauteng SOUTH AFRICA
Keywords:	Candida albicans; oxidative stress; antioxidant enzymes; gene expression; berberine
Abstract:	Invasive fungal infections arising from Candida species have significantly increased especially in immunocompromised patients. In addition, emergence of multidrug resistant species and forms of Candida are evolving, which advocates an urgent need for the development of new therapeutic strategies and antifungal drugs. Activation of antioxidant defense system has been known to be forefront mechanism to escape drug toxicity. This study was conducted to evaluate the role of antioxidant defense genes in the susceptibility to fluconazole (FLC) in C. albicans isolates. We also determine the effect of a plant-based alkaloid berberine (BER) on growth and antioxidant enzymes in C. albicans. Antifungal activity of BER was determined using microdilution method. Gene expression of SOD1, SOD2, GPx2, GLR1, GTT1, and CAT1 in untreatedand BER treated C. albicans cells was measured by RT-qPCR. The activity level of the corresponding enzymes in the presence of BER was determined spectrophotometrically. The key finding in this study revealed that drug resistance in Candida cells is not only caused by mutations in a particular gene/s but could also arise from proteomic modulations. Antifungal susceptibility showed BER MICs ranging from 125 to 500 µg/ml. Gene expression analysis showed an increase in mRNA expression levels of SOD1, SOD2, GPx2, GLR1 and GTT11 genes in fluconazole resistant isolates than in the susceptible group. BER treatment induced upregulation in the mRNA expression and enzymatic activities of major antioxidants. In fluconazole resistant C. albicans, excess concentrations caused downregulation of the targeted antioxidants indicating that BER at higher concentrations induced an intense oxidative stress. Overall results revealed an increase in the expression of antioxidant enzymesin response to BER induced oxidative stress, but the antioxidant capacity could not sufficiently combat the toxic effects occurring at higher concentrations. Therefore, BER could serve as a potent ROS-inducing agent in C. albicans.

Appendix 7.2: Abstract for poster presentation

Upregulation of oxidative stress enzymes confers fluconazole resistance in *Candida albicans* clinical isolates-Faculty of Health Science Biennial Research Day and Postgraduate Expo (2018), University of the Witwatersrand.

Evida Poopedi[1], Musa Marimani[1] and Aijaz Ahmad[1,2]

[1]Clinical Microbiology and Infectious Diseases, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand

[2]Infection Control, Charlotte Maxeke Johannesburg Academic Hospital, National Health Laboratory Service, South Africa

Introduction

Candidiasis is becoming a major health threat worldwide, particularly in the immunodeficient population leading to increased mortality rates. Currently available therapies have been in use for several decades and target mainly the biosynthesis or the integrity of the fungal cell envelop. This indicates an urgent need for the development of new antifungal drugs which will significantly impair the development of antifungal drug resistance, and have minimal side effects. Currently, anti-virulence therapeutics represents an attractive new approach to the development of novel classes of antifungal drugs. This approach intends to weaken specific virulence properties that promote or cause *C. albicans* infections in the host without compromising cell viability of the pathogen.This study aims to evaluate gene expression profile of major antioxidant enzymes mediating fluconazole resistance in clinical *C. albicans* isolates.

Method

A total of six isolates collected from HIV positive and other immunocompromised patients were provided by the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand. The isolates were cultured for species identification using API 20C AUX. Antifungal susceptibility to fluconazole was determined following the guidelines recommended by the CLSI M27-A3 with modifications. Real-time PCR was performed to evaluate gene expression patterns of antioxidant enzymes in fluconazole susceptible and resistant *C. albicans* isolates.

Results

The overall results showed an increase in mRNA expression level of *SOD1*, *SOD2*, *GLR1*, *GPx2* and *GTT11* genes in fluconazole resistant isolates. A significant upregulation of *SOD1* was revealed with 50.7-fold increase. The other genes showed a moderate increase in the expression with fold change ranging from 1.2 to 4.2.

Conclusion

Our findings speculate that drug resistance is not only caused by mutations in a particular gene but could also arise from proteomic modulations. Therefore, co-application of conventional antifungal agents with redox-potent natural compounds targeting the antioxidant system could serve as a new therapeutic strategy to overcome drug resistance.

Appendix 7.3: Abstract for poster presentation and flash talk

9th Wits Cross Faculty Postgraduate Symposium

The effect of berberine on gene expression of antioxidant enzymes in *Candida albicans* clinical isolates.

Evida Poopedi[1], Musa Marimani[1] and Aijaz Ahmad[1,2]

[1]Clinical Microbiology and Infectious Diseases, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand

[2]Infection Control, Charlotte Maxeke Johannesburg Academic Hospital, National Health Laboratory Service, South Africa

Despite the availability of antifungal drugs, *C. albicans* infections remain a major health threat worldwide particularly in immunocompromised patients leading to high mortality rates. This is mostly caused by emergence of resistant *C. albicans* isolates to conventional antifungal drugs indicating a need for the development of new therapeutic strategies and antifungal drugs. Phytomedicines are used throughout the world and are a major source of developing new therapeutic drugs. Berberine (BER) is a well-known isoquinoline alkaloid found in a variety of medicinal plant species. BER has shown to possess multiple biological and pharmacological properties including anti-inflammatory, antitumor, and antimicrobial activity. In this study, we assessed the effect of BER on gene expression of major antioxidant enzymes in fluconazole (FLC) susceptible and resistant *C. albicans* clinical isolates. *C. albicans* isolates were obtained from the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand. Species identification was confirmed using API 20C AUX. Antifungal susceptibility to BER was determined following CLSI M27-A3 guidelines. Gene expression profile of antioxidant enzymes in FLC susceptible and resistant *C. albicans* isolates were assessed using RT-qPCR. The susceptibility test showed MICs ranging from 0.125 to 0.5 mg/ml with a marked difference in the activity of BER between FLC susceptible and resistant *C. albicans*. RT-qPCR analysis showed an increase in mRNA expression level of *SOD1*, *SOD2*, *GPx2*, *GLR1* and *GTT11* genes in FLC resistant isolates than in the susceptible group. The most significantly expressed gene was *SOD1* with a 50.69-fold increase. The other genes showed moderate increase in the expression with fold change ranging from 1.2 to 4.2. Moreover, exposure of *C. albicans* to BER induced significant upregulation of oxidative stress responsive genes. *SOD1* showed an increase of (2.1 to 82.4-fold), *SOD2* (1.1 to 9.9-fold), *GPx2* (3.7 to 483.2-fold), *GLR1* (1 to 7.4-fold) and *GTT11* (1.6

to 13.8-fold). Based on the obtained data, BER induces a significant upregulation in mRNA expression of antioxidant enzymes indicating that BER achieves its antifungal activity in *C. albicans* by promoting an intense oxidative stress. Therefore, BER could serve as a potent ROS-inducing antifungal agent to overcome drug resistance.

Appendix 7.4: Ethical clearance

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

Rsearch Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 301, 29 Princess of Wales Terrace, Parktown, 2193 Tel +27 (0)11-717-1252 /1234/2656/2700
Private Bag 3, Wits 2050, email: zanele.ndlovu@wits.ac.za
Office email: hrec-medical.researchoffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CJ-170524-1 24/05/2017

TO WHOM IT MAY CONCERN:

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

Investigator: Poopedi Evida (student no. 350208), Dr A Ahmad.

Project title: Expression and activity of oxidative stress enzymes in mediating fluconazole resistance in *Candida albicans* and their regulation by berberine.

Reason: This is a laboratory study using existing clinical *Candida albicans* isolates collected under ethical clearance M000402 and a purchased reference culture *Canada albicans* SC5314. There are no human participants.

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

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

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

Appendix 7.5: Media, reagents and chemicals used in this study

Appendix 7.5.1: BER (10 mg/ml)

0.010 g BER was (Sigma-Aldrich, MO, USA) in 10 ml methanol (Merck Chemicals (Pty) Ltd, Darmstadt, Germany). The solution was further diluted in sterile distilled water in preparation of working solution.

Appendix 7.5.2: FLC (5 mg/ml)

20 ml of DMSO (Associated Chemical Enterprises (Pty) Ltd, South Africa) was added in 100 mg/ml FLC (Sigma-Aldrich, MO, USA) to make stock solution of 5 mg/ml. This was further diluted in sterile distilled water in preparation of working solution.

Appendix 7.5.3: 10 X DNase buffer

1 ml of 1 mol/L Tris-HCl buffer, pH 7.5

250 µl of 1 mol/L $MgCl_2$

50 µl of 1mol/L $CaCl_2$

8.7 ml sterile distilled water

1.47 g $CaCl_2$ (Merck Chemicals (Pty) Ltd, Darmstadt, Germany) and 2.03 g $MgCl_2$ (Merck Chemicals (Pty) Ltd, Darmstadt, Germany) were dissolved in different tubes each containing 10 ml sterile distilled water.

Appendix 7.5.4: 10 X Tris-acetate-EDT (TAE) buffer

48.4 g Tris base, 3.7 g EDTA and 11.4 ml glacial acetic acid (Sigma-Aldrich, MO, USA) were dissolved in 1000 ml distilled water. The solution was further diluted in sterile distilled water in preparation of 1 X TAE buffer.

Appendix 7.5.5: 250 mmol/L sucrose

85.87 g of sucrose (Sigma-Aldrich, MO, USA) was dissolved in 1000 ml sterile distilled water. The solution was filter sterilized and aliquots were stored at -20 ºC.

Appendix 7.5.6: 1 mol/L Tris-HCl buffer, pH 7.5

12.1 g Tris base (Tocris Bioscience, Bristol, UK) was dissolved in 80 ml sterile distilled water. Absolute HCl (Sigma-Aldrich, MO, USA) was added to adjust pH. The solution was allowed to cool down to room temperature before final adjustment to 100 ml with water and filter sterilized. To make 10 mmol/L Tris-HCl buffer, 1 ml of the stock solution was added to 99 ml sterile water.

Appendix 7.5.7: 10 mmol/L phenylmethane sulfonyl fluoride (PMSF)

0.0174 g PMSF (Roche Diagnostics, GmbH, Germany) was added in 10 ml isopropanol (Merck Chemicals (Pty) Ltd, Darmstadt, Germany). Aliquots were stored at -20 ºC.

Appendix 7.5.8: 50 mmol/L potassium phosphate buffer pH (6.5, 7.0 and 7.4)

1.701 g potassium phosphate monobasic (Sigma-Aldrich, MO, USA) was dissolved in 250 ml sterile distilled water. 200 mmol/L KOH (Associated Chemical Enterprises (Pty) Ltd, South Africa) was added to adjust pH of the solution.

Appendix 7.5.9: 50 mmol/L Tris buffer, pH 8.5

0.605 g Tris base was dissolved in 100 ml sterile distilled water. The pH of the solution was adjusted using 1 mmol/L EDTA.

Appendix 7.5.10: 20 mmol/L pyrogallol

0.025 g pyrogallol (Sigma-Aldrich, MO, USA) was added in 10 ml of 10 mmol/L HCl. The solution was freshly prepared at the time of the assay.

Appendix 7.5.11: 1 mmol/L sodium azide (NaN_3)

0.033 g NaN_3 (Sigma-Aldrich, MO, USA) was added in 500 ml sterile distilled water. The solution was covered with aluminum foil to protect against light.

Appendix 7.5.12: 1 mmol/L EDTA

0.146 g EDTA was dissolved in 500 ml sterile distilled water.

Appendix 7.5.13: 1 mmol/L tripeptide glutathione

0.015 g GSH (Tocris Bioscience, Bristol, UK) was added in 50 ml potassium phosphate buffer, pH 7.0. The solution was freshly prepared at the time of the assay.

Appendix 7.5.14: 0.2 mmol/L NADPH

0.017 g NADPH (Roche Diagnostics, GmbH, Germany) was added in 100 ml sterile distilled water. The solution was freshly prepared at the time of the assay.

Appendix 7.5.15: 0.1 mmol/L NADPH

0.008 g NADPH was added in 100 ml sterile distilled water. The solution was freshly prepared at the time of the assay.

Appendix 7.5.16: 0.5 mol/L EDTA, pH 8

1.86 g EDTA (Merck Chemicals (Pty) Ltd, Darmstadt, Germany) was added in 10 ml sterile distilled water. The pH was adjusted using 4 mol/L NaOH.

Appendix 7.5.17: 1mmol/L oxidized glutathione disulphide (GSSG)

0.031 g GSSG (Sigma-Aldrich, MO, USA) was added in 50 ml potassium phosphate buffer, pH 7.4. The solution was freshly prepared at the time of the assay.

Appendix 7.5.18: 100 mmol/L tripeptide glutathione (GSH)

0.307 g GSH was added in 10 ml potassium phosphate buffer, pH 6.5. The solution was freshly prepared at the time of the assay.

Appendix 7.5.19: 100 mmol/L 1-chloro-2.4-dinitrobenzene (CDNB)

0.203 g CDNB (Sigma-Aldrich, MO, USA) was dissolved in 6ml absolute alcohol (Diagnostic Media Products-NHLS, South Africa). 4 ml of sterile distilled water was added to the solution to make a final volume of 10 ml.

Appendix 7.5.20: Sabouraud dextrose broth

Mycological peptone	10 g/ L
Dextrose	20 g/ L

30 g of dehydrated powder (Sigma-Aldrich, MO, USA) was distilled in 1000 ml distilled water and autoclaved at 121 ºC for 15 min.

Appendix 7.5.21: Sabouraud 4% glucose agar

Peptone	10 g/ L
D (+)-Glucose	40 g/L
Agar	14 g/L

65 g of dehydrated powder (Sigma-Aldrich, MO, USA) was distilled in 1000 ml distilled water and autoclaved at 121 ºC for 15 min. The media was allowed to cool down to 50 ºC and poured into 90 mm sterile Petri dishes.

Appendix 7.5.22: 0.85% NaCl

8.5 g of NaCl (Merck Chemicals (Pty) Ltd, Darmstadt, Germany) was dissolved in 1000 ml water. The solution was autoclaved at 121 ºC for 30 min.

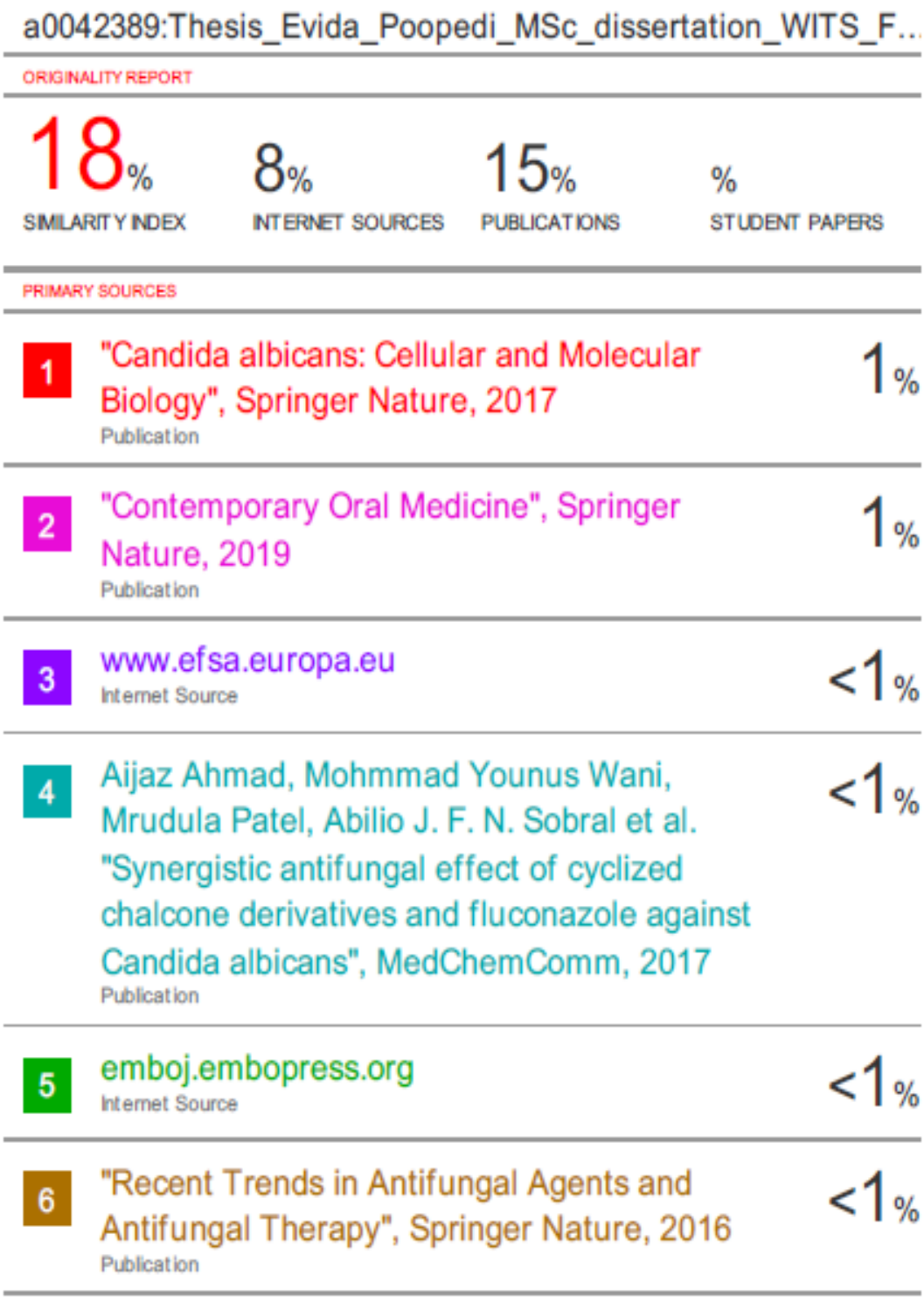
Appendix 7.5.23: 0.5 mmol/L EDTA

0.019 g EDTA was dissolved in 100 ml sterile distilled water.

Appendix 7.5.24: The composition of the API 20C AUX strips.

Test	**Ingredient**
0	None
GLU	D-Glucose
GLY	Glycerol
2KG	Calcium 2-Keto-Gluconate
ARA	L-Arabinose
XYL	D-Xylose
ADO	Adonitol
XLT	Xylitol
GAL	D-Galactose
INO	Inositol
SOR	D-Sorbitol
MDG	Methyl-αD-Glucopyranoside
NAG	N-Acetyl-Glucosamine
CEL	D-Cellobiose
LAC	D-Lactose (bovine origin)
MAL	D-Maltose
SAC	D-Saccharose (sucrose)
TRE	D-Trehalose
MLZ	D-Melezitose
RAF	D-Raffinose
GT	Germ tube

Appendix 7.6: Turnitin report



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