Expression and activity of antioxidant enzymes in *Candida auris* and their regulation by carvacrol

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Declaration

I, Mishka Ismail, 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.

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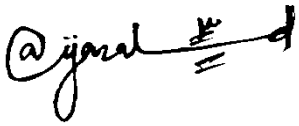

Dr Aijaz Ahmad

Supervisor

Dedication

This dissertation is dedicated to my two pillars of strength,

My mother, Tasneem Carrim,

&

My only sister, Nazia Ismail.

Presentations and Publications

Presentations

06 September 2019 – Clinical Microbiology and Infectious Diseases Departmental Research Day

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Publication

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Abstract

The occurrence of *Candida auris* has been described as a global health threat since its first official identification in 2009. High mortality rates have been observed which are owed to its ability to establish invasive infections in a nosocomial manner as well as expressing multi-drug resistance. The development of novel antifungal drugs with unique targets is a growing necessity to combat this frequently occurring fungal pathogen and its virulence. Natural compounds and agents have been utilized for many generations to overcome infections. Now, scientific investigations of the mechanisms of action of certain phytochemicals have gained attention. The phenolic monoterpenes commonly extracted and isolated from essential oils are well recognized for their noteworthy anti-*Candida* activity, both *in vitro* and *in vivo*. This study focused on the antifungal activity as well as the oxidative stress capability of carvacrol, a monoterpene phenol, against *C. auris*.

The antifungal susceptibility profile of 25 clinical isolates of *C. auris* against carvacrol was determined by the use of the micro dilution broth method following CLSI guidelines. Carvacrol displayed growth inhibition in the range of 125 – 500 µg/ml and a fungicidal effect in the range of 250 – 1 000 µg/ml. *In vitro* analysis of oxidative stress caused by carvacrol was determined by the spectrophotometric observation of antioxidant enzyme levels as well as the gene regulation of these enzymes by reverse transcription quantitative polymerase chain reaction (RT-qPCR). The activities of the primary antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) showed an average fold increase in a range of 1.93 – 7.16 with an upregulation of gene expression in the range of 1.18 – 2.6 after carvacrol treatment at minimum inhibitory concentration (MIC). Conversely, secondary antioxidant enzymes (glutathione transferase and glutathione reductase) exhibited an average fold decrease of 4.88 and 2.18 as well as a downregulation of gene expression by 0.66 and 0.93 folds, after carvacrol treatment at MIC respectively. Lipid peroxidation, an indicator of oxidative stress, was also observed to be increased with an average of greater than 2 fold, after cells were treated with carvacrol at MIC values. Furthermore, cytotoxicity assays revealed that carvacrol at MIC values can cause 2.8 – 6.4 % haemolysis in the horse red blood cells, advocating its safe use during *in vivo* studies.

This study presented the potential of carvacrol to inhibit and eradicate *C. auris* by inducing increased levels of oxidative stress. Low MICs and killing property of carvacrol against *C. auris* isolates indicate that carvacrol might be a potential candidate to develop into a novel antifungal agent.

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List of acronyms, symbols and abbreviations

•OH	hydroxyl radical
ALS	agglutinin-like sequence
API 20C	analytical profile index yeast
ATP	adenosine triphosphate
BSI	blood stream infection
Ca^{2+}	calcium ions
cAMP-PKA	cyclic adenosine monophosphate protein kinase A
CAT	catalase
CDC	Centers for Disease Control and Prevention
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
CFE	cell-free extracts
CFU	colony forming units
CHROMagar	chromogenic agar
CLSI	Clinical and Laboratory Standards Institute
CMC	chronic mucocutaneous candidiasis
CMID	Clinical Microbiology and Infectious Diseases
CO_2	carbon dioxide
CoQ	coenzyme Q
COX	cyclooxygenase
Cq	quantitation cycle
Cu-Zn	copper-zinc
DDC	diethyldithiocarbamate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ETC	electron transport chain

FDA	Food and Drug Administration
GPI	glycosylphosphatidylinositol
GPx	glutathione peroxidase
GR	glutathione reductase
GRAS	generally recognized as safe
GSH.	glutathione
GSSG	oxidized glutathione disulphide
GST	glutathione transferase
H^+	hydrogen ions
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HDC	hematogenously disseminated candidiasis
HPETE	hydroperoxyeicosatetraenoic acid
HWP	hyphal wall protein
ICU	intensive care unit
IgA	immunoglobulin A
LAMP	Loop-mediated Isothermal Amplification
LIP	lipase
LOX	lipoxygenase
LPO	lipid peroxidase
MALDI-TOF.MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MDR	multi-drug resistant
MFC	minimum fungicidal concentration
MIC	minimum inhibitory concentration
MMLV	Moloney Murine Leukemia Virus
Mn	manganese
mRNA	messenger RNA
MTL	mating type loci
NAC	non-*albicans Candida*

NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
$NADP^+$	oxidized nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaN_3	sodium azide
NICD	National Institute of Communicable Diseases
O_2	molecular oxygen
O_2^-	mitochondrial oxygen/superoxide anion
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PL.	phospholipase
PM	plasma membranes
PMSF	phenylmethane sulfonyl fluoride
qPCR	quantitative polymerase chain reaction
RBC	red blood cells
rDNA	ribosomal DNA
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rotations per minute
RT-qPCR	Reverse Transcriptase qPCR
SAPs	secreted aspartic proteases
SD	standard deviation
SDA	sabouraud dextrose agar
SDB	sabouraud dextrose broth
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid-reactive substances
TCA	trichloroacetic acid
VITEK 2 YST	VITEK® 2 Yeast identification card
WHO	World Health Organization
YCB–BSA	yeast carbon base– bovine serum albumin

Chapter 1) Introduction

1. 1) Background to the study

Candida species are among the most frequently encountered fungal pathogens that cause fungal infections worldwide. A variety of species within this genus cause different types of candidiasis. The Centers for Disease Control and Prevention (CDC) have reported that *Candida* species are the fifth most important hospital-acquired pathogens and the fourth most common among Blood Stream Infection (BSI) pathogens (CDC, 2020). Of the many species of *Candida* that have been discovered, *C. albicans* has been found to be the most common species causing nosocomial infections (Cortegiani *et al*., 2018). However, a considerable increase in infections due to non-*albicans Candida* (NAC) species has been recognized in recent years. *Candida auris* is one such species that has attained tremendous attention, due to its high morbidity and mortality rates.

Candida auris is relatively a new species of opportunistic fungi and has the/a unique ability to colonize the skin and non-human surfaces, resulting in fast and easy transmission between individuals. *C. auris* has also been found to infect the bloodstream, gut, wounds, or ears of hospitalized patients and persons with a weakened immune system (CDC, 2019). Cases of *C. auris* associated outbreaks have been reported globally with a mortality rate of approximately 32 – 66 % (Rossato & Colombo, 2018). In South Africa, several outbreaks of *C. auris* infections have been reported and this pathogen now accounts for approximately one in every 10 cases of candidemia (Govender *et al*., 2018).

Healthy individuals generally do not succumb to *C. auris* infection, as their immune system recognizes and engulfs the pathogen (Latocha, 2020). Being a primary line of defence, cells of the innate immune system bind to the fungal cell wall.via different receptors and activate an intracellular signalling cascade (Salazar and Brown, 2018). Activation of these signals initiates the production of reactive oxygen species (ROS) and therefore exposes the pathogen to toxic chemicals and ionic imbalance (Muralidharan and Mandrekar, 2013). The fungus is subjected to permanent cell damage due to the irreversible interaction of ROS with its cellular components, such as lipids, proteins, and nucleic acids (Sharma *et al.*, 2012). Therefore, immunocompromised patients remain highly susceptible to the fungal infections as their immune system is weakened and unable to detect and destroy an invading fungus (Puebla, 2012).

Aerobic organisms inevitably generate ROS at a carefully monitored rate where a loss of control can be detrimental. Subjecting a fungus to oxidative stress is critical for the effective termination of the pathogen from the host as it will prevent its ability to proliferate and spread. To overcome the damaging effect of oxidative stress caused by the host's immune system, *Candida* cells have evolved advanced and effective antioxidant mechanisms to reverse the oxidative attack (Bravim *et al*., 2016). Antioxidant mechanisms are very well defined in *C. albicans,* where the *CAP1* gene has been reported to produce vital enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductases and glutathione transferases, required for repairing the damages caused by host oxidative stress (Patterson *et al*., 2013). However, there is very limited knowledge related to the antioxidant defence system of *C. auris,* with nothing reported about the South African clade of *C. auris.*

Antifungal drugs are the next best defence against fungal infections. Among the antifungals, two major drug classes (polyenes and azoles) target different components of the fungal cell envelope (Ghannoum and Rice, 1999). However, in order to administer the appropriate drug, correct identification of the pathogen is required, which is an additional challenge with *C. auris.* Misidentification of *C. auris* with other infectious *Candida* species is common. This is due to phenotypic and phylogenetic similarities, which render commercially available methods of yeast identification insufficient and unreliable (Raiesi *et al*., 2019). This misidentification has further caused ineffective and inaccurate treatment strategies for infected patients.

Besides the diagnostic challenge, an additional challenge is posed by *C. auris* as it is multi-drug resistant. Strains of *C. auris* have been reported to be resistant to all of the three major antifungal drug classes (polyenes, azoles and echinocandins) (Kean and Ramage, 2019; Vallabhaneni *et al*., 2019). The multi-drug resistance therefore leads to additional restricted treatment options and warrants further investigation to develop innovative and effective antifungal therapeutics with different modes of action.

Phytomedicine has been globally practiced for centuries, whereby plant-derived compounds have a wide range of biological activities to treat and prevent communicable and non-communicable diseases (Prasad *et al*., 2015). Phytochemicals have also been reported to serve as vital sources of antifungal agents, where some of these natural compounds specifically induce oxidative stress to fungal cells with minimal side effects to the host (Sharifi-Rad *et al*., 2018). These could aid in the

development and potential success of new antifungal drugs, especially against drug resistant fungal pathogens. Carvacrol (Figure 2.1) is one such phytochemical that has been well studied for its medicinal values (Sharifi-Rad *et al*., 2018). It is a phenolic compound, found in the essential oils extracted from oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and other aromatic plants. Of all the volatile compounds present in essential oils, carvacrol has been proven to have a higher antimicrobial activity and has been reported to increase antioxidant enzyme activities and retard lipid oxidation (Sharifi-Rad *et al*., 2018). In recent studies, the efficacy of carvacrol alone and in combination with antifungal drugs against different clinical *Candida* isolates was analysed (Sharifzadeh *et al*., 2019; Khan *et al*., 2015). However, the oxidative effect that carvacrol might have on multi-drug resistant *C. auris* has not yet been investigated.

1. 2) Problem Statement

Candidiasis has become a worldwide health threat due to the emergence of new pathogenic species. New multi-drug resistant (MDR) *Candida* species and forms complicate the treatment of candidiasis, especially in immunocompromised individuals and thereby increases the associated mortality rates. Development of safer and effective antifungal drugs is extremely complex and challenging due to the high resemblance of the cells of this pathogen with the human host cells. Therefore, it is important to accurately identify a target within the invading fungus in order to design an appropriate fungal specific drug.

The recently identified *Candida* species, *C. auris*, is associated with several outbreaks around the globe. It has also been reported that *C. auris* is resistant to most of the commonly used classes of antifungal drugs including azoles, polyenes and echinocandins. In addition, being a newly identified *Candida* species, there is not much known about the structural, biochemical and pathogenic characteristics of this fungus. Therefore, it is important to understand this pathogen and find novel drug targets different from the cell envelope, which is the most utilized drug target by common antifungals. The antioxidant defence mechanism has recently been recognized as an emerging antifungal drug target. The determination of the presence of certain antioxidant enzymes in *C. auris* (in comparison with *C. albicans*) as well as their genetic profile and expression, could provide some knowledge of the intracellular mechanism this fungus utilizes to overcome oxidative stress. The identification of such enzymes could serve as potential markers for future drug targets. Therefore, instead of trying to exterminate the fungus externally (i.e. targeting the cell wall and

membrane), targeting the defence system of the pathogen may be an effective alternative strategy to counter fungal spread. Moreover, there are studies reporting the antifungal effect of carvacrol by impairing the fungal defence system against oxidative insults by hosts. However, to the best of our knowledge, nothing has been reported regarding the oxidative stress caused by carvacrol on *C. auris*.

1. 3) Aim of this study

To determine the presence of enzymes combating oxidative stress in *C. auris,* and to study the effect of carvacrol on the regulation of these enzymes and their respective genes.

1. 4) Objectives

a) To screen *C. auris* clinical isolates for the presence of antioxidant enzymes (i.e. catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione transferase).
b) To determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of carvacrol against *C. auris*.
c) To study the effect of carvacrol on the activity and gene expression of antioxidant enzymes in clinical isolates of *C. auris* by RT-qPCR.
d) To study the effect of carvacrol on the extent of lipid peroxidation.

The methodology that was utilised to fulfil the aforementioned objectives for this study is illustrated in Figure 1.1.

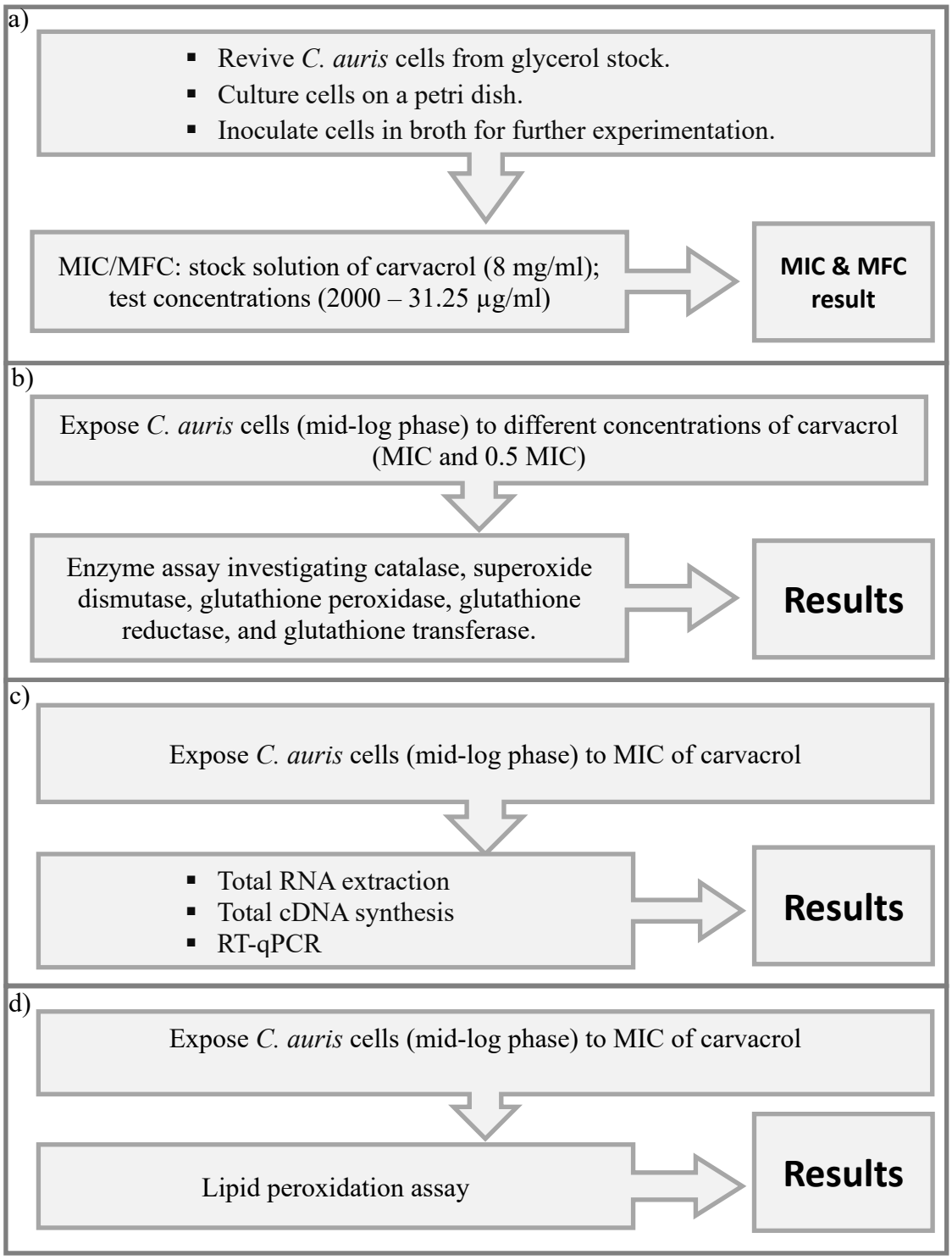

Figure 1.1: Flow-diagram illustrating the methodology used in this study. a) Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) determination. b) Enzyme Assays, c) RT-qPCR, d) Lipid Peroxidation.

Chapter 2) Literature Review

Microorganisms, or microbes, are organisms whose morphological structure can only be viewed through a microscope. They exist either as a single cell (unicellular) where all life processes occur in one cell or many cells (multicellular) where there is specialization of different cells for various functions (Ispolatov *et al*., 2012). Microbes are widespread in all kinds of environments. Most are beneficial to other organisms by providing nutrients or maintaining health. But some can be pathogenic and cause severe harmful infections/diseases. Microbes can be divided into seven major groups namely; algae, protozoa, slime moulds, bacteria, archaea, viruses and fungi (Pitt and Barer, 2012).

Cyanobacteria, better known as algae or blue-green algae, are eukaryotes that can be uni- or multicellular and are believed to hold the origin of green land plants. Protozoa are unicellular aerobic eukaryotes that make up the largest group of organisms globally regarding biomass, diversity and numbers. Slime moulds are unicellular eukaryotes that have a resemblance to both protozoa and fungi. Bacteria are unicellular prokaryotes and have been reported to potentially be the earliest life forms on land. Archaea are also unicellular prokaryotes that differ from bacteria through distinct molecular characteristics. Viruses are acellular particles that infect prokaryotic and eukaryotic cells to reproduce and cause disease. A fungus is a eukaryote which can be uni- or multicellular. The Fungi Kingdom include an incredible diversity of organisms that are neither plant nor animal (Lotha *et al*., 2019).

2. 1) Introduction to Fungi

The kingdom of fungi is inclusive of a broad assortment of eukaryotic organisms. Like humans, their cells contain a nucleus surrounded by a nuclear membrane. They differ in cell membrane composition in this respect that where mammalian cell membranes are cholesterol-rich, fungal cell membranes are principally composed of ergosterol. They further differ in that fungal cells have an additional layer of protection, a cell wall, which contains complex polysaccharides (i.e. chitin and glucans) (Bowman and Free, 2006).

All species of fungi can be identified as either filamentous fungi (i.e. moulds) or unicellular fungi (i.e. yeasts). The majority of fungi are filamentous which are multicellular as their morphological structure is composed of filaments (hyphae) consisting of microscopic tubular cells (Walker and

White, 2017). Few species of fungi are classified as yeasts as they grow as single cells. Fungi are omnipresent as they grow in almost all habitats on Earth. They interact and are essential for the survival of some of the many groups of organisms with which they form associations (Walker and White, 2017). Human and other animal bodies can also be sites of fungal colonization which can beneficially or detrimentally influence the overall well-being of the host (Ward *et al.*, 2017). Fungi have a multipurpose potential, where some are edible and are known to contain beneficial dietary minerals, some are used in the food industry for the production of numerous food products and beverages. Some are used in other industries in place of chemical processing to reduce the negative impact on the environment (Friedman, 2015). Scientific research studies also utilize fungi to conduct investigations of many biological phenomena as they are eukaryotic cells and are not costly to perpetuate under laboratory conditions.

The global number of fungal species that is generally accepted among mycologists is within the range of 1 to 5 million. Previously, species determination relied primarily on phenotypic observations whereby many of the previously described species that are known today were confirmed solely based upon morphology. With the introduction of obtaining taxon-specific molecular sequence data, Hawksworth and Lücking (2017) replaced previous estimations of global fungal species richness with an updated and well-accepted range of 2.2 to 3.8 million (Hawksworth and Lücking, 2017). Furthermore, Wu and co-workers (2019) deduced that a statistical ratio of 1:8.8 of the numbers of cultured fungi and the operational taxonomic units detected resulted in an estimate of around 12 million fungal species on earth (Wu *et al*., 2019). However, among the millions of fungal species, only a relative few are able to establish invasive human infection. The small minority of fungi, that can fulfil the four basic conditions necessary to infect humans, are; ability to tolerate high temperatures (> 37°C), successfully invade cells, metabolise tissues and supersede the immune system (Köhler *et al*., 2018).

Fungi successfully infect billions of people causing significant morbidity and mortality globally. Roughly 600 of the millions of existing fungal species are known to cause infections in humans that range from harmless to fatal diseases (Badiee and Hashemizadeh, 2014). Fungal infections can be described either as true pathogenic fungal infections or opportunistic fungal infections. A true pathogenic fungal infection is established in a host with a functional immune system whereas an opportunistic fungal infection manifests within a host with a compromised immunity. However,

the burden of fungal infections is largely undetermined due to limited availability of data required for burden estimates caused by the dearth of routine surveillance. It has been reported that approximately 200 of the 625 fungal species, that are able to infect vertebrates are associated with humans, either as commensal members of the microbiome or as pathogens that cause infectious diseases (Fisher *et al*., 2020). However, invasive fungal infections are rare in healthy individuals, but not impossible, owing to a sophistically evolved innate immune system that effectively responds to and terminates an invading fungus. In contrast, immunocompromised patients who are frequently diseased by fungi include those with a low white blood cell count, recent organ transplant, human immunodeficiency virus (HIV), cancer, corticosteroid dependence as well as new-borns and the elderly. There are several common fungal species that cause life-threatening diseases, these include; *Aspergillus*, *Cryptococcus*, *Pneumocystis*, *Histoplasma*, *Coccidiodes*, *Mucorales*, and *Candida* (Schelenz *et al*., 2015).

2. 2) *Candida* Species

The genus *Candida* belongs to the yeast family *Saccharomycetaceae* in the phylum *Ascomycota*. This genus includes an assortment of over 400 distantly related asexual yeasts, however only a fraction of *Candida* species are able to cause an invasive infection in humans (Lockhart *et al*., 2017). These dimorphic, opportunistic pathogens can grow in yeast form (unicellular) or hyphal form (multicellular). These diseases range from localized mild infections to deep-seated candidiasis. Mucosal membranes within the oral cavity, gastrointestinal tract and vagina are frequent sites of colonization. Incidences of *Candida* infections in patients have significantly inflated since the 1980s, with an increased prevalence in the immunocompromised (Hassan *et al*., 2019). A compromised immune system includes individuals who are; undergoing chemotherapy, taking immunosuppressant medications or broad-spectrum antibiotics, transplant recipients, HIV/acquired immunodeficiency syndrome (AIDS) positive or diabetic. The risk of severe life-threatening disseminated candidemia within these individuals is greater than that with the immunocompetent. Infections caused by the *Candida* species are ranked as the most frequent of hospital-acquired infections caused by fungi (Sabino *et al*., 2020). About 90% of invasive candidiasis in humans is most commonly caused by *Candida albicans, Candida glabrata*, *Candida tropicalis, Candida parapsilosis, Candida krusei* and the recently isolated *Candida auris* (Turner and Butler, 2014). *Candida albicans* maintains being the most frequently isolated human fungal

pathogen (Kim and Sudbery, 2011). The pathogenic repertoire of *Candida* comprises all features needed for a human fungal pathogen. Resistance of *Candida* to the human immune system is limited. Immunologically intact humans manifest robust defences against fungal diseases (Köhler *et al*., 2015).

2.2.1) *Candida albicans*

Candida albicans infections have been recognized since 400 BC, the time of Hippocrates as 'thrush'and this organism has since become a well-studied model organism (Kabir *et al*., 2012). *Candida albicans* is a polymorphic fungus that can grow as chlamydospores, filamentous pseudohyphae or true hyphae and/or spherical yeasts where each morphology has a discrete but flexible cell wall structure with key functions in pathogenicity (Köhler *et al*., 2018). This fungus primarily exists as a commensal in healthy individuals where it naturally and asymptomatically colonizes various sites of the human body like the skin, genital area and/or intestinal mucosa. It also forms part of the normal gut flora that reside in the human mouth and gastrointestinal tract (Hassan *et al*., 2019).

Since 80% of the human population is unaffectedly inhabited with *C. albicans*, carriage of this fungus is not necessarily indicative of an infection or disease (Hassan *et al*., 2019). However, in the dearth of competent immune recognition and response mechanisms, the inability to control *C. albicans* can cause candidiasis due to a transformation from harmless colonization to overgrowth on superficial tissues and invasion into deeper tissue (Köhler *et al*., 2018). This can further lead to disease manifestations such as disseminated candidiasis and chronic mucocutaneous candidiasis (CMC) (Hassan *et al*., 2019). Candidiasis is often observed in immunocompromised individuals where CMC is an infectious phenotype in patients with inherited or acquired T-cell deficiency (Hassan *et al*., 2019). Given that the human gut is a significant basin for *C. albicans*, recent investigations have found that specific populations of bacteria can encumber the overgrowth of this fungus, but administration of antibiotics/immunosuppressants can facilitate the switch of *C. albicans* from commensal to pathogen (Köhler *et al*., 2018).

The *Candida* species has been established as the most ubiquitous contributory agent of hospital-acquired invasive fungal infections with mortality rates associated with *C. albicans* approaching 40% (Fisher *et al*., 2020). The biology, genetics, and pathogenicity mechanisms of *C. albicans* are

the most well-studied of the disease-causing *Candida* species (Köhler *et al*., 2018). Owing to the frequent presence in medical settings, *C. albicans* (SC5314) was the first fungal pathogen elected for whole genome sequencing (Kabir, Hussain, and Ahmad, 2012). Even though *C. albicans* still remains the most common species of *Candida* to cause infections in humans, in current times, the global epidemiology of candidiasis is shifting. Other species of *Candida*, collectively called non-*albicans Candida* species, have significantly increased as emerging etiological agents of systemic diseases (Karkowska-Kuleta *et al*., 2020). In some areas NAC have surpassed *C. albicans* as the principal cause of nosocomial candidemia (Ding *et al*., 2015). Taking into consideration the diverse global allotment of the *Candida* species, local levels rather than worldwide investigations are recommended for the determination of the epidemiology of *Candida* infections (Ding *et al*., 2015). The ever-increasing number of NAC infections has become a serious public health challenge.

2.2.2) Non-*albicans Candida* species

Candida has been classified, by the CDC, as a severe risk to human health due to the sharp increase in drug-resistant infections caused by non-*albicans Candida* species (NAC) (Fisher *et al*., 2020). These species have recently gained clinical significance due to being the cause of nosocomial candidemia.

An overwhelming predominance (> 90%) of *Candida* infections are caused by only four of the numerous *Candida* species (Köhler *et al*., 2018). These are namely *C. albicans*, *C. glabrata, C. parapsilosis* and *C. tropicalis*. These *Candida* species are natural commensals of the microbiota of the skin (*C. parapsilosis*), mucosal surfaces (*C. albicans*, *C. parapsilosis* and *C. glabrata*) and general environment (*C. tropicalis*) (Köhler *et al*., 2018). In contrast to *C. albicans*, some are less susceptible to commercial antifungal drugs probably due to the frequent usage of fluconazole as a treatment and prophylactic in high-risk populations (Pfaller and Diekema, 2007; Lortholary *et al*., 2011). All four main human-associated *Candida* species are able to give rise to life threatening disseminated and systemic infections which are conditional to the degree of host immunocompromisation (Köhler *et al*., 2018). *Candida auris* has become known amongst these uncommon species as a vital and resistant pathogen. The infectious NAC species diversity is escalating as well as their drug resistance creating significant challenges in diagnostic settings, prophylactic approaches as well as in therapeutic management plans (Pfaller and Diekema, 2007).

2.2.2.1) *Candida glabrata*

Candida glabrata is the second most commonly investigated species of *Candida* after *C. albicans*. The mortality due to invasive *C. glabrata* infection surpasses that of *C. albicans* (Johnson *et al*., 2017). Of the 55% of the NAC species that account for diseases, *C. glabrata* is the most prevalent, particularly in Europe and North America (Johnson *et al*., 2017). This fungus has an increased intrinsic resistance to commonly used antifungals therefore posing obstacles in treatment strategies (Johnson *et al*., 2017). *Candida glabrata* and *C. albicans* vary genetically, however, both escape the immune response of the host though immune evasion strategies. An additional mechanism of immune evasion and drug resistance transpires by the production of biofilms, which also occurs on medical devices and mucosal surfaces (Köhler *et al*., 2018). The frequency of colonization with NAC species shifts according to age of the host where *C. glabrata* is more prevalent among older adults (Hameed *et al*., 2018).

2.2.2.2) *Candida parapsilosis*

Depending on underlying conditions, geography and age, *C. parapsilosis* ranks as the third cause of candidemia. It inhabits the gastrointestinal tract of 35 % of healthy individuals and is the most established *Candida* species gathered from fingernail infections (45.3 %), even higher than *C. albicans* (23.7 %) (Taei *et al*., 2019). Like *C. albicans*, *C. parapsilosis* is able to generate persistent biofilms. This accounts for its persistence in clinical settings and may be spread by negligent health care workers. *Candida parapsilosis* is among the most genetically homogenous *Candida* species and isolates from outbreaks can be more virulent than sporadic isolates. The use of highly resolutive typing techniques to differentiate outbreak from non-outbreak isolates is required (Arastehfar *et al*., 2020). The frequency of colonization with NAC species shifts according to age of the host where *C. parapsilosis* is more prevalent among children (Hameed *et al*., 2018).

2.2.2.3) *Candida tropicalis*

Candida tropicalis forms part of the naturally occurring microbiota in human skin, genitourinary, respiratory and gastrointestinal tracts (Zuza-Alves *et al.*, 2017). It arises as one of the predominant NAC species that cause invasive candidiasis and is one of the three most commonly isolated NAC species in Asia and South America (Paul *et al*., 2020; Silva *et al*., 2012; Taei *et al*., 2019). *Candida tropicalis* is often identified in patients with, neutropenia, cancer, catheters and who are admitted

to intensive care units (ICUs) and receiving broad-spectrum antibiotics (Silva *et al*., 2012). *C. tropicalis* has the highest genetic similarity to *C. albicans* when compared with other NAC species. However, it has a higher potential for dissemination and is associated with higher mortality which could be related to its virulence factors and a greater resistance to commonly used antifungals (Silva *et al*., 2012).

2.2.2.4) Other non-*albicans Candida* species

There are other NAC species which are less frequently, and even rarely, associated with human disease but still remain clinically significant (Moran *et al*., 2012). *Candida krusei, C. dubliniensis, C. guilliermondii, C. lusitaniae, C. rugosa, C. orthopsilosis* and *C. metapsilosis* are infrequently encountered NAC species. Some of these species are variably resistant to antifungal drugs, where most of them are susceptible and have the potential to develop resistance. They are responsible for < 1 - 5 % of cases of invasive candidiasis and mostly infect patients with HIV/AIDS or cancer (Turner and Butler, 2014; Moran *et al*., 2012).

The etiology/epidemiology of the diseases of the remaining NAC species associated with the cause of human disease is not well investigated as they are only rarely detected. These species include *C. famata, C. inconspicua, C. kefyr, C. lipolytica, C. norvegensis, C. sake, C. zeylanoides, C. bracarensis* and *C. nivariensis.* Clinical isolates of some of these species are resistant to various antifungal drugs; regardless of being rare, they have the potential to emerge as more significant pathogens like *C. auris* (Moran *et al*., 2012).

2. 3) ***Candida auris***

Candida auris is a frequently encountered fungal pathogen that has been found to cause severe invasive infections, with an approximate mortality ranging from 30% to 72% (Lone and Ahmad, 2019). It was initially isolated from the external ear canal (*auris*, Latin for “ear”) of a Japanese patient in 2009 and defined as a fungal pathogen that causes candidiasis in humans (Satoh *et al*., 2009). Within a few years of its identification, *C. auris* gained rigorous popularity due to its antifungal drug resistance and persistent ability to cause hospital outbreaks by tenaciously spreading in healthcare settings (Jeffery-Smith *et al*., 2017).

2.3.1) Clinical significance

Most *Candida* infections, including a *C. auris* infection, have similar clinical presentations. *C. auris* can be isolated from multiple body sites with linked clinical conditions (Lone and Ahmad, 2019). Clinical strains of *C. auris* have been reported to cause infections in several internal and external areas of the body, to name a few; the bloodstream (candidemia), vulva/vagina (vulvovaginitis), middle ear (otitis media), intra-abdominal candidiasis (IAC), bone (osteomyelitis), pericardium (pericarditis), ventricles in the brain (ventriculitis), and non-invasive sites like the respiratory tract, skin and urine. Colonization by *C. auris* persists for one to three months after initial infection, and it can survive for 14 days on plastic surfaces as well as for up to one week on steel surfaces and contaminated bedding (Lone and Ahmad, 2019).

In 2016, both the CDC and the European Centre for Disease Prevention and Control (ECDC) issued a clinical alert to the healthcare facilities in the United States and Europe regarding the worldwide emergence of *C. auris* (CDC, 2016; ECDC, 2018). After the hospital outbreaks of *C. auris*, the ECDC issued a rapid risk assessment of *C. auris* with the aim to prevent further hospital outbreaks, raise awareness of *C. auris* in European healthcare facilities, adapt laboratory testing strategies for adequate identification and establish enhanced control measures to prevent further outbreaks (ECDC, 2018). The main concerns for the CDC were the multi-drug resistance of this pathogen to commonly administered antifungal drugs, the ineffectiveness of conventional biochemical methods to accurately identify this pathogen and finally, the 70% mortality rate owing to healthcare-associated outbreaks and invasive infections (CDC, 2016; Horton *et al*., 2020).

2.3.2) Epidemiology

The first official identification of *C. auris* was in 2009 but the earliest known strain of *C. auris* was identified by retrospective testing of a sample from a *Candida* strain collection of unidentified yeasts from 1996 in South Korea (Satoh *et al*., 2009; Lee *et al*., 2011). Due to the phenotypical similarity with other *Candida* species, *C. auris* is commonly misidentified by commercially available diagnostic methods. This regular misidentification blurs the exact global prevalence of this organism therefore underestimating the true number of *C. auris* cases (Lone and Ahmad, 2019).

The first case of *C. auris* that caused candidemia was reported in 2011, in South Korea (Lee *et al*., 2011). Subsequently, from 2013 – 2018, reports of *C. auris* infections arose from across the globe from countries including South Korea, India, Pakistan, Bangladesh, Israel, Kuwait, Oman, Malaysia, China, United Arab Emirates, Saudi Arabia, Iran, Singapore, Thailand, South Africa, Kenya, Spain, Germany, France, Austria, Norway, Belgium, the United Kingdom, Switzerland, Netherlands, Russia, the United States, Canada, Panama, Colombia, Venezuela and Australia (Lone and Ahmad, 2019). Once again, owing to misidentification, *C. auris* may have emerged in other countries but has remained concealed due to incompetent commercially available identification systems as well as its phenotypical resemblance with *C. haemulonii, C. famata, C. sake* and *Saccharomyces cerevisiae* (Lone and Ahmad, 2019).

2.3.3) Phenotypic characteristics of *Candida auris*

The phenotypic characteristics of *C. auris* are similar to that of other *Candida* species therefore making it relatively difficult to identify. Furthermore, *C. auris* phenotypes vary by strain and origin. On Sabouraud Dextrose Agar (SDA), colonies appear smooth and creamy in colour whereas on chromogenic agar (CHROMagar), which is commonly used to differentiate *Candida* species, colonies appear as smooth, pale purple to pink (Kathuria *et al*., 2015; Lone and Ahmad, 2019). However, some *C. auris* strains show no characteristic colour on CHROMagar (Kumar *et al*., 2015). Regardless of *C. auris* being germ-tube negative, on cornmeal agar, some strains of *C. auris* form rudimentary pseudohyphae (Borman *et al*., 2016; Sherry *et al*., 2017).

When viewed under a microscope, cells are either single and/or in groups and appear ovoid, ellipsoidal to elongated (Kathuria *et al*., 2015; Lone and Ahmad, 2019). Borman and co-workers (2016) have observed that *C. auris* isolates present themselves in two forms, aggregate and non-aggregate (Borman *et al*., 2016). They reported that vigorous vortexing did not disrupt aggregate isolates and non-aggregating isolates were more pathogenic than the aggregating isolates. This pathogenicity did not correlate with the degree of virulence in clinical cases (Borman *et al*., 2016; Sherry *et al*., 2017)

Thermoresistance seems to be the most characteristic feature of *C. auris* whereby it can grow in temperatures ranging from 37 to 42 °C, unlike other *Candida* species that cannot survive at temperatures above 37 °C (Osei Sekyere, 2018). This thermtolerance contributes to the

understanding of the persistent survival rate in the host and the environment and could potentially be utilized to differentiate it from other *Candida* species (Chowdhary *et al*., 2014; Satoh *et al*., 2009)

2.3.4) Genomic characteristics of *Candida auris*

Five genetically diverse clades of *C. auris* have been discovered thus far originating from different locations around the globe namely: Clade I - South Asian (India and Pakistan); Clade II - East Asian (Japan); Clade III – African (South Africa); Clade IV - South American (Venezuela) and finally Clade V (Iran) (Chybowska *et al*.,). The first isolates to have their genomes assembled belong to clade I (Rhodes *et al*., 2019; Chatterjee *et al*., 2015). *C. auris* has 7 chromosomes, a genome size ranging between 12.1 – 12.7 Mb where predicted protein coding genes range from 5 288 to 5 601 (Muñoz *et al*., 2018; Chybowska *et al*., 2020). Like in *C. haemulonii* and *C. lusitaniae*, phylogenetically conserved mating type loci (MTL) are present in the genome of certain *C. auris* isolates, namely MTLa isolates (a1 and a2 genes) and MTLα isolates (single α1 gene) (Muñoz *et al*., 2018). Despite a lack of clear recombinants between or within clades, the presence of a conserved mating type suggests that *C. auris* can mate (Chybowska *et al*., 2020). Clades I and IV have so far all been identified as MTLa, whilst clades II and III have all been found to be MTLα. The mating type of Clade V has not yet been reported (Chybowska *et al*., 2020).

Gene function might be impacted by the genomic variation between and within clades of *C. auris*. The changes observed in the genes of *C. auris* and its closest relatives showed linkage to drug resistance and virulence as well as expanded families of transporters and lipases (Muñoz *et al*., 2018). However, the positive selection between clades as well as various features of population genetics within clades has yet to be elucidated (Chybowska *et al*., 2020).

The allelic variation within the *C. auris* species remains unclear, regardless of the rapid clinical emergence of *C. auris* and the newly identified Clade V in 2018 (Chybowska *et al*., 2020). The necessity to further sample *C. auris* is still very apparent, in terms of screening potentially mischaracterized historic samples and new sampling efforts in both clinical and environmental settings (Chybowska *et al*., 2020).

2.3.5) *Candida auris* in South Africa

The African continent is well-known for its heavy burden of infectious diseases. Owing to the tropical climate and global warming that facilitates the spread of diseases, the amount of immunocompromised individuals is increasing. The first incidences of *C..auris* caused candidemia in South Africa were initially misidentified as *C. haemulonii* in 2009 and five years later was confirmed as *C. auris* at the National Institute for Communicable Diseases (NICD), Johannesburg (Lone and Ahmad, 2019). Four cases of *C. auris* candidemia were detected in 2012-2013 and 10 more isolates were reported in 2017 (Magobo *et al*., 2014; Lone and Ahmad, 2019). During 2012-2016, more than 1 000 confirmed cases of *C. auris* were identified in both public and private hospitals countrywide. Infectious *C. auris* outbreaks in 2016 resulted in *C. auris* being reported as the second and fourth most common candidemia causing *Candida* species in the private and public sector hospitals, respectively, with the majority of cases identified in the Gauteng province (NICD, 2016; Lone and Ahmad, 2019). In 2017, the first neonate was reported to have *C. auris* candemia (NICD, 2017). South African patients of all ages can be subjected to infection. *Candida auris* now accounts for 10 % of cases of candidemia and has been isolated from more than 94 hospitals across the country (Lone and Ahmad, 2019).

2. 4) Laboratory methods to identify *Candida auris*

Molecular methods have drastically improved over the years allowing for broadened knowledge of fungi and accurate identification of fungal species (Blackwell, 2011). The formally described species known today were identified based on morphology as mycologists used to largely rely on the phenotypic characteristics for species determination (Blackwell, 2011). Currently, high-throughput methods are being used by mycologists for rapid and accurate identification (Blackwell, 2011). *Candida auris* has a solid history of being misidentified as other pathogenic *Candida* species. However, with the use of polymerase chain reaction (PCR) and mass spectrometry, *C. auris* identification is more accurate when compared to biochemical reactions (Iguchi *et al*., 2019). It is of vital importance to correctly identify a *C. auris* infection in order to prescribe the best treatment option. Fast and precise diagnosis of *C. auris* could aid in the prevention of the progression of severe infections as well as potentially lessen the associated morbidity and mortality rates.

There is a vast variety of fungal identification methods used by clinical and public health laboratories all over the world to detect *C. auris*. However, many of them use systems (e.g., VITEK 2, API 20C) for which misidentification with other yeasts has been reported. More reliable identification methods include ribosomal deoxyribonucleic acid (rDNA) sequencing, *C. auris*-specific PCR/quantitative PCR (qPCR), or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Kordalewska and Perlin, 2019).

2.4.1) Fungal culturing

Candida auris is characterized as having white to cream colonies with a smooth edge on SDA and light blue colonies with a blue halo on CHROMagar *Candida* medium (Bayona *et al.,* 2020). Therefore, identification by standard laboratory techniques is insufficient and requires further investigation to determine the species and antifungal susceptibility of the isolate.

2.4.2) Phenotypic

Phenotype switching in *C. auris* isolates has been identified where alternate colours: pink, white, and sectored (dark purple) of smooth and glossy colonies have been observed which are similar to other yeast, commonly *C. parapsilosis, C. haemulonii* and *C. duobushaemulonii* (Kordalewska and Perlin, 2019). Phenotypic identification of *C. auris* is by no means reliable and the results thereof cannot be considered as the final identification of *C. auris* (Kordalewska and Perlin, 2019).

2.4.3) Biochemical

The frequent misidentification of *C. auris* is persistent even in conventional diagnostic laboratories that utilize commercial biochemical identification systems for yeast identification namely VITEK® 2 Yeast identification card (VITEK 2 YST), analytical profile index yeast (API 20C), MicroScan and BD Phoenix yeast identification system (Kordalewska and Perlin, 2019). The lapse in identification by these techniques could be accounted for by a lack of *C. auris* references in their databases. Generally, these methods require visual observations obtained from the reaction between certain dehydrated substrates and the unknown yeast. The observations are then captured into a database (either automatically or manually) and the software identifies the organism based on the observed reactions. The need for careful attention is required when using these systems as all these databases have already misidentified *C. auris* as other *Candida* species due to a database

that is not up to date. Common species that *C. auris* has been misidentified as using these systems include *C. haemulonii*, *C. duobushaemulonii*, *C. famata, C. glabrata, C. sake, R. glutinis, S. cerevisiae, C. parapsilosis, C. catenulate, C. albicans, C. tropicalis, C..lusitaniae, C. guilliermondii* and *R. rubra* (Kordalewska and Perlin, 2019).

2.4.4) MALDI-TOF MS

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, better known as MALDI-TOF MS, is used for fast and precise identification of microorganisms. A uniquely characteristic mass spectrum is generated for each microorganism which is used as their fingerprint. In general, all microbial 'fingerprints' are collated into a database whereby the spectra acquired for tested samples are compared with a known spectrum within the database. The result returned is solely reliant on the presence of the sample organism spectrum in the database. However, databases that have been updated with *C. auris* and related species fingerprints render accurate identification of *C. auris* to the species level when using MALDI-TOF MS (Kordalewska and Perlin, 2019).

In comparison to conventional biochemical and molecular identification systems, MALDI-TOF MS has shown to present a noteworthy gain of both working time (sample preparation) and turnaround time (automated results returned) (Kordalewska and Perlin, 2019). With a database containing a comprehensive collection of accurately identified reference, MALDI-TOF MS provides high-throughput, accurate and rapid microbial identification at low costs if the instrument is already purchased.

2.4.5) Molecular Methods

The diagnosis and specific identification of microbes that cause infectious diseases has been significantly improved as a result of the use of specialized molecular techniques. Presently, precise identification of *C. auris* can be confirmed by MALDI-TOF MS or better yet, with the use of molecular methods like genetic sequencing and PCR. With proper validation, real time/qPCR assays possess unmatched potential for being the most efficient tool for screening of *C. auris* within several hours (Kordalewska and Perlin, 2019).

2.4.5.1) Sequencing of Genetic Loci

Accurate identification of *C. auris* and differentiation from other yeast is acquired by genetic sequencing of certain sections of the rDNA of the large subunit namely the internal transcribed spacer and D1/D2 region. However, owing to the overall cost, practical burden and prolonged turnaround times for those without the facility to perform in-house sequencing, this technique is incongruous for some diagnostic laboratories and therefore not routinely used to establish isolate identity (Kordalewska and Perlin, 2019).

2.4.5.2) Nucleic Acid-Based Identification Strategies

The delay generated by the requirement of culture for biochemical automated systems and MALDI-TOF MS has dramatically limited the opportunity to quickly respond to outbreaks. However, with the development of a variety of molecular-based assays, the ability to provide rapid results within several hours from the clinical sample delivery is present. Most of the developed assays (i.e. various PCR and more complex T2 magnetic resonance or Loop-mediated Isothermal Amplification (LAMP) assays) are extremely sensitive and specific with a detection limit of 1 – 10 *C. auris* colony forming units (CFU) per PCR reaction (Kordalewska and Perlin, 2019).

Unfortunately, depending on the diagnostic laboratory infrastructure, nucleic acid-based identification strategies may pose several technical (equipment and sample preparation) and cost (reagents and equipment) limitations. Furthermore, detection of DNA from both live and dead *C. auris* cells could either be considered as a benefit or a detriment, depending on the aim of the particular study. However, this should not be considered as a false positive result because it can serve as an indicator of *C. auris* pre-colonization (Kordalewska and Perlin, 2019).

2. 5) Antifungal therapy of *Candida* infections

In contrast to antibacterial agents, efficient antifungal drugs are difficult to develop owing to the phylogenetic origin and cellular similarity of fungal and human cells (Steinberg and Perez-Martin, 2008). It is therefore incredibly challenging to select pathogen-specific drug targets that will not cause harm to the host (Tsui *et al*., 2016). The pace of antifungal drug development is a leading concern since the most recently introduced antifungal drug class, echinocandins, took 30 years to enter clinical practices. The commonly administered commercial antifungal drugs either target substrates within the fungal cell wall and/or membrane, namely ergosterol or 1,3-β-glucan

(Pianalto and Alspaugh, 2016). Regardless of the type of *Candida* infection (superficial or invasive), three different classes of antifungal drugs commonly control and treat these infections. These classes are the polyenes, azoles and echinocandins. Although effective in their appropriate contexts, they differ in their bioavailability, spectrum of antifungal activity, side effects and are limitedly used for clinical purposes due to their high toxicity, narrow drug spectrum, etc. Other less common therapeutic agents for the treatment of candidiasis include pyrimidine analogs, allylamines, flucytosine and griseofulvin (Chandrasekar, 2010; Gulati and Nobile, 2016). A major cause of treatment failures is owed to the emergence of drug resistant *Candida* species.

2.5.1) **Polyenes**

Polyenes are the earliest developed family of antifungal drugs (Carolus *et al.,* 2020). They are cyclic amphiphilic organic molecules synthesized from *Streptomyces* bacteria. Of the more than 200 chemical molecules belonging to this class, only amphotericin B and nystatin are used for clinical purposes (Vandeputte *et al*., 2011). Polyenes are fungicidal, the fungal cell membrane component, ergosterol, is a shared target.

Polyenes create pores within the lipid bilayer of the fungal cell by binding to ergosterol within the membrane. The outcome is altered membrane permeability, metabolic disturbance, leakage of intracellular electrolytes and other cytoplasmic contents and, as a result, cell death (Mathew and Nath, 2009). The biosynthesis of ergosterol has become a popular target in the development of antifungal drugs owing to its major role in the persistence of yeasts by maintaining the structure, integrity and function of the yeast cell membrane (Dupont *et al*., 2012). Additionally, an accumulation of toxic reactive oxygen species due to polyenes can also cause cellular death (Mesa-Arango *et al*., 2014).

The earliest developed antifungal polyene is nystatin which has a broader antifungal activity spectrum than amphotericin B, but is limited to topical applications owing to its high toxicity. Therefore, the gold standard for the treatment of severe systemic fungal infections is amphotericin B which is administered intravenously. Amphotericin B exhibits the broadest spectrum of antifungal activity but its clinical use is severely hampered due to side effects associated with nephrotoxicity (Ellis, 2002). Resistance to this class of antifungals is rare even in within the

Candida genus where some are either intrinsically resistant or require higher doses (Spampinato and Leonardi, 2013; Bondaryk *et al*., 2013).

Novel amphotericin B formulations have revealed a lower occurrence of nephrotoxicity, improved efficacy and an improved safety profile when compared to its conventional form (Sanglard and Odds, 2002; Moen *et al*., 2009). These new formulations are lipid and/or liposomal based but are costly and not readily available in some regions (Hamill, 2013). Triazoles are less toxic than polyenes and have replaced amphotericin B in treatment of certain forms of systemic fungal infections. However, amphotericin B remains at the forefront of possessing the broadest antifungal activity spectrum (Ashley, 2006).

2.5.2) **Azoles**

Azoles are synthetic compounds and are the most important class of antifungal drugs. They have been grouped based on their five-membered azole ring, containing either two or three nitrogen atoms. These groups are the imidazoles (clotrimazole, miconazole, and ketoconazole) and the triazoles (fluconazole, itraconazole and voriconazole) (Maertens, 2004; Vandeputte *et al*., 2011). For systemic and superficial fungal infections, the triazoles and ketoconazole are appropriate (Mathew and Nath, 2009).

Briefly, azoles directly bind to their target which is an enzyme dependent called lanosterol 14-α-demethylase. This impedes the-conversion of-lanosterol to-ergosterol, a vital component-of the fungal cell membrane, and causes an accumulation of 14-α-methylated sterol-precursors. The diminution of-ergosterol and escalation of 14-α-methylated effectively alters normal fungal functions due to modified membrane fluidity which increases permeability (Prasad *et al*., 2016). The ability to proliferate as well as the activity of several other membrane-associated-enzymes are also negatively affected (Nigam, 2015).

The most extensively clinically administered azole is fluconazole (Arastehfar *et al.,* 2020). It possesses a high bioavailability, satisfactory pharmacokinetic properties, fitting tolerance, very mild side effects and displays proper absorption through the gastrointestinal tract as well as the cerebrospinal barrier (Poopedi *et al.,* 2020). Fluconazole can be administered either orally or intravenously; however, interactions with anticonvulsant and anticoagulant drugs have been reported.

It has been used as a first line therapy for the treatment of superficial, cutaneous and disseminated candidiasis as well as a prophylactic agent for high-risk patients (Spampinato and Leonardi, 2013; Whaley *et al*., 2017). Consequently, within the last few decades, drug resistance to azoles has been severely augmented owing to their frequent use. Mutations within the gene encoding lanosterol (*ERG11*), a greater expression of efflux pumps, as well as the formation of biofilms has led to the establishment of azole resistance in clinical *Candida* isolates (Srivastava *et al*., 2020).

2.5.3) Echinocandins

Echinocandins are a relatively recent addition to the cache of antifungals that safely and effectively combat invasive fungal infections (Denning, 2002; Roemer and Krysan, 2014). They are naturally synthesized semisynthetic lipopeptide compounds from several fungal species and are a suggested alternative to fluconazole and amphotericin B (Arendrup and Perlin, 2014). Caspofungin, micafungin and anidulafungin are drugs of this class licensed for clinical use. They inhibit the synthesis of 1,3-β-glucan synthase which impairs the production of a critical fungal cell wall component required to maintain integrity, 1,3-β-glucan (Bondaryk *et al*., 2013; Pierce and Lopez-Ribot, 2013). A deficiency of 1,3-β-glucan in the fungal cell wall causes the induction of osmotic instability and eventual cell death (Spampinato and Leonardi, 2013).

Caspofungin and micafungin have similar efficacy. Both have less adverse side-effects than amphotericin B when used for the treatment of invasive candidiasis (Villanueva *et al*., 2002; Wiederhold *et al*., 2008). Resistance to echinocandin drugs remains relatively uncommon within the *Candida* species (with the exception of *C. glabrata*) despite prolonged exposure and increasing usage (Perlin, 2015). Resistance is owed to point mutations in 1,3-β-glucan synthase subunits Rho1p and FKsp which is associated with advanced minimum inhibitory concentration values that regularly lead to failures in clinical treatment (Beyda *et al*., 2012).

Considering the nephrotoxicity of amphotericin B and fluconazole resistant strains of *Candida*, echinocandins have been put forward as the first-line therapy for invasive candidiasis. They are appealing substitutes in preference to other available antifungals owing to their confirmed efficacy, tolerance and inconsequential effects and drug interaction. Their limited use is mainly due to elevated cost of the drug (Bruynesteyn *et al*., 2007).

2. 6) Antifungal drug resistance

Antimicrobial drug resistance is an inevitable evolutionary process (Cort *et al*., 2016). The resistance to antifungal drugs, regardless of the variety of antifungal drug classes, is responsible for/has lead to increasing death rates and therefore cannot be ignored (Srinivasan *et al*., 2014). This resistance could be owed to the fact that the majority of antifungal drugs utilized clinically have been administered for several decades which prompts fungi into developing resistance to overcome intentionally lethal effects (Sanglard *et al*., 2009; Srinivasan *et al*., 2014).

Traditionally, the resistance to antifungals has been grouped into three types; firstly, intrinsic resistance, whereby isolates display resistance prior to drug exposure; secondly, acquired resistance whereby resistance is developed in initially susceptible isolates after drug exposure; and finally, clinical resistance whereby patients respond poorly to clinical treatment but *in vitro* the fungi are susceptible (Kontoyiannis and Lewis, 2002). There is a constant rising necessity for the development of novel antifungal therapeutics to impede drug resistance with minimal side effects (Parente-Rocha *et al*., 2017).

2.6.1) Resistance within the *Candida* species

Antifungal drug resistance can have fatal consequences for immunodeficient individuals rendering it as a severe clinical issue especially with candidiasis. Resistant *Candida* species have been associated with failure of therapeutic interventions which inevitably causes increased rates of morbidity and mortality specifically amongst those infected with invasive candidiasis (White *et al*., 1997)

The resistant *Candida* species display acquired and intrinsic resistance (Pfaller, 2012). The striking increase of infectious NAC species (*C. krusei* and *C. glabrata*) can be traced to the universal use of fluconazole in both prophylactic and therapeutic settings. These species are intrinsically resistant to fluconazole, unlike *C. albicans* which normally displays susceptibility.

Of the multiple mechanisms of fluconazole resistance, the most prominent mechanism is the active efflux of the antifungal from cells via efflux pumps (Pemán *et al*., 2009). These membrane bound pumps usually supply a mode of transport to export lipids and toxic compounds out of the cell. Through the development of drug resistance, they effectively reduce the intracellular burden of the

drug (Sanglard and Odds, 2002). Overexpression of the genes that encode these efflux pumps have been associated with intrinsic antifungal resistance to other azoles and this phenomenon has also been observed in *C. auris* (Grossman *et al*., 2015; Ben-Ami *et al*., 2017).

Unlike the thoroughly investigated mechanisms of azole resistance in resistant *Candida* species, the mechanisms of polyene and echinocandins resistance is under investigated. This neglect could be owed to the knowledge that the exceptional resistance to these two classes is mostly limited to infrequent species of *Candida*, such as *C. glabrata, C.krusei, C. lusitaniae* and recently, *C. auris*. These strains however, are emerging more frequently which further advocates the dire need for the development of novel antifungal drugs or, if nothing else, new approaches (like effective combination therapy) to control fungal resistance.

2. 7) Virulence markers as potential antifungal therapeutic drug targets

Mechanisms dedicated to colonise and cause infection have been developed within all opportunistic pathogens but resistance to conventional antifungal drugs, which inhibit proliferation or simply terminate fungi, is driven by an elevated extent of selective pressure on the fungus (Casadevall *et al*., 2002).

Therapeutics targeting the virulence factors/markers of pathogens poses a tempting approach in/for the development of novel antimicrobial drugs. In order to overcome antibiotic resistance, targeting virulence factors of bacterial cells have advanced the development of novel classes of antibacterial drugs (Clatworthy *et al*., 2007). That being said, a viable option would be to shift the focus from the current antifungal targets which are mostly external (i.e. within the fungal cell wall/membrane) and ineffective. Establishing internal targets pertaining to virulence in fungal pathogens can be promising in the development of a novel antifungal drug (Gauwerky *et al*., 2009). This stance would intend to effectively target specific virulence attributes which normally support the establishment of an infection. Total comprehension and identification of virulence factors is crucial for the development of antifungal drugs that target enzymes contributing to virulence in different *Candida* species.

A completely new class of antifungal drugs, with new mechanisms of action, could arise with increasing number of potential targets. By targeting virulence factors, fungal cells will be deprived of their virulence and extinguished with less selective pressure that could lead to resistance.

Familiarity of the virulence factors, in multi-drug resistant *C. auris*, will aid in the investigation of new targets and enhanced therapeutic regimens. Targeting pathogenicity markers or virulence factors have rewards which include the preservation of the host's natural microbiota (Pierce and Lopez-Ribot, 2013).

2.7.1) Virulence in *Candida* species

Virulence factors encompass enzymes within a pathogen that allow for the ability and activity to establish an infection within a host. The ability of *C. albicans* and other opportunistic *Candida* species to cause infection is attributed to their diverse virulence features (Casadevall and Pirofski, 2001). The mechanism of pathogenesis observed in *Candida* species to facilitate the establishment of an infection is a complex process. The fungal cells undergo multiple morphological and biochemical changes which foster the invasion of host tissues. The strength of the host's immunity plays a significant role in the process of infection. Incursion through the mucosal epithelia subsequently causing infection of the blood stream and internal organs (i.e. candidemia) may be initially elicited by a disproportion of the host's immunity and the invading opportunistic fungi (Larriba *et al*., 2000).

Candida albicans is an extremely well-studied organism by which the expression of multiple virulence factors that promote phylogeneticity have been detected. Disappointingly, there is a lack of significant elucidation of the virulence factors contributing to the pathogenicity of *C. auris* and other NAC species, where antifungal resistance is apparent (Shaban *et al*., 2020). However, molecular investigations have established that virulence genes of *C. albicans* and *C. glabrata* are similar to that of *C. auris* (Munoz *et al*., 2020; Sharma *et al*., 2016). Virulence features of pathogenic *Candida* are species specific but generally include adherence to host cells, morphogenesis, secretion of hydrolytic enzymes and production of biofilms (Ahmad *et al*., 2016). Virulence factors broaden the number of particular drug targets in fungi and pose an optimistic antifungal strategy. However, thorough comprehension of these factors is critical for the development of a viable antifungal drug.

2.7.2) Morphogenesis

Micro-organisms commonly utilize morphogenesis in order to swiftly adapt to environmental changes. Under particular environmental conditions, both bacterial and fungal species can

experience morphological transitions. Morphological transformations in *Candida* are displayed by the ability to transition from unicellular yeast cells to filamentous growth forms (true hyphae and pseudohyphae) (i.e. the yeast–hyphal transition) as well as the white-opaque switch (Du *et al*., 2020). Both forms, yeast and hyphal, differentially contribute to the virulence of *Candida* as well as disease development. Briefly, the yeast form provides adhesion and dissemination of the fungus whereas the hyphal form is the more invasive virulent form owing to being physically larger in size which causes elimination difficulty to the macrophages (Odds, 1988). These transformations are well-characterized and evoked either spontaneously or by environmental changes in pH, temperature, carbon dioxide (CO_2) concentrations, etc. The transduction of these changes is facilitated by different signalling pathways of *Candida* cells which result in the activation of a certain set of hyphal forming genes, namely; hyphal wall protein (HWP1), agglutinin-like sequence protein (ALS3), hypha associated proteins (ECE1 and HYR1) and the secreted aspartic proteases (SAP4, SAP5 and SAP6) (Yang *et al*., 2018).

Morphogenisis in certain *Candida species* is induced by multiple signaling pathways, a major one being the cyclic adenosine monophosphate protein kinase A (cAMP-PKA pathway). Exernal stimuli (i.e. CO_2 and temperature) activate the hypha-specific G1 cyclin-related protein Hgc1 by acting on adenylate cyclase Cyr1. Thereafter, Hgc1- cyclindependent kinase (CDK) complexes are formed which endorse growth and hyphal formation (Ahmad *et al*., 2016). However, only certain *Candida* species (*C. albicans* and *C. dubliniensis*) are able to form both true and pseudohyphae whereas a vast diversity of pathogenic *Candida* species (*C. parapsilosis*, *C. tropicalis* and *C. haemulonii*) thrive in their pseudohyphal form (Calderone and Fonzi, 2001; Silva *et al*., 2011).

Candida auris, like other pathogenic *Candida* species, has numerous morphological phenotypes. However, the function and regulatory mechanisms of each morphology is significantly uninvestigated. Multiple *C. auris* isolates are present in the single-cell, yeast form but there are some naturally occurring isolates that form large aggregates of pseudohyphal-like cells whereby the attachment of mother and daughter cells persists. This is probably due to a cell division defect supported by a study in which induction of DNA damage was demonstrated to induce pseudohyphal-like formation in *C. auris* (Du *et al*., 2020). Other potentially strain specific or media-dependant *C. auris* strains were shown to form rudimentary pseudohyphae (Borman *et al*., 2016; Sherry *et al*., 2017).

Interestingly, *C. auris* alters its morphology when treated with 10 % sodium chloride (NaCl) or after passage through animal models, like *C. albicans* that morphs into is hyphal form in the presence of serum *in vitro* (Du *et al.*, 2020). Owing to the disparity in morphological characteristics of filamentous growth forms, the product of morphogenesis of *C. auris* cells are described as filamentous growth or pseudohyphal-like form (Wang *et al*., 2018; Yue *et al*., 2018).

2.7.3) Adhesion molecules

Colonisation and infection establishment by a pathogen occurs in two steps, adherence to a host surface and invasion into the host cell. Adhesion is well investigated in *C. albicans* and other NAC species and has been discovered to play a critical part of the process of infection (Haynes, 2001). *Candida* species have been identified to adhere to and thrive in many biological cells and tissues as well as on inanimate surfaces where biofilm formation is prompted by adherence. The capability to adhere to a number of surfaces results in the assumption that adhesion is related to virulence and drug resistance (Hawser *et al*., 1994). Adhesins are a particular set of cell wall proteins produced by *C. albicans* which manage adherence. Well-investigated adhesins are the eight agglutinin-like sequence (ALS) proteins (ALS1 – 7 and ALS9) (Murciano *et al*., 2012).

After successful adhesion to a host cell, invasion into the cell is induced through either; endocytosis or active penetration. Endocytosis is mediated by the expression of invasions. These are pathogen cell wall proteins namely Ssa1 (a member of Hsp70) and the dual functional ALS3. Active penetration is promoted by the secretion of hydrolases, particularly SAPs (Ahmad *et al*., 2016).

This virulence factor has not been examined in *C. auris*, but adherence to catheter materials as well as the expression of genes encoding for adhesins has been reported (Larkin *et al*., 2017; Chatterjee *et al*., 2015).

2.7.4) Hydrolytic enzymes

The production of a variety of hydrolytic enzymes allows for the adaptation of *C. albicans* to varying internal niches as well as to mediate multiple pathogenic characteristics (Khan *et al*., 2010). Additionally, the primary function of these enzymes is to metabolise the host tissue into nutrients in order to improve *Candida* growth and survival (Naglik *et al*., 2003).

The best studied of these enzymes, owing to their function in fungal pathogenicity, are SAPs, phospholipases and lipases (Sachin *et al*., 2012; Mayer *et al*., 2013; Pawar *et al*., 2014). Other hydrolytic enzymes which are also involved in the pathogenicity of *C. albicans* include haemolysin, coagulase, chondroitinase and hyaluronidase (Tsang *et al*., 2007).

In general, these enzymes facilitate pathogenetic characteristics like adherence, invasion, host immunity interference, and penetration which can result in tissue damage, iron acquisition, and dissemination within the host. Furthermore, they allow the pathogen to overcome the host's immunity as well as rendering the immune system ineffective (Staniszewska *et al*., 2012; Tsai *et al*., 2013). Additionally, hyphal invasion in hematogenously disseminated candidiasis (HDC) is mediated by iron acquisition facilitated by the production of hemolysin by *C. albicans* (Sachin *et al*., 2012). Mutant *C. albicans* strains that have displayed impaired pathogenicity owing to the loss-of-function/knock-out of particular genes that would normally encode hydrolytic enzymes are easily subjected to phagocytosis (Aoki *et al*., 2011). Recently, *C. auris* has been confirmed to express numerous known virulence factors similar to *C. albicans*. These include SAPs and lipases in order to disrupt and assail host tissues (Du *et al*., 2020).

2.7.4.1) Secretion of aspartyl proteinases

Secreted aspartyl proteinases (SAPs) are proteolytic enzymes present in numerous fungal pathogens. They play a vital role in the host invasion process of pathogenicity. The SAP isoenzymes interfere with the host innate immune system by hydrolyzing critical immunological and structural defence proteins such as,secretory immunoglobulin A (IgA), complement component 3, albumin, haemoglobin and others (Silva *et al*., 2011; Aoki *et al*., 2011; Pawar *et al*., 2014). Furthermore, they disrupt membrane proteins (i.e. collagen, keratin and mucin) of epithelial and mucosal cells. This disruption aids in adherence subsequently causing tissue damage but also evasion of attack by antimicrobials (Deepa *et al*., 2015).

The SAPs are a family of ten different aspartic proteinase members (SAP1-SAP10) that are justifiably encoded by ten different aspartic proteinase genes (*SAP1-10*) (Feng *et al*., 2015; Dutton *et al*., 2016). Although the *SAP* genes encode enzymes with similar functionalities and characteristics, each possess uniquely distinctive molecular properties like molecular weight, pH and isoelectric point (Karkowska-Kuleta *et al*., 2009; Pawar *et al*., 2014). Differential regulation

of these genes is dependent on the morphology, surrounding environment and phase/type of infection.

Increased expression of SAP1-6 has been recognized in the yeast and hyphal phases, respectively. The SAP9 and SAP10 expression is produced by both yeast and hyphae forms as it encodes glycosylphosphatidylinositol (GPI) anchoring domains. This expression is indicative of SAPs association with the fungal cell wall integrity as these domains remain cell-surface bound, serving to maintain and preserve the membrane integrity (Khan *et al*., 2010; Aoki *et al*., 2011; Staniszewska *et al*., 2012). The functional roles of SAP7 and SAP8 are not yet sufficiently elucidated and therefore not entirely understood (Ahmad *et al*., 2016). Furthermore, owing to the differential expression levels during the course of infection, the precise participation of each individual SAP isoenzyme and activity within the pathogenesis of *C. albicans* is not definite (Naglik *et al*., 2008; Mayer *et al*., 2013; Kumar *et al*., 2015). Both *C. auris* yeast and filamentation-competent cells have been detected to produce SAPs, based on yeast carbon base– bovine serum albumin (YCB–BSA) assays (Du *et al*., 2020).

Demonstrations from murine investigations identified that the production of SAP1-3 proteins are essential in the early infection stages of skin and mucous membranes as well as for virulence in HDC (Correia *et al*., 2010; Staniszewska *et al*., 2012; Pawar *et al*., 2014). The SAP4-6 proteins are implicated within both mucosal and systemic infections as well as possess a role in organ invasion (Staniszewska *et al*., 2012; Kumar *et al*., 2015).

2.7.4.2) Phospholipases

Phospholipases are lipolytic enzymes that hydrolyze phospholipid substrates into fatty acids. They contribute to pathogenicity by adhering to the host cell membrane and ultimately cause cell lysis (De Luca *et al*., 2012). Elevated phospholipase activity is indicative of tissue damage since these enzymes hydrolyze one or more than one ester bonds of glycophospholipids within the host cell membranes (Karkowska-Kuleta *et al*., 2009; Sardi *et al*., 2013).

Each secreted phospholipase cleaves a different, but specific ester bond in the phospholipid molecule, based on this, they are classified into four different classes (phospholipase/PL A-D) (Ghannoum *et al*., 2000; Khan *et al*., 2010; Pereira *et al*., 2015). Five members of class B (PLB1-5) are associated with the pathogenicity of *C. albicans* and disease development (Niewerth and

Korting, 2001). Deteriorated virulence is observed in *C. albicans* as well as reduced capability of cell membrane penetration in a murine model owing to the inactivation of the *PLB1* and *PLB5* genes (Theiss *et al*., 2006). Moreover, *C. albicans* clinical isolates have displayed an increase of extracellular phospholipase production when compared to commensal isolates (Pinto *et al*., 2008; Mahmoudabadi *et al*., 2010).

Certain NAC species (*C. tropicalis, C parapsilosis, C. guilliermondii* and *C. auris*) also produce phospholipases at different enzyme activity degrees (Ghannoum *et al*., 2000; Larkin *et al*., 2017). *Candida auris* has been reported to produce proteinase and phospholipase in varying quantities and activities (Kumar *et al*., 2015; Larkin *et al*., 2017; Wang *et al*., 2018).

2.7.4.3) Lipases

Lipases are water-soluble enzymes that catalyze the hydrolysis of insoluble substrates like long-chain triglycerides (Gopinath *et al*., 2013). The role of extracellular lipases as a virulence factor has been exposed through *in vitro* investigations of *C. albicans*. Some of the significant features that this enzyme provide to the pathogenicity of *C. albicans* are; cytotoxicity, facilitation of adhesion to and invasion of host cells. It also serves as a trigger for local inflammatory disorders that cause damage to host cells (Hube *et al*., 2000; Nguyen *et al*., 2011).

Ten members make up the family of genes that encode lipases (LIP1-10) (Paraje *et al*., 2008). The level of *LIP* gene expression was determined to depend on the progress and type of infection where the participation of extracellular lipases in both cutaneous and systemic infections was confirmed in a study (Stehr *et al*., 2004). The genome of *C. auris* was determined to foster the lipase gene family where eight genes, orthologous to secreted lipases, were annotated and implicated in invasiveness (Chatterjee *et al*., 2015). Lipases have been unanimously neglected when compared to other hydrolytic enzymes (i.e. proteinases and PLs) resulting in inadequate data (Bhat *et al*., 2011).

2.7.5) **Biofilm formation**

An aggregate of microbial cells with adherence to each other, host cells or an inanimate surface, enclosed by a self-produced slimy extracellular polymeric matrix, is a biofilm (Mathé and Van Dijck, 2013; Gulati and Nobile, 2016). The matrix is responsible for the structural integrity of the

biofilm and is directly associated with virulence due to antifungal drug resistance (Martins *et al*., 2010). Internal biofilm formation begins with adherence of pathogenic cells to host tissue. Thereafter, cell proliferation, hyphal cell formation, accumulation of extracellular matrix results in the successful establishment of infection (Mayer *et al*., 2013). Mature biofilms release their pathogenic cells from the complex in order to spread and colonize new locations, like a metastatic cancer. Disseminated invasive candidiasis is suggestively caused by biofilm maturity (Uppuluri *et al*., 2010). In *C. albicans*, certain transcription factors (BCR1, TEC1 and EFG1) control and regulate the formation of biofilms (Harriott *et al*., 2010; Nett *et al*., 2010).

Candida biofilms are a potent virulence factor as they can be highly resistant to the host's immunity as well as conventional antifungal drugs (Fanning and Mitchell, 2012). They can thrive in diverse environments and on almost all surfaces, biotic or not. A major public health concern arises as cells from biofilms can colonize all kinds of medical equipment. The greatest threat is colonization of implanted medical devices such as urinary and central venous catheters, dentures, or orthopaedic implants and potentiate invasive candidiasis. This is probably a major factor contributing to increased mortality rates of hospitalized patients (Römling and Balsalobre, 2012; Nobile and Johnson, 2015). *Candida* biofilms that cause nosocomial device-related infections are fundamentally difficult to control and exterminate as they display resilience to a broad spectrum of antifungal drugs with extremely high minimum inhibitory concentrations (Taff *et al*., 2012; Silva-Dias *et al*., 2015; Sandai *et al*., 2016). Several factors could pertain to the well-developed resistance of *C. albicans* biofilms, namely; upregulation of drug efflux pumps, impermeable extracellular matrix and existence of intractable persister cells (Nobile and Johnson, 2015).

In *C. albicans*, both yeast and hyphal forms are required for the formation of a biofilm. However, *Candida* species that cannot produce true hyphae (*C. tropicalis*, *C. parapsilosis*, and *C. glabrata*) can still produce biofilms mainly comprised of yeast cells and extracellular matrix (Silva *et al*., 2012). *Candida auris* biofilms are significantly thinner and therefore inherently weaker when compared to *C. albicans* biofilms (Larkin *et al*., 2017). Cells isolated from *C. auris* biofilms have exhibited elevated resistance to antifungal agents when compared with free-floating cells. The ability to form biofilms is strain specific where biofilms formed by non-aggregated *C. auris* cell types are more robust when compared to aggregated *C. auris* cell types (Srivastava *et al*., 2020). The up-regulation of genes encoding putative adhesins, efflux pumps, and virulence factors were

noted during the development of *C. auris* biofilms. Regardless of the insufficient knowledge of the roles of *C. auris* biofilms, they undoubtedly contribute to the virulence, antifungal resistance and survival properties of *C. auris* (Du *et al*., 2020). Therapeutic approaches that target *C. auris* biofilms is a vital area for future research.

2.7.6) **Oxidative stress**

Oxidative stress is caused by the overproduction of reactive oxygen species (ROS) that can lead to intracellular damage. Oxygen radicals, are the naturally occurring by-products of cellular metabolism. These ROS can be beneficial by playing a role in protective reactions, but also detrimental whereby excess can lead to adverse modifications of cellular components (i.e. lipids, proteins, and nucleic acids) (Schieber and Chandel, 2014). Under normal circumstance, the antioxidant defence system of hosts is sufficient to combat oxidative stress, however, a shift in this delicate balance can be fatal (Birben *et al*., 2012; Fisher *et al*., 2012). The pathophysiology of numerous human diseases are associated with an increased level of ROS, these are inclusive of, but not limited to; 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 chemical molecules that contain one or more than one unpaired electron rendering these molecules as inherently unstable as unpaired electrons proportionately increase the affinity towards any other available substance (Betteridge, 2000; Lenaz, 2012). Radical species that hold physiological significance include, hydroxyl radical (•OH), superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) (Rahman *et al*., 2012; Wang *et al*., 2017).

Numerous external stimuli have the potential to trigger the production of free radicals, these consist of; ultraviolet radiation, gamma rays, air pollutants, heavy metal ions and chemotherapy drugs (Betteridge, 2000; Gu *et al*., 2015). There are numerous signalling pathways that are directly initiated and activated in response to environmental stresses. Activation of the C. albicans mitogen-activated protein kinase (MAPK) pathway by the transcription factor protein (Cap1p) and high osmolarity protein (Hog1p) majorly contributes to oxidative stress tolerance in *C. albicans*. Osmotic adaptation and glycerol accumulation is promoted by the transcriptional response of Hog1p and Cap1p (Enjalbert *et al*., 2006; Mayer *et al*., 2013; Kaloroti *et al*., 2014). However, a

dysfunctional resistance to stress and attenuation of *C. albicans* virulence has been described to the inactivation of these regulators (Cheetham *et al*., 2011; Jain *et al*., 2013; Tillmann *et al*., 2015).

The mitochondrial electron transport chain (ETC) constantly reduces molecular oxygen (O_2) to water during regular cellular respiration. Various respiratory burst enzymes (i.e. reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase) are mediators of this process. The majority of intracellular O_2 is produced via the respiratory chain within the inner mitochondrial membrane whereby 1-3 % of electron leakage to oxygen occurs during oxidative phosphorylation. This results in the reduction of mitochondrial oxygen (O_2^-), instead of O_2 to H_2O (Birben *et al*., 2012; Lenaz, 2012; Lone *et al*., 2013; Kim *et al*., 2015).

Five major multi-subunit complexes make up the mitochondrial ETC, these are; I – nicotinamide adenine dinucleotide (NADH)-coenzyme Q (CoQ) oxidoreductase; II – succinate-ubiquinone oxidoreductase; III – ubiquinol-cytochrome *c* oxidoreductase; IV – cytochrome *c* oxidase; and V – ATP synthase (Larosa and Remacle, 2018). Complexes I and II of the respiratory chain produce significant amounts of ROS, particularly O_2^- (Brand, 2010; Gupta *et al*., 2014). The transfer of electrons from NADH to ubiquinone is catalysed by Complex I, which also serves as an entry point for electrons from NADH into the mitochondrial ETC. Complex III funnels electrons from CoQ (QH2) to cytochrome C and, in the presence of a respiratory inhibitor (i.e. antimycin), this complex produces significantly high amounts of O_2^- . This complex also leaks electrons to O_2 which thereby enhances the formation of O_2^- (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).

2.7.6.1) Resistance to oxidative stress by production of antioxidant enzymes

Robust antioxidant defence mechanisms have been determined which allows a pathogen to thrive and establish infections within the host by evading or neutralizing ROS produced either by the host's innate immune system or antimicrobial drugs (Belenky and Collins, 2011; Bink *et al*., 2011; Miramón *et al*., 2012; Youseff *et al*., 2012). Endogenous antioxidant compounds that directly counterbalance the harmful effects of ROS and repair cellular damage are; primarily catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and secondary; glutathione transferase (GST) and glutathione reductase (GR) (Rahman *et al*., 2012; Gupta *et al*., 2014). Fungal

cells which are exposed to significant levels of ROS will eventually lead to death if not neutralized by the activity of antioxidant enzymes.

Briefly, SOD converts O^{2-} to a more stable oxidant H_2O_2 and O_2, this spontaneous dismutation of O^{2-} by SOD is the primary step for regulating intracellular ROS (Gupta *et al*., 2014; Wang *et al*., 2017). 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 disulphide (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; Miramón *et al*., 2014). The GST catalyses the conjugation of GSH to xenobiotics, thereby preventing the interaction with cellular proteins and biological membranes (Birben *et al*., 2012; Wang *et al*., 2017).

2.7.6.1.1) Superoxide dismutase

Two SOD types are solely expressed by a typical eukaryotic organism; copper-zinc (Cu-Zn) dependent SOD1 and manganese (Mn) dependent SOD2 (Miller, 2012). These are found in the cytosol and mitochondrial matrix. Four additional SOD isoforms have been identified in *C. albicans*, namely; MnSOD3 and copper only glycosylphosphatidylinositol (GPI) - anchored SOD4 to SOD6 (Bink *et al*., 2011; Peterson *et al*., 2016). Modification of the expression level of SOD genes has been identified when the organism is exposed to fungicidal compounds, natural and/or synthetic (Ojha *et al*., 2010; Yousuf *et al*., 2010; Linares *et al*., 2013; De Cremer *et al*., 2016). Investigations subjecting *C. albicans* mutant strains (i.e. SOD inactivation and deletion) to miconazole and SOD inhibitor N,N′-diethyldithiocarbamate (DDC) have reported a significant upregulation in all SOD genes, an increased antifungal activity of miconazole, and a rise in endogenous ROS production (Bink *et al*., 2011; De Cremer *et al*., 2016; Sun *et al*., 2017).

2.7.6.1.2) Catalase

The majority of aerobic organisms produce the catalase enzyme in their peroxisome. Catalase prevents the accumulation of toxic compounds, especially H_2O_2 where it efficiently uses iron as a co-factor to convert H_2O_2 to water and molecular oxygen. This enzyme has the highest conversion turnover whereby one catalase molecule can convert roughly six million H_2O_2 molecules in a single

second (Rahman, 2007). *Candida albicans* and *C. auris* express only one catalase unlike many other yeasts that encode two different catalase enzymes (Wysong *et al*., 1998; Nishimoto *et al*., 2016). Catalase provides resistance to peroxide stress and contributes to *C. albicans* infection establishment (Wysong *et al*., 1998; Nakagawa *et al*., 2003; Kaloroti *et al*., 2014). The susceptibility of *C. albicans* increases through the disruption of the *CAT1* gene in *C. albicans* (Wysong *et al*., 1998; Komalapriya *et al*., 2015). Catalase gene expression in *C. albicans* is induced by hydrogen peroxide and repressed by glucose.

2.7.6.1.3) Glutathione

Glutathione (GSH) is a low molecular weight tripeptide thiol which is found in abundance in living organisms (Lushchak, 2012). The intracellular concentration in yeast cells is approximately 10 mM as it contributes to multiple metabolic processes. However, there is an implication of its redox homeostasis imbalance in progression of many diseases (Yadav *et al*., 2011). It is synthesized by GSH synthase and γ-glutamylcysteinesynthetase and mostly exists in its reduced form which serves as an electron donor for disulphide bond reduction. The GSSG is its oxidized form which is then reduced back to GSH by GR which utilises NADPH as a cofactor. Redox status within *C. albicans* is affected by the disturbance of the GSH/GSSG ratio. This induces elevated ROS levels, auxotrophy and low virulence (Baek *et al*., 2004; Yadav *et al*., 2011; Zhu *et al*., 2011; Guedouari *et al*., 2014). The GSH functions in combating oxidative damage by the detoxification of free radicals and many other toxic compounds. Adenosine triphosphate (ATP) driven transporters are induced in response to antifungal drugs which are exported out of the cell after conjugation with GSH (Zhu *et al*., 2011).

A dose-dependent depletion of GSH levels and elevated ROS production have been observed after exposure to natural and synthetic antifungal compounds (Abegg *et al*., 2012; Bertóti *et al*., 2016; Thangamani *et al*., 2017). Moreover, a decrease in the overall GSH/GSSG ratio in *C. albicans* was observed after treatment of fluconazole and micafungin proving interference with the natural regulation of redox state. Furthermore, a significant increase in GSH content was observed in fluconazole resistant isolates when compared to fluconazole sensitive strains which suggest that levels of GSH participate in the removal of the drug from the cell (Maras *et al*., 2014).

2. 8) Antifungal drugs for treatment of *Candida auris* infections

A tentative breakpoint for selected antifungal drugs has been proposed by the CDC based on the susceptibility of closely related *Candida* species for antifungal susceptibility testing of *C. auris*. Drug resistance against fluconazole, amphotericin B and echinocandins individually has been observed in *C. auris* isolates, however, some isolates have proven resistant to all three. Echinocandins have proven to be the most effective antifungal class and therefore is recommended as the first-line therapy for treatment of *C. auris*. Regardless of combination therapies proving effectiveness, acquired resistance is still a great concern (Cortegiani *et al*., 2018; Shaban *et al*.,, 2020).

Antifungal drug resistance and a high treatment failure rate associated with *C. auris* infections causes anxiety amongst clinicians. Alternative strategies and new antifungal drugs with novel and/or multiple targets have become a crucial necessity for efficient treatment. This hunt for novel antifungal drugs has brought attention to plant secondary metabolites which, thus far, display promising results (Hyde *et al.,* 2019).

2. 9) Monoterpene phenols as antifungal agents

Plants are natural sources from which a variety of chemical compounds are derived owing to their medicinal and antimicrobial properties. Herbal medicine has gained popularity in health care around the world, especially in developing countries as they are often affordable and accessible. Around 80 % of global populations (especially African and Asian countries) maintain the use of herbal medicine, as reported by the World Health Organisation (WHO). The attempt to obtain the safety and effectiveness of phytomedicines and eventual integration into clinics, has recently gained much attention in research (Ahmad *et al*., 2016).

Aqueous extracts of *Salvadora persica L*. (Toothbrush Tree), *Satureja cuneifolia*, *Coridothymus capitatus* (Conehead thyme), *Satureja thymbra* (savory of Crete), *Thymus syriacus* (Zattar), and *Thymbra spicata* exhibit antimicrobial properties against several microbial species including fungi (Othman *et al*., 2019). Phenolic monoterpenes are generally produced as a response against plant pathogenic fungi (Wang *et al*., 2019). These compounds could potentially be used as natural preservative agents in food industries as they have proven to be effectively inhibitory against many food-borne pathogens (Juneja *et al*.,, 2012; Xu *et al*., 2009; Lima *et al*., 2013).

There are four phenolic compounds that have become widely popular in research. Two are phenyl propanoids (eugenol and methyleugenol) and two monoterpene phenols (carvacrol and thymol). These are major components in the essential oils of many aromatic plants that are used as food flavouring agents (Khan *et al*., 2010; Ahmad *et al*., 2010; Ahmad *et al*., 2011; Wang *et al*., 2018). Eugenol and methyleugenol are found in clove and basil oil, whereas carvacrol and thymol are found in oregano and thyme oil (Ahmad *et al*., 2010; Ahmad *et al*., 2011). Human exposure to these four compounds is mostly through foods, spices, and traditional medicine. Therefore, they are nontoxic and recognized as safe when used as food flavouring agents (Juneja *et al*., 2012). However, *in vivo* investigations have yet to be conducted to confirm whether their dose toxicity in their concentrated forms is safe. Contrastingly, *in vivo* investigations on their genotoxicity potential revealed no evidence of carcinogenicity in mice (Sanders *et al.,* 2009). Eugenol, methyleugenol, carvacrol and thymol have been experimentally investigated *in vitro* whereby all four compounds show antifungal activity against different species of *Candida* including clinical isolates of *C. auris* (Shaban *et al*., 2020). Additionally, some of these compounds have been shown to have anti-virulence effects against *C. albicans,* and recently, against *C. auris* as well (Shaban *et al*., 2020).

2. 10) Carvacrol

Carvacrol (5-isopropyl-2-methylphenol) is a phenolic compound that has proven to have a higher antimicrobial activity than other volatile compounds present in essential oils (Sharifi-Rad *et al*., 2018). The efficacy of carvacrol against clinical *Candida* isolates, including *C. auris*, established that it possesses potent antifungal activities (Sharifzadeh *et al*., 2019; Shaban *et al*., 2020). Observations of carvacrol's effectiveness include; inhibition of hyphal and biofilm formation and reduction of SAPs expression and proteinase production (Shaban *et al*., 2020; Hosseini *et al*., 2016). These anti-virulence effects in turn reduce pathogenicity and prevent and reduce infection severity.

Insufficient information is available on the mechanism of action of carvacrol, but it has been elucidated that it affects functional properties of the cell membrane in *Candida* species in a dose dependent manner, by inhibiting ergosterol biosynthesis which results in major membrane damage that induces cell leakage and inevitable cell death (Ahmad *et al*., 2010; Pinto *et al*., 2008; Lima *et al*., 2013). Furthermore, it has been observed to cause calcium (Ca^{2+}) and hydrogen ion (H^{+}) homeostasis disruption in the yeast as well as oxidative stress induction (Khan *et al*., 2015). In a

recent study on *C. auris*, carvacrol was found to inhibit adherence and proteinase production (Shaban *et al*., 2020).

In vivo studies confirmed that carvacrol does not produce harsh cytotoxic effects at a higher survival rate. No kidney burden or acute toxicity, was observed after the oral administration of carvacrol to mice with systemic candidiasis (Manohar *et al*., 2001). Therefore, carvacrol shows potential for therapeutic and clinical application. Further investigations of the mechanism of action, detailed cellular response and molecular basis yeast termination, would be an additional support to/for the therapeutic potential of carvacrol as a stand-alone drug or in combination with other antifungal agents or drugs.

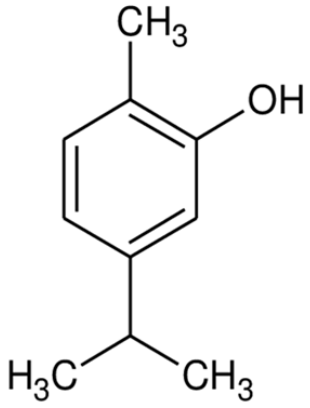

Figure 2.1: The chemical structure of carvacrol

2. 11) Justification

Candida auris has proven to be a multi-drug resistant pathogen in numerous investigations. It has been associated with candidemia and hospital outbreaks globally. The severe drug resistance, as well as ability to vigorously and persistently spread, has driven the urgency for the development of a novel antifungal drug that is biologically safe, efficient, and targets virulence properties or defence mechanisms. In order to determine if carvacrol has an effect on the oxidative stress defence system of *C. auris*, it is important to; firstly confirm the inhibitory and fungicidal effect of carvacrol on *C. auris* and secondly, confirm the presence of the antioxidant enzymes and if carvacrol has an effect on their activity and gene expression. Interfering with the regulation of antioxidant enzymes should not allow for survival pressure and therefore avoid resistance development.

Chapter 3) Materials and Methods

3. 1) Study population

Twenty five *C. auris* isolates that were preserved in glycerol stock at -80 °C were utilized for this study. These isolates are stored in the Department of Clinical Microbiology and Infectious Diseases (CMID), School of Pathology, University of the Witwatersrand, Johannesburg and were obtained from the Division of Mycology, National Institute of Communicable Diseases (NICD), Johannesburg, South Africa. All these isolates were previously identified as *C. auris* using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique. *C. albicans* SC5314 was used as a control strain, as it has been thoroughly studied for antioxidant enzymes. All the isolates were revived from glycerol stocks on Sabouraud Dextrose Agar (SDA) plates. These plates were incubated for 24 hours at 37 °C. A primary culture was prepared whereby a single colony was inoculated in Sabouraud Dextrose Broth (SDB) for 24 hours at 37 °C.

3. 2) Ethics clearance

Existing stock cultures of *C. auris* that were used in this study were stored in the Department of CMID, University of the Witwatersrand. To use these isolates, ethics clearance (Ref.: W-CBP-191103-01) was obtained from the Human Research Ethics committee of the University of the Witwatersrand, Johannesburg, South Africa, and performed according to the guidelines outlined in the Helsinki Declaration (Appendix A).

3. 3) Antifungal susceptibility testing

The antifungal activity of carvacrol was determined by performing the broth microdilution method following the recommended guidelines described by the CLSI M27-A3 (CLSI, 2008). A 100 mg/ml working stock solution (Appendix B1.1) was made from a 0.98 g/ml carvacrol stock (Sigma-Aldrich, USA) by dissolving in 1 % (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). The minimum inhibitory concentration (MIC) was observed as the lowest concentration of carvacrol that inhibits fungal growth. The minimum fungicidal concentration (MFC) was observed as the lowest concentration of carvacrol that kills the fungus. A 5 ml volume of a suspension (0.5 McFarland standard) of each strain was used in the following procedures.

3.3.1) Minimum Inhibitory Concentration

A working solution of 8 000 µg/ml of carvacrol (Appendix B1.1.2) and 8 µg/ml of amphotericin B (Sigma-Aldrich, USA) (Appendix B2) were prepared for this experiment. Separately, a 100 µl of SDB (Merck, RSA) was dispensed into each well of a 96 well microtiter plate (Thermo Fischer Scientific, USA). The first row of wells contained the sterility control (medium only). From the working solution (8 000 µg/ml carvacrol), 100 µl of carvacrol was added to the second row of wells that contained 100 µl of SDB and mixed properly by pipetting up and down three times. A serial two-fold dilution was performed by transferring 100 µl of the mixture from the second row of wells to the third. This process was successively repeated until the second last row of well where 100 µl was discarded. The last row of wells contained the growth control (no carvacrol). Aliquots of 100 µl exponentially growing *C. auris* cells were dispersed into all of the wells except for the sterility control wells (first row). Each experiment had a sterility control (SC - 200 µl of media), growth control (GC - 100 µl of medium and 100 µl culture), negative control (100 µl of 1 % (v/v) DMSO (in medium) and 100 µl culture) and a positive control (100 µl of 4 µg/ml amphotericin B (in medium) and 100 µl culture). The final concentrations in the plate ranged from 2 000 µg/ml to 31.25 µg/ml of carvacrol. All the microtiter plates were sealed with lids and were incubated at 37 ºC for 24 hours. The results were read using a microtiter plate reader (Bio-Rad iMark™ Microplate Reader). The lowest concentration with no visible growth was considered as the MIC value.

3.3.2) Minimum Fungicidal Concentration

Once the MIC was determined, SDA plates were prepared and marked whereby 10 µl from all the wells in the microtitre plates without visible growth were sub-cultured. All the SDA plates were allowed to dry followed by incubation at 37 ºC for 24 hours. After incubation, the MFC was determined as the concentration in the first well where no growth was observed on the SDA plates.

3. 4) Lipid peroxidation assay

Lipid peroxidation (LPO) products (thiobarbituric acid reactive substances (TBARS)/ malondialdehyde (MDA)) play a common role in the formation of membrane lacerations. Treatment with carvacrol was utilized to evaluate the extent of LPO of the cell membrane. The level of LPO that the cell membranes endured was estimated to confirm the state of oxidative stress.

Candida auris isolates were inoculated in SDB liquid medium at 37 °C, with shaking at 150 rpm for 8 hours. Post-incubation, yeast cells (mid-log phase) were washed with phosphate buffered saline (PBS) and re-suspended in fresh SDB in the absence (control) or presence of different concentrations of carvacrol (0.5 MIC, MIC and 4 MIC) and further incubated at 37 °C, with shaking at 150 rpm for 4 hours to allow for sufficient exposure.

3.4.1) Preparation of plasma membrane

Plasma membranes (PM) were prepared by the following the procedures described by Chikezie, 2016 with adjustments (Chikezie, 2016). Carvacrol treated and untreated isolates were suspended in a grinding medium consisting of 250 mM sucrose (Merck, RSA) (Appendix B3), 10 mM Trizima base buffer (pH 7.0; Sigma-Aldrich, USA) (Appendix B4.1), 1 mM phenylmethane sulfonyl fluoride (PMSF; Roche, Germany) (Appendix B5), 2 g glass beads (BDH Chemicals, England) and 1 g wet weight cells. For the disruption of the cells, the suspension was subjected to 9 cycles of cell homogenization (1 cycle is 30 sec homogenization) using a homogenizer. The homogenate was centrifuged (Eppendorf Centrifuge 5720 R, Germany) at 2 814 x g for 10 minutes at 4 °C. Subsequently, the cell debris and glass beads were discarded and the cell free supernatant was transferred into micro centrifuge tubes and further centrifuged (Eppendorf Centrifuge 5417 C) at 14 000 x g for 40 minutes in order to pellet the plasma membrane which was used for the determination of LPO.

3.4.2) Determination of lipid peroxidation

LPO was determined by following the procedures described by Chikezie, 2016, with adjustments (Chikezie, 2016). A reaction mixture containing 1.8 ml phosphate buffer (0.1 M, pH 7.4) (Appendix B6) and 0.2 ml of PM was incubated at 37 ºC in water bath shaker for 1 hour. The reaction was terminated by adding 1.0 ml of 10 % (w/v) trichloroacetic acid (TCA; Sigma-Aldrich, USA) (Appendix B7) followed by the addition of 1.0 ml of 0.67 % thiobarbituric acid (TBA; Sigma-Aldrich, USA) (Appendix B8). All the tubes were kept in boiling water for 20 minutes, followed by cooling on ice and centrifugation (Eppendorf Centrifuge 5417 C) at 2 500 x g for 10 minutes. The resulting supernatant containing TBARS was measured spectrophotometrically (SpectraMax iD3 multi-mode microplate readers (Molecular Devices, USA)) by taking the absorbance at

432 nm against a reagent blank (i.e. TCA and TBA). The extent of LPO is expressed as nmol of TBARS.

3. 5) Enzymatic activity

The imperative antioxidant enzymes released by different *Candida* species are catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione transferase (GST). To determine the presence and activity of these enzymes in *C. auris* isolates, cells were grown in SDB at 37 °C, with shaking at 150 rpm for 8 hours. Post-incubation, yeast cells (mid-log phase) were washed with PBS and resuspended in fresh SDB in the absence (control) or presence of different concentrations of carvacrol (0.5 MIC and MIC) and further incubated at 37 °C, with shaking at 150 rpm for 4 hours to allow for sufficient exposure.

3.5.1) Preparation of cell-free extract

Cell-free extracts (CFE) were prepared by following the procedures described by Khan and co-workers (2015), with adjustments (Khan *et al*., 2015). Carvacrol treated and untreated isolates were suspended in a grinding medium consisting of 250 mM sucrose (Merck, RSA), 10 mM Trizima base buffer (pH 7.0), 1 mM PMSF (Roche, Germany), 2 g glass beads (BDH Chemicals, England) and 1 g wet weight cells. For the disruption of the cells, the suspension was subjected to 9 cycles, each of 30 seconds, of cell homogenization by a homogoniser. The homogenate was then centrifuged (Eppendorf Centrifuge 5720 R, Germany) at 2 814 x g for 10 minutes at 4 °C. Subsequently, the cell debris and glass beads were discarded and the cell free supernatant was transferred into micro centrifuge tubes and further centrifuged (Eppendorf Centrifuge 5417 C) at 14 000 x g for 40 minutes in order to pellet the plasma membrane so that the supernatant, containing intracellular components, could be extracted and utilised.

3.5.2) Catalase (CAT)

Experimental procedures to determine catalase activity were performed by following the procedures described by Khan and co-workers (2015) with some modifications (Khan *et al*., 2015). An assay mixture containing 30 % v/v hydrogen peroxide (H_2O_2; SAARCHEM-HOLPRO Analytic, RSA) (Appendix 14), 50 mM phosphate buffer (pH 7.0) (Appendix B9) and CFE was scanned at 230 nm using a UV spectrophotometer (UV-1800 Shimadzu). The consumption of H_2O_2

was measured every 30 seconds over a period of 3 minutes. The catalase activity results were expressed as µmol H_2O_2 consumed per minute, using an extinction co-efficient of 0.0081 per M.

3.5.3) Superoxide dismutase (SOD)

The activity of SOD was investigated using a method described by Khan and co-workers (2015) with minor experimental alterations (Khan *et al*., 2015). The CFE (500 µl) was added to 50 mM Trizima buffer (pH 8.5) and a freshly prepared solution of 20 mM pyragallol (Sigma-Aldrich, USA) (Appendix B10) followed by 90 seconds induction at room temperature. The inhibition by pyragallol (i.e. SOD activity) was measured every 30 seconds over a period of 3 minutes at 420 nm using a UV spectrophotometer (UV-1800 Shimadzu). The inhibition of autoxidation of pyrogallol was calculated as follows:

$$\text{Unit of SOD per ml of sample} = (\text{A} - \text{B} / \text{A} \times 50 \times 100) \times 10 \text{ dilution factor}$$

Where A = control difference in absorbance per minute, and; B = absorbance of the test sample. The final results are expressed in U of SOD per ml.

3.5.4) Glutathione peroxidase (GPx)

Activity of the GPx was determined by using an experimental procedure employed by Yousuf and co-workers (2010) with some modifications (Yousuf *et al*., 2010). An assay mixure was prepared containing 50 mM phosphate buffer (pH 7.0), 1 mM sodium azide (NaN_3; Sigma-Aldrich, USA) (Appendix B11), freshly prepared solution of 1 mM glutathione (GSH; Tocris Bioscience, UK) (Appendix B12.2), 1 mM ethylenediaminetetraacetic acid (EDTA; pH 7.0; Merck, RSA), freshly prepared solution of 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH; Roche, Germany) (Appendix B13.1), 30 % v/v H_2O_2 (SAARCHEM-HOLPRO Analytic, RSA) and CFE (300 µl). The removal of NADPH (i.e. GPx activity) was measured every 30 seconds over a period of 3 minutes at 340 nm on a UV spectrophotometer (UV-1800 Shimadzu). The GPx activity results are expressed as µmol NADPH oxidised per minute, using an extinction co-efficient of 6.22×10^3 per M per minute.

3.5.5) Glutathione reductase (GR)

The activity of GR was assessed using a method described by Yousuf and co-workers (2010) with minor experimental changes (Yousuf *et al*., 2010). An assay mixure was prepared that comprised of 50 mM phosphate buffer (pH 7.4), freshly prepared solution of 1 mM oxidized glutathione disulphide (GSSG; Sigma-Aldrich, USA) (Appendix B15), 0.5 mM EDTA (pH 8.0; Merck, RSA), a freshly prepared solution of 0.1 mM NADPH (Roche, Germany) (Appendix B13.2) and CFE (300 µl). The rate of NADPH oxidation (GR activity) was measured every 30 seconds over a period of 3 minutes at 340 nm on a UV spectrophotometer (UV-1800 Shimadzu). The GR activity results are expressed as 1 µmol NADPH oxidised per minute.

3.5.6) Glutathione S-transferase (GST)

Activity of GST was evaluated using a protocol employed by Maras and co-workers (2014) with minor adjustments (Maras *et al*., 2014). An assay mixure was prepared that comprised of 50 mM phosphate buffer (pH 6.5), a freshly prepared solution of 100 mM GSH (Tocris Bioscience, UK) (Appendix B12.1), 0.1 mM 1-chloro-2,4-dinitrobenzene (CDNB; Sigma-Aldrich, USA) (Appendix B16) and CFE. The conjugation of GSH with CDNB (GST activity) was measured every 30 seconds over a period of 3 minutes at 340 nm on a UV spectrophotometer (UV-1800 Shimadzu). The GST activity result is expressed as 1 µmol glutathione conjugated per minute.

3. 6) Gene expression of antioxidant enzymes

To study the effect of carvacrol on the gene expression of the antioxidant enzymes, Reverse Transcriptase Quantitative real-time Polymerase Chain Reaction (RT-qPCR) was performed. *Candida auris* isolates, that were inoculated and incubated with shaking in SDB in the absence (control) and presence of carvacrol (MIC), were harvested by centrifugation. The supernatant was discarded appropriately and the pelleted cells were washed twice with PBS (pH 7.0). Total RNA was extracted with the use of an RNA extraction kit (detailed in section 3.6.1). A nanodrop was utilized to determine the concentration and purity of the extracted RNA (detailed in section 3.6.1.1). Thereafter, total complementary DNA (cDNA) was synthesized using a cDNA synthesis kit (detailed in section 3.6.2). Primers were specifically designed for this study with the use of whole genome sequencing data (Lockhart *et al*., 2017) and an online tool (Primer3 - https://primer3.ut.ee/). The RT-qPCR experiment was carried out whereby the following

antioxidant genes were targeted; *CATA, SOD4, GPX3, GST1* and *GSHR* together with a *C. auris* housekeeping gene *ACT1*. To confirm the amplification of specific products, analysis utilizing a melting curve was conducted once the RT-qPCR cycling had ended (detailed in section 3.6.4).

3.6.1) Extraction of RNA

Following the manufacturer instructions, total RNA of *C. auris* isolates were extracted using the Zymo Research Quick-RNA Fungal/Bacterial Miniprep Kit (Zymo Research Corp, USA). *C. auris* isolates that were inoculated and incubated with shaking in SDB in the absence (control) and presence of carvacrol (MIC) were harvested by centrifugation (2 814 x g for 3 minutes). The supernatant was discarded appropriately and the pelleted cells were washed with PBS (pH 7.0), re-suspended in 800 µl of RNA lysis buffer and transferred to ZR BashingBead lysis tubes. The samples were vortexed for 2 minutes and centrifuged at 1 308 x g for 1 minute (Eppendorf Centrifuge 5417 C, Germany). After centrifugation, 400 – 700 µl of the supernatant was transferred to a Zymo-Spin IIICG column in a 2 ml collection tube and centrifuged with the same conditions. Thereafter, 1 ml of absolute ethanol (Merck (Pty) Ltd, South Africa) was added to the flow-through in the collection tube and mixed gently. The mixture was transferred to a Zymo-Spin IIC column, in a new collection tube, and centrifuged at 1 308 x g for 1 minute. The flow through was discarded. The column was washed with 400 µl of RNA prep buffer, in a new collection tube, and centrifuged at 1 308 x g for 1 minute. The flow through was discarded, 80 µl of a DNase I Reaction mix composed of 5 µl DNase I and 75 µl of DNA Digestion Buffer was added to the column and incubated at room temperature for 15 minutes. Thereafter, 400 µl of RNA Prep buffer was added to the column and centrifuged at 1 308 x g for 1 minute. The flow through was discarded, 700 µl RNA wash buffer was added to the column, in a new collection tube, and centrifuged at 1 308 x g for 1 minute. The flow through was discarded, 400 µl RNA wash buffer was added to the column, in a new collection tube, and centrifuged at 1 308 x g for 2 minutes. The column was then carefully transferred into an RNase free tube where 52 µl of DNase/RNase-free water was added directly to the column matrix and centrifuged at 1 308 x g for 1 minute. The eluted RNA was stored at -80 °C for further use.

3.6.1.1) RNA quality assessment

The concentration and purity of the total RNA for each sample eluted was measured using a Nanodrop 2 000 Spectrophotometer (Thermo Scientific, USA) with the Nanodrop 2 000/2 000c software v1.5 (Thermo Scientific, USA). The purity of RNA was assessed by determining the A_{260}/A_{280} ratio whereby a ratio above 2 was used for further experimentation. The concentration was recorded as ng/µl.

3.6.2) **cDNA synthesis**

Following the manufacturer instructions, reverse transcription of the total RNA extracted and cDNA synthesis of each sample was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad, .USA). Firstly, total RNA was diluted to 1 µg using the following formula:

$$vol\ of\ total\ RNA\ to\ be\ used = \frac{1\mu g}{RNA\ (\frac{ng}{\mu l})} \times 1\ 000$$

A total reaction mixture of 20 µl volume consisting of 5X iScript reaction mix (containing oligo (dT) and random hexamer primers), iScript Reverse Transcriptase (composed of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase with RNase inhibitor), nuclease-free water and 1 µg of RNA template. The mixture was incubated in an Eppendorf Thermomixer Comfort (Germany) using the following conditions: priming for 5 minute sat 25 °C, reverse transcription for 20 minutes at 46 °C, and reverse transcription inactivation for 1 minute at 95 °C. cDNA samples were stored at – 20 °C for further use.

3.6.2.1) cDNA quality assessment

The concentration and purity of the total DNA for each sample synthesised was measured using a Nanodrop 2 000 Spectrophotometer (Thermo Scientific, USA) with the Nanodrop 2 000/2 000c software v1.5 (Thermo Scientific, USA). The purity of the cDNA of each sample was assessed by determining the A_{260}/A_{280} ratio, whereby a ratio of 1.7 – 2.0 was used for further experimentation. The concentration was recorded as ng/µl.

3.6.3) Gradient PCR

In order to confirm the optimal annealing temperature for each primer pair to the specific genes, a gradient PCR was performed. Aliquots of stock primers (Metabion, Germany) were diluted to 10 μM with nuclease free water. A list of the primer sequences and PCR product lengths can be found in Table 3.1.

Table 3.1: List of primer sequences designed for and used in this study.

Gene		Primer Sequences		Expected Product length (bp)
		Forward (5'-3')	Reverse (3'-5')	
Antioxidant	*CATA*	GTGGATCGACTCTGGGTTGT	CAAGAGGCTTCTCCACCAAG	197
	SOD4	TCAACCCTTACCACGGCTAC	CACCACCACAGACAAGTTGG	176
	GPX3	CTACATCTCAACCGCAGCAA	GCACTTTTCGCCTGAAGAAC	165
	GST1	GGGGTCCCAAATACCACTCT	CTTGAACAAGGGCAGAGGAG	167
	GSHR	CCATTGCCCAAAAACACTCT	CAACTTGGTCATTCGTGGTG	171
HK*	*ACT1*	ACGCACATCGACATCACATT	CCTCTCAGTCGTCCGCTATC	182

CATA: Peroxisomal catalase, *SOD4*: Cell surface superoxide dismutase [Cu-Zn], *GPX3*: Glutathione peroxidase-like peroxiredoxin HYR1, *GST1*: Glutathione S-transferase 1, *GSHR*: Glutathione reductase, *ACT1*: Actin. (*HK : House Keeping)

The cDNA of each sample was diluted to 200 ng/μl. A 25 μl PCR reaction mixture was prepared for each gene consisting of 12.5 μl of PCR master mix 2X (Fermentas, USA), 0.5 μl of 10 μM each primer (Metabion, Germany), 9.5 μl nuclease-free water where 2 μl of 200 ng/μl cDNA template would be added finally to the PCR tubes. Thermocycling conditions are displayed in Table 3.2.

Table 3.2: Thermocycling conditions for gradient PCR.

Step	**Temperature (°C)**	**Time**	**Cycles**
Initial denature	95	3 minutes	1
Denature	95	30 seconds	35
Anneal	53-60	30 seconds	
Extend	72	1 minute	
Final Extend	72	10 minutes	1
Hold	4	∞	1

MyCycler™ Thermal Cycler, Bio-Rad, USA

PCR products were mixed with 5 µl of loading dye and run on a 2 % (w/v) Agarose gel (2 g Agarose (BioLine, USA) and 100 ml of 1 X TAE Buffer) for 1 hour at 100 V (PowerPac™ Basic, Bio-Rad, USA) with GeneRuler 50 bp DNA Ladder (Fermentas, USA).

3.6.4) Reverse Transcription Quantitative PCR (qPCR)

The RT-qPCR was performed with PowerUp SYBR Green Master Mix (Thermo Fischer Scientific, USA) using the Bio-Rad CFX 96 qPCR System (USA). A reaction mixture of 20 µl was prepared consisting of 10 µl SYBR Green master mix, 0.5 µl of 10 µM each primer (Metabion, Germany), 7 µl nuclease-free water and 2 µl of 100 ng cDNA template. Cycling conditions are displayed in Table 3.3.

Table 3.3: Final PCR cycling conditions.

Step	**Temperature (°C)**	**Time**	**Cycles**
Uracil-DNA glycosylase activation	50	2 minutes	1
Initial denature	95	2 minutes	1
Denature	95	15 seconds	40
Anneal	55	30 seconds	
Extend	72	1 minute	
Hold	4	∞	1

Three blank and a no-template control for each gene and each strain was included in each run to identify PCR contamination. The transcription profile of genes encoding critical oxidative stress enzymes (i.e. *CATA, SOD4, GPx3, GST1* and *GSHR*) was determined with the use of RT-qPCR. In order to confirm the precise amplification PCR products, melting curve analysis was implemented. The level of antioxidant gene expression was quantified and analysed with respect to the housekeeping gene *ACT1*. The messenger RNA (mRNA) concentration of each gene was computed with the use of crossing-point analysis. The returned data was analysed and calculated with the use of the following formula:

$$\Delta Cq = 2^{\text{Cq target gene} - \text{Cq reference gene}}$$

Where the quantitation cycle (Cq) was the average Cq value of target gene minus the mean of the reference/housekeeping gene. Furthermore, the fold change in gene expression was calculated by relativizing the average $2^{Cq\ target\ gene}$ of treated samples to the average $2^{Cq\ target\ gene}$ in untreated samples (Marimani *et al*., 2020).

3. 7) Cytotoxicity studies

Carvacrol was evaluated for its cytotoxic activity by using horse red blood cells (NHLS, Johannesburg, South Africa). The haemolytic activity of carvacrol was tested by a method described previously (Lone *et al*., 2020), with some modifications. Briefly, 10 ml horse red blood cells (RBCs) was aliquoted in 50 ml sterile falcon tubes, centrifuged at 581 x g for 10 minutes. The pellet was secured, washed three times with chilled PBS and resuspended in chilled PBS to give 10 % (v/v) RBC suspension. Next the 10 % (v/v) RBC suspension was further diluted to 1:10 in PBS, 100 µl of this suspension was added to different concentrations of carvacrol (0 – 4 000 µg/ml) in 1.5 ml tubes. The tubes were incubated for 1 hour at room temperature and centrifuged at 581 x g for 10 minutes. From each tube, 200 µl of the supernatant was transferred to a 96-well microtiter plate and absorbance was observed at 450 nm spectrophotometrically using a SpectraMax iD3 multi-mode microplate reader (Molecular Devices, USA). Triton X-100 (1 % v/v) and PBS were used as positive and negative controls respectively. The percentage of haemolysis was calculated by the following equation:

$$\%\ Haemolysis = \frac{[(\text{A450 of treated sample}) - (\text{A450 of negative control})]}{[(\text{A450 of positive control}) - (\text{A450 of negative control})]} \times 100$$

3. 8) Statistical analysis

The represented data and graphs were made and statistically analysed using one-way ANOVA test by GraphPad Prism software, version 9.0.0 (Poopedi *et al.*, 2020). All the experiments were carried out independently in triplicates, and the data obtained were presented as means ± standard deviation (± SD). The statistical analysis was performed using values of (**** = $p < 0.0001$; *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.1$) were considered as statistically significant.

Chapter 4) Results

Candida auris has gained much attention since its identification in 2009 owing to several *C. auris* outbreaks around the globe, its multi-drug resistance and increased associated mortality rates (Lone *et al.,* 2019). Being a newly identified *Candida* species, investigations of its structural, biochemical and pathogenic characteristics have yet to be elucidated. Unique drug targets different from the cell envelope, like the antioxidant defence mechanism, have recently been recognized as promising options for the development and identification of antifungal drugs. Carvacrol has demonstrated its antifungal activity aganst multiple species of *Candida* and other fungi by aiding the infected host to deteriorate the fungal defence system against oxidative insults (Poopedi *et al.,* 2020). In addition, carvacrol has also shown low levels of cytotoxicity *in vitro* and *in vivo* (Aydın *et al.,* 2014). This study aimed to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of carvacrol against 25 *C. auris* clinical isolates (Table 4.1). Furthermore, the presence of vital antioxidant enzymes in these isolates, the effect of carvacrol on the activities (Figures 4.2 – 4.6) and relative gene expression (Figures 4.8) of these enzymes was studied. Additionally, the effect of carvacrol on lipid peroxidation (Figure 4.1) as well as haemolysis (Figure 4.9) was also studied.

4. 1) Antifungal activity of carvacrol against *C. auris*

To determine the antifungal activity of carvacrol against 25 isolates of *C. auris* and a control laboratory strain of *C. albicans* (SC5314), the MIC and MFC were determined following CLSI recommended guidelines (CLSI, 2008). All the MIC and MFC results are shown in Table 4.1, and it is evident that carvacrol was active against all 25 *C. auris* strains/isolates as well as against *C. albicans* SC5314. All the clinical isolates of *C. auris* used in this study were found to be sensitive to carvacrol within an MIC range of 125 – 500 μg/ml. For ease of reference, the isolates were grouped according to their MIC values. Out of the 25 tested *C. auris* isolates, 40 % (10/25) displayed an MIC of 125 μg/ml (group A), 40 % (10/25) displayed an MIC of.250 μg/ml (group B), and 20 % (5/25) showed an MIC of 500 μg/ml (group C). Based on these results, a single *C. auris* isolate, MRL6015, MRL5762 and MRL2921, from each group (A, B and C) respectively. There were used as a reference isolate for further experimental procedures.

Furthermore, carvacrol was also been found to have fungicidal activity against the 25 tested *C. auris* isolates, with an MFC values ranging from 250 – 1 000 µg/ml (Table 4.1). The MFC values were generally observed to be 2 – 4 folds higher than the MIC values.

Table 4.1: The minimum inhibitory and fungicidal concentrations of carvacrol against *Candida auris* isolates and a *Candida albicans* isolate.

Isolates		MIC (µg/ml)	MFC (µg/ml)
SC5314.		250	500
Group A	MRL6015	125	250
	MRL6173	125	250
	MRL6125	125	250
	MRL6183	125	250
	MRL6059	125	250
	MRL6333	125	250
	MRL6338	125	250
	MRL5418	125	250
	MRL6065	125	250
	MRL2397	125	500
Group B	MRL6057	250	500
	MRL4587	250	500
	MRL3758	250	500
	MRL4000	250	500
	MRL6194	250	500
	MRL6277	250	500
	MRL5765	250	500
	MRL5762	250	1 000
	MRL6005	250	1 000
	MRL6334	250	1 000
Group C	MRL4888	500	500
	MRL3499	500	1 000
	MRL2921	500	1 000
	MRL6326	500	1 000
	MRL6339	500	1 000

4. 2) Lipid peroxidation

Lipid peroxidation is the characteristic marker of oxidative stress generated by reactive oxygen species (ROS). With the use of, TBARS is an indicator to estimate LPO, the results of this study showed a gradual incline in the rate of TBARS formation when cells were treated with increasing concentrations of carvacrol (Figure 4.1). In the control *C. auris* cells, an average of 5.43×10^{-6} µmol of TBARS formed without exposure which increased with increasing concentrations of carvacrol. At 0.5 MIC, MIC and 4 MIC, the average values increased to 7.93×10^{-6}, 10.20×10^{-6} and 14.52×10^{-6} µmoles of TBARS formed respectively. These results indicated that a greater than 150 % increase of TBARS formation was determined when *C. auris* cells were treated with MIC values in comparison to negative (untreated) control cells.

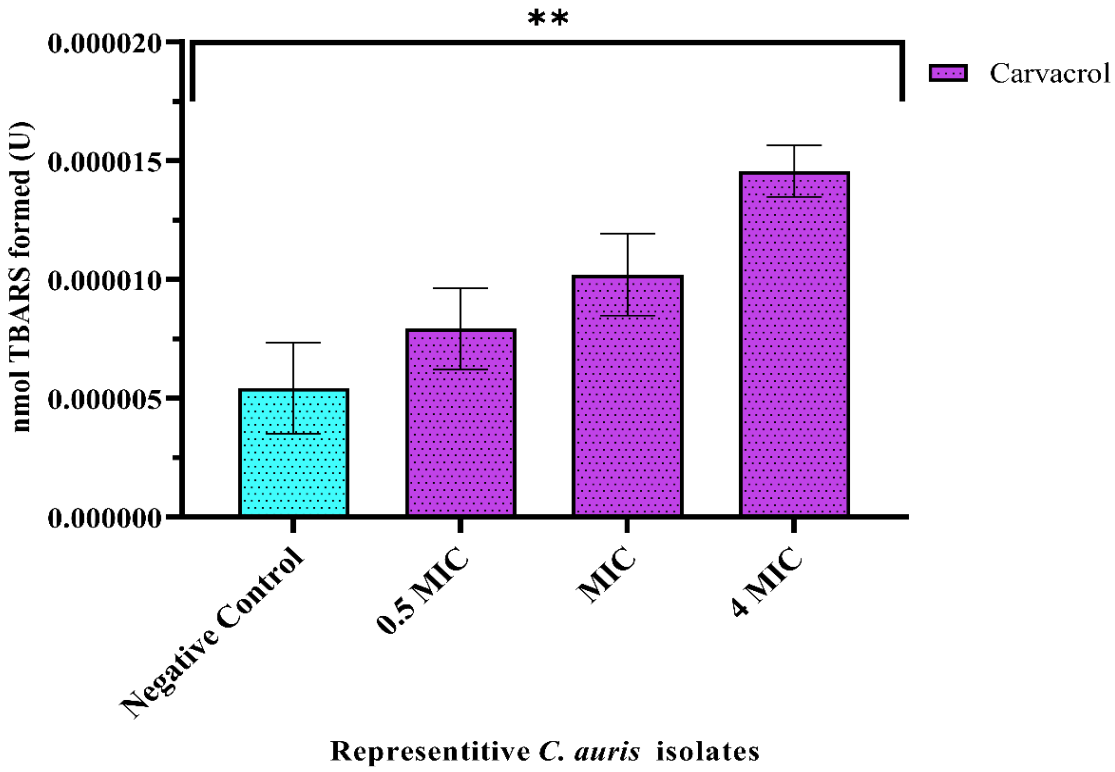

Figure 4.1: Effect of carvacrol on lipid peroxidation in *C. auris*. Figure displays an overall increase in the formation of TBARS in *C. auris* isolates after exposure to different concentrations of carvacrol (0.5 Minimum Inhibitory Concentration, Minimum Inhibitory Concentration and 4 Minimum Inhibitory Concentration; with respect to each strain's Minimum Inhibitory Concentration). The bars represent the means ± Standard Deviation of three replicates of each isolate.

4. 3) The effect of carvacrol on antioxidant enzymes

Antioxidant enzymes are very well studied in *C. albicans*, however there is no study reporting the antioxidant enzymes in *C. auris*. This study is the first of its type to report the presence of these antioxidant enzymes in this newly emerged *Candida* species. Due to the lack of literature related to the antioxidant enzymes in *C. auris,* it was important to screen for the presence of vital antioxidant enzymes prior to the assay procedures. Confirmation of the production of these enzymes rendered the continuation of the assays of these enzymes and the effect of carvacrol on their activity. Specific enzymatic activities of total catalase, SOD, GPx, GR and GST were assessed spectrophotometrically, 3 hours after carvacrol exposure.

4.3.1) Catalase

The enzyme activity of one of the primary oxidative defence enzymes, catalase, has been studied in untreated (control) and carvacrol exposed cells. The average enzyme activity of untreated *C. auris* isolates and the *C. albicans* SC5314 was 7.27 and 10.1 μmol of H_2O_2 consumed/minute, respectively (Figure 4.2). The *C. auris* cells exposed to 0.5 MIC carvacrol increased to 8.23 – 16.05 μmoles of H_2O_2 consumed/minute and MIC carvacrol increased to 13.58 – 32.1 μmoles of H_2O_2 consumed/minute (Figure 4.2). This is in accordance with the increase of catalase activity observed in the *C. albicans* control strain (SC5314) which showed 17.28 and 24.69 μmoles of H_2O_2 consumed/minute at 0.5 MIC and MIC carvacrol, respectively. Thus the overall increase of the catalase enzyme activity within *C. auris* was observed to be 56.52 % (1.57 fold increase) at 0.5 MIC and 180.97 % (2.81 fold increase) at MIC.

4.3.2) Superoxide dismutase

The enzyme activity of another primary oxidative defence enzyme, SOD, was also studied in the presence and absence of carvacrol. As in the case of catalase, SOD enzyme activity was also observed to be increased when cells were exposed to increasing concentrations of carvacrol. The average of *C. auris* and the *C. albicans* SC5314 control, SOD enzyme activity was determined as 113.77 and 180.43 U of SOD/ml, respectively. These values gradually rose with increasing concentration of carvacrol (Figure 4.3). At the lowest concentration (0.5 MIC), the enzyme activity of SOD within *C. auris* was observed to be 60.65 – 255.76 U of SOD/ml which increased to 119.44 – 327.67 U of SOD/ml at MIC. This increase of SOD activity was also observed for the

control strain (SC5314) which was 214.81 and 300 U of SOD/ml at 0.5 MIC and MIC, respectively. Overall, an average of 1.38 fold (37.69 %) and 1.98 (98.35 %) fold increase in SOD enzyme activity was observed within *C. auris* at 0.5 MIC and MIC, respectively.

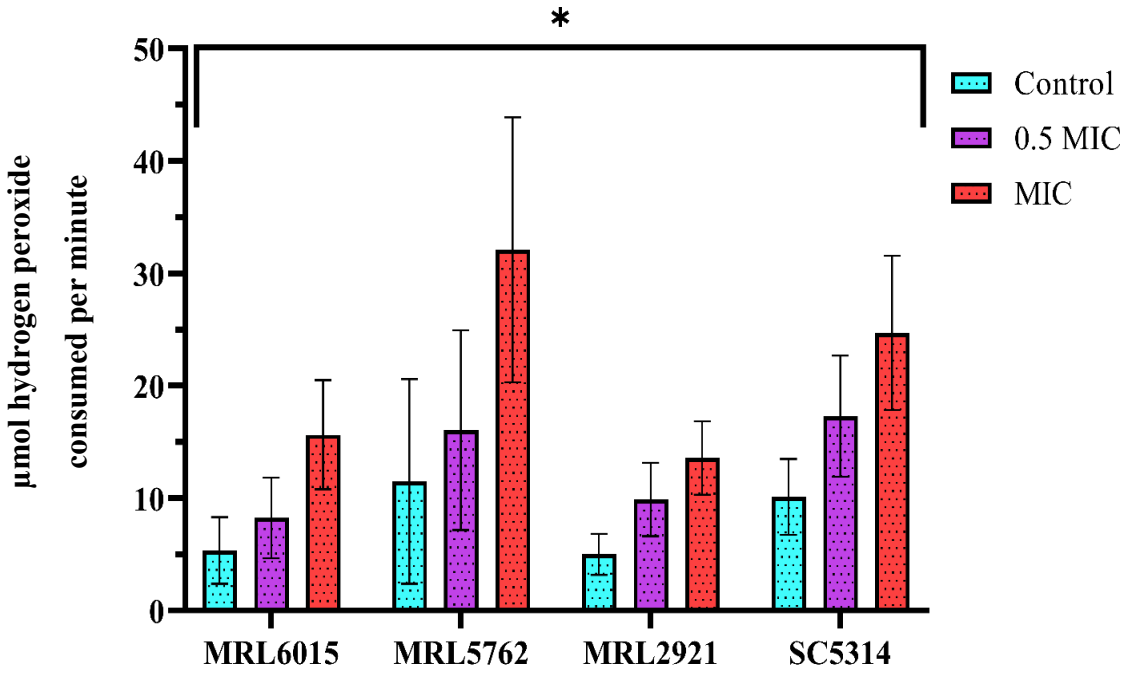

Figure 4.2: Catalase enzyme activity. *Candida auris* cells were exposed to 0.5 Minimum Inhibitory Concentration and Minimum Inhibitory Concentration of carvacrol (in respect to each strain's Minimum Inhibitory Concentration). Untreated cells were used as a control. An increase in catalase activity was observed with increasing carvacrol concentrations.

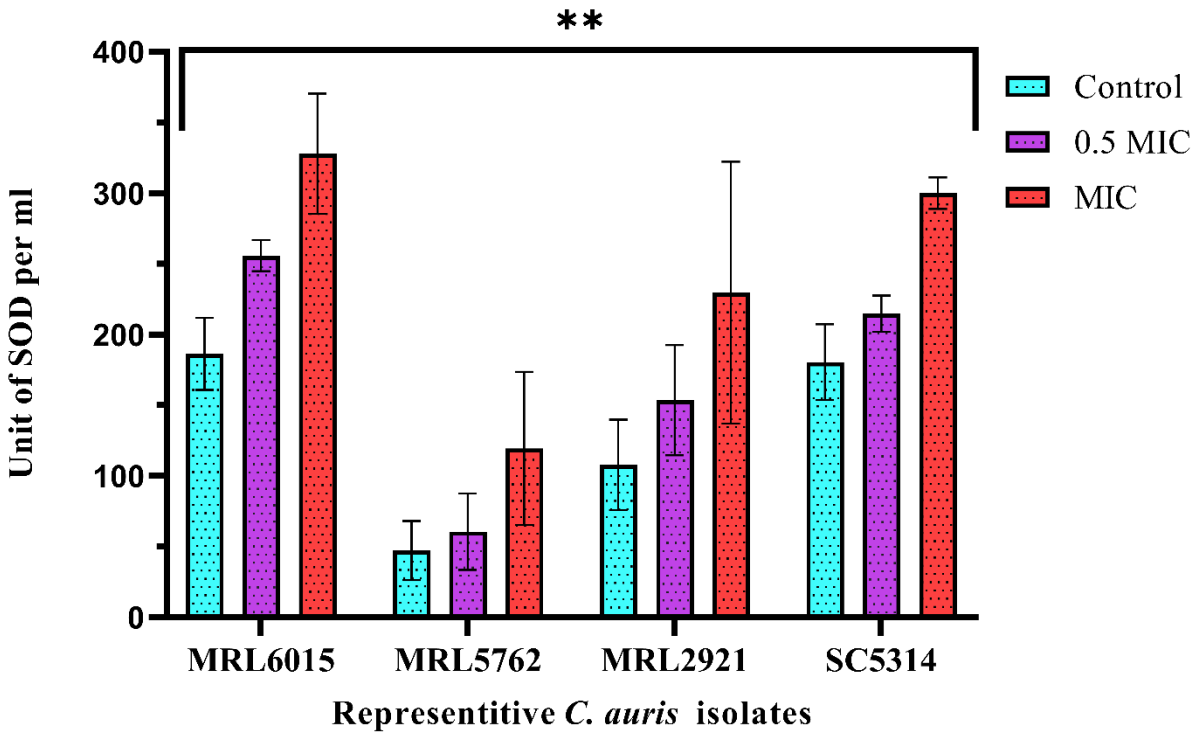

Figure 4.3: Superoxide dismutase enzyme activity. *Candida auris* cells exposed to 0.5 Minimum Inhibitory Concentration and Minimum Inhibitory Concentration of carvacrol. The control represents untreated cells. An increase in enzyme activity was observed with increasing carvacrol concentrations.

4.3.3) Glutathione peroxidase

The enzyme activity of GPx in *Candida* cells was calculated as NADPH oxidized/min protein. The *C. albicans* and the average of the *C. auris* controls exhibited a GPx activity of 23.33×10^{-6} and $10.18 - 23.33 \times 10^{-6}$ µmol of NADPH oxidized/min. There was an increase in activity when cells were exposed to increasing concentrations of carvacrol (Figure 4.4). An increase in enzyme activity by $4.02 - 35.38$ to $7.23 - 65.38 \times 10^{-6}$ µmol of NADPH oxidized/min were observed when *C. auris* cells were exposed to 0.5 MIC and MIC, respectively. These values for laboratory control strain *C. albicans* SC5314 were also found to be increasing from 39.94 to 47.17×10^{-6} µmol of NADPH oxidized/min when exposed to 0.5 MIC and MIC of carvacrol, respectively. An overall average of 1.51 fold (51.2 %) and 2.67 (> 150 %) fold increase in GPx enzyme activity was observed within *C. auris* at 0.5 MIC and MIC, respectively.

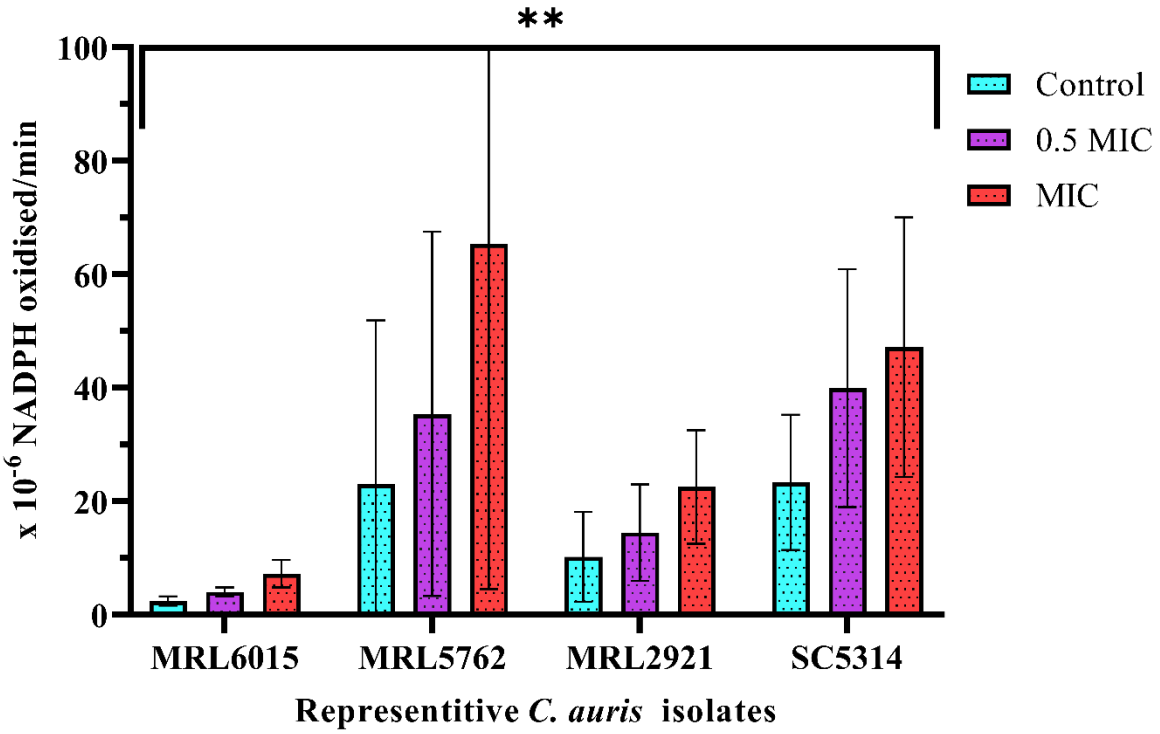

Figure 4.4: Glutathione peroxidase enzyme activity. Exponentially growing cells were exposed to 0.5 Minimum Inhibitory Concentration and Minimum Inhibitory Concentration of carvacrol. Untreated cells were used as a control. An increase in enzyme activity was displayed when cells were treated with different concentrations of carvacrol.

4.3.4) Glutathione reductase

The GR is an important enzyme required for maintaining the intracellular glutathione redox ratio of GSH/GSSG. The GR assay showed a decrease of the activity of enzyme with increasing concentration of carvacrol. The *C. albicans* (SC5314) and average *C. auris* control GR activity showed a value of 0.97×10^{-5} and $1.69 – 3.86 \times 10^{-5}$ µmoles of NADPH oxidized/minute (Figure 4.5). Along with 0.5 MIC and MIC values dropped to $1.21 – 3.06 \times 10^{-5}$ and $0.72 – 1.61 \times 10^{-5}$ µmoles of NADPH oxidized/minute within *C. auris*, respectively. This trend was also observed in SC5314 whereby a drop from 0.59 at 0.5 MIC to 0.32 at MIC was observed (Figure 4.5). Thus an overall percent decrease in enzyme activity within *C. auris* was determined as 24.32 % ≈ 0.76 folds at 0.5 MIC and 54.99 % ≈ 0.45 folds at MIC.

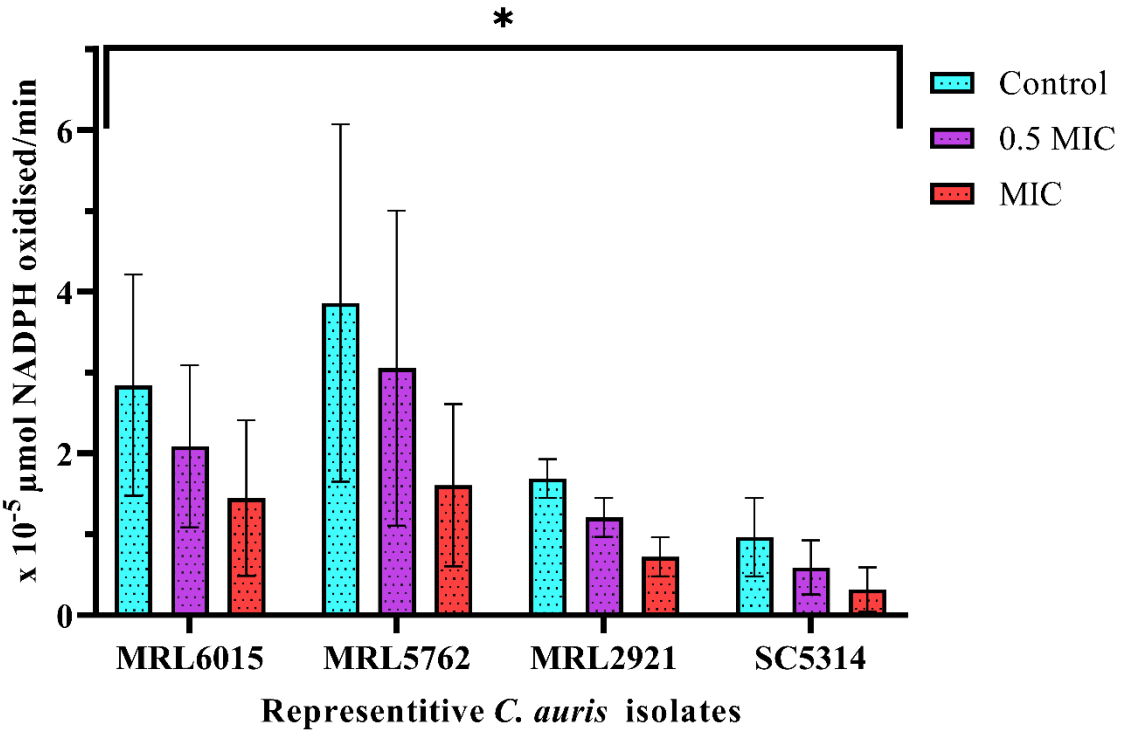

Figure 4.5: Glutathione reductase enzyme activity. Exponentially grown cells were exposed to 0.5 Minimum Inhibitory Concentration and Minimum Inhibitory Concentration of carvacrol with respect to each strain's Minimum Inhibitory Concentration. Untreated cells were used as a negative control. A decrease in glutathione reductase enzyme activity was observed with the increasing concentrations of carvacrol.

4.3.5) Glutathione transferase

The GST is a key antioxidant enzyme, which helps in the detoxification of foreign agents. In this study, a decrease was observed in GST activity when *Candida* cells were exposed to increasing concentrations of carvacrol. The average *C. auris* control GST activity was found to be 5.31×10^{-5} µmol glutathione conjugated/minute where the GST activity within untreated *C. albicans* was 9.48×10^{-5} µmol glutathione conjugated/minute (Figure 4.6). *Candida auris* exposure to carvacrol with 0.5 MIC and MIC values decreased from $5.31 – 27.97 \times 10^{-5}$ to $0.59 – 6.29 \times 10^{-5}$ µmol glutathione conjugated/minute, respectively. With exposure to carvacrol,

an **overall** percent decrease of 52.04 % (0.48 fold decrease) for 0.5 MIC and 76.39 % (0.24 fold decrease) for MIC in enzyme activity was observed for *C. auris*. In *C. albicans* SC5314, exposure at 0.5 MIC showed a decrease of GST activity from 6.88×10^{-5} µmol glutathione conjugated/minute to 3.09×10^{-5} µmol glutathione conjugated/minute when exposed to MIC carvacrol (Figure 4.6).

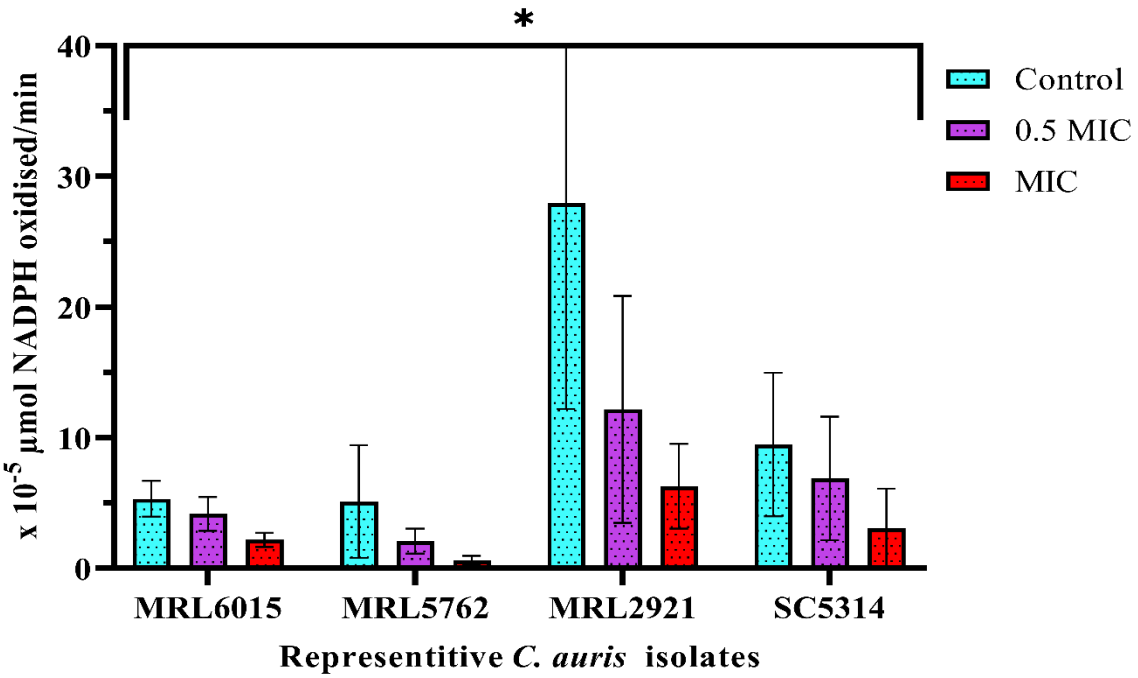

Figure 4.6: Glutathione transferase enzyme activity. *Candida auris* cells were exposed to 0.5 Minimum Inhibitory Concentration and Minimum Inhibitory Concentration of carvacrol. Untreated cells were used as a control. A decrease in GST enzyme activity was observed when cells were exposed to different concentrations of carvacrol.

4. 4) Gene expression of antioxidant enzymes in *C. auris*

It is evident from the above results that carvacrol has significant effect on the activity of different antioxidant enzymes in *C. auris*. In order to study whether the effect is also at the genetic level, mRNA expression of antioxidant genes (*CATA, SOD4, GPX3, GST1, GSHR* and *ACT1*) in untreated (control) and carvacrol treated (at MIC), *C. auris* isolates was studied by means of RT-qPCR. The RNA of untreated and treated cells were extracted and quantified, followed by cDNA synthesis and quantification. Primers were specifically designed for this investigation and the determination of the optimal annealing temperatures were obtained by means of gradient PCR. Thereafter, RT-qPCR was performed and the gene expression fold changes were calculated for each gene.

4.4.1) RNA and cDNA quantification

The integrity and purity of the total RNA extracted from each sample was assessed using a Nanodrop 2 000 Spectrophotometer. The RNA yielded was of high quality with adequate concentrations (60.2 – 288.1 ng/µl) and the purity (A_{280}/A_{260} ratio) was greater than 2. With these results, it was acceptable to continue with cDNA synthesis as 100 x 10^{-7} – 1 µg of total RNA template was required for this procedure. The integrity and purity of the synthesized cDNA products were also assessed using a Nanodrop 2 000 Spectrophotometer. The cDNA yielded concentrations (1 469.7 – 2 606.4 ng/µl) that were adequate and the purity (A_{260}/A_{280} ratio) was in the range of 1.7 – 2. With these results, it was acceptable to continue with PCR as 100 ng/µl of cDNA template was required for this procedure and further downstream applications.

4.4.2) Determination of the optimal annealing temperature of primers

Primers were specifically designed for this project and therefore their annealing temperatures were requir. The primer melting temperatures ranged from 56 – 63 °C, as indicated by the manufacturer. The optimal annealing temperature for each primer pair was determined to be 55 °C with the use of agarose gel electrophoresis (Figure 4.7).

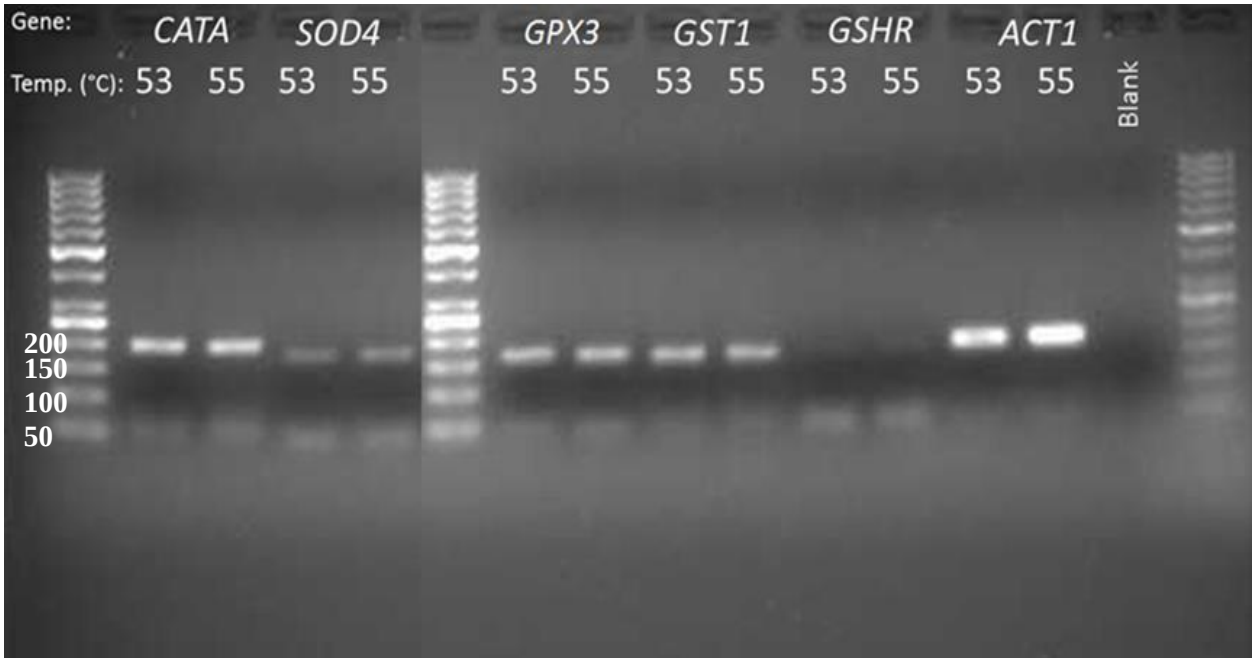

Figure 4.7: Agarose gel electrophoresis image for the gradient PCR of antioxidant enzyme primers in *Candida auris*. The optimal annealing temperature for all antioxidant enzymes including one housekeeping gene (actin - *ACT1*) was established as 55 °C. The antioxidant genes; catalase (*CATA*), superoxide dismutase (*SOD4*), glutathione peroxidase (*GPX3*), glutathione transferase (*GST1*) and glutathione reductase (*GSHR*), were specifically designed for this investigation. A 50 bp molecular weight marker (Fermentas, USA) was utilised as a DNA ladder.

4.4.3) Effect of carvacrol on the gene expression of antioxidant enzymes

The transcription profile of genes encoding critical oxidative stress enzymes (*CATA, SOD4, GPX3, GST1* and *GSHR*) was determined with the use of RT-qPCR. Data analysis was performed with the replicates of carvacrol treated and untreated samples. The genes encoding the primary enzymes (*CATA, SOD4* and *GPX3*) were found to have increased fold expression ranging from 1.18 to 2.6, whereas the genes encoding the secondary enzymes (*GSHR* and *GST1*) were found to have decreased fold expression of 0.93 and 0.66 respectively. Among the genes that displayed an increase of expression, the most significantly expressed gene was *CATA* with a fold expression increase of 2.6. The gene that displayed the lowest level of expression was *GST1* with a fold expression decrease of 0.66. A summary of the difference in fold change is displayed in Figure 4.8.

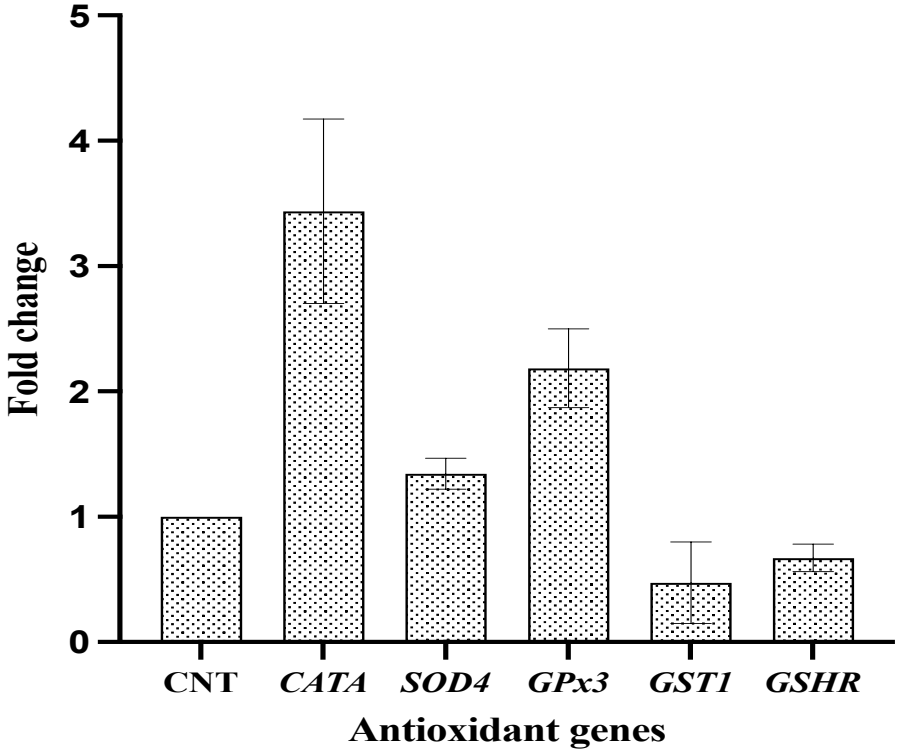

Figure 4.8: Antioxidant gene expression in carvacrol exposed *Candida auris* isolates. Overall fold change in antioxidant gene expression of carvacrol treated *C. auris* isolates. Total RNA was isolated from carvacrol treated (MIC) *C. auris* isolates where 1 µg of RNA was used for cDNA synthesis. Certain *C. auris* antioxidant genes were analysed following quantification by RT-qPCR using primers specific for *CATA, SOD4, GPX3, GST1* and *GSHR* target genes. CNT represents a controlhousekeeping gene.

4. 5) Haemolysis Assay

Based on the above results, it is evident that carvacrol has potent antifungal activity and effects the antioxidant enzyme expression in *C. auris*. Therefore, it was crucial to determine the cytotoxic effects of carvacrol. In this study, *in vitro* cytotoxicity of carvacrol was determined by haemolytic assay using horse red blood cells (RBCs). From the results, it was observed that the effect of carvacrol on horse RBCs is minimal at different concentrations tested in comparison to the Triton-X, which was used a positive control (Figure 4.9). At MIC concentrations (125, 250 and 500 µg/ml) of carvacrol, only 2.8 %, 3.6 % and 6.4 % haemolysis was observed. These *in vitro* results advocate that carvacrol is safe to use for *in vivo* studies using animal models.

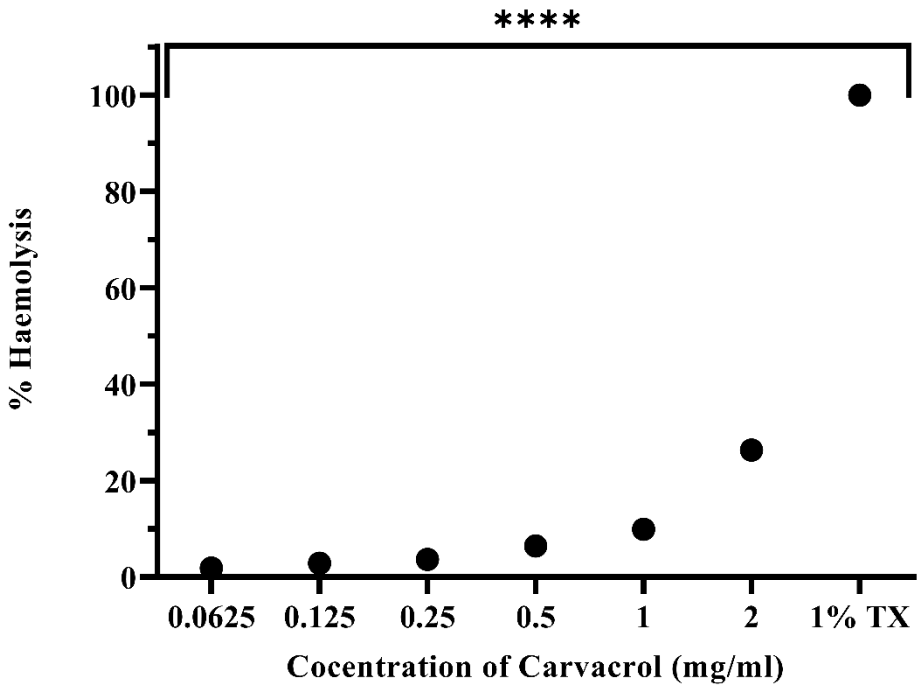

Figure 4.9: Cytotoxicity assay of carvacrol by haemolytic activity. Carvacrol at varying concentrations (0 – 2 mg/ml) showing different percentages of haemolysis in horse red blood cells compared to positive control (1 % Triton X-100).

Chapter 5) Discussion

Candida auris has become globally prevalent in the past decade since its discovery in 2009 and this prevalence is continuously increasing around the world (Lone and Ahmad, 2019). With high prevalence and mortality rates, this multi-drug resistant pathogen renders several available commercial antifungal drugs inefficient. Therefore these drugs are ineffective in combating the spread of these severe infections. Additionally, investigations have documented that *C. auris* strains from different regions have variations, phenotypically and genomically (Lone and Ahmad, 2019). This further emphasizes the urgency to develop a novel drug to promote the prevention and treatment of *C. auris* infections. Accordingly, the rush to discover innovative but viable and effective therapeutic drugs has gained traction. However, the development of a new antifungal drug is significantly slow-going and challenging due to the fact that these fungal cells are eukaryotic and have a high resemblance with the human host cells. Furthermore, as little is known about the characteristics and mechanisms of *C. auris*, drug development against this pathogen is even more complicated. A good start would be to delve into examining and comprehending the pathogenicity of this organism by thoroughly investigating the mode of actions used to overcome antifungal agents, for example; overproduction of the target enzymes, target alteration, efflux pumps, entry prevention, bypass pathways, drug degradation, etc. Thereafter, new potential intra/extracellular components of the fungus can be considered as targets for new, more effective antifungal drugs with the intention to overcome drug resistance. Hitherto, phytochemicals are proving to be propitious potentials for novel antifungal agents. Nevertheless, it is vital to be aware of the implemented mechanism of action that these natural compounds utilize in order to evade the potential of fungal resistance development.

This study investigated the effect that carvacrol ($C_{10}H_{14}O$), a phenolic phytochemical, has against *C. auris* isolates of the South African clade. Furthermore, we also screened for the presence of different antioxidant defence enzymes in these isolates and determined the regulation of expression and activity of these enzymes by carvacrol. Numerous studies have reported the antifungal activity of carvacrol, including the effect on the oxidative stress response, of *C. albicans* and various NAC species (Ahmad *et al*., 2011; Sharifzadeh *et al*., 2019; Chaillot *et al*., 2015; Marchese *et al*., 2018; Niu *et al*., 2020). The inhibitory and fungicidal efficacy of carvacrol on *C. auris* proved to be variable in this study. The production of certain antioxidant enzymes was confirmed and thereby a

significant effect of carvacrol on the activity of these enzymes as well as their gene regulation was shown in this study. These results can be supported by previous studies which investigated the effect of carvacrol and other natural compounds on different NAC species (de Oliveira Santos *et al*., 2018; Ahmad *et al*., 2011).

5. 1) Carvacrol activity against *Candida auris* cells

The antifungal activity of a compound is determined by antifungal susceptibility testing which defines the optimum therapeutic concentration of the compound of interest on a certain fungus, as well as the antifungal drug resistance (Rex *et al.,* 2001). An MIC assay has been standardized by the Clinical Laboratory Standards Institute (CLSI) using the microdilution method which is the gold standard technique to determine antifungal susceptibility. The CLSI have defined MIC breakpoints of commercial antifungal drugs and agents in order to group susceptible and resistant fungal strains. This is performed by growing the fungus in known drug dilutions over time and thereafter measuring the fungal growth. Regrettably, these breakpoints are not always accurate as they do not consider *in vivo* response, yet clinicians still rely on these to administer the appropriate concentration of antifungal drug. For *C. auris* infections, owing to inadequate clinical data, the CLSI have recommended certain susceptibility breakpoints for particular antifungal drugs based on the susceptibility of closely related *Candida* species (CLSI, 2008).

The treatment options for *C. auris* infections are narrow owing to its antifungal drug resistance leading to higher mortality rates due to inadequately treated invasive infections (CDC, 2019). Higher MIC values of fluconazole and amphotericin B have been revealed for a large quota of *C. auris* isolates (Shaban *et al*., 2020). As such, echinocandins have been recommended as the first-line of therapy for the treatment of *C. auris* infections (CDC, 2016). However, isolates with an increased echinocandin MIC value raise the concern of the potential of acquired resistance development during the course of treatment (Cortegiani *et al*., 2018). Hence, it is a growing necessity to ascertain novel and effective antifungal agents in order to successfully combat this persistently vicious pathogen.

Phytochemicals showed potential through their useful therapeutic characteristics including antifungal effectiveness. A recent study investigated the antifungal resistance of *C. auris* against four antifungal drugs as well as efficacy of carvacrol and other phenolic monoterpenoids on

C. auris (Shaban *et al*., 2020). Whereas, *C. albicans* showed susceptibility to all the four drugs, *C. auris* was highly resistant to two antifungal drugs (fluconazole and nystatin) and most susceptible to the echinocandin, caspofungin. All four of the investigated monoterpenoids (eugenol, methyleugenol, carvacrol and thymol) displayed varying antifungal activity against *C. auris* strains with carvacrol being the most potent having the lowest MIC values (Shaban *et al*., 2020). Furthermore, the clinical potential of carvacrol has been thoroughly investigated (Klein *et al*., 2013; Costa *et al*., 2015; Sharifi-Rad *et al*., 2018). Carvacrol displays a wide bioavailability that mainly emphasises its antimicrobial, antioxidant and anticancer properties (Sharifi-Rad *et al*., 2018). However, the oxidative effect that carvacrol might have on the multi-drug resistant *C. auris* has not yet been investigated. The present study investigates the antifungal potential of carvacrol along with its effect on antioxidant enzyme activities of a variety of *C. auris* isolates.

The MIC of carvacrol against *C. auris* has been reported as 125 µg/ml in various investigations (Shaban *et al*., 2020; Kumar *et al*., 2020). The present study determined a carvacrol MIC range of 125 - 500 µg/ml and an MFC range of 250 to 1 000 µg/ml against *C. auris* isolates. These results concur with previous findings where carvacrol has been reported to have antifungal activity with similar MIC and MFC values (de Oliveira Santos *et al*., 2018; Ahmad *et al*., 2011; Shaban *et al*., , 2020; Kumar *et al*., 2020).

The antifungal activity of carvacrol could be owed to the presence of a free hydroxyl group on its aromatic ring (de Oliveira Santos *et al*., 2018). Carvacrol has a weaker antifungal activity against *C. albicans* than seen for some *C. auris* isolates (Doke *et al*., 2014). This could potentially indicate that *C. auris* has an increased sensitivity to carvacrol, which might be owed to the smaller cell size and low fractions of cell wall proteins when compared to *C. albicans* that causes an effect on the chemical interaction between carvacrol and the fungal cell (Navarro-Arias *et al*., 2019). Carvacrol causes fungal cell death by the disruption of membrane integrity which results in an increased cell permeability (Ahmad *et al*., 2010).

The *C. auris* isolates investigated in this study were grouped based on their MIC values displayed in Table 1 (group A - 125 µg/ml; group B – 250 µg/ml and, group C - 500 µg/ml) and one isolate from each group (MRL6015, MRL5762 and MRL2921 respectively) was used for further experimental procedures.

5. 2) Lipid Peroxidation

Oxidative stress is caused by the disproportion of ROS generation and detoxification within a cell. The production of ROS occurs by standard physiological processes and plays vital roles within cell signalling. However, detrimental modifications of cell components (i.e. lipids, proteins and DNA damage) as well as amplified pathogenesis can occur by the overproduction of ROS (Schieber and Chandel, 2014). The excess production of ROS can be caused by internal factors, such as depletion of glutathione and reduced antioxidant enzyme activity, or by external factors such as drug treatment (Bhattacharyya *et al.*, 2014). Polyunsaturated fatty acids (PUFAs), highly concentrated within cellular and organelle membranes, are particularly vulnerable to ROS damage, called lipid peroxidation (LPO) (Ayala *et al.*, 2014).

In vitro studies have elucidated the mechanisms, dynamics and products of LPO fairly well. Conversely, the biological effects as well as the physiological levels of LPO and its products have not been well investigated as yet (Niki, 2014). The process of LPO occurs when potentially toxic reactive intermediates are produced by the removal of electrons from lipids by free radical species. Furthermore, the integrity, fluidity, permeability and function of biomembranes are lost and fine structures are disturbed (Niki, 2014; Niki, 2009). Since LPO directly impairs phospholipids, it can act as an oxidative stress signal as well as a signal for cell death (Su *et al*., 2019).

The most inevitable expression of oxidative stress brought about by ROS is LPO (Khan *et al*., 2015). The main products of LPO are the 3-carbon dialdehyde species malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), whereby MDA can be spectrophotometrically measured by the thiobarbituric acid reactive substances (TBARS) assay. The production of these lipid peroxides deforms the structure of the bilayer and overall functionality of the membrane. This lipid damage is represented by the build-up of thiobarbituric acid (TBA) reactive substances (TBARS) in the cytoplasm.

The TBARS and MDA are appropriate biomarkers of LPO and have been utilized as standard biomarkers of LPO *in vivo* for over 30 years (Grotto *et al.*, 2014). These markers are frequently utilized owing to simplicity and low cost. Regardless of the specificity dearth and post-sampling formation issues, they continue to be the most regularly applied indicators of oxidative damage. The TBARS assay spectrophotometrically measures the reaction of MDA with TBA under strong

acidic condition and heating (Niki, 2014). To further support the induction of oxidative stress endured by *C. auris* when exposed to carvacrol, the TBARS method provided evidence of an increase in LPO. In comparison with the untreated *C. auris* cells, on average, the quantity of TBARS formed increased nearly 1.5 fold at 0.5 MIC carvacrol and nearly 2 fold at MIC carvacrol. (Figure 4.1). This study proves that exposure to carvacrol causes LPO in *C. auris* strains which is indicative of membrane disintegration. These observations are in accordance with previous findings of antifungal activity testing which investigated the LPO in other *Candida* species (Khan *et al*., 2015). This is the first study to provide attestation of LPO induced by carvacrol in *C. auris*.

5. 3) Regulation of antioxidant enzymes and genes by carvacrol in *C. auris*

All aerobic organisms constantly produce and are exposed to ROS at controlled levels. However, the elevation of the concentration of ROS beyond the fragile antioxidant capacity within an organism can be detrimental, subjecting the cell to oxidative stress (Bhattacharyya *et al.*, 2014). Some conventionally administered antimicrobial drugs stimulate endogenous ROS production which results in apoptosis (Delattin *et al*., 2014; Mesa-Arango *et al*., 2014). Consequently, oxidative stress can begin a downstream cascade of intracellular events that cause structural and molecular modifications, such as DNA disorganization and lipid oxidation. Additionally, oxidative stress is also a uniquely sensitive predictor of cell death.

The concentration of oxidants within a cell are regulated by several free radical scavenging enzymes which control ROS levels by detoxification and serve as a defence mechanism against oxidative attack (Lobo *et al.*, 2010). An undeviating reflection of internal oxidative stress is observed by the activation of these antioxidant enzymes (Kaloriti *et al*., 2014). The activity of these enzymes serve as a defensive mechanism utilized to; survive in hostile environments, thrive and establish infections, promote drug tolerance and biofilm-associated persistence (Linares *et al*., 2006; Belenky and Collins, 2011; Bink *et al*., 2011; Van Acker *et al*., 2013; Kaloriti *et al*., 2014). Furthermore, transformation of the antioxidant defense system can arise on activation of antioxidant enzymes in response to particular stress conditions. Accordingly, precise mechanisms have been employed in response to ROS for tolerance, survival, and virulence.

The development of drug resistance could be owed to the accumulation of ROS induced by some classes of antifungal drugs, such as azoles (Bink.*et.al*.,.2011; Ferreira.*et.al*.,.2013). The response

to oxidative stress is a key mechanism of defence in *C. albicans*. In order to successfully endure stressful conditions and to express virulence and cause pathogenesis, the mediation of oxidative resistance is of vital importance (Ahmad *et al*., 2016). Consequently, these virulence properties and markers show potential as novel therapeutic drug targets owing to their participation in the progression of *Candida* infections. Therefore, instead of exterminating the pathogen from the host's system, the aim of this approach would be to attack and deactivate the specialised defence armoury of the invading *Candida* resulting in the eventual deceleration in the progression of the infection (Cegelski *et al*., 2008).

Antioxidant enzymes are the main line of defence against oxidative stress. These enzymes are categorised into primary, secondary, and tertiary antioxidants based on their mechanisms of action (Aziz *et al.*, 2019). Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) are ROS scavenging enzymes that minimise the damaging effects of produced ROS throughout the free radical reaction. A precursor for most ROS is the superoxide anion radical (O^{2-}) which is also an intermediary of oxidative reactions. The SOD induces free radical dismutation and produces less toxic hydrogen peroxide (H_2O_2) that is reduced by CAT and GPx to water (H_2O) and oxygen (O_2) (Isakova *et al*., 2020; Lee *et al*., 2019).

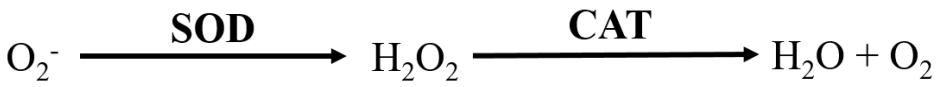

Figure 5.1: The reduction of reactive oxygen species by catalase and superoxide dismutase.

Secondary antioxidant enzymes, such as glutathione reductase (GR) and glutathione-S-transferase (GST), continuously supply glutathione (GSH) and nicotinamide adenine dinucleotide phosphate (NADPH) to the primary antioxidant enzymes (Ahmad *et al*., 2016). An increased level of ROS within aerobic organisms induces the activity of the primary antioxidant enzymes, which are the first line of defence against oxidative stress (Angelova *et al*., 2005). The fundamental oxidative resistance and stress adaptation in *C. albicans* is owed to the upregulation of the gene expression of these chief antioxidant enzymes (Kaloriti *et al*., 2014). They act by metabolizing ROS before it causes permanent damage to a cell.

Candida auris has been found to display an increased tolerance to oxidative stress when compared to *C. albicans* (Pathirana *et al*., 2018). This ROS resistance appears to be a significant factor to terminate *C. auris*. Regrettably, the antioxidant stress enzymes and oxidative stress defence mechanism has not been well investigated or reported. However, this study shows that carvacrol appears to have a noteworthy effect on the antioxidant enzyme activity of *C. auris* after exposure at applicable concentrations (0 – 500 μg/ml) and the necessary treatment time. Previous mechanistic insights revealed that carvacrol exerts its antifungal activity by disrupting endoplasmic reticulum (ER) integrity and other intracellular vesicular trafficking (Chaillot *et al*., 2015). Disruptions of the ER have an effect on multiple features of the membrane such as permeability, ergosterol biosynthesis and other lipid content which in turn evidently affects other cellular mechanisms and induces stress.

This study revealed that, in comparison with the untreated *C. auris* cells, strains exposed to carvacrol showed an increase in gene expression and activity of the primary enzymes. Conversely, a decrease in gene expression and activity of the secondary enzymes was observed. The enzyme quantities of CAT, SOD and GPx increased with increasing carvacrol concentration (0 – 500 μg/ml). An overall fold increase of 2.39 (CAT), 1.46 (SOD) and 1.89 (GPx) was observed at 0.5 MIC, where a fold increment of 7.76 (CAT), 1.93 (SOD) and 2.79 (GPx) at MIC was observed for the activity of primary antioxidant enzymes (Figures 4.2, 4.3 and 4.4). Additionally, the genes of these primary enzymes (i.e. *CATA*, *SOD4* and *GPX3*) also showed an increased fold expression ranging from 0.18 to 1.6 at MIC (Figure 4.8). The secondary enzymes, GR and GST, showed an overall fold decrease in enzyme activities when exposed to 0.5 MIC (0.79 fold and 0.5 fold) and MIC (0.46 fold and 0.21 fold) carvacrol, respectively (Figures 4.5 and 4.6). Furthermore, it was noted that a decrease fold expression of the genes of these secondary enzymes (i.e. *GSHR* and *GST1*) were 0.07 and 0.34 respectively (Figure 4.8). Therefore, the present results clearly signifies that exposure with carvacrol resulted in building oxidative stress in *C. auris* strains and inspite of increased gene and protein expression of primar antioxidant enzymes, critical for combating oxidative stress the *C. auris* cells were not able to survive. These observations are in accordance with previous findings of antifungal activity testing which investigated these enzymes in other *Candida* species (Lee *et al*., 2019; Sun *et al*., 2017; Abegg *et al*., 2012; Guirao-Abad *et al*., 2017; Maras *et al*., 2014; Owsiak *et al*., 2010; de Olieveira *et al*., 2013; Mahl *et al*., 2015).

Candida albicans has several signalling components, transcriptional regulatory factors, and antioxidant enzyme systems in order to defend cells from oxidative stress (Lee *et al.*, 2019). The SOD, CAT and GPx enzymes represent the antioxidant system (Lee *et al.*, 2019). These enzymatic antioxidants are able to decompose ROS and therefore provide sufficient protection against oxidative attack. Superoxide produces the most ROS which renders SOD vital for living cells (Khan *et al*., 2011). This enzyme acts as a primary antioxidant enzyme which eases the effects of ROS by the catalysing the translation of superoxide into oxygen and hydrogen peroxide (Khan *et al*., 2011). The SOD is therefore considered as a key antioxidant enzyme in the regulation of ROS build-up in living organisms. This study revealed an average of 1.18 and 1.93 fold increase in mRNA expression of *SOD4* and enzyme activity, respectively; in *C. auris* isolates after being treated with carvacrol. The rise in the gene expression of SOD, within *C. albicans*, has been noted in previous investigations (Khan *et al*., 2015, Poopedi *et al*., 2020; Lee *et al*., 2019). However, *SOD4* was the least expressed primary antioxidant gene in this study and, the antioxidant enzyme that showed the least activity.

The decomposition of H_2O_2 in most fungal cells is due to the chief H_2O_2 scavenging enzymes, CAT and GPx (de Olieveira *et al*., 2013). The CAT enzyme significantly reduces oxidative stress by converting hydrogen peroxide into molecular oxygen and water (Su *et al*., 2019). This study revealed an average of 2.6 and 7.16 fold increase in mRNA expression of *CATA* and activity of CAT, respectively: in *C. auris* isolates. Additionally, *CATA* was the most significantly expressed antioxidant gene in this study and the antioxidant enzyme that showed the most activity. Multiple studies have revealed the overexpression of CAT in *C. albicans* and other NAC species as it is considered a major H_2O_2 scavenging enzyme in most fungal cells (Yousuf *et al*., 2010; Khan *et al*., 2011; Sun *et al*., 2017; Román *et al*., 2016; Lee *et al*., 2019). However, this is the first study to investigate the expression of CAT in *C. auris*. CAT displayed the highest level of enzymatic activity as well as gene expression demonstrating the significance of CAT when a cell is under oxidative stress.

Persistent oxidative stress results in the expression of GPx which becomes the main H_2O_2-scavenging enzyme (Su *et al*., 2019). After *C. auris* isolates were subjected to treatment with carvacrol, a significant increase in the mRNA expression of *GPX3* and enzyme activity of GPx was observed in this study. The GPx enzyme was among the most active antioxidant enzymes to

show significant activity in *C. auris* treated cells. By using glutathione as an electron donor, GPx reduces the harmful oxidizing agent hydrogen peroxide to water and molecular oxygen. It is evident from this study that, even though CAT can drive the reaction that degrades H_2O_2, GPx is essential for the maintenance of H_2O_2 content when there is an overload within a system. The results from this study support that GPx is activated in the antioxidant defence response as a somewhat "back-up" for CAT against stress induced by carvacrol. It is therefore suggested that low expression of *SOD4* may be correlated to the increased *CATA* and *GPX3* expression.

The secondary enzymes (glutathione S-transferase (GST) and glutathione reductase (GR)), also play a significant role within the antioxidant defence system in *C. auris*. The intracellular glutathione redox ratio of GSH/GSSG is maintained either by GR which recycles GSH from GSSG or by S-conjugates which utilize membrane efflux pumps to export and deplete glutathione. Additionally, GST sustains the detoxification of foreign compounds by driving the spontaneous conjugation of reduced glutathione (GSH) and xenobiotics (Gostimskaya and Grant, 2016). These supplementary enzymes are vital to the survival of yeast cells that are subject to oxidative stress (Gostimskaya and Grant, 2016). The imbalance of the GSH redox state is implicated in severe cell damage and reduced virulence (Guedouari *et al*., 2014). In order to preserve cellular homeostasis, the GSH dependent enzymes regulate the level of GSH. However, the observations from this study exhibited a downregulation of GST and GR enzyme activities with a 4.88 and 2.18 fold decrease after carvacrol treatment. Furthermore, a downregulation of these genes were detected where a 0.66 and 0.93 fold change in expression was observed for *GST1* and *GSHR* gene expression, respectively after carvacrol treatment. The observed reduction of GST and GR activity could be due to the fact that carvacrol is a lipophile which has the ability to conjugate with GSH (Khan *et al*., 2015). Furthermore, carvacrol probably disturbs the GSH/GSSG ratio by raising the intracellular GSSG level. Nonetheless, oxidation by ROS seems to be the cause of the GSH level decline. The results of this study are in agreement with previous findings that have described the reduction of GR and GST (González-Párraga *et al*., 2003).

The induction of oxidative stress by carvacrol treatment in *C. auris* is characterised by the increase in activity as well as genetic expression of primary antioxidant enzymes (CAT/*CATA*, SOD/*SOD4*, and GPx/*GPX3*) and a decrease of secondary antioxidant enzymes (GST and GR) activity and gene expression (*GST1* and *GSHR*). Carvacrol triggered effective oxidative stress in *C. auris* resulting

in a clear antioxidant response by the production of antioxidant enzymes. This proves that carvacrol has the ability to render *C. auris* antivirulent by deforming its antioxidant defence response by causing the primary enzymes as insufficient to combat oxidative stress. Furthermore, carvacrol could serve as a potent ROS-inducing agent and aid in overcoming antifungal drug resistance observed in *C. auris* by disrupting the antioxidant defense system. This strategy has the potential to significantly lower the cost and risks of severe side effects when treating infected patients. To the best of our knowledge, this is the first study to evaluate the effect of carvacrol on the antioxidant defence system in *C. auris* as a potential antifungal target.

5. 4) Cytotoxicity of carvacrol

In vitro studies of carvacrol have proven its interference with microorganism physiology by diverse mechanisms of action. It meddles with membrane functions (i.e. depolarization and permeability), production of virulence factors, fragmentation of DNA, formation of bacterial and fungal biofilms as well as induces apoptosis (Marchese *et al*., 2018; Srivastava *et al*., 2020). Furthermore, this study has shown that carvacrol has the ability to induce oxidative stress and lipid peroxidation in *C. auris*. However, the effect of carvacrol *in vivo* might have adverse effects on the host when being administered to terminate a fungal infection. Accordingly it was considered imperative to determine the cytotoxicity of this compound. A previous investigation has provided evidence that carvacrol shows a lower cytotoxcity in eukaryotic cells in comparison to conventional antifungals and other natural compounds namely cinnamaldehyde and thymol as well as other antiseptics such as chlorhexidine (García-Salinas *et al*, 2018).

This study utilised horse red blood cells (RBCs) in a haemolytic assay in order to determine the cytotoxicity of carvacrol at varying concentrations (i.e. 0 – 1 000 µg/ml). Triton X-100 was used as a positive control that causes 100 % haemolysis. Carvacrol displayed cell haemolysis with a range of 2.1 % to 36.3 % (Figure 4.9). These results indicate that at low concentrations carvacrol is much less toxic when compared to triton X-100. Therefore, future *in vivo* animal studies could utilize these findings in order to combat *C. auris* infections. These results are in accordance with former examinations (Ahmad *et al*., 2011). Previous investigations have also reported that carvacrol has been observed to have no cytotoxic effect on human cells at 250 µg/ml (Shaban *et al.*, 2020; Bandeira Junior *et al*., 2018). Additionally, carvacrol is currently used as a flavouring agent in food products (beverages, sweets, chewing gum, condiment relish, frozen dairy) and has

been classified by the Food and Drug Administration among the 'Generally Recognized As Safe (GRAS substances' (Marchese *et al*., 2018). All these factors advocated the use of carvacrol for further *in vivo* studies.

5. 5) Clinical implications

Carvacrol has exhibited its inhibitory effect against many *Candida* species, and now also to *C. auris*. This study confirms that carvacrol has the ability to induce oxidative stress in *C. auris*, which will result in a reduced number of cells and potential termination of this fungus. This study, in accordance with many others, proves the preventive and therapeutic potential exhibited by this naturally occurring monoterpene against *C. auris* colonization and infections. However, further research is necessary for the administration to human patients with confirmed *C. auris* infections.

5. 6) Limitations

The foremost limitation of this study was the limited number of *C. auris* isolates investigated. A greater numbers of *C. auris* isolates are required to accurately determine carvacrol antifungal breakpoints and the efficacy of carvacrol on the virulence of *C. auris*. In addition, higher intensity *in vivo* investigations are required alongside *in vitro* studies in order to precisely establish the internal effect of carvacrol, both on *C. auris* as well as the host.

Moreover, the virulence of the isolates utilized within this study could have been compromised due to the fact that the experimental strains were revived from previously frozen isolates. This could result in discrepancies when investigating the *in vivo* effect of carvacrol on these isolates.

Continued research should be driven by these limitations in order to ascertain the virulence characteristics of *C. auris* as well as the veiled potential of carvacrol as an antifungal agent. This investigation only reveals the tip of the mechanisms of pathogenicity displayed by the newly emerged, persistent *C. auris* and the promising potential that carvacrol has against this multi-drug resistant fungal pathogen.

5. 7) Future research

Owing to the different clades of *C. auris* identified from different geographical regions, investigations on the effect of carvacrol on.the diversity of *C. auris* strains will present invaluable information on the overall virulence, pathogenesis and resistance of *C. auris* as well as the prospect

of drug development that could include carvacrol. The phenotypic and genotypic characteristics of *C. auris* can also be cumulated which could be utilized in innumerable studies.

Possible investigations include:

- The *in vivo* and *in vitro* study of the efficacy of carvacrol on *C. auris* from diverse sites of infection. This would provide knowledge on the *in vivo* effect of carvacrol on *C. auris* as well as the patient response to administration of carvacrol.
- Irrespective of carvacrol being regularly utilized as a flavoring agent in food products, as well as the observed non-toxicity rates, it is imperative to confirm the safety, cytotoxicity and required concentrations of carvacrol before being administered to immunocompromised patients with a *C. auris* infection.
- Comparative *in vitro* and *in vivo* investigations are required to determine the efficacy and optimal concentration of carvacrol when used with or as an antifungal drug in clinical settings.

Chapter 6) Conclusion

Candida auris mainly establishes infections in immunocompromised hospital patients. Due to limited options for the treatment of *C. auris* infections in these individuals, there is a crucial need for an alternative antifungal strategy to overcome the drug resistance exhibited by *C. auris*. Since the anti-virulence effect of carvacrol on *C. albicans* has already been reported (Hosseini *et al*., 2016), this investigation determined the effect of carvacrol on a certain virulence attributes of *C. auris*. This study investigated the oxidative effect of carvacrol on 3 different *C. auris* isolates of a South African clade. All isolates showed susceptibility to carvacrol with an MIC ranging from 125 – 500 µg/ml and an MFC range of 250 – 1 000 µg/ml. Carvacrol successfully induced oxidative stress in *C. auris* at MIC as seen by an increase in LPO and the activity of primary antioxidant enzymes as well as the upregulation of their gene expression (catalase, superoxide dismutase and glutathione peroxidase). Cytotoxicity studies have proven that carvacrol poses no threat to mammalian cells.

In conclusion, the results from this analysis further advocates that the phytochemical carvacrol could be a potential antifungal drug as it efficiently inhibits the growth of multi-drug resistant *C. auris* isolates. These finding suggest that carvacrol is a promising antifungal. To the best of our knowledge, this is the first study that has inspected the effect of carvacrol on oxidative stress and LPO in *C. auris*. The data from this study can be useful for further experimentation to develop appropriate prescriptions and establish concentrations which would be most advantageous in a clinical setting to prevent and treat candidiasis caused by *C. auris*.

Chapter 7) References

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Chapter 8) Appendices

Appendix A: Ethical Clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

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

TO: Mlles M/V Ismail/Srivastava
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FROM: Iain Burns
Human Research Ethics Committee (Medical)
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E-mail: Iain.Burns@wits.ac.za

DATE: 04/11/2019

REF: *R14/49*

PROTOCOL NO: W-CBP-191103-01 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: *Expression and activity of antioxidant enzymes in Candida auris and their regulation by carvacrol*

Please find attached the Ethics Waiver Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/ClearScanWaiver.wps

Appendix B: Reagents

Appendix B1: Carvacrol dilutions

Appendix B1.1: 100 mg/ml carvacrol

100 µl of 100 % DMSO (Sigma-Aldrich, USA) was diluted in 9.9 ml of sterile distilled water to make 1 % DMSO. This solution was utilised for further dilutions in order to prepare further working solutions. The solution was stored at 4 ºC.

512.3 µl of 976 mg/ml carvacrol (Sigma-Aldrich, USA) was diluted in 1 000 µl of 1 % DMSO. Thereafter, 3 487.7 µl of sterile distilled water was added to make up a 100 mg/ml carvacrol solution.

Appendix B1.2: 8 mg/ml carvacrol

0.8 ml of the 100 mg/ml stock carvacrol was diluted with 3.ml of 1 % DMSO and.made up.to 10 ml with 6.2 ml of sterile distilled water to make an 8 mg/ml carvacrol solution. The solution was stored at 4 ºC

Appendix B2: Amphotericin B dilution

0.64 ml of the 125 µg/ml stock amphotericin B (Sigma-Aldrich, USA) solution was diluted with 1.ml of 1 %.DMSO and made up to.10 ml with 8.36 ml of sterile distilled water to make an 8 µg/ml Amphotericin B solution. The solution was stored at - 20 ºC.

Appendix B3: Sucrose

8.587 g of sucrose (Merck, RSA) was dissolved in 100 ml of sterile distilled water to make a 250 mM sucrose solution. The solution was stored at - 20 ºC.

Appendix B4: Trizima base buffer

12.1 g of Trizima (Sigma-Aldrich, USA) was dissolved in 80 ml of sterile distilled water. Hydrochloric acid (HCl; Merck, RSA) was used to adjust the pH. Thereafter, sterile distilled water was added to make up the 100 ml 1 M tris base solution. This solution was utilised for further dilutions in order to prepare further diluted solutions. The solution was stored at room temperature.

Appendix B4.1: 10 mM Trizima

0.5 ml of 1 M Trizima (Sigma-Aldrich, USA) was added to 50 ml of sterile distilled water to make a 10 mM tris base solution. The solution was stored at room temperature.

Appendix B4.2: 50 mM Trizima

2.5 ml of 1 M Trizima (Sigma-Aldrich, USA) was added to 50 ml of sterile distilled water to make a 50 mM tris base solution. The solution was stored at room temperature.

Appendix B5: Phenylmethane sulfonyl fluoride (PMSF)

0.0174 g PMSF (Roche, Germany) was added in 10 ml 2-propanol (Merck, RSA) to make a 1 mM PMSF solution. The solution was stored at - 20 ºC.

Appendix B6: Phosphate buffer

2.8055 g of potassium hydroxide (KOH; Merck, RSA) was dissolved in 250 ml of sterile distilled water to make a 200 mM KOH solution. The solution was stored at room temperature.

3.402 g of potassium phosphate monobasic (KH_2PO_4; Sigma-Aldrich, USA) was dissolved in 250 ml sterile distilled water to make 100 mM of phosphate buffer. The 200 mM KOH was utilized to adjust the pH accordingly. This solution was utilised for further dilutions in order to prepare further diluted solutions. The solution was stored at room temperature.

250 ml of the 100 mM phosphate buffer was diluted in 250 ml sterile distilled water to make 50 mM phosphate buffer. The pH was adjusted accordingly with hydrochloric acid (HCl) and KOH. The solution was stored at room temperature.

Appendix B7: Trichloroacetic acid (TCA)

5 g of trichloroacetic acid (TCA; Sigma-Aldrich, USA) was dissolved in 50 ml of sterile distilled water to make a 10 % TCA solution. The solution was stored at room temperature.

Appendix B8: Thiobarbituric acid (TBA)

335 mg of thiobarbituric acid (TBA; Sigma-Aldrich, USA) was dissolved in 40 ml of sterile distilled water. The solution was heated in a water bath at 50 °C for 45 minutes until the TBA was compleely dissolved. Sterile distilled water was added to make up a 50 ml 0.67 % TBA solution. The solution was stored at room temperature.

Appendix B9: Phosphate buffered saline (PBS)

8 g of sodium chloride (NaCl; Merck, RSA), 0.2 g of sodium chloride (KCl; Merck, RSA), 1.44g of sodium phosphate dibasic (Na_2HPO_4; Merck, RSA), 0.24 g of potassium phosphate monobasic (KH_2PO_4; Sigma-Aldrich, USA) was dissolved in 1 litre of distilled water and autoclaved for 20 minutes at 121 °C. The solution was stored at room temperature.

Appendix B10: Pyrogallol

36.46 mg of hydrochloric acid (HCl; Merck, RSA) was added to 100 ml or sterile distilled water to make a 10 mM HCl solution to make 20 mM pyrogallol. The solution was stored at room temperature.

0.025 g pyrogallol (Sigma-Aldrich, USA) was.added to 10 ml of 10.mM.HCl. The solution was freshly prepared at the time of the assay. The solution was stored at - 20 ºC.

Appendix B11: Sodium azide (NaN_3)

0.033 g NaN_3 (Sigma-Aldrich, USA) was added to 500 ml sterile distilled water to make 1 mM NaN_3. The solution was covered with aluminium foil to protect it against the light and stored at 4 ºC.

Appendix B12: Glutathione (GSH)

Appendix B12.1: <u>100 mM GSH</u>

0.307 g GSH (Tocris Bioscience, UK) was added to 10 ml phosphate buffer, pH 6.5. This solution was freshly prepared at the time of the assay. The solution was stored at 4 ºC.

Appendix B12.2: <u>1 mM GSH</u>

100 µl of 100 mM GSH (Tocris Bioscience, UK) was added to 9.9 ml of phosphate buffer, pH 7.0. This solution was freshly prepared at the time of the assay. The solution was stored at 4 ºC.

Appendix B13: Reduced nicotinamide adenine dinucleotide phosphate (NADPH)

Appendix B13.1: <u>0.2 mM NADPH</u>

0.017 g of NADPH (Roche, Germany) was added to 100 ml sterile distilled water to make 0.2 mM NADPH. The solution was freshly prepared at the time of the assay. The solution was stored at 4 ºC.

Appendix B13.2: 0.1 mM NADPH

5 ml of 0.2 mM NADPH was diluted in 5 ml of sterile distilled water to make 0.1 mM NADPH. The solution was freshly prepared at the time of the assay. The solution was stored at 4 ºC.

Appendix B14: Hydrogen peroxide (H_2O_2)

30 ml of H_2O_2 (SAARCHEM-HOLPRO Analytic, RSA) was added to 70 ml of sterile distilled water to make 30 % v/v H_2O_2. The solution was stored at room temperature.

Appendix B15: Oxidized glutathione disulphide (GSSG)

0.031 g GSSG (Sigma-Aldrich, USA) was added to 50 ml phosphate buffer, pH 7.4, to make 1 mM GSSG. The solution was freshly prepared at the time of the assay. The solution was stored at 4 ºC.

Appendix B16: 1-chloro-2,4-dinitrobenzene (CDNB)

0.203 g of CDNB (Sigma-Aldrich, USA) was added to 6 ml absolute alcohol (Merck, RSA) and 4 ml of sterile.distilled water.to make a final volume of 10.ml of 0.1 mM of CDNB. The solution was then heated until CDNB had fully dissolved. The solution was stored at room temperature.

Appendix C: TurnItIn Report

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Appendix D: Publication

PLOS ONE

Expression and activity of antioxidant enzymes and their regulation by carvacrol in *Candida auris*

--Manuscript Draft--

Manuscript Number:	PONE-D-20-26479
Article Type:	Research Article
Full Title:	Expression and activity of antioxidant enzymes and their regulation by carvacrol in *Candida auris*
Short Title:	Effect of carvacrol on antioxidant enzymes in *C. auris*
Corresponding Author:	Aijaz Ahmad University of the Witwatersrand Johannesburg, South Africa
Keywords:	*C. auris*; carvacrol; antioxidant enzymes; oxidative stress; lipid peroxidation; gene expression
Abstract:	Outbreaks associated with the 'new' multidrug resistant Candida species (i.e. C. auris) has notably increased around the globe in patients who are immunocompromised. The evolutionary characteristics of this fungus are unexplored which renders a crucial need for novel therapeutic strategies. A common mechanism to escape drug toxicity is by the activation of the antioxidant defense system. This system has recently been recognized as an emerging antifungal drug target. The present study was conducted in order to assess the anti-Candida activity of carvacrol on the growth and survival of C. auris, the gene expression and production of antioxidant enzymes, as well as the effect that carvacrol would have on lipid peroxidation (LPO). The antifungal activity of carvacrol was determined using microdilution method. The activity of major antioxidant enzymes and LPO after carvacrol exposure was determined spectrophotometrically. The effect of carvacrol on the gene expression of antioxidant enzymes was determined by RT-qPCR. The findings from this study revealed that carvacrol has a potential inhibitory effect over proliferation of C. auris (MIC range 125 to 500 µg/ml). Furthermore, carvacrol caused an adequate amount of oxidative stress which was clearly demonstrated by the significant increase in LPO, the activity and gene expression of primary antioxidant enzymes. This is the first study to provide attestation of oxidative stress induced by carvacrol in C. auris.
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Opposed Reviewers:	
Additional Information:	
Question	Response
Additional data availability information:	Tick here if your circumstances are not covered by the questions above and you need the journal's help to make your data available.; Tick here if the URLs/accession numbers/DOIs will be available only after acceptance of the manuscript for publication so that we can ensure their inclusion before publication.