IDENTIFICATION OF VIRULENCE FACTORS IN *CANDIDA AURIS* AND ANTIFUNGAL ACTIVITY OF MONOTERPENE PHENOLS AGAINST THIS PATHOGEN

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DECLARATION

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

..... (Signature of candidate)

...21..... day of .....Jan..... 2020.

DEDICATION

To my family, your unconditional love brought me through.

PUBLICATIONS

Siham Shaban, Mrudula Patel and Aijaz Ahmad. Improved efficacy of antifungal drugs in combination with monoterpene phenols against *Candida auris*, published in the International Journal of Scientific Reports (Appendix D).

ABSTRACT

Emergence of *Candida auris* has been described as a global health threat due to its ability to cause invasive infections with high mortality rate and its multidrug resistance propensity. Alternative antifungal strategy along with novel antifungal drugs are required to target this newly emerged fungal organism and its pathogenicity. Anti-virulence approach and combination therapy have been proposed in recent years. Essential oils rich in phenolic monoterpenes are well known for their significant *in vitro* and *in vivo* anti *C. albicans* activity. They have also been reported to synergize with the conventional antifungal drugs and have shown anti-virulence effect against *Candida* species other than *C. auris*. This study investigated the virulence factors in *C. auris*, evaluated the antifungal activity of selected monoterpene phenols (eugenol, methyl eugenol, carvacrol and thymol) against *C. auris*, combination antifungal activity of the most effective phenolic compound with antifungal drugs, and determined effect of the most active compound on positive pathogenicity markers of *C. auris.*

Antifungal susceptibility profile of 25 clinical isolates of *C. auris* against antifungal agents as well as against phenolic compounds was obtained using micro broth dilution method following CLSI guidelines. Combination of the most active phenolic compound with antifungal drugs was determined by calculating the fractional inhibitory concentration index (FICI) based on the zero-interaction theory of Loewe additivity. The virulence factors such as (adherence, morphogenesis, phospholipase and proteinase production) of *C. auris* isolates were characterized using *in vitro* virulence assays. Effect of carvacrol on the virulence factors was also studied.

Eugenol, methyl eugenol, carvacrol and thymol had antifungal activity against *C. auris* clinical isolates. Carvacrol was the most active phenol with median MIC of 0.125 mg/ml and MFC of

0.25 mg/ml, and its combination with fluconazole, amphotericin B, nystatin and caspofungin resulted synergistic and additive effects in 68%, 64%, 96% and 28% of the strains, respectively. Combination also reduced the original MIC values of the drugs. All test strains showed adherence ability to epithelial cells and 96% of strains showed strong proteinase activity. None of the strains produced hyphae and phospholipase. Carvacrol at MIC value significantly inhibited the proteinase production in *C. auris* ($p<0.01$), while at subinhibitory concentrations (0.063 mg/ml) it significantly inhibited its adherence to epithelial cells ($p<0.02$).

This study showed that *C. auris* possess various virulence factors such as the ability to adhere to epithelial cells and proteinase activity. Monoterpene phenols have antifungal activity against this pathogen. Carvacrol has the most inhibitory effect. It also showed an enhanced antifungal activity in combination with antifungal agents and anti-virulence activity against *C. auris*. Therefore, it has potential to be developed into a novel antifungal agent.

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LIST OF ABBREVIATIONS AND ACRONYMNS

AFLP:	Amplified fragment length polymorphism
ALS:	Agglutinin-like Sequence
ANI:	Average nucleotide identity
ANOVA:	Analysis of Variances
API:	Analytical Profile Index
AST:	Antifungal susceptibility testing
BEC:	Buccal Epithelial Cells
BMD:	Broth microdilution
BSA:	Bovine serum albumin
BSI:	Blood Stream Infection
CDC:	The Centres for Diseases Control and Prevention
CDK:	Cyclin-dependent kinase
CFU/ml:	Colony Forming Units per millilitre
CLSI:	The Clinical and Laboratory Standards Institute
DMSO:	Dimethyl sulfoxide
ECDC:	The European Centre for Disease Prevention and Control
FICI:	Fractional inhibitory concentration index
g:	Gram
GPI:	Glycosylphosphatidylinisotol

GRSA: Generally recognized as safe

HIV: Human Immunodeficiency Virus

HWP1: Hyphal wall protein 1

ITS: Internal Transcribed Region

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

MDR: Multi-drug resistant

MFC: Minimum Fungicidal Concentration

mg/ml: Milligram per millilitre

MIC: Minimum inhibitory concentrations

ml: Millilitre

MLST: Multilocus sequence typing

mm: Millimetre

MRSA: Methicillin-resistant *Staphylococcus aureus*

µl: Microlitre

µm: Micrometre

NAC: Non-albicans *Candida*

NAG: N-acetyl glucosamine

NICD: The National Institute for Communicable Diseases

NTP: National Toxicology Program

OPC: Oropharyngeal/esophageal candidiasis

rpm: Revolutions per minute

SAP: Secreted Aspartyl Proteinase

SD: Standard Deviation

SDA: Sabouraud dextrose agar

SDB: Sabouraud dextrose broth

VVC: Volvovaginal candidiasis

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Candida species are major human fungal pathogens that cause a wide range of infections varying from mucocutaneous candidiasis to sever life-threatening disseminated candidemia in hospitalized patients. The Centres for Diseases Control and Prevention (CDC) have reported that *Candida* species are the fifth among hospital-acquired pathogens and the fourth among Blood Stream Infection (BSI) pathogens (Yapar, 2014). With approximately 30% mortality rate among patients with invasive candidiasis, in which about 19-24% of deaths are directly attributable to candidemia. The most prevalent species involved in invasive candidiasis is *Candida albicans*. However, the incidence of infections due to non-albicans *Candida* (NAC) species is increasing in recent years and gaining more clinical significance especially in hospital setups (Pfaller and Diekema, 2007). Among (NAC) species, a newly reported *Candida auris* is an emerging multi-drug resistant (MDR) *Candida* species. *C. auris* has been associated with highly invasive BSI, hospital outbreaks, treatment failures and high mortality rate. Previous studies have reported this pathogen as highly resistant to all the major classes of available antifungal drugs (CDC, 2016). Therefore, new drugs along with new strategies are required to deal with this newly emerged (MDR) fungal pathogen and most importantly to identify drug target virulence factors.

1. LITERATURE REVIEW

The kingdom of fungi is made up of diverse eukaryotic organisms, characterized by the presence of chitin in their cell walls. Fungi are heterotrophic organisms, can be either unicellular or multicellular. An estimated 250,000 species of fungi have been described (Mueller and Schmit, 2007). Of these, at least 300 fungal species are known to cause infections in humans (Taylor and Berbee, 2006). With the exception of few species such as *Candida*, which forms part of the normal flora of humans, most of these fungi are free-living in nature (Dixon *et al,* 1980).

There are two main types of fungal infections described, true pathogenic fungal infections and opportunistic fungal infections. The opportunistic fungal infections are caused by organisms which are normally of low virulence. However, disease manifestation is dependent on a reduced host resistance to infection. *Aspergillus*, *Cryptococcus* and *Candida* species are common fungi involved in opportunistic infections. Among these, *Candida* species are the most common cause of fungal infections worldwide (Pfaller and Diekema, 2007).

1.1 *Candida* species

Candida is a dimorphic organism, which can grow in yeast form or hyphal form, and the most common cause of opportunistic fungal infections called candidiasis. It is also a frequent colonizer of the mucous membranes of the oral cavity, vagina and gastrointestinal tract of most healthy humans, and to a lesser extent on the human skin ((Pfaller and Diekema, 2010).

Candida is a genus of *ascomycetous* fungi in the family *Saccharomycetaceae*. The genus *Candida* includes more than 200 species, but only few of them are of medical relevance. Besides *C. albicans*, which is the most commonly isolated species, non-albicans *candida* (NAC) species such as *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. krusei* as well as *C.*

dubliniensis are also clinically important species. They are frequently isolated as causative agents of candidiasis among hospitalized patients, especially those who are cared for in intensive care units (ICUs) with serious underlying diseases or those who may be immunocompromised (Wingard, 1995; Shin *et al,* 2002).

The clinical spectrum of candidiasis is extremely diverse, which can either be superficial localized or deep-seated disseminated systemic infection. Etiologically candidiasis can be oropharyngeal/oesophageal (OPC), genital/vulvovaginal (VVC), or invasive candidiasis/ candidemia, when the fungus *Candida* enters the blood causing bloodstream infection (Haynes, 2001).

Candida species now rank as the fifth most common hospital-acquired pathogens and the fourth-most common cause of nosocomial bloodstream infections among hospitalized patients in the United States, with mortalities reaching up to 40% (Pfaller and Diekema, 2007; Yapar, 2014). Incidences of infection have risen with the increased prevalence of patients with compromised immune systems such as patients undergoing chemotherapy for cancer or immunosuppressive therapies for chronic medical conditions or organ and bone marrow transplants, those with underlying disease states such as AIDS or diabetes mellitus, those born prematurely, and those whose normal flora have been eliminated by the use of broad-spectrum antibiotics are particularly at high risk of developing sever life-threatening disseminated candidemia (Wright and Wenzel, 1997). Other predisposing factors include the use of in-dwelling medical devices such as catheters and heart valves, total parental nutrition and disruption of mucosal barriers due to surgery or radiotherapy (Odds, 1988).

Although *Candida* infections, from superficial to invasive infections, are commonly controlled by using three different classes of antifungal drugs which are; azoles, polyenes and echinocandins, problems of toxicity and the emergence of drug resistant *Candida* species has

been a major cause of treatment failures, especially among severely ill patients with invasive candidiasis (Odds *et al*, 2003).

Importantly, there has been a recent increase in the proportion of the nosocomial candidemia due to non-*C. albicans* species and they have gained clinical significance. They are frequently less susceptible to the currently used antifungal drugs in contrast to *C. albicans* (Pfaller and Diekema, 2007). It has been suggested that this is highly related to the use of Fluconazole, the most commonly used azole drug, as a prophylactic antifungal drug in high-risk populations (Lortholary *et al,* 2011).

Nevertheless, the diversity of NAC species that are encountered in infections is expanding and their antifungal resistance is increasing. The emergence of uncommon species that were rarely in play in the past is now pose considerable challenge for both diagnostic, prophylactic strategies and therapeutic management (Pfaller and Diekema, 2007). Among these uncommon species, *Candida auris* has emerged as an important and potentially resistant fungal pathogen.

1.2 *Candida auris*

Candida auris is an emerging species of the *Candida* genus, it was first described as a fungal pathogen that cause candidiasis in humans in 2009 (Satoh *et al*, 2009). This previously unknown yeast has attracted most attention and public health efforts, within only a few years of its identification, mainly due to its tendency to spread in healthcare settings causing hospital outbreaks and its antifungal drug resistance (Jeffery-Smith *et al*, 2017).

1.2.1 Epidemiology of *Candida auris*

The first descriptions of *Candida auris* came from Japan after it was isolated from the external ear canal of a 70-year-old Japanese woman at the Tokyo Metropolitan Geriatric Hospital in 2009 (Satoh *et al*, 2009). The name, *auris* comes from the Latin word for ear. Around the same time it was isolated from ear specimens of 15 patients with chronic otitis media in South Korea (Kim *et al*, 2009). However, retrospective testing of *Candida* strain collections discovered that the earliest known strain of *C. auris* was found in a stored sample of unidentified yeasts from 1996 in South Korea (Lee *et al,* 2011).

Thereafter reports of bloodstream infections due to C. *auris* followed quickly. The first case of *C. auris* candidemia were reported from South Korea in 2011, (Lee *et al,* 2011) followed by reports from India in 2013 (Sarma *et al,* 2013; Chowdhary *et al,* 2013). Subsequently, it has been reported from many countries across Asia, Europe and Africa, including South Africa (Magobo *et al*, 2014), Kenya (Okinda *et al,* 2014), Kuwait (Emara *et al*, 2015), the United Kingdom (Schelenz *et al*, 2016), Germany (ECDC, 2016) and Norway (ECDC, 2016), to arrive in the United States and South America in 2016, USA (Vallabhaneni *et al*, 2016). Venezuela (Calvo *et al,* 2016), and Brazil (Prakash *et al,* 2016). More recently it has been reported from Colombia (Morales-López *et al,* 2017), Pakistan (Lockhart *et al*, 2017), Canada (Schwartz and Hammond, 2017), Spain (Ruiz Gaitán *et al*, 2017), and Oman (Al-Siyabi *et al,* 2017). However, due to the difficulties in *C. auris* identification using commercial laboratory methods, it is likely that it has emerged in other countries with a limited laboratory capacity but has remained undetected (Kathuria *et al,* 2015).

1.2.2 Clinical significance of *Candida auris*

In June 2016, the Centres for Disease Control and Prevention (CDC) issued a clinical alert to the healthcare facilities in the United States about global emergence of invasive infections caused by *C. auris* (CDC, 2016). The CDC was concerned about *C. auris* for three main reasons:

- It is often resistant to multiple antifungal drugs commonly used to treat *Candida* infections, meaning that it is a multidrug-resistant (MDR) yeast.
- It is difficult to identify using conventional biochemical methods, and it can be misidentified in laboratories without specific technology.
- It has caused healthcare-associated outbreaks and invasive infections with high mortality of up to 70%.

Later, the European Centre for Disease Prevention and Control (ECDC) has issued a rapid risk assessment on *C. auris* in December 2016, this was after hospital outbreaks caused by *C. auris* have occurred in the UK between 2015 and 2016. The main aim was to prevent further hospital outbreaks by raising awareness of *C. auris* in European healthcare facilities, adapting laboratory testing strategies and establishing enhanced control measures (ECDC, 2016).

Clinically, *C. auris* has been isolated from different specimens such as; blood samples, ear discharge, catheter tips, bone, sputum, skin, vaginal secretions and body fluids (cerebrospinal fluid, pancreatic fluid, pericardial fluid, peritoneal fluid, pleural fluid). Furthermore, it has been reported as a causative agents of several infections, for example; candidemia, osteomyelitis, otitis media, intra-abdominal infections, pericarditis, pleural effusions, ventriculitis and vulvovaginitis (Satoh *et al*, 2009; Lee *et al,* 2011; Sarma *et al*, 2013; Magobo *et al,* 2014; Schelenz *et al,* 2016; Borman *et al*, 2016; Tsay *et al,* 2017).

1.2.3 *Candida auris* in South Africa

In South Africa, *C. auris* was first reported as a causative agent of candidemia in 2014, after the notice of the report by Chowdhary *et al* from India in 2013, about persistent fungemia caused by *C. auris*. Four isolates, were initially identified as *C. haemulonii*, have been confirmed later as *C. auris* at the National Institute for Communicable Diseases (NICD), Johannesburg (Magobo *et al,* 2014). These isolates were collected from 4 patients with candidemia who had been admitted to different public and private hospitals from October 2012 to October 2013.

In December 2016, the NICD reported that *C. auris* was the second most common species of *Candida* causing candidemia in the South African private sector hospitals, and the fourth most common cause of candidemia in public sector hospitals in 2016, with most cases occurring in Gauteng province (NICD, 2016). After large outbreaks caused by *C. auris* have occurred at several hospitals in Johannesburg and Pretoria from 2015 onwards, again with most cases occurring in private sector, the NICD has requested all the South African healthcare facilities to notify urgently if a new outbreak in a hospital is suspected.

Recent national laboratory-based surveillance for candidemia have reported that *C. auris* accounts for 10% of all cases of candidemia in South Africa (Van Schalkwyk *et al,* 2017). *C. auris* infections have been found in South African patients of all ages, with the first neonatal *C. auris* bloodstream infection were reported in August 2017 (NICD, 2017).

Govender *et al*, (2018) have reported that the dramatic increase in cases of *C. auris* isolation, from 18 isolates in 2013 to 861 isolates in 2016, is of a major concern in South Africa. Most cases were admitted to private sector hospitals in Gauteng Province (Govender *et al,* 2018) and they have suggested that the overuse of fluconazole for prophylaxis and treatment has caused

this C. *auris* endemic, this setting was previously associated with the emergence of azole-resistant *C. parapsilosis* causing hospital outbreaks in Gauteng province (Govender *et al,* 2016).

1.2.4 Identification and diagnosis of *Candida auris*

Candida infections are usually diagnosed by fungal blood culture or culture of other body fluids (Tortora *et al*, 2010). Unlike other more common species of *Candida, C. auris* is difficult to identify in the routine diagnostic laboratory. It is often misidentified as other fungi in clinical laboratories using commercial biochemical methods for yeast identification such as VITEK 2 YST, API 20C, and MicroScan, as it has not been included in their database. For example, it can be misidentified as *Candida haemuloni,* the phenotypically closely related-spices, and also several other yeast species, such as *Candida parapsilosis,* C*andida famata, Candida sake, Candida guilliermondii, Candida lusitaniae, Saccharomyces cerevisiae,* and *Rhodotorula glutinis* (Kathuria *et al,* 2015). If the yeast was identified by standard laboratory tests as one of these fungi, further identification tests to determine the species and antifungal susceptibility testing (AST) of the isolate should be done, especially if the fungus is fluconazole resistant. Special laboratory techniques that use molecular methods or mass spectrometry are needed to differentiate *C. auris* from other related species (Britz and Govender, 2016). Currently, the accurate identification of *C. auris* can be confirmed by using the diagnostic technology based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry or molecular identification based on sequencing the D1-D2 region of the 28s ribosomal DNA (rDNA) or the Internal Transcribed Region (ITS) of rDNA (Girard *et al,* 2016).

1.2.5 Phenotypic characteristics of *Candida auris*

*C. auris i*s difficult to distinguish from other species of *Candida* by the shape, size and colour of its colonies growing on different culture media. *C. auris* appears smooth, pale purple to pink colonies on CHROM agar, which is the most common media used in differentiation between *Candida* species (Kathuria *et al,* 2015; Mohsin *et al,* 2017). However, some strains show no characteristic colour on CHROM agar (Kumar *et al,* 2015).

On microscopy, *C. auris* is ovoid, ellipsoidal to elongate cells, that either be single and/or in groups (Kathuria *et al*, 2015, Mohsin *et al,* 2017). An average cell size is about (2.0–3.0) × (2.5–5.0) μm which is smaller than *C. albicans* and more comparable to *C. glabrata* (Borman *et al.* 2016). However, its growth pattern is similar to that of *C. albicans* (Larkin *et al.* 2017). Interestingly, observation of two phenotypes of *C. auris* was reported by Borman *et al*, (2016), they have divided *C. auris* strains into aggregate-forming and nonoaggregate isolates, the aggregate cells could not be physically disrupted by vigorous vortexing, and they have also reported that nonaggregating isolates are more pathogenic than aggregating isolates in the *Galleria mellonella* infection model. However, the results of this study did not correlate with the virulence degree in clinical cases (Borman *et al*. 2016; Sherry *et al*. 2017).

The characteristic feature of *C. auris* is the thermoresistance, while other candida species do not grow at higher temperature beyond 37°C, *C. auris* grows between 37 and 42°C. This unique feature can explain the high survival rate of this pathogen in the host and the environment and can also help to differentiate it from other species such as *C. haemulonii* which fails to grow at 42°C (Chowdhary *et al*, 2014; Satoh *et al,* 2009).

Furthermore, other important marker used to determine the species of the pathogenic yeast is the germ tube formation on cornmeal agar. *C. auris* is germ tube negative and does not form

pseudohyphae and chlamydospores. On the other hand, *C. haemulonii* form pseudohyphae (Satoh *et al,* 2009; Lee *et al*, 2011; Kumar *et al,* 2015; Larkin *et al,* 2017). However, more recently researchers found that some strains of *C. auris* form rudimentary pseudohyphae (Borman *et al,* 2016; Sherry *et al,* 2017). This strain specific features will need further studies with a larger number of isolates to fully describe these differences between strains.

Remarkably, phenotypic differences exist among *C. auris* isolates from the same or different countries. For example; the ability to assimilate the N-acetyl glucosamine (NAG), unlike Japanese and Korean *C. auris* isolates, all South African, Indian and Brazilian isolates assimilated NAG (Chowdhary *et al,* 2014). Further, while most *C. auris* strains form biofilms to different degrees, some do not form biofilms (Chowdhary *et al.* 2013, Chatterjee *et al.* 2015).

Moreover, genetic diversity between *C. auris* strains from different geographic areas have been also reported by Sharma *et al*, (2015) and Prakash *et al*, (2016). The whole genome sequencing analyses of the clinical isolates from Asia, South Africa, Europe and South America have identified four distinct clades, which suggest that an independent and nearly simultaneous emergence of different clonal populations has occurred within each region (Sharma *et al,* 2015; Prakash *et al,* 2016; Lockhart *et al,* 2017).

1.2.6 Genomic characteristics of *Candida auris*

Only a minor proportion of the *C. auris* genome have been characterized (Sharma *et al,* 2015; Chatterjee *et al,* 2015; Sharma *et al,* 2016; Vallabhaneni et al, 2016; Prakash *et al,* 2016; Tsay *et al,* 2017; Lockhart *et al,* 2017). The major proportion of the whole genome, however, is still uncharacterized.

Molecular study has investigated a number of 104 clinical *C. auris* isolates from India, South Africa and Brazil along with control strains from Korea. Three techniques have been used

which include Multilocus sequence typing (MLST), amplified fragment length polymorphism (AFLP) fingerprinting and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Notably, the three techniques have shown evidence of geographical clustering of the population structure of *C. auris*. The internal transcribed spacer (ITS) sequence revealed two major clusters including Indian and Brazilian isolates, while the South African isolates were randomly distributed between the two clusters, suggesting different genotypes. However, limited number of only 14 *C. auris* isolates from countries other than India were used in this study (Prakash *et al*, 2016).

Comparative analysis using the whole genome sequencing of five *C. auris* isolates and other eight species (include *C. dubliniensis*, *C. albicans, C. guillermondii, C. lusitaniae, C. tropicalis, S. cerevisiae, C. parapsilosis* and *C. glabrata)* has shown that, *C. auris* genome was closest to that of *C. lusitaniae* with average nucleotide identity (ANI) value (85.9%–86.4%), and their genomes were highly related to each other than to other *Candid*a species with only 0.2% variation (Sharma *et al,* 2016).

Moreover, when *C. auris* genome was analysed with the reference genome of the well-studied species *C. albicans* showed that, more than 99.5 % of the *C. auris* reads did not align to the genome sequences of *C. albicans*. However, *C. auris* genome shares significant virulence traits common to *C. albicans*, such as ion transporters, amino acid and metabolite transporters, adhesins, secreted aspartyl proteases, lipases, phospholipases, mannosyl transferases and genes involved in biofilm formation (Chatterjee *et al,* 2015).

1.3 Virulence factors of *Candida* species

The pathogenicity of *C. albicans* and other *Candida* spp. and its ability to cause infection in humans, has been attributed to its different virulence features, which are referred to as virulence factors. Virulence factors are the components or the activity of the pathogen that damage the host and help in establishment of infection (Casadevall and Pirofski, 2001).

The pathogenicity mechanism of *Candida* spp. is a complex process. In order to establish an infection*, Candida* cells undergoes several morphological and biochemical changes, these changes enable *Candida* cells to invade host tissues, interfere with the immune system and damage the host. However, host immunity is an important factor in the infection process. The imbalance between the host immunity and these opportunistic fungi may trigger invasion of the mucosal epithelia by *Candida* followed by dissemination via the blood stream and infection of internal organs (Larriba *et al,* 2000).

The expression of several virulence factors is species specific. While *C. albicans* has been established for the pathogenicity mechanisms study, there is relatively less information available about *C. auris* and other (NAC) species. However, molecular study, as mentioned above, has established that *C. auris* shares significant virulence genes common to that of *C. albicans* (Chatterjee *et al*. 2015; Sharma *et al.* 2016).

C. albicans expresses several virulence factors that contribute to the pathogenesis. The primary virulence factor is host recognition, followed by adhesion to host cells, morphogenesis (the reversible transition between unicellular yeast cells and hyphal forms), secretion of tissue damaging hydrolytic enzymes such as secreted aspartyl proteases, phospholipases and haemolysins and biofilm formation (Ahmad *et al,* 2016).

1.3.1 Adhesion and invasion

The initial step for colonization and subsequent infection by a pathogen is its adherence to a host surface. Yeast cells adherence to host tissue and its critical role in the infection process is well recognized in *C. albicans* and other (NAC) species (Haynes, 2001). *Candida* species can adhere to epithelial cells, endothelial cells and other host tissues as well as to abiotic surfaces such as medical devices. Adherence is also an essential and early step in biofilm formation, which is thought to be positively associated with the virulence degree and the antifungal resistance of *Candida* species (Hawser *et al,* 1994).

C. albicans expresses a specialized set of cell wall proteins which mediate adherence referred to as adhesins. The best studied adhesins are the agglutinin-like sequence (ALS) proteins, which form a family consisting of eight members (ALS1–7 and ALS9). In addition, Hyphal wall protein (Hwp1) is another important adhesin in *C. albicans*, which is a hypha-associated Glycosylphosphatidylinisotol (GPI) -linked protein, it was originally isolated as a germ tube specific protein (Staab *et al,* 1999; Murciano *et al,* 2012).

The second step following adherence to host cells is invasion, *C. albicans* invades into the host cells two different mechanisms which are: induced endocytosis and active penetration. Induced endocytosis mediated by the expression of cell wall proteins invasins, namely Als3 (which also functions as an adhesin) and Ssa1 (which is member of the heat shock protein 70 (Hsp70) family). On the other hand, hydrolases secretion specifically secreted aspartic proteases (Saps) have been proposed to promote active penetration in *C. albicans* (Ahmad *et al,* 2016).

Few studies have investigated this virulence factor in *C. auris*. In a recent study by Larkin *et al,* (2017), they have studied two *C. auris* isolates and have observed that *in vitro* adherence ability of *C. auris* to catheter materials is reduced by 25% as compared to *C. albicans* (Larkin

et al, 2017). Study by Chatterjee *et al*, (2015) has shown that *C. auris* expresses adhesins similar to that of *C. albicans* (Chatterjee *et al*, 2015).

1.3.2 Morphogenesis

Morphogenesis refers to the reversible transformation between unicellular yeast cells and a different filamentous growth forms, including true hyphae and pseudohyphae. Both yeast and filamentous form have their own function to support virulence of *Candida* and development of disease, whereas the yeast form is primarily involved in adhesion and dissemination of the fungus. The hyphal form is proposed to be more invasive virulent form, as it is bigger and more difficult to be eliminated by the macrophages (Odds, 1988).

The conversion between the two morphologies is promoted by several environmental factors such as Ph, temperature, CO_2 and the presence of serum or N-acetyl glucosamine. *Candida* cells transduce these external signals to the interior of the cell via different signalling pathways, resulting in activation of a subset of genes, such as hyphal wall protein (HWP1), agglutinin-like sequence protein (ALS3), hypha associated proteins (ECE1 and HYR1) and the secreted aspartic proteases (Sap4, Sap5 and Sap6), and subsequent hyphae formation (Yang *et al,* 2003).

Several signalling pathways induce morphogenesis in *Candida*. For example; CAMP–protein kinase A (PKA) is the major pathway, in which different stimuli such as CO_2 and temperature act on the adenylate cyclase Cyr1, leads to activation of the hypha-specific G1 cyclin-related protein Hgc1 through the transcription factor Efg1. Hgc1 form complexes with the cyclin-dependent kinase (CDK). These complexes promote polarized growth and hyphal formation. On the other hand, depletion of Hgc1 result in failure to produce hyphae (Ahmad *et al,* 2016). Hyphae formation can be also induced *in vitro* by incubating *Candida* in medium containing

serum. However, serum-induced morphogenesis pathway is not yet known (Shepherd *et al*, 1980).

In fact, not all of the *Candida* species are capable to form both types of filamentous growth, only *C. albicans* and *C. dubliniensis* can grow in both true hyphal and/or pseudohyphal form (Calderone and Fonzi, 2001). On the other hand, a wide range of pathogenic *Candida* species, such as *C. parapsilosis*, *C. tropicalis* and *C. haemulonii*, can only grow in pseudohyphal form (Silva *et al,* 2011). With regards to *C. auris,* most reports from different geographical areas suggesting that it does not germinate or form true hyphae or significant pseudohyphae *in vitro* (Lee *et al*. 2011, Chowdhary *et al.* 2013; Kumar *et al.* 2015; Larkin *et al*, 2017). However, recent studies found that some *C. auris* strains form rudimentary pseudohyphae, which might be a strain specific feature or due to different conditions used (Borman *et al*. 2016; Sherry *et al*. 2017). More recently, two reports from china observed that the first isolate of *C. auris* in China undergoes some morphological changes as a response to different environmental factors. Unlike *C. albicans*, which switches to hyphal form in the presence of serum *in vitro*, *C. auris* switches when it was treated with 10% NaCl or after passage through animal model. However, filamentous growth and pseudohyphal-like form were used to describe *C. auris* cells in these studies rather than true hyphae, due to the differences in morphological characteristics between the two cell forms (Wang *et al,* 2018; Yue *et al,* 2018).

1.3.3 Secretion of extracellular hydrolytic enzymes

C. albicans produce many hydrolytic enzymes, which have been proposed to facilitate its pathogenic characteristics such as; adherence, invasiveness, active penetration and interference with the host immune system. In addition, these enzymes are essential for *Candida* survival and their primary function is to enhance *Candida* growth by degrading complex materials of the host tissue into nutrients (Naglik *et al,* 2003).

The two major classes of extracellular hydrolytic enzymes secreted by *C. albicans* are secreted aspartyl proteinases (SAPs) and phospholipase, which are the most studied enzymes as well. Others, such as haemolysin, Coagulase, chondroitinase, hyaluronidase and lipase are also involved in *C. albicans* pathogenicity (Tsang *et al*, 2007).

1.3.3.1 Secreted aspartyl proteinases (SAPs)

SAPs contribute to pathogenicity of *Candida* by degrading important structural and immunological human proteins such as albumin, haemoglobin and secretory IgA. They also disrupt host epithelial and mucosal membrane proteins which include collagen, keratin and mucin, this aid *Candida* adherence to the human epithelium (Borst and Fluit, 2003).

Studies have identified ten aspartic proteinase (Saps) which are encoded by ten (SAP1–10) genes. While Sap9 and Sap10 are secreted and remain bound to cell surface preserving the membrane integrity of yeast cells, Sap1–8 are released to the surrounding medium. (Albrecht *et al,* 2006) These enzymes have been shown to play various roles during different phases of infection. SAP1-3 have been expressed in the yeast form of *C. albicans,* while a hyphal form expresses SAP4-6. On the other hand, SAP9-10 have been expressed in both forms. The roles of SAP7-8 are not yet fully understood (Ahmad *et al*, 2016). An animal study model has shown that, Saps1–6 are associated with invasive infections and required for disease development, and the most important enzymes are Sap1–3 and -6, which were detected early within 48 h post-infection of tissue (Hube and Naglik*,* 2001).

1.3.3.2 Phospholipases

Phospholipases contribute to pathogenicity of *C. albicans* by hydrolysing phospholipids in the host cell membranes to fatty acids facilitating *Candida* invasion to the host tissues. Phospholipases are classified into four classes: phospholipase A, B, C and D, of which only

five members of class B (PLB1–5) have been associated with virulence and disease development (Niewerth and Korting, 2001).

Saps and phospholipases are not only produced by *C. albicans*, certain NAC species have also shown different degree of enzyme activity, such as *C. tropicalis*, *C parapsilosis* and *C. guilliermondii* as well as *C. auris* (Ghannoum *et al,* 2000; Larkin *et al,* 2017).

Few studies have investigated the proteinase and phospholipase production in *C. auris*. Investigator have reported a strong proteinase and phospholipase activity in *C. auris* isolate from a case of vulvovaginitis (Kumar *et al,* 2015). Whereas Larkin *et al,* (2017) with a larger number (n = 16) of isolates, have reported that proteinase and phospholipase production is strain dependent and a limited number of the tested isolates (37.5%; n = 16) produce phospholipase with weak enzyme activity compared to *C. albicans.* A higher number of *C. auris* isolates (64%; n = 14) were proteinases producers. Recently Wang *et al,* (2018) have tested the first isolate of *C. auris* in China for proteinase production and reported that it exhibited high proteinase activity.

1.3.4 Biofilm formation

Another potent virulence factor of *C. albicans* is biofilm formation. Biofilms are complex biological communities which form on biotic (host cells) or abiotic (e.g., catheters, dentures) surfaces, and characterized by its high resistance to host immune system and antifungal drugs (Fanning and Mitchell, 2012).

The primary step in biofilm formation is adherence of yeast cells to the host tissue, followed by cell proliferation, formation of hyphal cells in the upper part and accumulation of extracellular matrix (Mayer *et al,* 2013). Later, when the biofilm becomes mature, yeast cells

released from the complex spread to colonize new location. This is suggested to be responsible for the subsequent disseminated invasive candidiasis (Uppuluri *et al,* 2010).

Furthermore, biofilm matrix has been shown to directly contribute to virulence, as it is responsible for biofilm structural integrity and its resistance to antifungal drugs (Martins *et al*, 2010). Biofilm formation is controlled by several transcription factors such as; *BCR1*, *TEC1* and *EFG1*, these factors are critical regulators of *C. albicans* biofilm in both *in vitro* and *in vivo* animal models (Harriott *et al,* 2010; Nett *et al*, 2010).

Although both yeast cells and hyphal form are required for biofilm formation in *C. albicans,* other *Candida* species, which do not produce true hyphae, such as; *C. tropicalis, C. parapsilosis, and C. glabrata* are able to form biofilms which are composed mainly of yeast cells and extracellular matrix (Silva *et al,* 2012). Recently, Larkin *et al,* (2017) have reported that *C. auris* isolates were able to form biofilms composed mainly of yeast cells and less extracellular matrix and significantly thinner in comparison to *C. albicans* biofilms, and their biofilms have been shown to be resistant to antifungal drugs (Sherry *et al,* 2017).

1.4 Role of virulence factors in antifungal drug development

Understanding of factors and activities which contribute to virulence in different *Candida* species is crucial for antifungal drug development. The identification of virulence factors can ultimately increase the number of potential targets that can be exploited for drug development, thereby leads to discovery of entirely new classes of antifungal drugs with new mechanisms of action. In the bacterial field, targeting virulence factors has emerged as an attractive alternative in the development of novel classes of antibacterial drugs and as a potential solution to overcome antibiotic drug resistance (Clatworthy *et al,* 2007). Therefore, the concept of targeting virulence factors has been proposed as an alternative and promising antifungal

strategy, since the development of new antifungal drugs is restricted by several factors such as the limited number of selective drug targets in fungi (Gauwerky *et al*, 2009).

In contrast to the conventional antifungal drugs, which act by inhibiting the growth or killing the fungal cells, thereby exerting a high degree of selective pressure on the fungus which is responsible for the emergence of drug resistance (Casadevall *et al,* 2002). By targeting virulence factors, fungal cell virulence can be affected without threatening its existence, which should exert a less selective pressure for drug resistance mutations. However, unlike conventional antifungal drugs which have a broad spectrum activity against most, if not all, pathogenic fungi, anti-virulence strategies display a narrow spectrum antifungal activity, which are effective against only a limited range of fungi which express the same virulence factor (Odds *et al,* 2003).

In particular, knowledge of these virulence factors in *C. auris*, which has been resistant to one or more of the three major classes of antifungal drugs, will help to explore new antifungal drug targets and to develop more effective antifungal agents for improved therapeutic regimens. In addition, it will be an important tool to improve laboratory diagnostic for rapid and accurate detection of *C. auris*, which also remains a major challenge.

1.5 Antifungal therapy

As a matter of fact, the development of antifungal drugs is slower than that of antibacterial drugs for several reasons. Perhaps most importantly, the significant structural and metabolic difference between fungi and bacteria, the cellular structure of bacteria offers different targets, in contrast to fungi, which are eukaryotes, and most agents target fungal cell membrane (Ghannoum and Rice, 1999). Currently available antifungal drugs for the treatment of systemic fungal infections are limited to only three main classes, which include: polyenes, azoles and

the newest class echinocandins (Mohr *et al,* 2008). The newly introduced class, echinocandins, took 30 years to enter clinical practice, leading to concerns about the ability of the current pace of antifungal drug development to keep up with the clinical needs. In addition to the restricted number of currently available antifungal agents, problems of drug toxicity, undesirable side effect as well as the emergence of resistant strains have been a major cause of treatment failure and also have been reported more frequently (Thompson *et al,* 2009). Recently, various other agents are being added to the antifungal therapy such as flucytosine, allylamines and griseofulvin. Flucytosine has been used in combination with amphotericin B for treatment of certain invasive fungal infections (Chandrasekar*,* 2010). furthermore, all of the three antifungal drug classes target components of the fungal cell wall at different sites, however, they differ in their antifungal spectrum of activity, side effects, and bioavailability, hence, detailed knowledge of each drug class pharmacological properties is necessary (Thompson *et al*, 2009).

1.5.1 Antifungal drugs

1.5.1.1 Polyenes

Polyenes are the oldest family of antifungal drugs, which have been isolated from *Streptomyces* species. The currently available polyenes are amphotericin B and nystatin. Polyenes act by binding to ergosterol within fungal cell membranes. This results in an increase in membrane permeability, metabolic disturbance, leakage of cell molecules and, as a result, cell death (Mathew and Nath, 2009). Ergosterol is an essential structure of yeast cell membrane, which maintains the membrane integrity and function, and due to its major role in the persistence of yeasts, ergosterol synthesis has become an important target in antifungal drug development (Dupont *et al,* 2012).

Amphotericin B has been considered the gold standard treatment of severe fungal infections and primarily used intravenously. The broad antifungal spectrum and greater therapeutic response with the use of amphotericin B accounts for its widespread use despite the potential nephrotoxicity concerns (Ellis, 2002). However, new formulations of amphotericin B such as lipid and liposomal formulation have shown lower incidence of nephrotoxicity and greater efficacy than the conventional form (Sanglard and Odds, 2002. Moen *et al,* 2009).

Nystatin was the first polyene antifungal to be developed. (Hazen *et al,* 1950) Although antifungal activity of nystatin is broader than that of amphotericin B, its high toxicity has limited its application to topical use only. Importantly, liposomal formulation of nystatin, which is currently in late phase III clinical trials, has shown a greater antifungal activity with significant reduction in nephrotoxicity than amphotericin B and its formulation when administrated intravenously, and also has shown effectiveness against amphotericin B resistant infections (Arikan *et al,* 2002).

1.5.1.2 Azoles

The azole antifungal class includes two drug groups, the imidazole group such as clotrimazole, miconazole, and ketoconazole, and the triazole group such as fluconazole, itraconazole and voriconazole. Of these, only the triazoles and ketoconazole are applicable in both systemic and superficial fungal infections (Mathew and Nath, 2009). Importantly, the triazoles, which is less toxic than polyenes, have replaced amphotericin B in treatment of certain forms of systemic fungal infections. However, no single agent has the broad spectrum antifungal activity of amphotericin B (Dodds-Ashley, 2006).

Generally, azoles are fungistatic drugs, unlike polyenes which are fungicidal drugs. However, they both target fungal cell membrane. Azoles inhibit the cytochrome P450 dependent enzyme

(14-**α** lanosterol demethylase), which interferes with the conversion of lanosterol to ergosterol in fungal cell membrane, and this leads to depletion of ergosterol and inhibition of fungal growth and replication (Chandrasekar *et al*, 2010).

Fluconazole is one of the most commonly used azoles in clinical practice, mainly because of its high bioavailability, well tolerance and less common side effects. However, drug interactions with other medications such as; anticonvulsant and anticoagulant drugs have been reported. Fluconazole can be administered either orally or intravenously. It has been used as a first line therapy for the treatment of oropharyngeal candidiasis (OPC) and also as a prophylactic agent in high risk patients for invasive fungal infections (Debruyne and Ryckelynck, 1993).

1.5.1.3 Echinocandins

Echinocandins are semisynthetic lipopeptides compounds that represent the newest class of antifungal drugs, they include caspofungin, micafungin and anidulafungin. Echinocandins inhibit glucan synthesis, which is an important component to the structure and function of the fungal cell wall, by blocking the pathway of β (1, 3)-D- glucan synthase enzyme, this leads to cell death. Generally, they are potent fungicidal against all *Candida* species and available only as intravenous formulations (Odds *et al,* 2003). Caspofungin was the first agent of this class to be developed. Comparative studies have shown that caspofungin and micafungin have similar efficacy and fewer adverse effects than amphotericin B in the treatment of invasive candidiasis and they are safer in renal patients (Villanueva *et al,* 2002; De Wet *et al*, 2004).

Furthermore, the emergence of fluconazole resistant strains of *Candida* and the well-known nephrotoxicity of amphotericin B has prompted to recommend echinocandins as first-line therapy for invasive candidiasis. Additionally, their proven efficacy, favourable tolerability and

less common adverse effects and drug interaction make them attractive alternatives over other available antifungals in clinical practice. However, the higher cost of these drugs in comparison to azoles has limited their use to azole resistant *Candida* infections (Bruynesteyn *et al,* 2007).

1.5.2 Antifungal drug resistance in *Candida* spieces

Antifungal drug resistance has become an important clinical problem with *Candida* infections in certain populations, especially those with human immunodeficiency virus (HIV) infection, cancer chemotherapy, and organ and bone marrow transplantation. The emergence of resistant *Candida* isolate has been associated with therapeutic failure, which consequently leads to high levels of morbidity and mortality among patients with invasive candidiasis (White *et al*, 1997).

There are two patterns of resistance in *Candida* species. Species were initially susceptible but mutates post exposure to certain types of antifungal drug and becomes resistant which known as acquired resistance, and species were initially resistant pre exposure to the drug which known as intrinsic resistance. Acquired resistance may develop during the treatment course due to several factors such as; the improper use of antifungal drugs (Pfaller, 2012).

Furthermore, The wide spread use of fluconazole for both prophylactic and therapeutic purposes due to its high safety profile, has been associated with the dramatic increase in infections with NAC species, such as *C. krusei and C. glabrata,* which are often more resistant to fluconazole intrinsically, compared with *C. albicans* which is known to be susceptible to this drug (Rex *et al,* 1995). However, the development of fluconazole resistance in *C. albicans* isolates during the course of fluconazole preventive therapy is common (Pittet *et al,* 1994).

Several mechanisms involved in fluconazole resistance have been identified for *Candida* species (White *et al,* 1997; Peman *et al*, 2009). The most important mechanism is active efflux of the drug from fungal cells, efflux pump, which normally serve to transport lipids and toxic

compounds across fungal cell membranes, could participate in the development of fluconazole resistance by reducing the intracellular accumulation of the drug (Sanglard and Odds, 2002). In addition to fluconazole, several studies have correlated overexpression of the efflux pump genes with the development of resistance in other azoles such as itraconazole and ketoconazole. (Grossman *et al,* 2015) Recently, study by Ben-Ami *et al,* (2017) has reported drug efflux pump as a major mechanism of the intrinsic resistance of *C. auris* to azoles (Ben-Ami *et al,* 2017).

Although, the mechanisms of azole resistance have been extensively studied in resistant *Candida* isolates, limited numbers of studies have addressed the mechanisms of polyene and echinocandins resistance. This is due to the fact that resistance to these two classes is rare and being restricted mostly to the less common species of *Candida*, such as *C. glabrata, C. krusei and C. lusitaniae* as well as *C. auris*. However, the emergence of these strains has been reported more frequently. Therefore, the development of new approaches such as combined antifungal therapy has been effective alternative to control resistant infections (Ghannoum *et al,* 1995).

1.5.3 Combination antifungal therapy

The concept of combination therapy has been used successfully in antibacterial therapy, and more recently in antiretroviral therapy, using two or more antimicrobial agents with a synergistic mode of action together can improve their efficacy and broaden their spectrum of activity, as well as, overcome antimicrobial resistance and reduce the toxicity of these agents (Ghannoum *et al,* 1995). Theoretically, combination therapy should also be a useful strategy in antifungal therapy. However, it is uncommon in clinical practice, except for a few situations where this combination has been shown to be superior to monotherapy (Mukherjee *et al,* 2005).

For example; the combination of amphotericin B and flucytosine was seen to be superior to monotherapy in cryptococcal meningitis. Also, the combination of fluconazole and flucytosine has increased the survival of patients with *Cryptococcus neoformans* meningitis in randomised clinical trials (Bennett *et al,* 1979).

Clinical data supporting the use of combined antifungal therapy for invasive *Candida* infection are still lacking. Although theses combinations have been studied *in vitro* and in animal models, no clinical trials on these combinations have been reported (Chaturvedi *et al,* 2011). *In vitro* studies have suggested that a combination of amphotericin B and flucytosine may be better than amphotericin B monotherapy in *Candida* meningitis (Smego *et al,* 1984). In addition, the combination of echinocandins with amphotericin B or azoles has been applied by clinicians for treatment of resistant and invasive *Candida* infection that are not responding to monotherapy. However, this has not been fully supported from clinical trials (Campitelli *et al,* 2017). Despite the growing interest of combination antifungal therapy as a treatment strategy for refractory *Candida* infections, controlled clinical trials are still needed to exclude the possible antagonism and/or toxicity of some of these combinations.

1.5.4 Antifungal drugs for treatment of *Candida auris* infections

Although, there are currently no established specific susceptibility clinical breakpoints for *C. auris*, the CDC has proposed a tentative breakpoint for selected antifungal drugs based on susceptibility of closely related *Candida* species, which has been used to interpret the antifungal susceptibility testing of *C. auris*. Based on this, several studies have reported that almost all *C. auris* isolates were resistant to fluconazole, up to one-third were resistant to amphotericin B, about 5% were resistant to echinocandins, and, alarmingly, some of *C. auris* isolates were resistant to all three classes of antifungal drugs. Therefore, echinocandins are the most effective antifungal class against this pathogen. However, acquired resistance to

echinocandins can develop during the treatment course, this is especially concerning as echinocandins are currently recommended first-line therapy for treatment of *C. auris* infections. Furthermore, echinocandins are also used in combination therapies with the other antifungal drugs against multi drug resistant isolates in difficult to treat cases (CDC, 2017b).

In conclusion, it is remarkable that antifungal drug resistance and high treatment failure rate are particular problems with *C. auris* infections, this raises major concerns among microbiologist and clinicians about the ability of the existing antifungal drug to combat infections caused by this emerging multi-drug resistant fungal pathogen. Therefore, it is currently agreed that alternative strategies as well as discovery of new antifungal drugs with multiple targets have become an urgent need for efficient treatment of patients. Moreover, the search for novel antifungal drugs from plant secondary metabolites has been a major interest of the field.

1.6 Plant essential oils and their potential applications as antifungal agents

Drug development from natural sources such as plants and microorganisms continue to play a highly important role in the treatment of many human diseases. In the field of infectious diseases, natural product compounds have been a major source for the antimicrobial drug discovery, about 75% of commercial antimicrobial drugs were produced from natural sources. Natural products are being developed as drugs directly or templates for drugs to treat infections caused by resistant pathogens, it also leads to the discovery of new targets and pathways involved in the disease process (Vincent *et al,* 2006).

There are various examples of antimicrobial drugs discovered from natural sources, which have certainly revolutionized medicine. Examples include penicillin, tetracyclines, erythromycin, , micafungin and caspofungin. Moreover, different drug compounds discovered this way in other

therapeutic areas, include anticancer, anticoagulant and immunosuppressant agents (Harvey *et al,* 2008; Katiyar *et al*, 2012).

Furthermore, natural plant extract, especially essential oils, are of great interest to researchers in food, cosmetics and pest control industries, (Isman *et al*, 2000; Bakkali *et al,* 2008) as well as in the pharmaceutical industries. Many essential oils and its biologically active compounds have been reported to have antiseptic, (Bajpai *et al,* 2012) local anaesthetics, (Bakkali *et al,* 2008) antioxidant, (Roby *et al*, 2013) antibacterial (Rosato *et al,* 2008*)* and antifungal activities (Ahmad *et al,* 2011; Khan *et al*, 2012).

In addition*,* several published research articles demonstrated the antifungal activity of the most active components of the essential oils, specifically (phenolic derivatives) monoterpene phenols, against different *Candida* species. They have also reported the synergistic in vitro activity of monoterpene phenols in combination with the antifungal drugs against resistant *Candida* isolates (Pinto *et al,* 2006; Ahmad e*t al,* 2010; Khan *et al,* 2010), using the active component is preferable upon using the essential oil to avoid any potential influence or side effect of other minor component.

According to our knowledge no studies has investigated the effect of these compounds against *C. auris*. On this basis, it attracted our interests to study the antifungal activity of selected monoterpene phenols against *C. auris*, which has been reported to be highly resistant to all the major classes of antifungal drugs. Therefore, the search for alternative therapies from natural sources featured by new mechanisms of action along with new strategies have become an indispensable medical priority to maintain control of this multidrug resistant fungal pathogen.

1.6.1 Monoterpene phenols

Monoterpene phenols are major components of plant essential oils. It is evident that compounds containing phenol groups are the most active constituents, which contribute to the overall antimicrobial properties of essential oils. Essential oils rich in phenolic monoterpenes are known to be active against many microorganisms, including gram-positive and gram-negative bacteria and fungi, such as methicillin-resistant *Staphylococcus aureus (*MRSA), *Pseudomonas aeruginosa* and *Candida* species (Medicinal Plants: Biodiversity and Drugs, 2012; Cowan, 2009; Trombetta *et al,* 2005).

Generally, monoterpene phenols are produced from plants as a defence mechanism against plant pathogenic fungi (Numpaque *et al,* 2011). In addition, the effectiveness of these compounds in reducing fungal growth in fruits during storage have been reported, as well as their inhibitory effect against many food-borne pathogens in food products artificially contaminated with *Vibrio cholera* and *Escherichia coli*. Therefore, it is suggested that they might be used as natural preservative agents in food industries (Ultee *et al*, 2002; Burt, 2004; Xu *et al,* 2008; Lima e*t al*, 2013).

Four phenolic compounds have attracted much attention, which are; Eugenol [2-methoxy-4-(2-propenyl) phenol] and methyleugenol [1, 2-dimethoxy-4-(2-propenyl) phenol] are two phenyl propanoids, and carvacrol (5-isopropyl-2-methylphenol) and thymol (2-isopropyl-5-methylphenol) are two monoterpene phenols. These are especially known for their significant antifungal activity against *Candida* species (Khan *et al,* 2010; Ahmad *et al*, 2010; Ahmad *et al,* 2011; Wang *et al,* 2018).

These substances are found as major components in the essential oils of many aromatic plants. Eugenol and methyleugenol are found in the clove and basil oil, whereas carvacrol and thymol

are found in the oregano and thyme oil. These essential oils are used as a food flavouring agents (Ahmad *et al,* 2010; Ahmad *et al*, 2011).

1.6.1.1 Antifungal activity of monoterpene phenols

Several studies have reported the specific in vitro and in vivo antifungal activity of eugenol, methyleugenol, carvacrol and thymol against clinical fluconazole-sensitive and fluconazole-resistant *Candida* isolates other than *C. auris* (Suresh *et al,* 1997; Manohar e*t al,* 2001; Pina-Vaz *et al,* 2004; Chami e*t al*, 2005; Braga e*t al,* 2007; Guo e*t al*, 2009; Ahmad *et al,* 2010; Ahmad *et al*, 2011; Ahmad *et al,* 2012).

These compounds exhibited a potent *in vitro* fungicidal activity against different species of *Candida*, namely *C. albicans,* C. *tropicalis*, *C. glabrata*, *C. krusei*, *C. guillermondii* and C. *parapsilosis.* In contrast, fluconazole, which is the most widely used azole for treatment and prevention of candidiasis and the least toxic agent among the currently available antifungals, acts as fungistatic agent rather than fungicidal. The fungistatic nature of filuconazole possess a selective pressure on the fungus and consequently leads to the development of fluconazole resistance *Candida* species (Pina-Vaz *et al,* 2003; Ahmad *et al,* 2010).

In addition, some of these compounds have also been reported to possess anti-virulence effects against *C. albicans*. Carvacrol and thymol have shown inhibitory effect against hyphal formation in *C. albicans* at sub inhibitory concentrations (Pina-Vaz e*t al,* 2003; Braga e*t al,* 2007). Carvacrol have been shown to be effective in reduction of the Secreted Aspartyl Proteinase (SAP) gene expression in both susceptible and resistant *C. albicans* isolates with a higher effect against the resistant isolates (Hosseini *et al,* 2016). Carvacrol also was able to inhibit biofilm formation of different *Candida* species, including *C. albicans*, *C. glabrata*, and

C. parapsilosis (Dalleau *et al,* 2008). Such an anti-virulence effect will reduce the pathogenic potential of these fungi, thereby preventing or reducing the severity of infection.

Furthermore, in oral candidiasis animal study, local treatment with eugenol and carvacrol to experimentally infected immunosuppressed rats has shown a significant reduction of this infection in the treated group when compared to the untreated group, and more comparable to the control group treated with nystatin (Chami e*t al,* 2005). In other systemic candidiasis animal model, oral administration of carvacrol to the infected mice resulted in higher survival rate compared to the untreated mice (Manohar *et al,* 2001). Importantly, no acute toxicity nor kidney burden was observed in the treated group of mice in both studies.

Therefore, the *in vivo* therapeutic efficacy of these compounds on experimental oral and systemic candidiasis leads up to consider them as potential antifungal agents. However, further *in vivo* studies and comparative studies on the therapeutic efficacy with other fungi will assess the prospect of these compounds for clinical applications.

1.6.1.2 Mechanism of action

Although the specific fungicidal effect of these active compounds against different species of *Candida* is well established, there is relatively little information available on the mechanism involved in the antifungal activity. Little is known about their mechanism of action against other microorganisms as well (Pina-Vaz e*t al,* 2003; Chami *et al*, 2005; Ahmad *et al,* 2010).

Thymol and carvacrol affect the structural and functional properties of *Candida* cell membrane. They inhibit the ergosterol biosynthesis within the fungal cell membrane that leads to major membrane damage, induction of cell leakage and consequently cell death (Pinto e*t al,* 2001; Lambert e*t al,* 2001; Lima e*t al,* 2013).

Furthermore, Ahmad *et al,* (2010) observed that monoterpenes affect the ergosterol biosynthesis in a dose dependent manner and decrease the membrane ergosterol content at their sub-inhibitory concentrations. In addition, Rao *et al*, (2010) described the ionic stress response of *Saccharomyces cerevisiae* model to monoterpenes. Both carvacrol and thymol disrupt Ca^{2+} and H^{+} homeostasis in yeast and that lead to increase cell permeability and loss of metabolic activity, which ultimately causes cell death. Recently, their ability to induce oxidative stress and impair the defence system in *C. albicans*, even at sub-inhibitory concentrations, has also been reported (Khan *et al,* 2015).

Further investigation on the detailed cellular response and the molecular basis of the killing mechanism of these compounds would improve their therapeutic potential as a drug by itself or in combination with conventional antifungal drugs.

1.6.1.3 Synergism with antifungal drugs

Several studies have shown that essential oils and its major constituents have a synergistic effect in combination with conventional antifungal drugs against different *Candida* species (Suresh e*t al*, 1996; Khan *et al,* 2010; Khan e*t al*, 2012). Combination therapy has become an interesting area in developing new therapeutic strategies against fungal infections. They can improve the efficacy, overcome drug resistance and reduce the toxicity of antifungal drugs (Ghannoum *et al*, 1995; Mukherjee *et al,* 2005).

In particular, eugenol, methyleugenol, carvacrol and thymol possess a synergistic in vitro antifungal activity in combination with fluconazole and other antifungal drugs against different *Candida* isolates, including both susceptible and resistant *Candida* strains (Guo *et al,* 2009; Ahmad *et al,* 2010; Ahmad *et al,* 2012). These compounds attack the *Candida* cell at different targets from the antifungal drugs, which enable them to interact synergistically and augment

the efficacy of the antifungal agents. Furthermore, Thymol and carvacrol have shown a high potency in reversal of drug resistance in *Candida* species. The two compounds have inhibited the drug efflux pump gene expression in *C. albicans*, *C. glabrata* and *C. krusei*, which is the main mechanism of azole resistance in *Candida*. Therefore, they can restore the sensitivity of the resistant *Candida* isolates to the drugs (Ahmad *et al*, 2012).

Although these phenolic compounds possess a potent *in vitro* synergistic antifungal effect, when combined with antifungal drugs, against different *Candida* isolates. Further researches based on animal models focus on the mechanism of action of these combinations and possible side effects and toxicity in humans is needed in order to expand the knowledge on clinical application of such combination.

1.6.1.4 Safety of monoterpene phenols *in vivo*

Humans are exposed to eugenol, methyleugenol, carvacrol and thymol through its widespread use in food as spices and in traditional medicine, and based on the available data from several reports, these naturally occurring compounds are considered as nontoxic and generally recognized as safe (GRAS) to be administrated as food flavouring agents (EAFUS, 2006). However, the available *in vivo* study data on the safety and dose toxicity of these compounds in humans are insufficient (Rompelberg *et al*, 1996).

Moreover, the genotoxicity potential of these compounds was assessed by the National Toxicology Program (NTP) in an *in vivo* short-term assay in rodents and have reported that, these compounds showed no evidence of carcinogenicity in rats (NTP, 1983; NTP, 2008). However, hepatotoxicity and raised liver enzymes, which has been associated with the development of the hepatocellular tumours, following administration of very high doses of eugenol in mice was observed by some reporters, which present some concern that eugenol

might play role in liver cancer development by non-genotoxic mechanisms (Allavena *et al,* 1992; Rompelberg *et al,* 1993).

In order to use these compounds as antifungal agent in a rational way, a good knowledge of its biochemical properties, absorption, distribution, metabolism, and excretion is required, and much more work needs to be performed to exclude any long-term adverse effects or chronic toxicity in humans.

1.7 Justification for this study

Despite being the newly emerged *Candida* spp, *C. auris* is a multi-drug resistant pathogen associated with highly invasive bloodstream infections and hospital outbreaks. This species has been reported to be highly resistant to all the major classes of antifungal drugs used to treat candidiasis. Therefore, there is an urgent need to develop new and safe strategy to deal with this pathogen and one important approach is to target virulence factors. In order to target virulence factors, it is important to identify and compare these factors with other *Candida* species. Inhibiting virulence factors will not pose survival pressure on the microbes and thereby bypass resistance development. Furthermore, focus on developing new and safer antifungal compounds from natural sources and use these compounds in combination with antifungal drugs will be an alternative and effective approach to deal with multidrug resistance of *C. auris*.

1.8 Aims

The primary aim of this study was to investigate the pathogenic characteristics of *C. auris* and study the antifungal activity of monoterpene phenols against *C. auris* isolates.

1.8.1 Objectives

- To study the characteristic virulence factors (adherence, morphogenesis and secretion of hydrolytic enzymes) of *C. auris* isolates.
- To evaluate the minimum inhibitory concentrations and minimum fungicidal concentrations of selected monoterpene phenols against *C. auris.*
- To evaluate the antifungal activity of the most active monoterpene phenol in combination with the commonly used antifungal drugs against *C. auris.*
- To determine the effect of the most active monoterpene phenols on the pathogenicity markers of *C. auris*.

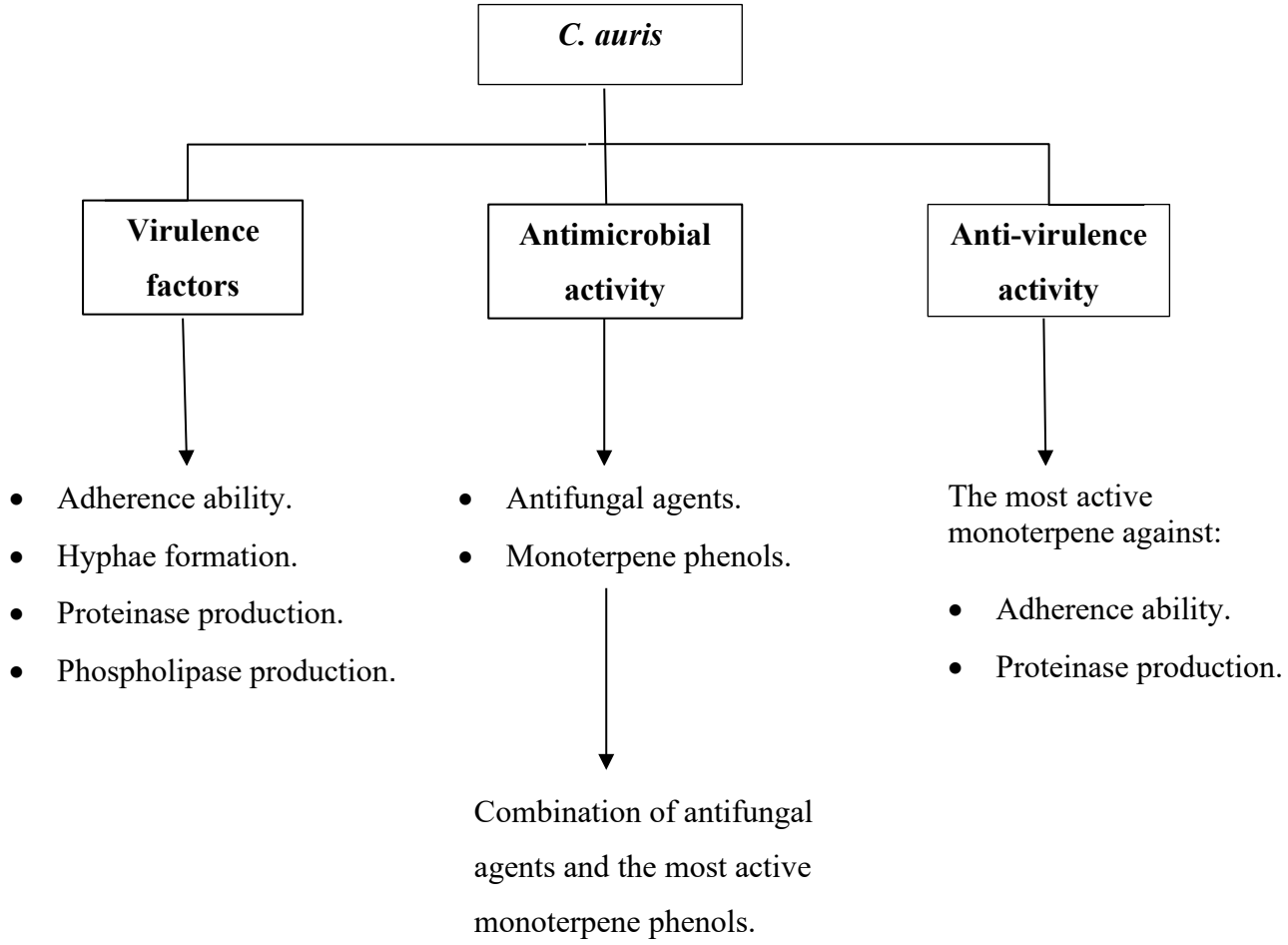

Figure 1: Diagram illustrating the study outline.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals, reagents and media

All the reagents used in this study were of molecular or analytical grade. A list of all reagents used as well as the composition of media, buffers and solutions are found in appendices (Appendix B, page 120). All reagents used were purchased from Sigma Aldrich, South Africa.

2.2 Natural compounds and antifungal drugs

The following phenolic compounds: eugenol (≥ 97.5 % purity), methyl eugenol (≥ 98.0 % purity), thymol (≥ 98.5 % purity) and carvacrol (≥ 98.0 % purity) were used in this study (Sigma-Aldrich, South Africa). A stock solution of 5000 µg/ml for all the four compounds was made using 1% dimethyl sulfoxide (DMSO) and stored at 4 ± 2°C in the dark until required. The following antifungal agents were used in this study: fluconazole, amphotericin b, caspofungin and nystatin (Sigma-Aldrich, South Africa). All antifungals were prepared at a stock concentration of 2000 µg/ml in sterile water and stored at -20°C until required.

2.3 *Candida* strains and inocula

In order to accomplish the objectives defined, a total of 25 clinical *C. auris* isolates were used along with a control laboratory strain *C. albicans* SC5314. The clinical isolates were obtained from the Division of Mycology, National Institute of Communicable Diseases, Johannesburg, South Africa, which were identified using Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) technique. The fungal strains used are listed in Appendix C. *C. albicans* SC5314 was selected as a positive control because its virulence factors have been well characterized. The isolates were stored in glycerol stock at -80 ºC in the Department of Clinical Microbiology and Infectious Diseases. For the use of these isolates ethical clearance was

obtained from The Committee for Research on Human Subjects (Medical), University of the Witwatersrand, Johannesburg (certificate number W-CBP-180529-4).

Prior to the experiments, cells from glycerol stocks for each *Candida* isolate was streaked onto a Sabouraud dextrose agar (SDA) plate and incubated overnight at 37 ± 1°C. A colony was then inoculated into 5 ml Sabouraud dextrose broth (SDB). This suspension was then incubated at 37 ± 1°C for 18 hrs and was used for all the experiments throughout the study

2.4 Antifungal susceptibility

Antifungal susceptibility testing to determine minimum inhibitory concentrations (MIC) of the antifungal drugs and the test compounds against *Candida* isolate was obtained using the Broth microdilution (BMD) method following the Clinical and Laboratory Standards Institute (CLSI) guidelines reference document M-27A with slight modifications (CLSI, 2008).

A standardised inoculum of *Candida* isolates ($1.5x10^6$ cfu/ml) was prepared by suspending colonies from the *Candida* cells cultures in 5 ml of 0.85% saline, then vortexing for 1 minute to allow cells to disperse properly. The 96 well microtitre plate was prepared by adding 100 µl of media to all the rows. Followed by the addition of 100 µl of each antifungal drug/ phenolic compound from the initial concentration of the test compounds to the first row of the plate and serial 2-fold dilutions were made. To each of these dilutions, 100 µl of the standardised inoculum of each *Candida* isolate was added, giving a final volume of 200 µl.

The range of concentrations for each test agent used were: fluconazole (FLU; 0.25–500 µg/ml), amphotericin B (AMP; 0.03–16 µg/ml), nystatin (NYS; 0.03–64 µg/ml), caspofungin (CAS; 0.03–16 µg/ml), carvacrol (CAR; 4–500 µg/ml), thymol (THY; 10–1250 µg/ml), eugenol (EUG; 20-2500 µg/ml) and methyleugenol (MEUG; 20-2500 µg/ml).

In every set of experiment, a culture control well i.e. “yeast with no drug/ compound”, a media control well i.e. “media with no yeast” and a negative control well i.e. “yeast with 1% DMSO” were added into the plates to confirm the viability and sterility of the experiments. The microtitre plates were then incubated at 37°C ± 1°C for 24hrs, and evaluated visually, by eye, observing the presence or absence of visible growth. The MIC was defined as the lowest concentration of the test drug/compound that resulted in the complete inhibition of visible growth or a decrease of growth by ≥90% relative to that of the culture control. The reference strain *C. albicans* SC5314 were used for quality control.

The results of antifungal susceptibility test were interpreted as sensitive (S) and resistant (R) for each antifungal drug used. Due to the lack of defined *C. auris* specific susceptibility breakpoints by the Clinical Laboratory Standard Institute (CLSI), tentative breakpoints recommended by the CDC were used, which were based on the established MICs for closely related *Candida* species. The tentative MIC breakpoints were for fluconazole <32 µg/ml considered sensitive; ≥32 µg/ml resistant, amphotericin B S <2 µg/ml; R ≥2 µg/ml and caspofungin S <2 µg/ml; R ≥2. No breakpoints have been determined for nystatin. We considered using breakpoints for amphotericin B since close MIC values were obtained for both nystatin and amphotericin B against *C. albicans* SC5314.

The minimum fungicidal concentrations (MFCs) were determined after determining the 24 hours MIC values. All wells in the microtitre plates without visible growth were sub-cultured onto SD agar plates, by spreading 10 µl onto agar plate. The plates then were incubated at 37°C for 24hrs to 48 hrs. Concentration in the first well with no growth on the plate was taken as MFC.

These experiments were repeated three times for each strain to validate the results. In addition, median MIC/MFC were determined, which represents the MIC/MFC obtained by ≥50% of the *C. auris* isolates tested in this study.

On the basis of the antifungal susceptibility testing results, the MIC and MFC values were used to identify the most active compound among the four phenolic compounds used in this study. The most active compound was determined as the one with the lowest median MIC/MFC values against *C. auris* isolates.

Carvacrol was selected as the most active compound and used throughout the study. The median MFC, the median MIC and two subinhibitory concentrations were selected and used in the subsequent virulence assays, these concentrations were 0.25, 0.125, 0.063, 0.031 mg/ml.

2.5 Combination assay

To determine the combination interaction of the most active phenolic compound (carvacrol) with fluconazole, amphotericin B, nystatin and caspofungin, a method described by Ahmad *et al*. (2014) was followed with some modifications. All four combinations (carvacrol-fluconazole, carvacrol- amphotericin B, carvacrol- nystatin and carvacrol- caspofungin) were tested against 25 clinical *C. auris* isolates and one control strain.

The test agent was combined with the antifungal drugs in a 1:1 volume ratio using their previously determined MICs. The microtiter plates were prepared by adding 100 µL of media into each of the wells followed by an addition of carvacrol (50 µl) and antifungals (50 µl) in the first row and were then serially diluted. 100 µl of the inoculum suspension prepared as mentioned above (in section 2.3.1) were added directly to the wells. All the controls as described above were included in this assay. The plates were then incubated at 37 ± 1 ºC for

24 h and MICs of both test agents in combination were determined visually. The experiment was performed in triplicate to validate the results.

2.5.1 Determination of the fractional inhibitory concentration index (FICI)

For each combination, we calculated the fractional inhibitory concentration index (FICI) based on the zero-interaction theory of Loewe additivity (LA) model (Loewe, 1953), which is the reference model used for measuring the effects of drug combinations. FICI value was calculated as the sum of the FICs of the test agents. The FIC for each agent was determined by establishing the MIC (a or b) in the combination (MIC in combination) divided by the MIC of the test agent acting alone (MIC tested alone). The formula used to calculate the FICI is the following:

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

Where, MICa and MICb were the MICs of the most active compound (carvacrol) and antifungal drugs, respectively.

2.5.2 Interpretation of the fractional inhibitory concentration index (FICI)

The FICI data were interpreted using the following criteria:

- Synergism: when FICI ≤ 0.5.
- Additive: when FICI between 0.5 and 1.0.
- Indifference: when FICI between 1.0 and 4.0.
- Antagonism: when FICI >4.0.

The term “synergism” means that the interaction between the two agents causes the final effect to be greater than the sum of the effects of the individual agents. An “additive” term, also

known as “partial synergy”, indicates that the total effect is equal to the sum of the effects of the two agents. The term “indifference” means that the effect of a combination is equal to the effect of the most effective agent. The term “antagonism” indicates that the interaction between the two agents causes reduced effect in comparison with the effect of the most effective agent.

2.6 Virulence assays

A comparative study was conducted to understand the similarities and differences of virulence factors between *C. albicans* and *C. auris*, particularly those that have been well described for *C. albicans*. They are adherence, morphogenesis, phospholipase and proteinase production. In addition, determined the effect of the most active compound (carvacrol) on these markers. All isolates expressing these markers were treated with different concentrations (0.25, 0.125, 0.063, 0.031 mg/ml) of carvacrol.

2.6.1 Adherence assay and the effect of carvacrol on the adherence

The ability of *C. auris* to adhere to the epithelial cells were determined using a technique described by Patel *et al.* (2009) with modifications.

Buccal epithelial cells were gathered from the investigator (myself) by use of a sterile swab, cells were suspended, washed three times and re-suspended in 2 ml sterile distilled water. With the use of a hemocytometer, epithelial cells count was adjusted to 10^5 cells/ml. Five millilitres of SD broth were inoculated with test culture and incubated overnight at 37 °C. Yeast cells were then harvested by centrifugation, washed three times with distilled water and re-suspended into 2 ml of SD broth, *Candida* cells count were standardized to 10^7 cells/ml using a haemocytometer. Subsequently, two milliliters of yeast cells and 2 ml of oral epithelial cells were mixed and incubated at 37 °C for 2 h while shaking at 60 rpm. A 20 µm pore nylon filter (Sigma-Aldrich, South Africa) were used to separate the epithelial cells from non-adherent

yeast cells. Cells were then washed twice with distilled water and re-suspended in 1 ml of sterile distilled water. Slides were prepared and stained with the gram stain. The number of yeast cells adhering to 100 epithelial cells was counted under a light microscope. Three slides were prepared from each sample and read as three results.

To study the effect of the most active compound, the four concentrations of carvacrol were inoculated into the media before incubation. A negative control without carvacrol was also included. Thereafter, adherence assay was followed as described above. A control culture of *C. albicans* was included in the assays.

2.6.2 Evaluation of morphogenesis

The ability of *C. auris* to form hyphae was assessed using a protocol mentioned by Yousuf *et al,* (2011) with some modifications. *Candida* cells were sub-cultured up to the late log phase of their growth at 37 °C, with gyratory shaking at 200 rpm. Cells were then transferred into another flask containing fresh media and incubated for 48 h at 37 °C, which resulted in a synchronised cell population. To induce hyphal formation, ten microliters of above grown cells were transferred into 5 ml of fresh SD broth supplemented with 10% foetal bovine serum (Sigma-Aldrich, South Africa) at pH 6.5. Cells were incubated at 37 °C and the pH was adjusted every half hour to the pH of 6.5. Then aliquots of ten microliters were transferred onto a glass slide at different time interval, after every 1 hour for 3 hours then every 3 hours till 24 hours, a coverslip was added, the slide was then viewed under the light microscope at a ×40 magnification and at least 200 cells were examined for hyphal formation. The percentage of hyphae was obtained by taking the ratio of the number of hyphae to total number of cells. A control culture of *C. albicans* was included in the assays.

These experiments were repeated three times for each strain. The effect of four concentrations of carvacrol on morphogenesis in *C. auris* isolates has not been done since all *C. auris* strains tested in this study did not show any true hyphal and/or pseudohyphal cells.

2.6.3 Determination of proteinase and phospholipase activity

All the *C. auris* isolates were initially screened for proteinase and phospholipase secretions using plate assay containing bovine serum albumin (BSA) and egg yolk respectively (Yousuf *et al.,* 2011). Fresh cultures of *Candida* grown on SD agar were harvested and sub-cultured in flasks containing 5 ml SD broth and incubated at 37 °C for 18 h. The cells were then centrifuged at 300 ×g for 5 minutes and a suspension of fresh culture in SD broth adjusted to approximately 10^6 cfu/ml using spectrophotometer was prepared. Enzyme assays were then performed as per the description below..

Extracellular proteinase activity of *C. auris* strains was evaluated by measuring the size of the zone of degradation after growth on BSA agar plates (2% agar, 2g BSA, 1.45 g yeast nitrogen base without amino acids, 1.45 g ammonium sulphate and 20g glucose, 1000 ml distilled water). Aliquots of two microliters of the culture solution was carefully spotted at equidistant points on the surface of proteinase agar plates. Agar plates were allowed to get dry by placing them in the incubator half open for 30 minutes. The plates were then closed and incubated at 37 °C for 3-4 days.

Plates were examined for proteinase activity. The isolates showing a clear zone of proteolysis around the colonies were considered to be positive for proteinase production. *C. albicans* SC5314 was used as positive control. The diameters of the colonies and the diameters of the clear zone around the colonies were measured and an average of four readings was calculated.

Thereafter, proteinase activity index (the P_z value) was calculated by dividing colony diameter with colony plus clear zone diameter.

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

C. auris isolates were screened for extracellular phospholipase activity by measuring the size of the zone of precipitation after growth on egg yolk agar plates (2 % agar, 10g peptone, 30g glucose, 57.3g NaCl, 0.55g $CaCl_2$ and 10% egg yolk emulsion to 900 ml of distilled water). Aliquots of two microliters of test suspension were carefully placed at equidistant points on the surface of the egg yolk agar plates. This suspension was allowed to get dry and the plates were incubated at 37 °C for 7 days.

After incubation, plates were examined for phospholipase activity, which were considered positive when there were visible precipitation zones around the colonies. *C. albicans* SC5314 was used as positive control. The diameter of opaque zone and the diameter of the colony were measured, and an average of four readings was taken. Phospholipase activity index (the P_z value) was assessed by measuring the ratio of the diameters of the colonies to the diameters of the colonies plus the precipitation zone around the colonies.

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

The results were interpretated as follow: a Pz value of 1 denoted no enzyme activity by the isolate, Pz < 1 indicated positive enzyme activity by the isolate. The lower the Pz value, the higher the enzyme activity. Enzymes activity levels were classified into five classes as described by Kantarcioglu *et al*. (2002). When P_z = 1, −, no enzyme activity, P_z = 0.90 to 0.99, +, weak enzyme activity, Pz = 0.80 to 0.89, ++, medium enzyme activity, P_z = 0.70 to 0.79, +++, strong enzyme activity, P_z = < 0.69, ++++, very strong enzyme activity.

2.6.3.1 Effect of carvacrol on the hydrolytic enzymes

To study the effect of the most active compound on secretion of proteinase enzyme, cells from isolates showing positive enzyme activity were exposed to the four concentrations of carvacrol for 2 hours at 37 °C. Cells without any treatment served as the controls and *C. albicans* SC5314 served as positive control. To minimize experimental error, the assays were conducted on three separate occasions for each yeast isolate tested. The proteinase activity was measured as described previously (section 2.7.3). The effect of carvacrol on phospholipase activity in *C. auris* isolates has not been done since all *C. auris* strains tested in this study did not show positive phospholipase activity.

Flow diagram described the study outline in figure 1.1, page 37.

2.7 Statistical analysis

All the experiments were performed in triplicate. Statistical analysis was done using the GraphPad Prism (version 5) software. A descriptive statistical analysis of the antifungal susceptibility and the combination study was performed and the median MIC/MFC and FICI were calculated. Descriptive statistics of the virulence assays were shown as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to analyse the effect of the four concentrations of carvacrol and the control on adherence and proteinase activity of *C. auris* isolates, and then the treated groups were compared to the untreated control groups using the Dunnett's multiple comparison test. A P-value of < 0.05 was considered statistically significant.

CHAPTER 3: RESULTS

3.1 Antifungal drug susceptibility profile of *C. auris*

The results of the MICs and MFCs values of the four antifungal drugs obtained for each of *C. auris* isolates are shown in Tables 1. There were three repeats done for each strain and the median MIC/MFC was recorded. The median MIC for caspofungin against all *C. auris* isolates (0.25 µg/ml) was lower than that of amphotericin B and nystatin (AMP: 0.5 µg/ml, NYS: 2 µg/ml). For fluconazole (FLU), the median MIC was 125 µg/ml with the range of 16-500 µg/ml (Table 2).

The median MFCs for caspofungin (CAS), amphotericin B (AMP) and nystatin (NYS) were 0.5, 1 and 4 µg/ml, respectively. Caspofungin (CAS) and amphotericin B (AMP) were also highly active against *C. albicans* SC5314 (MIC = 0.125 µg/ml and MFC = 0.5 µg/ml for both), while higher values for nystatin (NYS; MIC = 0.5 µg/ml and MFC = 1 µg/ml) and fluconazole (FLU; MIC = 4 µg/ml) were detected (Table 2).

The MIC results were interpreted for the sensitive or resistant status (Table 3). only one *C. auris* isolate (4%) was resistance to caspofungin (CAS), this isolate showed resistance to all antifungal drugs tested. Five *C. auris* isolates (20%) were resistant to amphotericin B (AMP). Resistance to fluconazole (FLU) was observed in 22 *C. auris* isolates (88%).

Table 1: Antifungal drug susceptibility profile of *C. auris* and *C. albicans*

Candida sp.	Strains	Repeats	Fluconazole		Amphotericin B		Caspofungin		Nystatin	
			MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)
C. auris	CAU 1	1	125	NS	0.25	0.5	0.25	0.5	0.5	1
		2	62.5	NS	0.125	0.25	0.25	0.5	1	2
		3	125	NS	0.25	0.5	0.5	1	1	2
	CAU 2	1	250	NS	0.5	1	0.5	1	2	4
		2	125	NS	0.5	2	0.25	0.5	1	2
		3	250	NS	0.25	1	0.25	0.5	1	2
	CAU 3	1	16	NS	0.5	2	0.125	0.25	1	2
		2	16	NS	2	1	0.25	0.5	1	2
		3	16	NS	1	2	0.25	0.5	1	2
	CAU 4	1	250	NS	0.5	2	0.25	0.5	2	4
		2	500	NS	2	4	0.125	0.5	2	4
		3	125	NS	2	4	0.25	0.5	1	2
	CAU 5	1	16	NS	0.25	0.5	0.5	1	4	4
		2	16	NS	0.5	1	0.25	0.5	2	2
		3	8	NS	0.5	1	0.25	0.5	2	2
	CAU 6	1	16	NS	0.125	0.25	0.25	0.5	2	4
		2	31	NS	0.25	0.5	0.5	0.5	1	2
		3	16	NS	0.125	0.25	0.25	1	2	4
	CAU 7	1	32	NS	0.5	0.5	0.5	1	2	4
		2	63	NS	0.25	0.5	0.25	0.5	2	2
		3	32	NS	0.5	1	0.25	1	1	4
	CAU 8	1	250	NS	2	4	0.5	1	2	4
		2	250	NS	2	4	0.5	1	4	8
		3	250	NS	1	2	0.25	1	2	4
	CAU 9	1	250	NS	1	1	0.25	0.5	1	2
		2	500	NS	1	2	0.25	0.5	1	2
		3	500	NS	0.5	1	0.25	0.5	2	4
	CAU 10	1	125	NS	0.5	1	0.25	0.5	2	4
		2	250	NS	0.5	1	0.5	0.5	1	2
		3	125	NS	0.25	1	0.25	0.5	1	2
	CAU 11	1	125	NS	0.5	1	0.25	0.5	1	2
		2	125	NS	0.5	1	0.5	0.5	1	2
		3	125	NS	1	1	0.25	0.5	0.5	1
	CAU 12	1	250	NS	0.5	1	0.5	0.5	2	4
		2	250	NS	0.25	0.5	1	1	2	4
		3	125	NS	0.5	1	0.5	1	1	2

- NS: Not specific.

Table 1: Antifungal drug susceptibility profile of *C. auris* and *C. albicans* - continue

Candida sp.	Strains	Repeats	Fluconazole		Amphotericin B		Caspofungin		Nystatin	
			MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)
C. auris	CAU 13	1	63	NS	0.25	0.5	0.125	0.5	2	4
		2	125	NS	0.25	0.25	0.125	0.5	2	4
		3	63	NS	0.5	0.5	0.25	0.5	1	2
	CAU 14	1	125	NS	0.5	0.5	0.5	1	2	4
		2	125	NS	0.25	0.25	0.5	1	2	4
		3	125	NS	0.25	0.5	1	2	4	8
	CAU 15	1	125	NS	0.25	0.25	0.5	1	0.5	1
		2	250	NS	0.25	0.5	0.5	1	1	2
		3	125	NS	0.5	0.5	0.5	1	0.5	1
	CAU 16	1	250	NS	0.5	0.5	0.5	0.5	1	1
		2	250	NS	0.25	0.5	0.25	1	1	2
		3	250	NS	0.25	0.5	0.5	0.5	2	2
	CAU 17	1	500	NS	2	4	0.125	0.5	2	4
		2	500	NS	1	2	0.25	0.5	1	4
		3	500	NS	2	4	0.25	0.5	2	8
	CAU 18	1	500	NS	0.5	1	0.25	0.5	2	4
		2	250	NS	2	2	0.5	1	4	8
		3	500	NS	2	4	0.25	0.5	4	8
	CAU 19	1	500	NS	0.5	1	0.25	0.5	1	2
		2	250	NS	1	1	0.5	1	2	4
		3	500	NS	0.5	1	0.25	1	2	4
	CAU 20	1	500	NS	1	2	0.25	0.5	1	2
		2	500	NS	0.5	1	0.125	0.5	2	4
		3	250	NS	1	2	0.25	1	1	2
	CAU 21	1	63	NS	0.25	0.5	0.5	1	1	2
		2	63	NS	0.5	1	0.25	0.5	1	2
		3	63	NS	0.25	0.5	0.5	1	1	4
	CAU 22	1	125	NS	0.5	1	1	1	2	4
		2	250	NS	0.5	1	0.5	1	1	2
		3	125	NS	0.25	0.5	0.5	1	1	2
	CAU 23	1	125	NS	0.5	1	0.25	0.5	1	2
		2	125	NS	1	2	0.25	0.5	1	2
		3	250	NS	1	2	0.25	1	0.5	1

- NS: Not specific.

Table 1: Antifungal drug susceptibility profile of *C. auris* and *C. albicans* – continue

Candida sp.	Strains	Repeats	Fluconazole		Amphotericin B		Caspofungin		Nystatin	
			MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)
C. auris	CAU 24	1	125	NS	4	8	1	2	1	2
		2	250	NS	2	4	2	4	2	4
		3	125	NS	4	8	2	4	2	4
	CAU 25	1	32	NS	0.5	1	0.25	0.5	2	4
		2	32	NS	0.25	1	0.25	1	2	4
		3	63	NS	0.25	0.5	0.25	0.5	2	4
C. albicans	SC5314	1	4	NS	0.125	0.5	0.125	0.5	0.25	1
		2	4	NS	0.25	0.5	0.125	0.5	1	2
		3	8	NS	0.125	0.5	0.125	0.5	0.5	1

- NS: Not specific.

Table 2: Summary results of antifungal drug susceptibility

***Candida* sp.**	**Antifungal**	**MICs values in µg/ml (n=3)**	**MFCs values in µg/ml (n=3)**
		Median (range)	**Median (range)**
C. auris (25)	Fluconazole	**125** (16-500)	**NS**
	Amphotericin B	**0.5** (0.125-4)	**1** (0.25-8)
	Nystatin	**2** (0.5-4)	**2** (1-8)
	Caspofungin	**0.25** (0.125-2)	**0.5** (0.5-4)
C. albicans (1)	Fluconazole	**4** (4-8)	**NS**
	Amphotericin B	**0.125** (0.125-0.25)	**0.5** (0.5)
	Nystatin	**0.5** (0.25-1)	**1** (1-2)
	Caspofungin	**0.125** (0.125)	**0.5** (0.5)

- NS: Not specific.

Table 3: Interpretation of antifungal drug susceptibility profile of *Candida* sp

***Candida* sp.**	**Test agents**	**No. of isolates (%)**	
		S	**R**
C. auris (25)	Fluconazole	3 (12)	22 (88)
	Amphotericin B	20 (80)	5 (20)
	Nystatin	12 (48)	13 (52)
	Caspofungin	24 (96)	1 (4)
C. albicans (1)	Fluconazole	1 (100)	0 (0)
	Amphotericin B	1 (100)	0 (0)
	Nystatin	1 (100)	0 (0)
	Caspofungin	1 (100)	0 (0)

- S, sensitive; R, resistance.
- Classification based on CDC guidelines (Tentative MIC Breakpoints); FLU (S<32 µg/ml; R ≥32 µg/ml); AMP (S<2 µg/ml; R ≥2); NYS (S<2 µg/ml; R ≥2) CAS (S<2 µg/ml; R ≥2).

3.2 Antifungal susceptibility of phenolic compounds against *C. auris*

Antifungal susceptibility testing of the phenolic compounds against *C. auris* are shown in table 4. The results showed that carvacrol was the most active agent tested with lowest median MIC and MFC values of 0.125 mg/ml and 0.25 mg/ml respectively. Eugenol (median MIC= 0.625 mg/ml) and methyleugenol (median MIC= 1.25 mg/ml) were the least active agents. Carvacrol (CAR) was also the most active agent against *C. albicans* SC5314 (median MIC = 0.25 mg/ml), however, MIC value was higher than the MIC for *C. auris* (Table 5).

Table 4: Antifungal susceptibility of phenolic compounds against *C. auris*

Candida sp.	Strains	Repeats	Carvacrol (mg/ml)		Thymol (mg/ml)		Eugenol (mg/ml)		Methyleugenol (mg/ml)	
			MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. auris	CAU 1	1	0.125	0.25	0.312	1.25	0.625	2.5	1.25	2.5
		2	0.125	0.5	0.156	1.25	1.25	2.5	0.625	1.25
		3	0.25	0.5	0.156	1.25	0.625	2.5	1.25	2.5
	CAU 2	1	0.125	0.25	0.312	1.25	0.625	2.5	1.25	2.5
		2	0.125	0.5	0.156	1.25	0.625	2.5	0.625	2.5
		3	0.125	0.5	0.312	1.25	1.25	2.5	1.25	2.5
	CAU 3	1	0.063	0.25	0.312	0.625	0.625	2.5	0.625	2.5
		2	0.125	0.25	0.156	0.625	0.312	1.25	0.312	1.25
		3	0.125	0.25	0.156	0.312	0.625	2.5	0.625	2.5
	CAU 4	1	0.125	0.25	0.312	1.25	0.625	2.5	1.25	2.5
		2	0.063	0.125	0.312	1.25	0.625	2.5	1.25	2.5
		3	0.125	0.25	0.156	0.625	0.312	1.25	1.25	2.5
	CAU 5	1	0.125	0.25	0.312	0.625	1.25	2.5	1.25	2.5
		2	0.125	0.25	0.312	1.25	1.25	2.5	1.25	2.5
		3	0.25	0.5	0.156	0.625	1.25	2.5	1.25	2.5
	CAU 6	1	0.125	0.25	0.312	1.25	1.25	2.5	1.25	2.5
		2	0.125	0.25	0.312	1.25	1.25	2.5	1.25	2.5
		3	0.25	0.5	0.156	0.625	0.625	1.25	0.625	2.5
	CAU 7	1	0.063	0.25	0.312	0.625	0.625	1.25	1.25	2.5
		2	0.125	0.25	0.312	1.25	0.312	1.25	0.625	2.5
		3	0.125	0.25	0.312	0.625	0.625	2.5	1.25	2.5
	CAU 8	1	0.063	0.25	0.156	0.625	1.25	2.5	0.625	1.25
		2	0.125	0.25	0.156	0.625	0.625	1.25	1.25	2.5
		3	0.063	0.25	0.312	1.25	1.25	2.5	1.25	2.5
	CAU 9	1	0.125	0.25	0.312	0.625	1.25	2.5	1.25	2.5
		2	0.063	0.25	0.312	0.625	1.25	2.5	1.25	2.5
		3	0.125	0.25	0.156	0.312	0.625	1.25	2.5	2.5
	CAU 10	1	0.063	0.25	0.312	1.25	0.625	1.25	1.25	2.5
		2	0.125	0.25	0.312	1.25	0.625	1.25	1.25	2.5
		3	0.125	0.25	0.156	0.625	1.25	2.5	0.625	1.25
	CAU 11	1	0.125	0.25	0.312	0.625	0.625	1.25	0.625	2.5
		2	0.125	0.5	0.312	0.625	0.625	1.25	1.25	2.5
		3	0.125	0.5	0.312	0.625	1.25	2.5	1.25	2.5
	CAU 12	1	0.125	0.5	0.312	1.25	0.625	1.25	1.25	2.5
		2	0.125	0.5	0.312	0.625	0.312	0.625	1.25	2.5
		3	0.125	0.25	0.312	0.625	1.25	2.5	1.25	2.5

Table 4: Antifungal susceptibility of phenolic compounds against *C. auris* - continue

Candida sp.	Strains	Repeats	Carvacrol (mg/ml)		Thymol (mg/ml)		Eugenol (mg/ml)		Methyleugenol (mg/ml)	
			MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. auris	CAU 13	1	0.125	0.5	0.312	1.25	0.625	1.25	1.25	2.5
		2	0.063	0.5	0.312	0.625	0.625	2.5	1.25	2.5
		3	0.125	0.5	0.312	1.25	1.25	2.5	1.25	2.5
	CAU 14	1	0.063	0.125	0.156	0.625	0.156	0.312	0.625	2.5
		2	0.063	0.25	0.312	0.625	0.312	0.625	0.625	1.25
		3	0.125	0.5	0.156	0.625	0.312	0.625	1.25	2.5
	CAU 15	1	0.125	0.25	0.312	1.25	0.625	2.5	1.25	2.5
		2	0.125	0.25	0.312	0.625	0.625	1.25	1.25	2.5
		3	0.125	0.25	0.312	1.25	0.625	1.25	0.625	2.5
	CAU 16	1	0.125	0.5	0.312	1.25	0.625	2.5	0.625	2.5
		2	0.125	0.25	0.625	1.25	0.625	2.5	1.25	2.5
		3	0.125	0.5	0.312	0.625	0.625	1.25	1.25	2.5
	CAU 17	1	0.125	0.25	0.625	1.25	0.625	2.5	0.625	2.5
		2	0.125	0.5	0.625	1.25	1.25	2.5	1.25	2.5
		3	0.25	0.5	0.312	0.625	0.625	2.5	1.25	2.5
	CAU 18	1	0.125	0.5	0.312	1.25	0.625	2.5	0.625	2.5
		2	0.250	0.5	0.625	1.25	0.625	2.5	1.25	2.5
		3	0.125	0.25	0.312	0.625	1.25	2.5	1.25	2.5
	CAU 19	1	0.125	0.25	0.312	1.25	0.625	1.25	1.25	2.5
		2	0.063	0.25	0.312	1.25	0.625	2.5	1.25	2.5
		3	0.125	0.25	0.156	0.625	1.25	2.5	1.25	2.5
	CAU 20	1	0.125	0.25	0.312	1.25	0.625	1.25	0.625	1.25
		2	0.125	0.5	0.312	0.625	1.25	2.5	1.25	2.5
		3	0.125	0.5	0.312	1.25	1.25	2.5	1.25	2.5
	CAU 21	1	0.125	0.25	0.312	0.625	0.625	1.25	1.25	2.5
		2	0.125	0.25	0.312	1.25	0.625	2.5	0.625	2.5
		3	0.25	0.25	0.312	0.625	1.25	2.5	1.25	2.5
	CAU 22	1	0.25	0.5	0.625	1.25	1.25	2.5	2.5	2.5
		2	0.25	0.5	0.625	1.25	1.25	2.5	1.25	2.5
		3	0.25	0.5	0.625	1.25	1.25	2.5	1.25	2.5
	CAU 23	1	0.125	0.5	0.312	1.25	0.625	2.5	1.25	2.5
		2	0.125	0.5	0.312	1.25	1.25	2.5	1.25	2.5
		3	0.125	0.25	0.156	0.625	1.25	2.5	0.625	2.5

Table 4: Antifungal susceptibility of phenolic compounds against *C. auris* - continue

Candida sp.	Strains	Repeats	Carvacrol (mg/ml)		Thymol (mg/ml)		Eugenol (mg/ml)		Methyleugenol (mg/ml)	
			MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. auris	CAU 24	1	0.25	0.5	0.625	1.25	0.625	2.5	1.25	2.5
		2	0.25	0.5	0.625	1.25	0.625	2.5	0.625	2.5
		3	0.125	0.25	0.312	0.625	1.25	2.5	1.25	2.5
	CAU 25	1	0.125	0.25	0.312	1.25	1.25	2.5	1.25	2.5
		2	0.25	0.5	0.312	0.625	1.25	2.5	0.625	2.5
		3	0.125	0.25	0.312	1.25	1.25	2.5	1.25	2.5
C. albicans	SC5314	1	0.25	0.5	0.625	1.25	0.625	1.25	1.25	2.5
		2	0.25	0.5	0.625	1.25	1.25	2.5	1.25	2.5
		3	0.25	0.5	0.312	0.625	0.625	1.25	0.625	2.5

Table 5: Summary results of the antifungal susceptibility of phenolic compounds against *C. auris*

Candida sp.	Test agents	MICs values in mg/ml (n=3)	MFCs values in mg/ml (n=3)
		Median (range)	Median (range)
C. auris (25)	Carvacrol	**0.125** (0.063-0.25)	**0.25** (0.25-0.5)
	Thymol	**0.312** (0.156-0.625)	**1.25** (0.625-1.25)
	Eugenol	**0.625** (0.312-1.25)	**2.5** (0.625-2.5)
	Methyleugenol	**1.25** (0.625-1.25)	**2.5** (≥ 2.5)
C. albicans (1)	Carvacrol	**0.25** (0.25)	**0.5** (0.5)
	Thymol	**0.625** (0.312-0.625)	**1.25** (0.625-1.25)
	Eugenol	**0.625** (0.625-1.25)	**1.25** (1.25-2.5)
	Methyleugenol	**1.25** (0.625-1.25)	**2.5** (≥ 2.5)

3.3 Antifungal activity of carvacrol in combination with antifungal drugs

The results of *in vitro* susceptibility of *C. auris* isolates against carvacrol (CAR) in combination with fluconazole (FLU). Amphotericin B (AMP), Nystatin (NYS) and Caspofungin (CAS) are shown in Table 6-10.

When CAR was combined with FLU, the median MIC for FLU and CAR decreased from 125 to 32 µg/ml and from 125 to 63 µg/ml respectively, the combination exhibited synergistic activity in 4 isolates (16%), additive in 13 isolates (52%) and 8 isolates (32%) showed indifferent activity (Table 6).

When CAR was combined with AMP, the median MIC for CAR and AMP decreased from 125 to 4 µg/ml and 0.5 to 0.25 µg/ml respectively. Synergistic activity was demonstrated in 7 isolates (28%), 9 isolates (36%) showed additive and 9 isolates (36%) showed indifference (Table 7).

When CAR was combined with CAS, the median MIC for CAR decreased from 125 to 4 µg/ml and the median MIC for CAS remains 0.25 µg/ml, however, the median MIC for CAS resistance strain decreased from 2 to 0.5 µg/ml, the combination was synergistic in only one isolate (4%), additive in 6 isolates (24%) and 18 isolates (72%) showed indifference (Table 8).

When CAR was combined with NYS, the median MIC for CAR and NYS decreased from 125 to 4 µg/ml and 2 to 0.5 µg/ml respectively, the combination was synergistic in 7 isolates (28%), additive in 17 isolates (68%) and only 1 isolate (4%) showed indifference (Table 9).

For *C. albicans* SC5314, the interaction was indifference between CAR and FLU. AMP, NYS and CAS with FICI values of 1.03, 1.01, 1.02 and 1.01 respectively.

Overall, all combinations demonstrated some degree of synergy, and no antagonistic effects were observed against the *C. auris* isolates tested. Synergistic activity was higher in drug

resistance strains. Synergistic effects of CAR with CAS were shown against the one isolate (4%) which showed resistance to CAS and all other antifungal drugs tested. Among AMP resistance isolates (n=5), three isolates (60%) showed synergistic interaction with the CAR and AMP combination. Among NYS resistance isolates (n=13), 7 isolates (54%) showed synergistic interaction with the CAR and NYS combination. Among FLU resistance isolates (n=22), four isolates (18%) showed synergistic interaction with the CAR and FLU combination.

Table 6: ***In vitro*** **antifungal activity of carvacrol in combination with Fluconazole against *C. auris* and *C. albicans* SC5314.**

Candida **sp**	**Strains**	**Median MIC (µg/ml) n=3**				**FICI**	**INT**
		Alone		**In combination**			
		CAR	**FLU**	**CAR**	**FLU**		
C. auris (25)	CAU 1	125	125	63	32	0.75	ADD
	CAU 2	125	250	63	32	0.63	ADD
	CAU 3	125	16	16	8	0.63	ADD
	CAU 4	125	250	63	32	0.63	ADD
	CAU 5	125	16	32	16	1.26	IND
	CAU 6	125	16	32	16	1.26	IND
	CAU 7	125	32	63	32	1.50	IND
	CAU 8	63	250	63	32	1.13	IND
	CAU 9	125	500	32	16	0.29	SYN*
	CAU 10	125	125	63	32	0.75	ADD
	CAU 11	125	125	125	63	1.50	IND
	CAU 12	125	250	125	63	1.25	IND
	CAU 13	125	63	32	16	0.51	ADD
	CAU 14	63	125	63	31	1.24	IND
	CAU 15	125	125	63	32	0.75	ADD
	CAU 16	125	250	63	32	0.63	ADD
	CAU 17	125	500	125	63	1.13	IND
	CAU 18	125	500	32	16	0.29	SYN*
	CAU 19	125	500	32	16	0.29	SYN*
	CAU 20	125	500	63	32	0.57	ADD
	CAU 21	125	63	32	16	0.51	ADD
	CAU 22	250	125	125	63	1.00	ADD
	CAU 23	125	125	32	16	0.38	SYN*
	CAU 24	250	125	125	63	1.00	ADD
	CAU 25	125	32	32	16	0.76	ADD
C. albicans (1)	SC5314	250	4	8	4	1.03	IND

- FICI, fractional inhibitory concentration index; CAR, carvacrol; FLU, fluconazole; INT, interpretation; SYN*, synergy: IND, Indifference; ADD, additive.

Table 7: ***In vitro*** **antifungal activity of carvacrol in combination with amphotericin B against *C. auris* and *C. albicans* SC5314.**

Candida **sp**	**strains**	**Median MIC (µg/ml) n=3**				**FICI**	**INT**
		Alone		**In combination**			
		CAR	**AMP**	**CAR**	**AMP**		
C. auris (25)	CAU 1	125	0.25	4	0.25	1.03	IND
	CAU 2	125	0.5	4	0.25	0.53	ADD
	CAU 3	125	1	4	0.25	0.28	SYN*
	CAU 4	125	2	8	0.5	0.31	SYN*
	CAU 5	125	0.5	4	0.25	0.53	ADD
	CAU 6	125	0.125	2	0.125	1.02	IND
	CAU 7	125	0.5	4	0.25	0.53	ADD
	CAU 8	63	2	32	2	1.50	IND
	CAU 9	125	1	4	0.25	0.28	SYN*
	CAU 10	125	0.5	4	0.25	0.53	ADD
	CAU 11	125	0.5	4	0.25	0.53	ADD
	CAU 12	125	0.5	4	0.25	0.53	ADD
	CAU 13	125	0.25	4	0.25	1.03	IND
	CAU 14	63	0.25	4	0.25	1.06	IND
	CAU 15	125	0.25	4	0.25	1.03	IND
	CAU 16	125	0.25	4	0.25	1.03	IND
	CAU 17	125	2	8	0.5	0.31	SYN*
	CAU 18	125	2	8	0.5	0.31	SYN*
	CAU 19	125	0.5	4	0.25	0.53	ADD
	CAU 20	125	1	4	0.25	0.28	SYN*
	CAU 21	125	0.25	4	0.25	1.03	IND
	CAU 22	250	0.5	4	0.25	0.53	ADD
	CAU 23	125	1	4	0.25	0.28	SYN*
	CAU 24	250	4	32	2	0.63	ADD
	CAU 25	125	0.25	4	0.25	1.03	IND
C. albicans (1)	SC5314	250	0.125	2	0.125	1.01	IND

- FICI, fractional inhibitory concentration index; CAR, carvacrol; AMP, amphotericin B; INT, interpretation; SYN*, synergy: IND, Indifference; ADD, additive.

Table 8: ***In vitro*** **antifungal activity of carvacrol in combination with Caspofungin against *C. auris* and *C. albicans* SC5314.**

Candida **sp**	**Strains**	**Median MIC (µg/ml) n=3**				**FICI**	**INT**
		Alone		**In combination**			
		CAR	**CAS**	**CAR**	**CAS**		
C. auris (25)	CAU 1	125	0.25	4	0.25	1.03	IND
	CAU 2	125	0.25	4	0.25	1.03	IND
	CAU 3	125	0.25	4	0.25	1.03	IND
	CAU 4	125	0.25	4	0.25	1.03	IND
	CAU 5	125	0.25	4	0.25	1.03	IND
	CAU 6	125	0.25	4	0.25	1.03	IND
	CAU 7	125	0.25	4	0.25	1.03	IND
	CAU 8	63	0.5	8	0.5	1.12	IND
	CAU 9	125	0.25	4	0.25	1.03	IND
	CAU 10	125	0.25	4	0.25	1.03	IND
	CAU 11	125	0.25	4	0.25	1.03	IND
	CAU 12	125	0.5	4	0.25	0.53	ADD
	CAU 13	125	0.125	2	0.125	1.02	IND
	CAU 14	63	0.5	4	0.25	0.56	ADD
	CAU 15	125	0.5	4	0.25	0.53	ADD
	CAU 16	125	0.5	4	0.25	0.53	ADD
	CAU 17	125	0.25	4	0.25	1.03	IND
	CAU 18	125	0.25	4	0.25	1.03	IND
	CAU 19	125	0.25	4	0.25	1.03	IND
	CAU 20	125	0.25	4	0.25	1.03	IND
	CAU 21	125	0.5	4	0.25	0.53	ADD
	CAU 22	250	0.5	4	0.25	0.52	ADD
	CAU 23	125	0.25	4	0.25	1.03	IND
	CAU 24	250	2	8	0.5	0.28	SYN*
	CAU 25	125	0.25	4	0.25	1.03	IND
C. albicans (1)	SC5314	250	0.12	2	0.12	1.01	IND

- FICI, fractional inhibitory concentration index; CAR, carvacrol; CAS, caspofungin; INT, interpretation; SYN*, synergy: IND, Indifference; ADD, additive.

Table 9: ***In vitro*** **antifungal activity of carvacrol in combination with Nystatin against *C. auris* and *C. albicans* SC5314.**

Candida sp	Strains	Median MIC (µg/ml) n=3				FICI	INT
		Alone		In combination			
		CAR	NYS	CAR	NYS		
C. auris (25)	CAU 1	125	1	4	0.5	0.53	ADD
	CAU 2	125	1	4	0.5	0.53	ADD
	CAU 3	125	1	4	0.5	0.53	ADD
	CAU 4	125	2	4	0.5	0.28	SYN*
	CAU 5	125	2	8	1	0.56	ADD
	CAU 6	125	2	8	1	0.56	ADD
	CAU 7	125	2	4	0.5	0.28	SYN*
	CAU 8	63	2	8	1	0.62	ADD
	CAU 9	125	1	4	0.5	0.53	ADD
	CAU 10	125	1	4	0.5	0.53	ADD
	CAU 11	125	1	4	0.5	0.53	ADD
	CAU 12	125	2	4	0.5	0.28	SYN*
	CAU 13	125	2	8	1	0.56	ADD
	CAU 14	63	2	8	1	0.63	ADD
	CAU 15	125	0.5	4	0.5	1.03	IND
	CAU 16	125	1	4	0.5	0.53	ADD
	CAU 17	125	2	4	0.5	0.28	SYN*
	CAU 18	125	4	8	1	0.31	SYN*
	CAU 19	125	2	4	0.5	0.28	SYN*
	CAU 20	125	1	4	0.5	0.53	ADD
	CAU 21	125	1	4	0.5	0.53	ADD
	CAU 22	250	1	4	0.5	0.53	ADD
	CAU 23	125	1	4	0.5	0.53	ADD
	CAU 24	250	2	8	1	0.53	ADD
	CAU 25	125	2	4	0.5	0.28	SYN*
C. albicans (1)	SC5314	250	0.5	4	0.5	1.02	IND

- FICI, fractional inhibitory concentration index; CAR, carvacrol; NYS, nystatin; INT, interpretation; SYN*, synergy: IND, Indifference; ADD, additive.

Table 10: *In vitro* antifungal activity of carvacrol in combination with fluconazole, amphotericin B, nystatin and caspofungin against *C. auris* and *C. albicans* SC5314.

***Candida* sp.**	**Test agent**	**Strains (n)**	**MIC in µg/ml**				**FICI**	**INT No. of isolates (%)**		
			MIC A	**CAR A**	**MIC B**	**CAR B**	**Mean ±SD (range)**	**SYN**	**ADD**	**IND**
C. auris (25)	CAR-FLU	Total (25)	125	125	32	63	0.83 ±0.37 (0.29-1.51)	4 (16)	13 (52)	8 (32)
		S (3)	16	125	16	32	1.05 ±0.30 (0.63-1.26)	0 (0)	1 (4)	2 (8)
		R (22)	125	125	32	63	0.80 ±0.37 (0.29-1.51)	4 (16)	12 (48)	6 (24)
	CAR-AMP	Total (25)	0.5	125	0.25	4	0.67 ±0.34 (0.28-1.50)	7 (28)	9 (36)	9 (36)
		S (20)	0.5	125	0.25	4	0.68 ±0.30 (0.28-1.06)	4 (16)	7 (28)	9 (36)
		R (5)	2	125	0.5	8	0.61 ±0.46 (0.31-1.50)	3 (12)	2 (8)	0 (0)
	CAR-NYS	Total (25)	2	125	0.5	4	0.49 ±0.16 (0.28-1.03)	7 (28)	17 (68)	1 (4)
		S (12)	1	125	0.5	4	0.57 ±0.14 (0.53-1.03)	0 (0)	11(44)	1 (4)
		R (13)	2	125	1	8	0.42 ±0.15 (0.28-0.63)	7 (28)	6 (24)	0 (0)
	CAR-CAS	Total (25)	0.25	125	0.25	4	0.88 ±0.25 (0.28-1.12)	1 (4)	6 (24)	18 (72)
		S (24)	0.25	125	0.25	4	0.91 ±0.22 (0.52-1.12)	0 (0)	6 (24)	18 (72)
		R (1)	2	250	0.5	8	0.28 ±0.00 (0.28)	1 (4)	0 (0)	0 (0)
C. albicans (1)	CAR-FLU	(1)	4	250	8	4	1.03 ±0.00 (1.03)	0 (0)	0 (0)	1 (100)
	CAR-AMP	(1)	0.125	250	2	0.125	1.01 ±0.00 (1.01)	0 (0)	0 (0)	1 (100)
	CAR-NYS	(1)	0.5	250	4	0.5	1.02 ±0.00 (1.02)	0 (0)	0 (0)	1 (100)
	CAR-CAS	(1)	0.125	250	2	0.12	1.01 ±0.00 (1.01)	0 (0)	0 (0)	1 (100)

- FICI, fractional inhibitory concentration index; CAR, carvacrol; FLU, fluconazole; AMP, amphotericin B; CAS, caspofungin; NYS, nystatin; INT, interpretation; SYN, synergy: IND, Indifference; ADD, additive. S, sensitive; R, resistance.
- MIC is the median MIC of three independent experiments. MIC A and MIC B are the median MIC of the drug alone and in combination respectively. CAR A and CAR B are the median MIC of the cravacrol alone and in combination respectively.

3.4 Virulence assays

3.4.1 Adherence to buccal epithelial cells

The adherence ability is expressed as the number of yeast cells attached to each of 100 buccal epithelial cells (Figure 2). The adherence values to BECs for the *Candida* sp are shown in Table 12 and Figure 3. There were three repeats done for each strain shown in table 11 and the mean value was recorded. Overall, 100% of *C. auris* isolates showed adherence to BECs. However, the extent of adherence appears to be unequal among the strains. The mean number of yeast cells was 141 ±40. *C. albicans* SC5314 showed higher ability of adherence to BECs, almost double than *C. auris*, with the mean value 311 ±14.

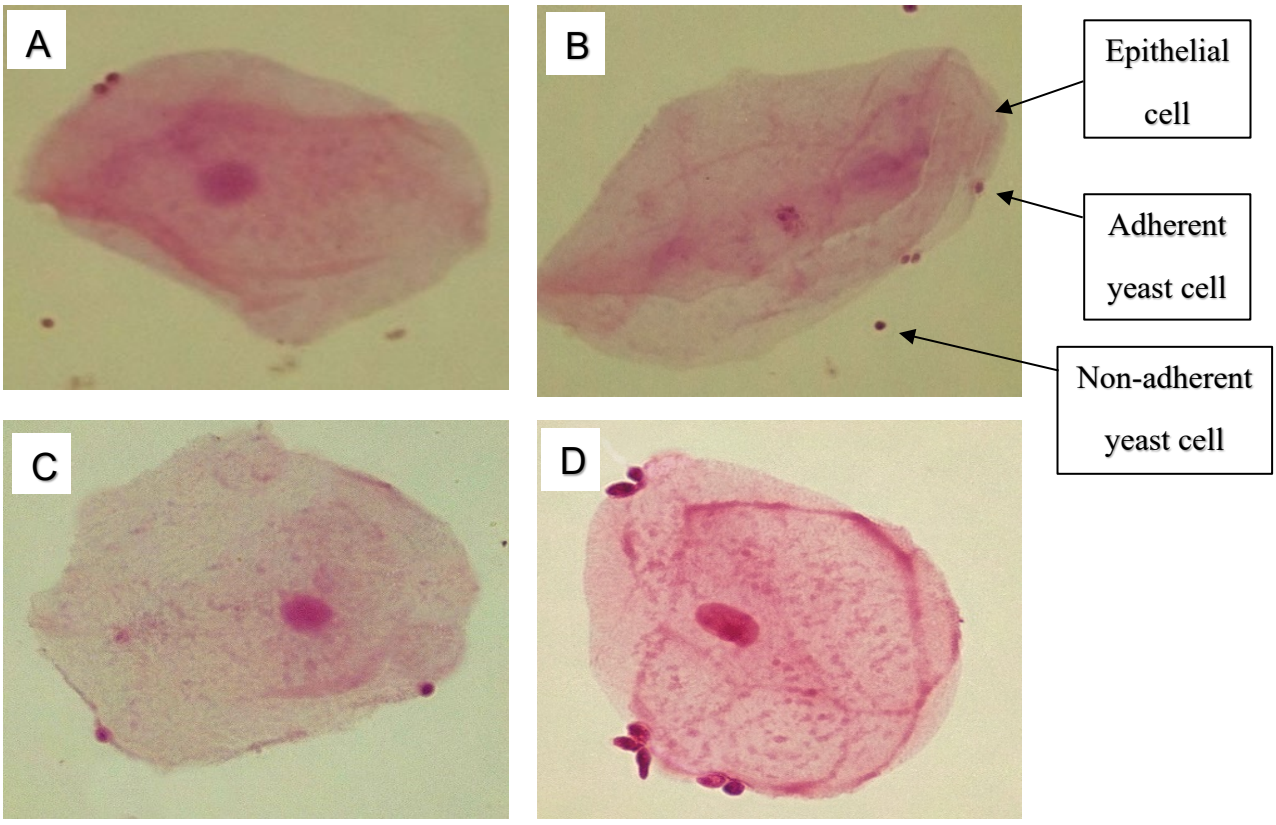

Figure 2: Adherence of *C. auris* and *C. albicans* SC5314 to buccal epithelial cells. A, *C. auris* CAU 1; B, *C. auris* CAU 24; C: *C. auris* CAU 25 and D; *C. albicans* SC5314 (magnification 400X)

Table 11: Adherence activity of *C. auris*

***Candida* sp.**	**Strain**	**No. of yeast cells adherent to 100 BECs**			
		1	**2**	**3**	**Mean ± SD**
C. auris (25)	CAU 1	162	160	170	164 ±04
	CAU 2	108	112	102	107 ±04
	CAU 3	78	88	68	78 ±08
	CAU 4	180	194	200	191 ±08
	CAU 5	182	180	168	177 ±06
	CAU 6	104	88	94	95 ±06
	CAU 7	180	196	182	186 ±07
	CAU 8	100	110	102	104 ±04
	CAU 9	122	116	108	115 ±06
	CAU 10	166	160	180	169 ±08
	CAU 11	140	128	138	135 ±05
	CAU 12	120	142	140	134 ±10
	CAU 13	180	166	164	170 ±07
	CAU 14	158	178	164	167 ±08
	CAU 15	74	88	84	82 ±06
	CAU 16	68	60	74	67 ±06
	CAU 17	170	180	164	171 ±07
	CAU 18	188	180	176	181 ±05
	CAU 19	102	122	118	114 ±09
	CAU 20	128	118	132	126 ±06
	CAU 21	166	176	160	167 ±07
	CAU 22	190	182	206	193 ±10
	CAU 23	98	80	100	93 ±09
	CAU 24	198	206	188	197 ±07
	CAU 25	134	120	140	131 ±08
	Combined				141 ±40
C. albicans (1)	SC5314	324	316	292	311 ±14

Table 12: Summary results of adherence of *C. auris* and *C. albicans* SC5314 to buccal epithelial cells

***Candida* sp**	**Strains**	**No. of yeast cells adherent to 100 BECs**
	No. (%)	**Mean ± SD (range) n=3**
C. auris (25)	25 (100)	141 ±40 (67-197)
C. albicans (1)	1 (100)	311 ±14 (292-324)

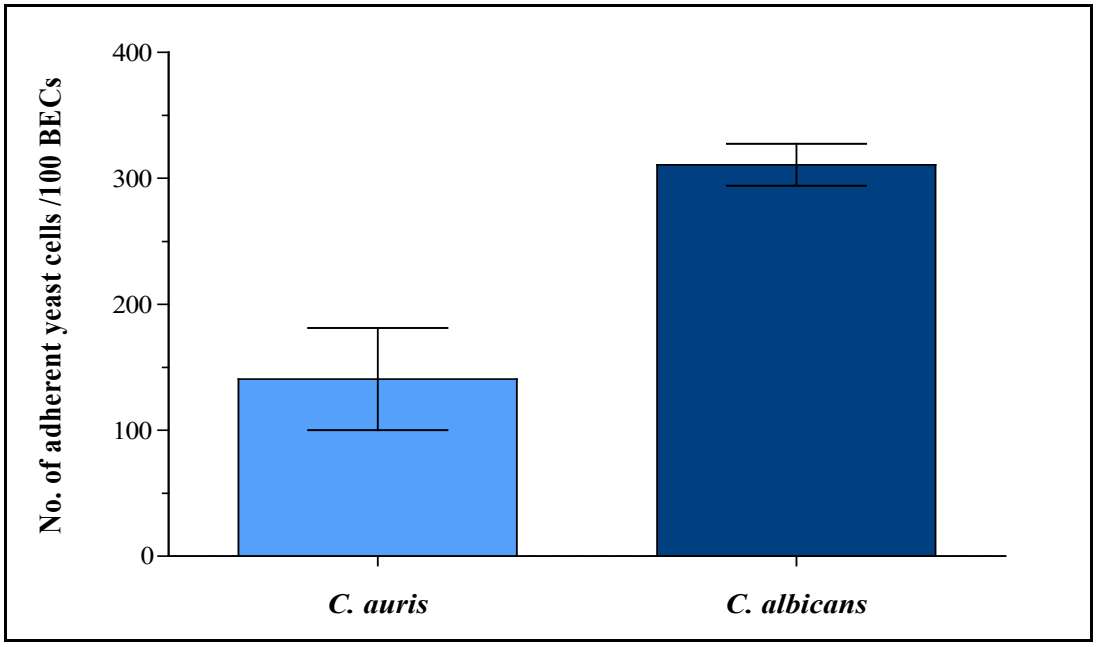

Figure 3: Adherence of *C. auris* and *C. albicans* to buccal epithelial cells. Bars represent the means (with standard deviations) number of yeast cells adherent to 100 BECs, which represents the adherence ability of *C. auris* and *C. albicans.*

3.4.2 Evaluation of morphogenesis in *C. auris*

In order to induce filamentation in *Candida* cells, all the isolates were incubated with 10% FBS at 37°C for 24 hours. Slides were prepared and viewed under microscope at 1h, 2h, 3h, 6h, 9h and 24h.

Microscopically, no hyphal and/or pseudohyphal cells were observed in all *C. auris* strains under this condition. In contrast, 90% of the cells in control strain *C. albicans* SC5314 showed true hyphae within 2 hours incubation (Table 13, Figure 4).

Table 13: Germ tube formation in *C. auris* and *C. albicans* SC5314

***Candida* sp**	**Strains**	**No. of *Candida* cells with germ tube/200 cells (%) n=3**					
		1 h	**2 h**	**3 h**	**6 h**	**9 h**	**24 h**
C. auris (25)	CAU 1-25	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
C. albicans (1)	*C. albicans* SC5314	0 (0)	180 (90)	190 (95)	198 (99)	++++	++++

- The experiment was done three times for each strain.

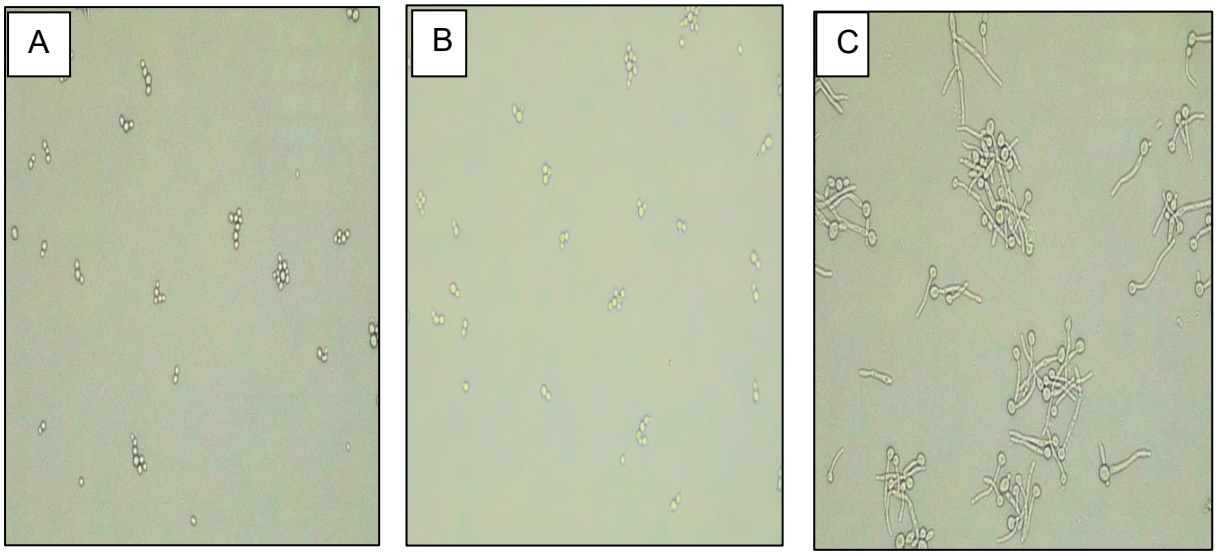

Figure 4: Morphology of *C. auris* and *C. albicans* SC5314 under a light microscopy after incubation for 3 hours with 10% FBS at 37°C (magnification 400X). A, *C. auris* CAU 1 and B, *C. auris* CAU 25 show yeast cells with no hyphae formation; C, *C. albicans* SC5314 shows yeast cells with positive hyphae formation.

3.4.3 Proteinase production in *C. auris* and *C. albicans* SC5314

The amount of extracellular proteinase activity (P_z value) was indicated by the halo surrounding the fungal colony on the BSA plates (Figure 5).The results are shown in Table 14 and 15. Out of 25 *C. auris* isolates tested, 24 (96%) isolates were proteinase producers and only 1 isolate (4%) showed no proteinase activity (CAU 3). As demonstrated in table 14 and 15. The 24 strains which tested positive for proteinase activity showed widely varying results of enzyme activity. Medium proteinase activity (P_z: 0.82 – 0.85) was observed in 6 isolates (25%), 4 isolates (17%) showed strong proteinase activity (P_z: 0.72 – 0.78), while the majority of the isolates, 14 (58%) showed very strong proteinase activity (P_z: 0.57 – 0.65). The mean proteinase production by the tested *C. auris* isolates (P_z =0.70) was comparable to the reference strain *C. albicans* SC5314 (P_z =0.60) proteinase production (Figure 6).

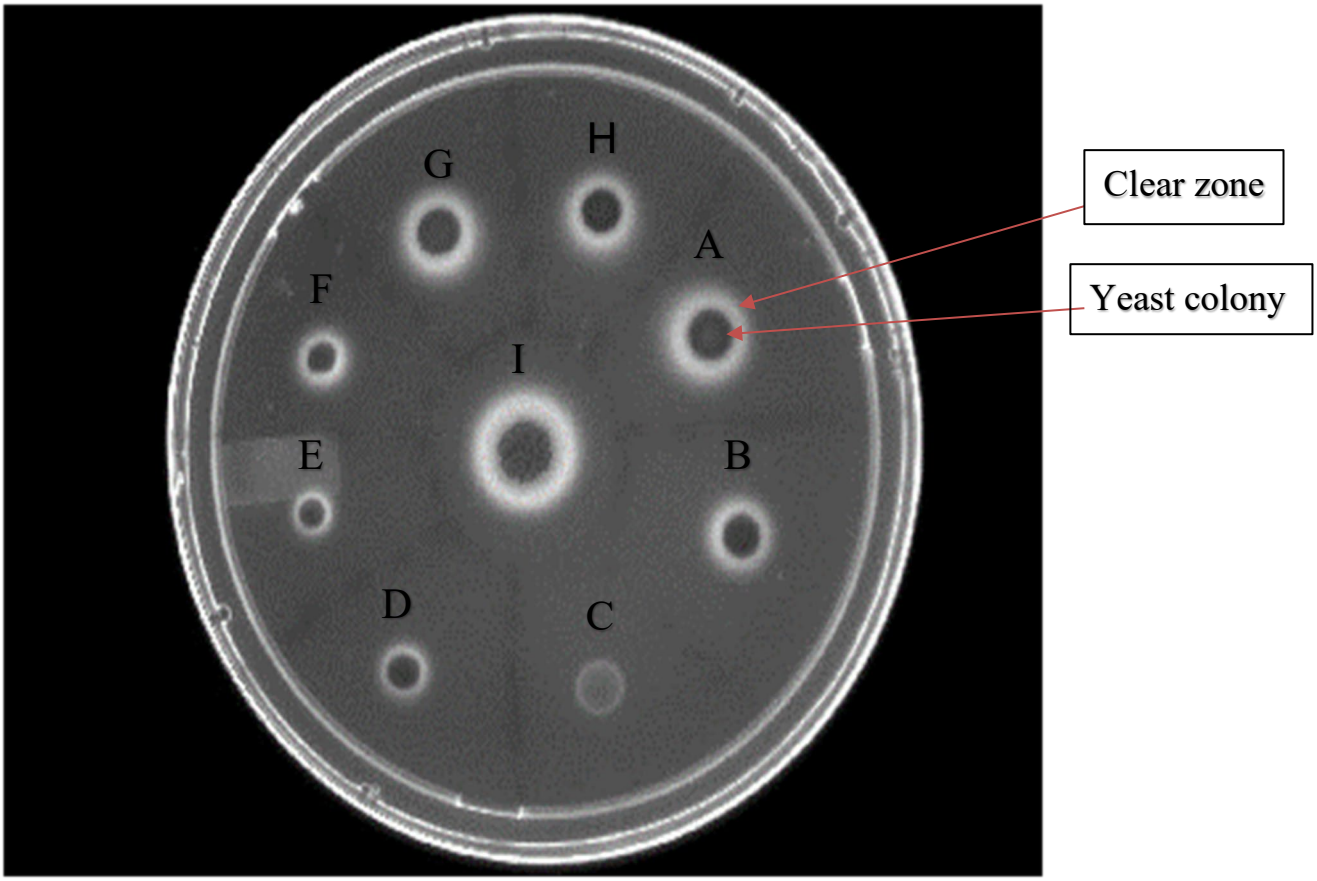

Figure 5: Proteinase activity of *C. auris* and *C. albicans* SC5314 on BSA plates. Zone of clearance around the colonies indicate positive proteinase activity. A, *C. auris* CAU 1; B, *C. auris* CAU 2; C, *C. auris* CAU 3; D, *C. auris* CAU 4; E, *C. auris* CAU 5; F, *C. auris* CAU 6; G, *C. auris* CAU 7; H, *C. auris* CAU 8 and I; A reference strain of *C. albicans* SC5314 served as a positive control.

Table 14: Proteinase activity of *C. auris* isolates.

Candida sp.	Strain	Proteinase production (*Pz* value)				Proteinase activity level
		1	2	3	Mean ± SD	
C. auris (25)	CAU 1	0.70	0.70	0.75	0.72 ± 0.01	+++
	CAU 2	0.80	0.85	0.80	0.82 ± 0.02	++
	CAU 3	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 4	0.64	0.64	0.67	0.65 ± 0.01	++++
	CAU 5	0.70	0.70	0.75	0.72 ± 0.02	+++
	CAU 6	0.80	0.85	0.80	0.82 ± 0.02	++
	CAU 7	0.60	0.66	0.56	0.61 ± 0.05	++++
	CAU 8	0.60	0.66	0.57	0.61 ± 0.04	++++
	CAU 9	0.66	0.67	0.55	0.63 ± 0.05	++++
	CAU 10	0.66	0.60	0.63	0.63 ± 0.02	++++
	CAU 11	0.60	0.63	0.60	0.61 ± 0.01	++++
	CAU 12	0.66	0.60	0.62	0.63 ± 0.03	++++
	CAU 13	0.57	0.60	0.61	0.59 ± 0.02	++++
	CAU 14	0.61	0.60	0.60	0.60 ± 0.01	++++
	CAU 15	0.80	0.88	0.77	0.82 ± 0.05	++
	CAU 16	0.88	0.80	0.77	0.82 ± 0.05	++
	CAU 17	0.50	0.60	0.66	0.59 ± 0.07	++++
	CAU 18	0.60	0.50	0.60	0.57 ± 0.05	++++
	CAU 19	0.80	0.88	0.80	0.83 ± 0.04	++
	CAU 20	0.88	0.88	0.80	0.85 ± 0.04	++
	CAU 21	0.78	0.75	0.77	0.77 ± 0.01	+++
	CAU 22	0.60	0.67	0.60	0.62 ± 0.03	++++
	CAU 23	0.80	0.80	0.75	0.78 ± 0.02	+++
	CAU 24	0.50	0.66	0.60	0.59 ± 0.07	++++
	CAU 25	0.60	0.66	0.50	0.59 ± 0.07	++++
	Combined				0.70 ± 0.01	+++
C. albicans (1)	SC5314	0.54	0.60	0.67	0.60 ± 0.05	++++

- Activity level: −, Pz = 1 (no proteinase activity); +, Pz = 0.90 to 0.99 (weak proteinase activity); ++, Pz = 0.80 to 0.89 (medium proteinase activity); +++, Pz = 0.70 to 0.79 (strong proteinase activity); ++++, Pz = <0.69 (very strong proteinase activity).

Table 15: Summary results of Proteinase activity of *C. auris* isolates

Candida sp.	Proteinase activity	No. of strains (%)
C. auris (25)	++++	14 (56)
	+++	4 (16)
	++	6 (24)
	+	0
	None	1 (4)
C. albicans (1)	++++	1 (100)

- Activity level: +, (weak proteinase activity); ++, (medium proteinase activity); +++, (strong proteinase activity); ++++, (very strong proteinase activity).

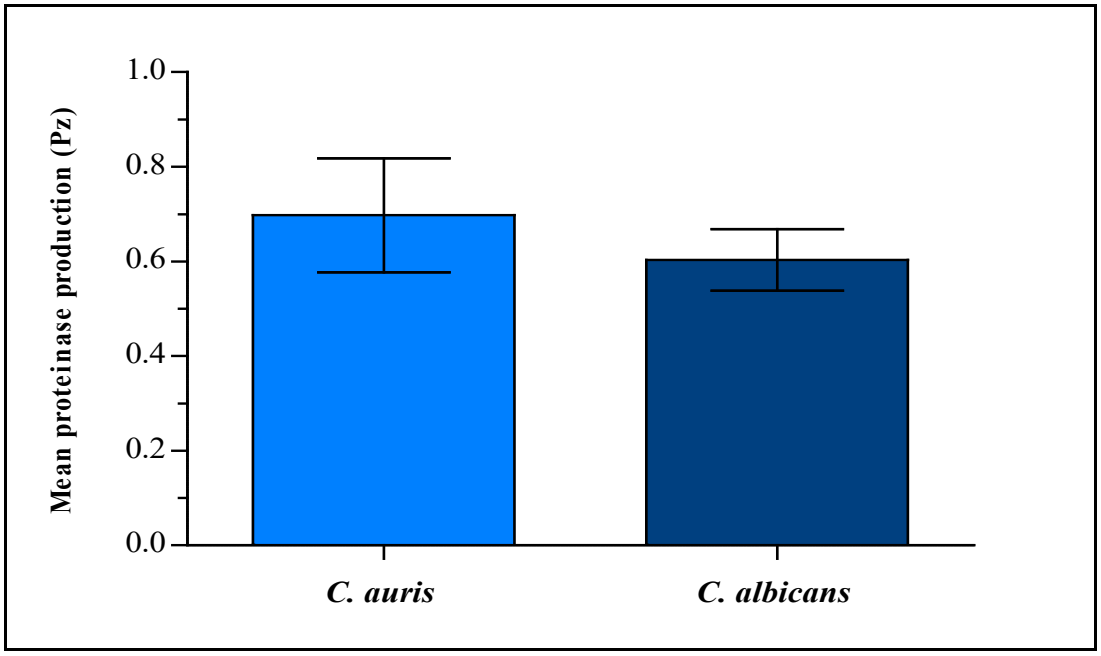

Figure 6: A proteinase production in *C. auris* and *C. albicans* SC5314. Bars represent the means (with standard deviations) of *Pz* values.

3.4.4 Phospholipase activity in *C. auris* and *C. albicans* SC5314

Phospholipase activity (P_z value) was evaluated by measuring the white precipitation zones around the colonies on the egg yolk agar plates. All *C. auris* isolates tested showed negative phospholipase activity ($Pz = 1$), where no visible white zone was observed around the fungal colony (Figure 7, table 16). In contrast, the control strain *C. albicans* SC5314 showed very strong phospholipase activity $P_z = 0.66$ (Figure 8).

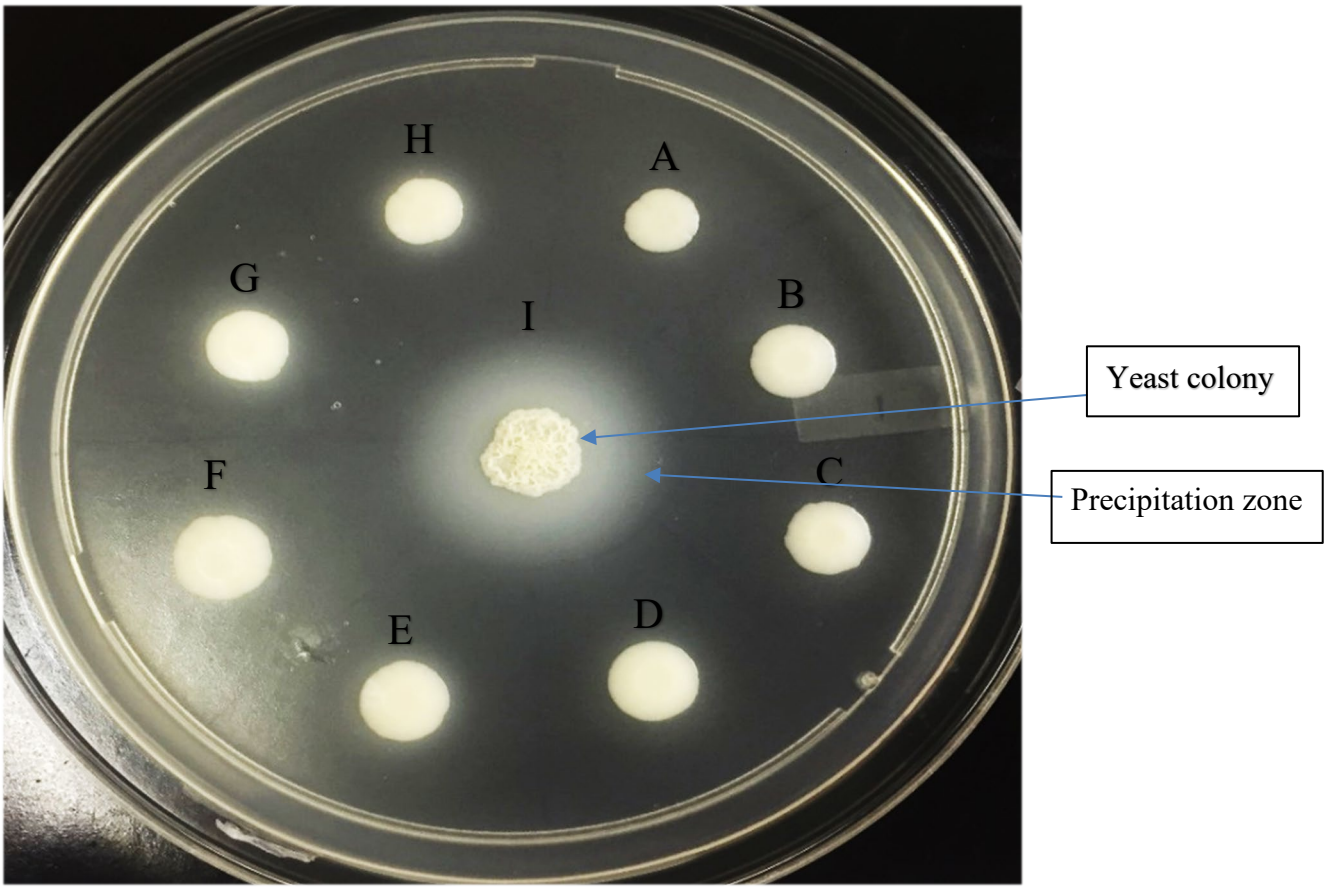

Figure 7: Phospholipase activity in *C. auris* and *C. albicans* SC5314 on egg yolk agar plates. Precipitation zones around the colonies indicate positive phospholipase activity. A, *C. auris* CAU 1; B, *C. auris* CAU 2; C, *C. auris* CAU 3; D, *C. auris* CAU 4; E, *C. auris* CAU 5; F, *C. auris* CAU 6; G, *C. auris* CAU 7; H, *C. auris* CAU 8 and I; A reference strain of *C. albicans* SC5314 served as a positive control.

Table 16: Phospholipase activity of *C. auris* isolates.

Candida sp.	Strain	Phospholipase production (*Pz* value)				Phospholipase activity level
		1	2	3	Mean ± SD	
C. auris (25)	CAU 1	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 2	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 3	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 4	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 5	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 6	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 7	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 8	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 9	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 10	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 11	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 12	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 13	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 14	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 15	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 16	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 17	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 18	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 19	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 20	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 21	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 22	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 23	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 24	1.00	1.00	1.00	1.00 ± 0.00	-
	CAU 25	1.00	1.00	1.00	1.00 ± 0.00	-
	Combined				1.00 ± 0.00	-
C. albicans (1)	SC5314	0.70	0.60	0.67	0.66 ± 0.04	++++

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

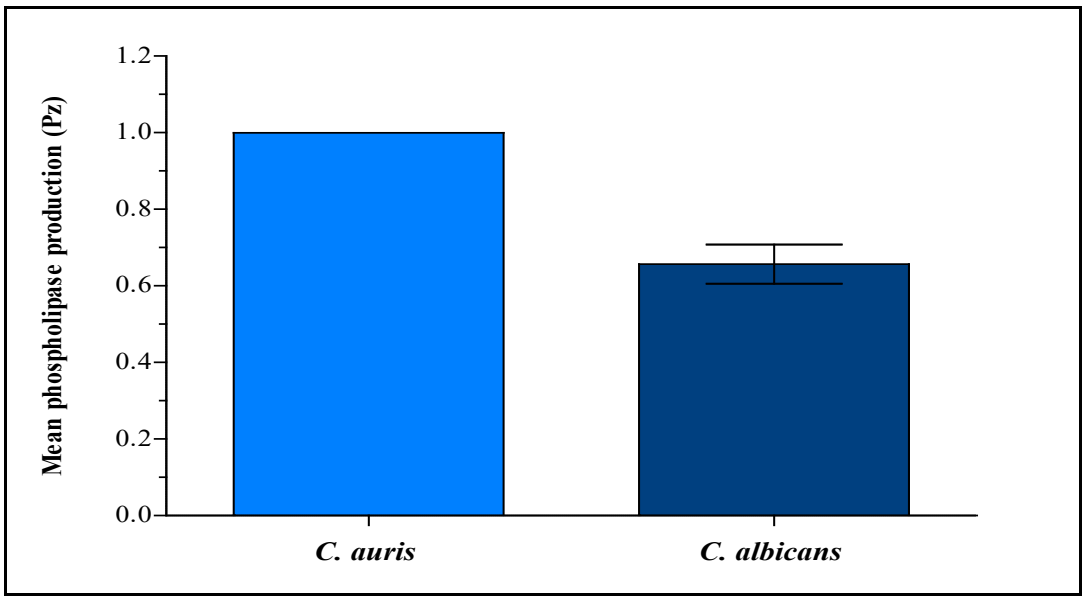

Figure 8: A phospholipase production in *C. auris* and *C. albicans* SC5314. Bars represent the means (with standard deviations) of *Pz* values.

3.5 Anti-virulence assay

3.5.1 Effect of carvacrol on *C. auris* adherence

Having shown that carvacrol was the most active compound with the lowest MICs against *C. auris*, *C. auris* isolates were exposed to various concentration of carvacrol to determine its effect on the adherence activity. The results of *C. auris* adherence ability at four concentrations of carvacrol are shown in Figure 9, Table 17 and 18. The adherence ability of *C. auris* decreased at the four concentration tested 0.25, 0.125, 0.063 and 0.031 mg/ml with the mean adherence value 83, 98, 115 and 128 respectively (Table 18). Overall this reduction was significant compared to the control with a P value of < 0.01 (Table 21). further statistical analysis showed significant inhibition at carvacrol concentration 0.25, 0.125 and 0.063 mg/ml (P = < 0.05). No significant inhibition was observed after exposure to 0.031 mg/ml carvacrol (Figure 10, Table 21). For *C. albicans* SC5314 adherence ability was also reduced at all carvacrol concentration when compared to untreated cells (Table 18).

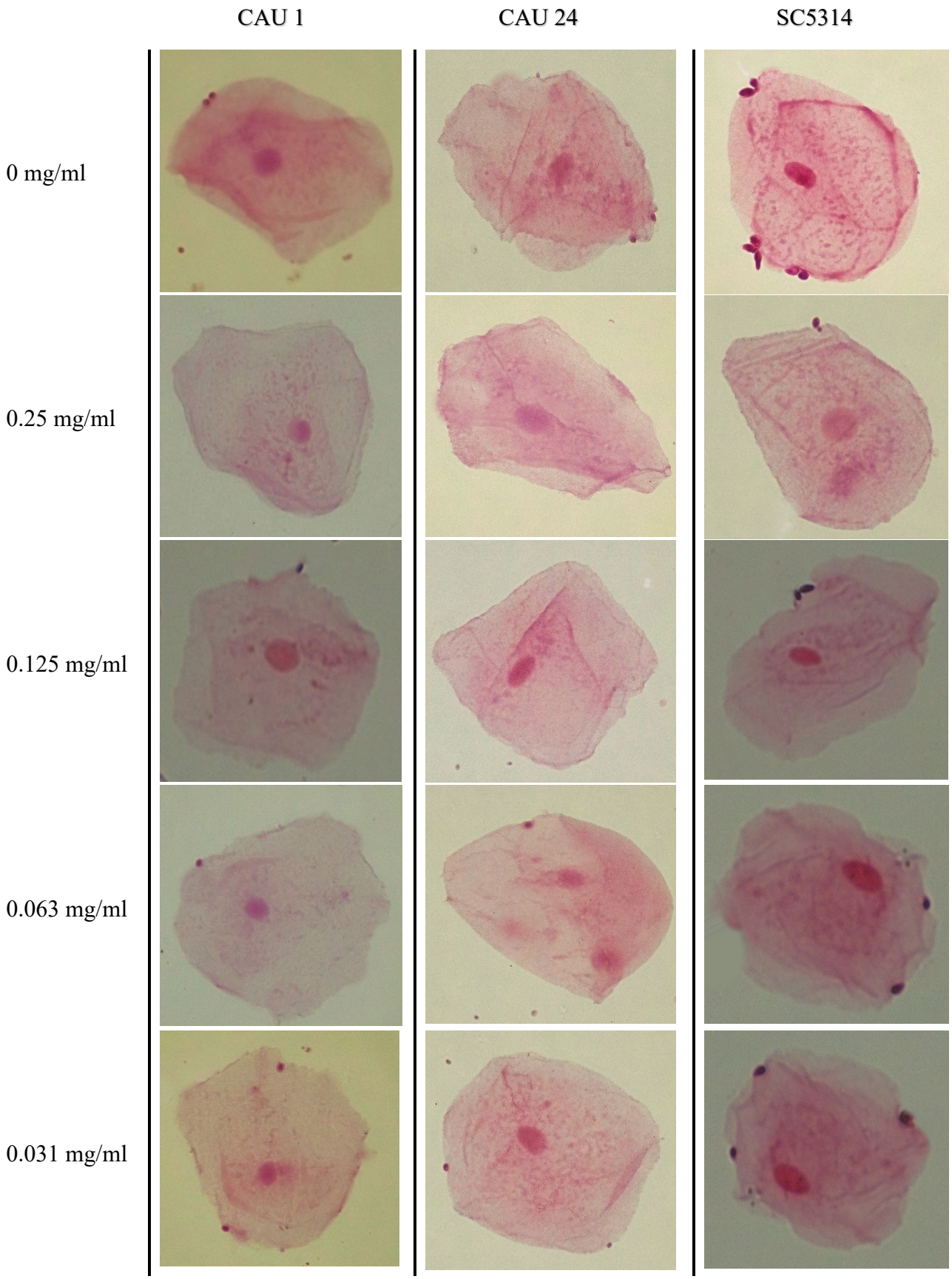

Figure 9: The effect of various concentration of carvacrol on adherence in *C. auris* strains (CAU 1 and CAU 24) and *C. albicans* SC5314.

Table 17: The effect of various concentrations of carvacrol on the adherence of *C. auris*

***Candida* sp.**	**Strains**	**Repeats**	**No. of adherent yeast cells to 100 BECs at various concentrations of carvacrol (mg/ml)**				
			0	**0.25**	**0.125**	**0.063**	**0.031**
C. auris (25)	CAU 1	1	162	108	122	144	150
		2	160	102	120	138	158
		3	170	112	148	156	160
	CAU 2	1	108	50	60	80	98
		2	112	56	68	88	90
		3	102	40	62	80	96
	CAU3	1	78	36	40	54	64
		2	88	48	56	60	70
		3	68	42	46	50	58
	CAU 4	1	180	106	120	146	160
		2	194	100	138	160	168
		3	200	120	140	150	178
	CAU 5	1	182	104	116	146	168
		2	180	122	128	154	170
		3	168	102	130	150	166
	CAU 6	1	104	60	68	50	80
		2	88	40	58	66	76
		3	94	42	50	64	70
	CAU 7	1	180	96	122	148	160
		2	196	102	132	144	166
		3	182	100	138	158	172
	CAU 8	1	100	ND	48	64	88
		2	110	ND	40	72	90
		3	102	ND	38	68	80
	CAU 9	1	122	56	86	100	110
		2	116	48	92	102	112
		3	108	58	82	100	110
	CAU 10	1	166	90	102	124	150
		2	160	98	108	130	148
		3	180	100	112	146	152
	CAU 11	1	140	88	100	118	128
		2	128	94	102	116	126
		3	138	98	100	114	124
	CAU 12	1	120	88	100	110	120
		2	142	98	112	122	132
		3	140	94	120	130	138
	CAU 13	1	180	112	138	156	166
		2	166	106	140	148	158
		3	164	108	128	144	154

- ND: Not done due to low median MFC/MIC values for those strains.

Table 17: The effect of various concentrations of carvacrol on the adherence of *C. auris*-continue

***Candida* sp.**	**Strains**	**Repeats**	**No. of adherent yeast cells to 100 BECs at various concentrations of carvacrol (mg/ml)**				
			0	**0.25**	**0.125**	**0.063**	**0.031**
C. auris	CAU 14	1	158	ND	102	122	146
		2	178	ND	112	138	148
		3	164	ND	108	140	144
	CAU 15	1	74	58	60	64	68
		2	88	50	72	72	82
		3	84	56	70	76	80
	CAU 16	1	68	44	46	52	62
		2	60	38	48	50	58
		3	74	42	52	58	68
	CAU 17	1	170	108	120	130	168
		2	180	102	130	140	150
		3	164	112	124	144	150
	CAU 18	1	188	100	120	150	166
		2	180	110	146	158	170
		3	176	102	140	162	162
	CAU 19	1	102	52	60	98	100
		2	122	56	68	80	110
		3	118	60	70	108	116
	CAU 20	1	128	66	100	110	120
		2	118	80	96	100	108
		3	132	86	102	118	128
	CAU 21	1	166	100	120	140	154
		2	176	116	122	142	158
		3	160	120	134	148	150
	CAU 22	1	190	108	120	150	174
		2	182	112	136	166	166
		3	206	118	144	164	192
	CAU 23	1	98	52	64	88	90
		2	80	60	70	78	80
		3	100	68	74	82	92
	CAU 24	1	198	100	110	140	160
		2	206	108	120	160	188
		3	188	102	142	152	164
	CAU 25	1	134	75	86	98	120
		2	120	66	90	100	130
		3	140	68	92	90	128
C. albicans (1)	SC5314	1	324	140	168	208	298
		2	316	134	160	192	286
		3	292	150	174	216	274

- ND: Not done due to low median MFC/MIC values for those strains.

Table 18: Summary results of the effect of various concentration of carvacrol on adherence in *C. auris* and *C. albicans.*

***Candida* sp.**	**Adherent yeast cells to 100 BEC at various concentrations of carvacrol (mg/ml)**				
	Mean ± SD (n=3)				
	0	**0.25**	**0.125**	**0.063**	**0.031**
C. auris (25)	141 ±40	83 ±26	98 ±32	115 ±36	128 ±37
C. albicans (1)	311 ±14	141 ±07	167 ±06	205 ±10	286 ±10

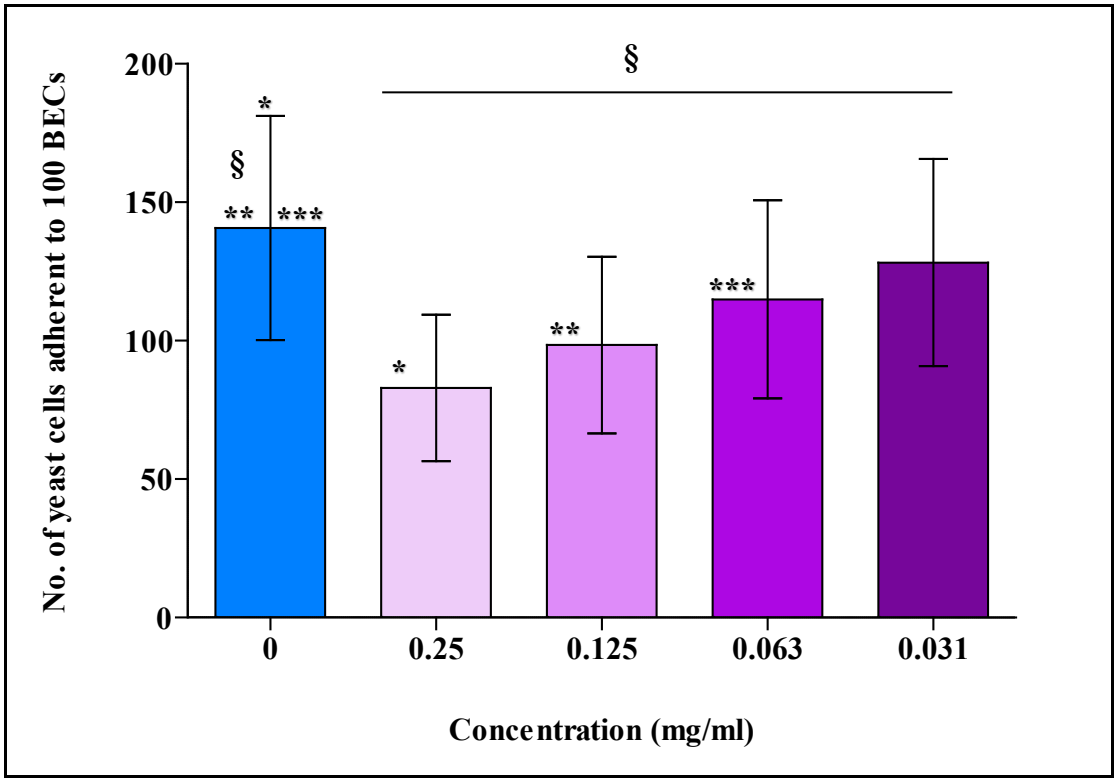

Figure 10: The effect of various concentration of carvacrol on adherence in *C. auris.* Bars represent the means (with standard deviations) number of yeast cells adherent to 100 BECs, which represents the adherence ability of *C. auris*. §, *, ** P = < 0.01; *** P = < 0.02.

3.5.2 Effect of carvacrol on *C. auris* proteinase production

Candida auris isolates and *C. albicans* SC5314 were exposed to various concentration of carvacrol to determine whether this compound possesses anti-proteinase activity. The results are depicted in Table 19, 20 and Figure 11, 12. At concentrations 0.25, 0.125, 0.063 and 0.031 mg/ml, *C. auris* proteinase activity was 0.81, 0.77, 0.72 and 0.69 (*Pz*). The control showed *Pz* of 0.68. When the overall comparison of carvacrol on the proteinase to the control was performed, the results showed a significant reduction ($P =< 0.01$). When the individual concentrations were compared to the control, concentration of 0.25 mg/ml and 0.125 mg/ml significantly reduced the proteinase production. Concentrations 0.063 and 0.031 mg/ml had no significant effect on the proteinase production (Table 21).

For *C. albicans* SC5314, proteinase activity was reduced only at carvacrol concentration of 0.25, 0.125 mg/ml with the mean *Pz* value (0.70, 0.62) respectively, when compared to untreated cells (*Pz* = 0.60). No Inhibition in enzyme was observed at carvacrol concentration of 0.063 and 0.031 mg/ml.

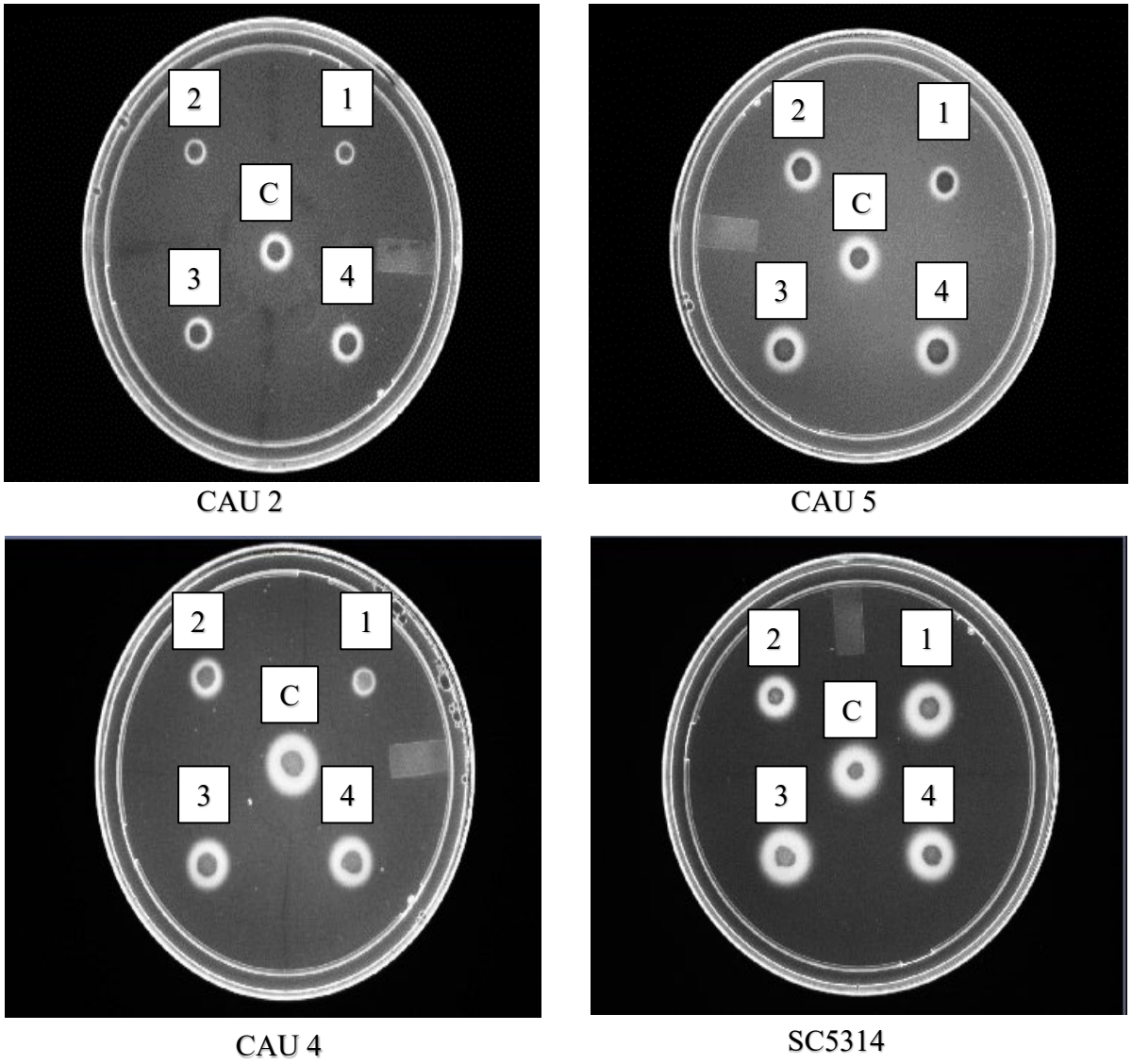

Figure 11: The effect of various concentrations of carvacrol (mg/ml) on the proteinase production of *C. auris* strains (CAU 2, CAU 4 and CAU 5) and *C. albicans* SC5314. 1, 2, 3 and 4 represent the effect of carvacrol at concentrations 0.25, 0.125, 0.063 and 0.031 mg/ml respectively; C, represents untreated cells served as a control.

Table 19: The effect of carvacrol on the proteinase production of *C. auris.*

***Candida* sp.**	**Strains**	**Repeats**	**Proteinase production (*Pz*) at various concentrations of carvacrol (mg/ml)**				
			0	**0.25**	**0.125**	**0.063**	**0.031**
C. auris (24)	CAU 1	1	0.70	0.88	0.77	0.70	0.70
		2	0.70	0.72	0.70	0.70	0.70
		3	0.75	0.86	0.77	0.77	0.75
	CAU 2	1	0.80	0.90	0.85	0.80	0.80
		2	0.85	0.90	0.90	0.85	0.85
		3	0.80	0.90	0.85	0.80	0.80
	CAU 4	1	0.64	0.90	0.80	0.67	0.67
		2	0.64	0.80	0.78	0.75	0.64
		3	0.67	0.90	0.80	0.75	0.67
	CAU 5	1	0.70	0.88	0.80	0.75	0.70
		2	0.70	0.88	0.80	0.75	0.75
		3	0.75	0.80	0.80	0.75	0.70
	CAU 6	1	0.80	0.88	0.80	0.80	0.80
		2	0.85	0.86	0.86	0.85	0.85
		3	0.80	0.88	0.80	0.80	0.80
	CAU 7	1	0.60	0.67	0.64	0.62	0.60
		2	0.66	0.72	0.67	0.66	0.66
		3	0.56	0.66	0.66	0.62	0.56
	CAU 8	1	0.60	ND	0.75	0.71	0.63
		2	0.66	ND	0.70	0.70	0.66
		3	0.57	ND	0.71	0.71	0.66
	CAU 9	1	0.66	0.75	0.75	0.66	0.66
		2	0.67	0.83	0.75	0.64	0.64
		3	0.55	0.75	0.70	0.61	0.61
	CAU 10	1	0.66	0.70	0.70	0.66	0.66
		2	0.60	0.77	0.70	0.66	0.60
		3	0.63	0.72	0.70	0.66	0.62
	CAU 11	1	0.60	0.88	0.70	0.66	0.60
		2	0.63	0.88	0.80	0.77	0.69
		3	0.60	0.70	0.70	0.69	0.60
	CAU 12	1	0.66	0.88	0.70	0.69	0.67
		2	0.60	0.88	0.88	0.61	0.60
		3	0.62	0.80	0.70	0.60	0.61
	CAU 13	1	0.57	0.67	0.67	0.67	0.57
		2	0.60	0.75	0.75	0.66	0.60
		3	0.61	0.77	0.77	0.66	0.61

- ND: Not done due to low median MFC/MIC values for those strains**.**

Table 19: The effect of carvacrol on the proteinase production of *C. auris*- Continue

***Candida* sp.**	**Strains**	**Repeats**	**Proteinase production (*Pz*) at various concentrations of carvacrol (mg/ml)**				
			0	**0.25**	**0.125**	**0.063**	**0.031**
C. auris	CAU 14	1	0.61	ND	0.75	0.70	0.66
		2	0.60	ND	0.75	0.70	0.66
		3	0.60	ND	0.77	0.75	0.66
	CAU 15	1	0.80	0.88	0.88	0.80	0.80
		2	0.88	0.90	0.88	0.88	0.88
		3	0.77	0.80	0.80	0.77	0.77
	CAU 16	1	0.88	0.90	0.90	0.88	0.88
		2	0.80	0.90	0.88	0.88	0.80
		3	0.77	0.88	0.88	0.77	0.77
	CAU 17	1	0.50	0.67	0.67	0.57	0.57
		2	0.60	0.75	0.66	0.57	0.57
		3	0.66	0.75	0.75	0.66	0.66
	CAU 18	1	0.60	0.72	0.72	0.66	0.60
		2	0.50	0.66	0.66	0.57	0.50
		3	0.60	0.72	0.72	0.66	0.66
	CAU 19	1	0.80	1.00	1.00	0.82	0.80
		2	0.88	1.00	1.00	0.81	0.88
		3	0.80	1.00	1.00	0.88	0.80
	CAU 20	1	0.88	1.00	1.00	0.90	0.88
		2	0.88	1.00	1.00	0.88	0.88
		3	0.80	1.00	1.00	0.88	0.81
	CAU 21	1	0.78	0.81	0.81	0.77	0.77
		2	0.75	0.81	0.77	0.77	0.75
		3	0.77	0.80	0.81	0.77	0.77
	CAU 22	1	0.60	0.80	0.66	0.60	0.60
		2	0.67	0.69	0.66	0.66	0.66
		3	0.60	0.69	0.69	0.63	0.63
	CAU 23	1	0.80	0.90	0.80	0.73	0.75
		2	0.80	0.90	0.80	0.80	0.80
		3	0.75	0.80	0.88	0.75	0.75
	CAU 24	1	0.50	0.67	0.60	0.50	0.50
		2	0.66	0.72	0.66	0.66	0.66
		3	0.60	0.72	0.66	0.60	0.60
	CAU 25	1	0.60	0.71	0.71	0.66	0.60
		2	0.66	0.75	0.71	0.66	0.66
		3	0.50	0.60	0.60	0.57	0.50
C. albicans (1)	SC5314	1	0.54	0.63	0.55	0.54	0.54
		2	0.60	0.63	0.60	0.60	0.60
		3	0.67	0.86	0.70	0.67	0.67

- ND: Not done due to low median MFC/MIC values for those strains.

Table 20: Summary results of the effect of carvacrol on the proteinase production of *C. auris* and *C. albicans* SC5314.

***Candida* sp.**	**Proteinase production (*Pz*) at various concentrations of carvacrol (mg/ml) n=3**				
	0	**0.25**	**0.125**	**0.063**	**0.031**
C. auris (24)	0.68 ± 0.10	0.81 ± 0.10	0.77 ± 0.10	0.72 ± 0.09	0.69 ± 0.10
C. albicans (1)	0.60 ± 0.05	0.70 ± 0.11	0.62 ± 0.06	0.60 ± 0.05	0.60 ± 0.05

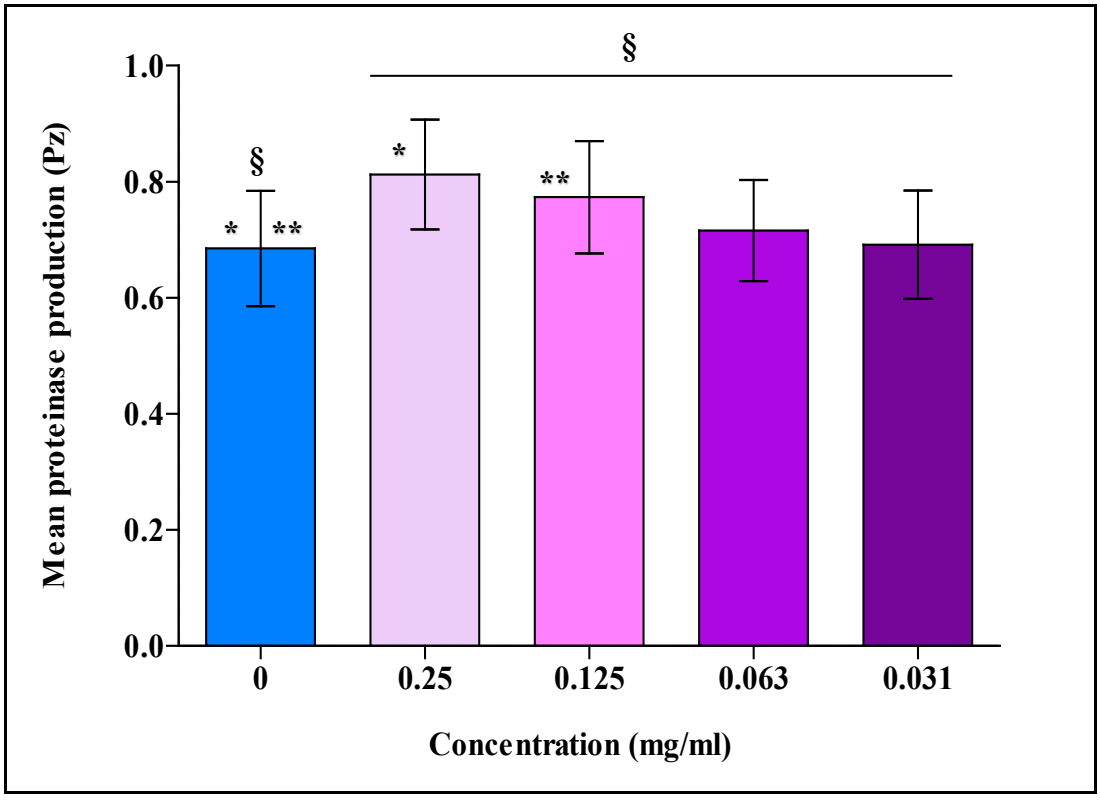

Figure 12: The effect of various concentrations of carvacrol (mg/ml) on the proteinase production of *C. auris*. Bars represent the means (with standard deviations) of *Pz* values, which represents proteinase activity of *C. auris*. §, *, ** P = < 0.01.

Table 21: Statistical analysis of the results of the anti-virulence assays.

Compound	Test	Comparison	P value
Carvacrol	Adherence	Control to overall	< 0.0001
		Control to 0.25 mg/ml	< 0.0001
		Control to 0.125 mg/ml	0.0002
		Control to 0.063 mg/ml	0.0212
		Control to 0.031 mg/ml	0.2638
	Proteinase	Control to overall	< 0.0001
		Control to 0.25 mg/ml	< 0.0001
		Control to 0.125 mg/ml	0.0031
		Control to 0.063 mg/ml	0.2587
		Control to 0.031 mg/ml	0.8156

CHAPTER 4: DISCUSSION

Although *C. auris* first isolated in 2009 in Japan, 10 years later it has become widespread across the globe. *C. auris* has been associated with sever invasive infections, poor outcomes and high mortality rates of up to 72%. The inability of several available commercial antifungal drugs to control *C. auris* infections illustrates the increased need to develop new drug to prevent and treat these infections. Research in antifungal drug discovery is slow and challenges with the newly emerging species of *Candida,* has made the development of new antifungal compounds and therapies a priority. Furthermore, the capacity of *C. auris* to possess virulence factors may be responsible for its underlying sever infections and high mortalities. Investigation to understand the pathogenicity of this organism would identify new drug targets, overcome drug resistance and develop more effective antifungal agents. Therefore, discovery of new antifungal drugs acting on new targets can be used by itself or in combination with other antifungals is crucial for the survival of patients.

In this study, the antifungal activity of four phenolic compounds namely, eugenol, methyleugenol, carvacrol and thymol, against *C. auris* was investigated. Several studies have reported the antifungal activity of these compounds against different *Candida* species (Ahmad *et al*, 2010). This study demonstrated that these compounds have antifungal activity against 25 *C. auris* clinical isolates. However, their levels of efficacy were highly variable in terms of the MIC/MFC values. Among them, carvacrol displayed the most inhibitory activity (0.125 mg/ml). Previous studies have shown that carvacrol is the most effective agent against other *Candida* species as well (Rao *et al*, 2010; Ahmad *et al*, 2011). In addition, carvacrol was selected to test synergism with four antifungal drugs from different classes against *C. auris* isolates. Several studies have reported the synergistic activity of carvacrol in combination with antifungal drugs against *C. albicans* and NAC species (Doke *et al*, 2014).

Furthermore, several reports have established the presence of phenotypic and genomic variations between different *C. auris* strains from the same or different regions. Therefore, this study investigated the pathogenicity of 25 different *C. auris* isolates obtained from South Africa. The capacity to express different virulence factors, such as adherence, germination and proteinase and phospholipase production was studied. A study on *C. auris* isolates which obtained from other countries have reported that the ability of *C. auris* to express these virulence factors is strain dependent and weaker than that of other *Candida* species (Larkin *et al,* 2017). Present study showed that *C. auris* has an ability to adhere to buccal epithelial cells and produces proteinase enzyme. None of the tested isolates, however, were able to form germ tube or produce phospholipase enzyme.

Moreover, because of limited treatment options for *C. auris* infections, the need for an alternative antifungal strategy to overcome drug resistance along with a novel antifungal drug acting on new targets has increased. A novel approach of targeting virulence factors will reduce the pathogenic potential of this yeast, thereby preventing or reducing the severity of its infections. Using this approach, this study investigated the effect of carvacrol on the virulence attributes of *C. auris*. The anti-virulence effect of carvacrol on *C. albicans* have already been reported (Braga e*t al,* 2007; Hosseini *et al,* 2016). Our results showed that carvacrol can also inhibit the adherence ability and proteinase production in *C. auris*. the results are discussed in detail, for a summary of the results refer to Figure 12, Page 104.

4.1 Antifungal susceptibility

In vitro antifungal susceptibility testing has been used to determine the best therapeutic concentration for a specific fungus and to identify antifungal drug resistance. Microdilution methods, as was used in this study, are the gold standard technique. Clinical Laboratory Standards Institute (CLSI) has standardized an MIC assay to perform antifungal susceptibility

testing, in which the growth of the fungal pathogen was measured with a series of drug concentrations over time, and has developed MIC breakpoints of some antifungal drugs to categirize susceptible and resistant strains. Although clinicians rely on these methods to select the drug of choice for fungal infections, they are not always accurate predictors of the *in vivo* response to the drug treatment. Several factors such as, pharmacokinetics of the drug and host-pathogen interactions can affect the clinical outcomes.

The CLSI has proposed specific susceptibility breakpoints for all *Candida* species except for *C. auris*, due to limited availability of clinical data. However, CDC has proposed a tentative breakpoint for selected antifungal drugs based on susceptibility of closely related *Candida* species, which has been used in this study.

4.1.1 Antifungal drug susceptibility against *C. auris*

Antifungal drug resistance is a relevant problem with *C. auris* infections, which limits the treatment options that can be administered to patients, leading to sever invasive infections with high rates of mortality. High proportion of C. *auris* isolates exhibit higher MICs for fluconazole and amphotericin B, this has led to the recommendation for the use of echinocandins as first-line therapy for *C. auris* infections (CDC, 2017b). However, acquired resistance to this class can develop during the treatment course and isolates with high MICs to echinocandins have also been reported (Cortegiani *et al*, 2018). Therefore, the identification of new antifungal agents that are effective against this pathogen is critical.

Data from this study showed that 88% of *C. auris* isolates have been resistant to fluconazole (MIC ≥32 µg/ml) and 20% have been resistant to amphotericin B (MIC ≥2 µg/ml). These results are in agreement with studies from the United States that reported about 90% of *C. auris* isolates to be resistant to fluconazole and 30% to be resistant to amphotericin B (CDC,

2017b). Other study have reported about 93% of *C. auris* isolates, including South African clad of *C. auris,* were resistant to fluconazole and 35% were resistant to amphotericin B (Lockhart *et al*, 2017). Moreover, in this study *C. auris* isolates are generally susceptible to caspofungin, only 4% have been resistant to this agent (MIC ≥2 µg/ml). This is in agreement with those of others reporting high susceptibility of echinocandins against *C. auris* (Borman et al, 2016; Larkin *et al,* 2017; Lockhart *et al*, 2017). However, therapeutic failure with this class has been reported for echinocandins sensitive isolates in different geographic regions, possibly explained by the development of extrinsic resistance after exposure of *C. auris* isolates to these agents (Cortegiani *et al*, 2018).

Regarding nystatin, although it has been used in cases of *C. auris* oropharyngeal colonization, which resulted in successful decolonization, currently no published data on the MICs and breakpoints of nystatin against *C. auris* available. This study found low MICs of nystatin against *C. auris,* which ranged from 0.5 to 4 µg/ml, showing sensitive nature against this antifungal agent.

4.1.2 Antifungal susceptibility of phenolic compounds against *C. auris*

To identify new therapeutic agent that may be effective against *C. auris*, in this study four phenolic compounds were tested, which has been shown to possess some antifungal activity against various other *Candida* species. Essential oils rich in phenolic monoterpenes are especially known for their significant *in vitro* fungicidal activity against clinical fluconazole-sensitive and fluconazole-resistant *Candida* isolates (Guo e*t al*, 2009; Ahmad *et al,* 2010). In addition, the *in vivo* therapeutic efficacy of these active compounds on experimental oral and systemic candidiasis is well established (Chami e*t al,* 2005). Therefore, phenolic monoterpenes have the potential to be developed into an antifungal agent for clinical applications. Antifungal activity of these phenolics against *C. auris* has not been studied previously.

Data from this study indicates that carvacrol, thymol, eugenol and methyl eugenol had antifungal activity against 100% of *C. auris* isolates tested. Previous studies have reported their potent antifungal activity against *C. albicans* and various NAC species (Ahmad *et al,* 2010). Among the four compounds, carvacrol was found to be the most active agent against *C. auris* strains tested, with an MIC of 0.125 mg/ml and MFC of 0.25 mg/ml. These results are in agreement with studies that reported carvacrol as the most effective inhibitor against other *Candida* species as well (Rao *et al*, 2010; Ahmad *et al*, 2011). The antifungal activity of carvacrol might be related to its structural characteristics. Rao *et al*. (2010) have reported that the presence of a free hydroxyl group on the aromatic ring in carvacrol increases its antifungal activity.

Interestingly, in this study, the median MIC/MFC of carvacrol against the reference strain *C. albicans* SC5314 (0.25 mg/ml and 0.5 mg/ml respectively) was higher than those of *C. auris.* Previous studies have also reported high MIC/MFC values for the same agent against different *Candida* species, with MIC ≥ 0.25 mg/ml and MFC ≥ 1 mg/ml (Doke *et al*. 2014). Our observation of lower MIC of carvacrol against *C. auris* than *C. albicans,* means *C. auris* is more sensitive to this compound, which could be explained by the differences in the cell size and cell wall composition between the two species. *C. auris* is relatively smaller and showed low percentages of cell wall proteins when compared to *C. albicans* (Navarro-Arias *et al*. 2019). These differences are probably to affect the chemical interaction between carvacrol and the fungal cell. As carvacrol cause fungal cell death by disrupting membrane integrity leads to increase cell permeability (Ahmad *et al,* 2010), and low proteins level in the cell wall lessen its rigidity and strength and make *C. auris* more susceptible to carvacrol.

The *in vitro* antifungal activity of carvacrol against *C. auris* clinical isolates, including the resistant strains, encouraged us to test its activity in combination with the conventional

antifungal drugs. Phenolic compounds have shown synergistic effects when combined with fluconazole and other antifungal drugs against different *Candida* species (Guo *et al,* 2009; Ahmad *et al,* 2010; Ahmad *et al,* 2012).

4.1.3 Combination study

Combination therapy is one of the strategies that could reduce toxicity and increase antifungal potential of the existing antifungal agents (Ghannoum *et al,* 1995). Considering the difficulties regarding the treatment of *C. auris* infections, the combination of antifungal drugs represents an important alternative to the conventional monotherapy approach. The combination of echinocandins with the other antifungal drugs against multi drug resistant isolates has been recommended in difficult to treat cases (CDC, 2017b).

Alternatively, the combination of essential oils or its active compounds with antifungal drugs were recently proposed to improve treatment of fungal infections (Khan *et al,* 2012). The combination of natural compounds with antifungal drug can represent nontoxic, cost-effective and more efficient alternative to the combination of two conventional antifungals. Synergistic potential was observed when phenolic compounds were combined with fluconazole and other antifungal drugs against different *Candida* isolates, such as *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. parapsilosis* (Guo *et al,* 2009; Ahmad *et al,* 2010). No studies have reported results of combination studies on *C. auris*.

In this study, carvacrol was combined with the commonly used antifungal agents and tested against *C. auris* strains. The highest synergistic effects were obtained from the combination between carvacrol and amphotericin B or nystatin (28% of *C. auris* strains). The combination of carvacrol and caspofungin showed synergism in one *C. auris* strain only, interestingly, this isolate is the one which exhibited resistance to the all antifungals used. In addition, carvacrol increased the action of fluconazole, by reducing its MIC, in high percentage of strains through

additive (52% of strains) and synergistic (16% of strains) effect. Moreover, results also show that the required dose is much less in combination compared to the test drug and carvacrol alone. All combinations, except with fluconazole, have reduced the MICs of the antifungal drugs to the sensitive levels, particularly in resistance strains. Therefore, carvacrol can help to sensitize *C. auris* isolates to these drugs. Here we hypothesis that carvacrol may be useful as a candidate antifungal chemosensitizing agent, which can restore the sensitivity of the resistant *C. auris* strains to the antifungal drugs or even reverse drug resistance. Reversal of drug resistance mechanisms offers the hope of increasing the efficacy of conventional antifungal drugs. Ahmad *et al*, (2012) reported that carvacrol have shown a high potency in reversal of azole resistance in *C. albicans* by inhibiting the drug efflux pump gene expression. Further *in vitro* and *in vivo* studies are necessary to confirm the practical utility of these combinations.

4.2 Virulence assays

Although *C. auris* has attracted great attention in the scientific field in the past 10 years, knowledge of the virulence attributes of this pathogen is still limited. Few studies have been conducted to investigate the expression of virulence factors in *C. auris* strains from different geographical clades (Kumar *et al.* 2015; Larkin *et al*, 2017; Wang *et al,* 2018). To the best of our knowledge, however, none of the studies have included strains from the South African clade. Therefore, the purpose of this study was to determine the virulence factors of clinical *C. auris* isolates from South Africa using *in vitro* virulence assays. Several virulence factors have been well characterized in *C. albicans* such as: adherence, morphogenesis and phospholipase and proteinase production (Ahmad *et al,* 2016).

4. 2.1 Adherence

Adherence of yeast cells to host tissue is the initial step for colonization and subsequent development of *Candida* infection (Haynes, 2001). Our results showed that all *C. auris* strains

possesses an ability to adhere to BECs. The adherence capacity of *C. auris* seems to be partially responsible for its ability to colonize human host and persist in the hospital environments. Adhesins, especially the ALS proteins, play a key role in *C. albicans* adherence. The *C. auris* genome was also found to encode members of ALS and other proteins belonging to the adhesin gene families. *C. auris,* however, expresses a highly reduced number of adhesins compared to *C. albicans* (Munoz *et al.* 2018). This could explain our finding of lower adherence ability of *C. auris* strains (141 yeast cells adherent to 100 BECs) compared to the reference strain *C. albicans* SC5314 (311 yeast cells adherent to 100 BECs). Larkin *et al,* (2017) have observed that *in vitro* adherence ability of *C. auris* to catheter materials is also reduced by 25% as compared to *C. albicans.* More interestingly, *C. auris* strains showed different levels of adherence ability. It has been reported that adherence to epithelial cells was significantly higher in *C. albicans* isolated from cancer patients and denture wearer than the healthy individuals (Mothibe and Patel, 2017). Therefore, other host and environmental factors may influence the adherence capacity of *C. auris*.

4.2.2 Morphogenesis

Morphogenesis is well known virulence feature of *C. albicans.* The filamentous growth thought to promote the ability of the fungus to invade and damage epithelial cell layers (Odds, 1988). The ability to produce true hyphae has been reported in *C. albicans* and the closely related species, *C. dubliniensis.* While other NAC species are either only able to produce pseudohyphae or do not undergo any morphological transition (Silva *et al,* 2011). Previous studies as well as our results showed that *C. auris* does not produce true hyphae and/or pseudohyphae when incubated with serum *in vitro* (Lee *et al*. 2011, Chowdhary *et al.* 2013; Kumar *et al.* 2015; Larkin *et al*, 2017). Molecular study have also confirmed that *C. auris* do not express genes such as, *ECE1* and *HWP1*, which are expressed and strongly associated with

hyphal formation in *C. albicans* (Munoz *et al*. 2018). Moreover, Borman *et al*. (2016) have reported that some *C. auris* isolates were able to form rudimentary pseudohyphae. *C. auris,* however, did not undergo significant filamentation in the *Galleria mellonella* infection model in the same study. More recently, *C. auris* were able to undergo some morphological changes and form filaments growth and pseudohyphal-like growth, when it was treated with 10% NaCl or after passage through animal model (Wang *et al,* 2018; Yue *et al,* 2018). Thermotolerance and salt tolerance are known characteristic feature of *C. auris* (Satoh *et al,* 2009). Therefore, it would be interesting to investigate *C. auris* response to different environmental conditions, other than incubation with serum which is well known to stimulate hyphal growth in *C. albicans*.

4.2.3 Proteinase activity

Proteinases are one of the most significant extracellular hydrolytic enzymes produced in *C. albicans and* many other pathogenic *Candida* species. The production of proteinases has been recognized as an important virulence factor contributing to the pathogenicity of *Candida* species. These enzymes were considered to play a role in the degradation of important structural and immunological proteins in the host tissue. They also disrupt host epithelial and mucosal membrane proteins and enable the fungal cells to adhere and hence penetrate into the host (Ahmad *et al*, 2016).

Production of proteinases is very well studied in *C. albicans*, however, very little is known in *C. auris*. In this study, we have shown that 96% of tested *C. auris* strains produced proteinases and these results are in line with the previous study by Larkin *et al*, (2017) where they reported 64% of the strains of *C. auris* producing proteinases. Our results also showed that none of the strains of *C. auris* produced hyphae which suggest that SAPs 4-6 may not be produced by this organism, because SAPs 1-3 are expressed by the yeast phase only while as SAPs 4-6 are

expressed in the hyphal phase. Most interestingly, among the *C. auris* strains which tested positive for proteinase activity, 58% of the strains showed very strong (++++) proteinase activity, which was comparable to the reference strain *C. albicans* SC5314 proteinase production. These results suggested that *C. auris* seems to be strong proteinases producer, and these enzymes are likely to contribute to the pathogenicity and rapid spread of this pathogen. However, the enzymes are not characterised and not much is known about their pattern of expression.

4.2.4 Phospholipase activity

Another important group of extracellular hydrolytic enzymes, the phospholipases, have been shown to be produced by multiple pathogenic *Candida* species. Phospholipases play an important role in *Candida* invasion to the host tissues by hydrolysing phospholipids in the host cell membranes. Results in this study revealed that none of the strains of *C. auris* produced phospholipase. In contrast, a study by Larkin *et al.* (2017) has reported some weak phospholipase activity in 37.5% (6/16 isolates) of tested strains. These differences can be related to the geographical variations as the tested strain in our study belong to South African clade while as the strains in Larkin and co-workers belong to American and Asian clade. However, genetic differences between strains from the same clade have also been reported. Therefore, large number of *C. auris* strains need to be studied for their ability to produce phospholipases.

4.3 Effect of carvacrol on virulence

Natural plant extract, especially essential oils and their active compounds have demonstrated some inhibitory effect against the growth of pathogenic *Candida* species (Suresh e*t al*, 1996; Pinto *et al,* 2006; Khan *et al,* 2010). In addition, several of these plant products have shown anti-virulence effects against different *Candida* species. Anti-virulence approach provides new

alternative option for antifungal drug discovery by extending the range of potential drug targets. For example, at subinhibitory concentration the crude extract of *Dodonaea viscosa* can inhibite the adherence of *C. albicans* to oral epithelial cells (Patel *et al*. 2009). Essential oils rich in monoterpene phenols have been shown to inhibit the hyphal growth and biofilm formation in *C. albicans (*De Toledo *et al.* 2016). Furthermore, carvacrol at subinhibitory concentration was also able to inhibit the formation of hyphae (Braga e*t al,* 2007), eradicate the mature biofilms (Dalleau *et al,* 2008) and down regulate genes of aspartyl proteinase (Hosseini *et al,* 2016). Therefore, we evaluated the activity of carvacrol against the virulence factors such as adherence ability and proteinases production in *C. auris* strains.

4.3.1 Carvacrol possesses activity against *C. auris* adherence

The importance of adherence in the virulence and the pathogenesis of *Candida* infections is widely recognized. Adherence ability and subsequent colonization in *Candida* species are highly dependent on cell surface expressed adhesins (Ahmad *et al*, 2016). Therefore, compounds that modulate *Candida* adherence ability or target the specific proteins responsible for that could potentially reduce *Candida* virulence and considered as an attractive candidate for antifungal drug development.

In the present study, results showed that carvacrol can inhibit the adherence ability of *C. auris* at all concentrations tested (0.25, 0.125, 0.063 and 0.031 mg/ml). However, statistically significant inhibition was observed at the concentration of 0.25, 0.125 and 0.063 mg/ml. In this study, *C. auris* cells were first treated with carvacrol for 2 hours, washed and then exposed to the epithelial cells, which suggests that the effect of carvacrol was on the yeast cell surface. Carvacrol may have blocked these adhesins or interfered with their synthesis and thereby reducing the adherence of *C. auris* to host cells. Essential oils and their compounds are known to cause damage to biological membranes, disrupting membrane permeability and inhibit

respiration (Ahmad *et al*, 2011). Similar phenomenon has been observed in *C. albicans* treated with *D. viscosa* (Patel *et al*. 2009).

4.3.2 Inhibitory effect of carvacrol on *C. au*ris proteinase production

Proteinases are major *C. albicans* secreted enzymes which contributes to invasiveness during infections. Results in this study revealed that *C. auris* is also able to produce proteinase. Therefore, proteinase inhibitors could be attractive agents for antifungal drug development. For example: the HIV proteinase inhibitors, primarily used to target HIV, have shown potency in the cure of mucosal candidiasis in HIV infected patients, due to a direct inhibitory activity of these drugs against proteinases secreted from *C. albicans* (Korting *et al*. 1999).

The present study showed significant reduction in collective *in vitro* proteinase production with the treatment of carvacrol at concentration of tested 0.25 and 0.125 mg/ml. In contrast, the crude extract of *Dodonaea viscosa* had no effect on the production of proteinase and phospholipase in *C. albicans*. This could be due to higher inhibitory activity of the purified compound of plant extract, such as carvacrol in our study, than the crude extract of the plant. Furthermore, Hosseini et al. (2016) have reported that carvacrol is effective in reduction of aspartyl proteinase genes expression, with a higher effect against fluconazole resistant isolates. Therefore, this property makes carvacrol a better candidate as an antifungal agent. Although adherence and proteinase production are interlinked, in our study the effect must have been independent to one another because *Candida* proteinases generally act on the epithelial tissues and enable the fungal cells to adhere and hence penetrate the host tissues.

4.4 Clinical implications

This study has shown that carvacrol has inhibitory effect against *C. auris,* and the effect is different at different concentrations. At high concentrations carvacrol would kill *C. auris* cells

therefore it would reduce the number of cells. In addition, it will inhibit the proteinase secreted from the surviving cells, thereby reducing their pathogenicity. While at low concentrations it would reduce the adherence of *C. auris* to host cells, which is crucial in the pathogenesis of infection, rendering this pathogen avirulent. Furthermore, the combination of carvacrol and fluconazole or amphotericin B presented a potentiated antifungal effect on *C. auris*, these combinations will reduce the required dose of the two drugs and restore the sensitivity of the resistance *C. auris* strains to the drugs. This study showed that this monoterpene has preventive and therapeutic potential against *C. auris* colonization and infections. However, further research is necessary.

4.5 Limitation

The main limitation of the present study was *in-vitro* nature of the experiments with small number of isolates. Both *in-vivo* and *in-vitro* studies with larger number of isolates are needed to determine the efficacy of phenolic compounds on *C. auris* while actively infecting animals, and the effect of carvacrol on the organism and its virulence.

In addition, the experimental strains were collected from previously frozen isolates and this could have resulted in pathogen that were not as virulent compared to the initial isolation of these fungal pathogens. These limitations; however, provide areas for continued research. This study is just beginning to shed light on the mechanisms of pathogenicity of this newly emerged fungal pathogen.

4.6 Future research

- In light of the considerable diversity among the *C. auris* strains from different geographical areas, investigations to understand the effect of monoterpenes on the different clads will be valuable. Moreover, it will be fascinating to see how these

phenotypic and genetic variations influence virulence and pathogenicity of this pathogen.

- Both *in-vivo* and *in-vitro* studies for the efficacy of phenolic compounds on *C. auris* from different sites of infection is required. The possibility of studying not only the effects of the compounds on *C. auris*, but also on how the patient responds to these compounds remains to be determined.
- Although carvacrol is commonly used as a food flavouring agent and considered as nontoxic to humans, and oral administration of this monoterpene has shown no signs of toxicity in rates. Nevertheless, the safety, cytotoxicity and the required concentrations of this compound needs to be established.
- Comparative studies on the efficacy of carvacrol with the currently used antifungal drugs and determination of the optimal concentration for clinical application need further investigations.
- The efficacy and safety of these combinations based on animal models and clinical trials need to be carried out. In addition, the mode of actions of these synergistic combinations should be deciphered.

CHAPTER 5: CONCLUSION

This study showed that *C. auris* expresses various virulence factors such as adherence ability and proteinase activity. All tested strains showed ability to adhere to buccal epithelial cells and 96% of the strains produced proteinase. None of the isolates produce hyphae and phospholipase. Furthermore, the results showed that carvacrol, thymol, eugenol and methyl eugenol had antifungal activity against *C. auris* with carvacrol being the most effective monoterpene phenol. Carvacrol at high concentration (0.125 mg/ml) has inhibitory activity against the growth of *C. auris* and it also inhibits the proteinase production, while at subinhibitory concentrations (0.063 mg/ml), it significantly inhibited the adherence to epithelial cells in *C. auris*. This suggests that at MIC concentrations carvacrol will inhibit the growth of *C. auris* and as it becomes diluted it will render them avirulent providing additional long lasting effect. In addition, combination of carvacrol with four antifungal drugs reduces the effective concentrations of both the agents with high synergistic to additive effects particularly in carvacrol combined with fluconazole, amphotericin B and nystatin. Therefore, carvacrol has potential to be developed into an antifungal agent for the prevention and treatment of *C. auris* infections.

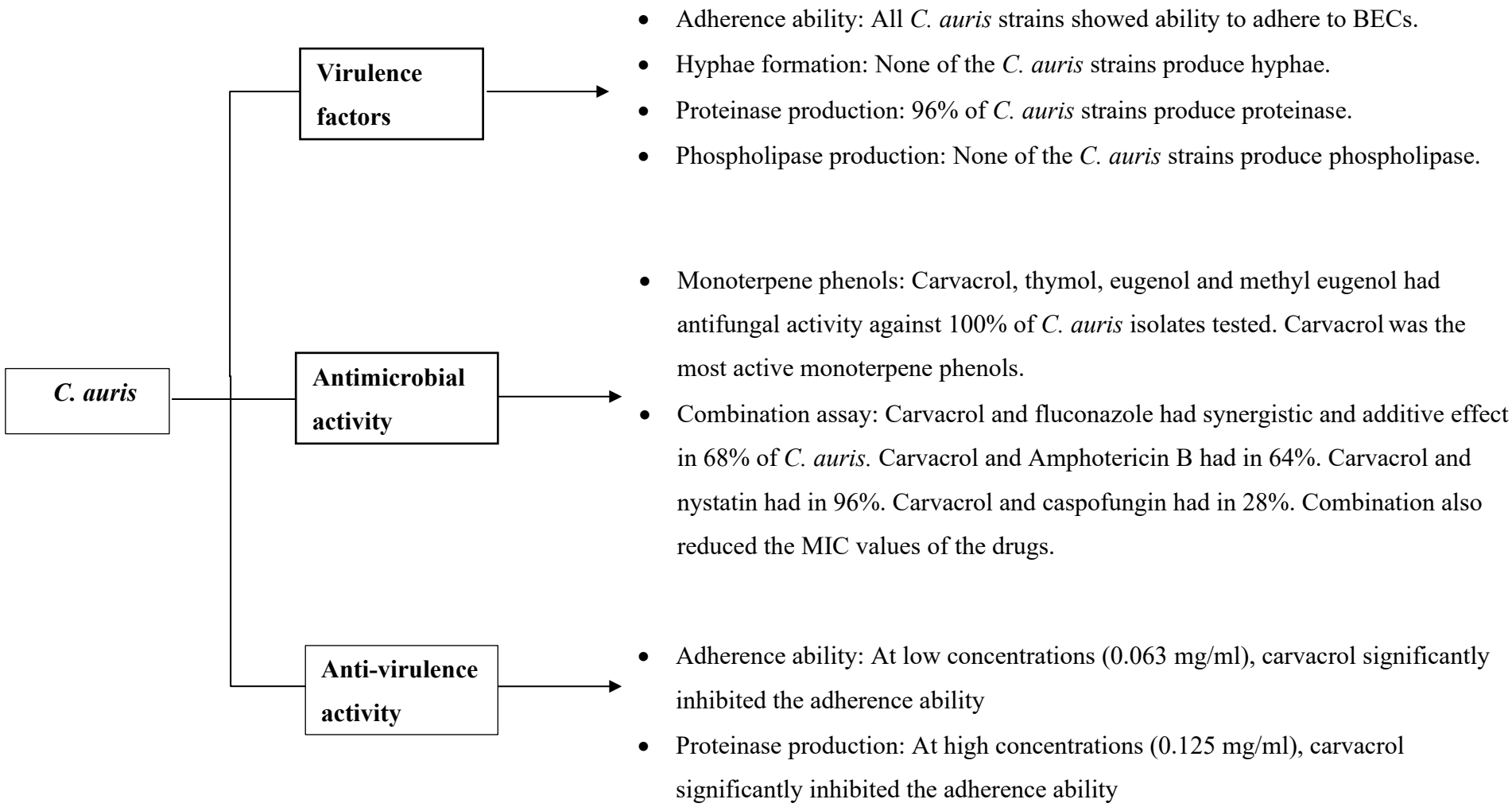

Figure 13: Diagram illustrating the summary of results.

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APPENDICES

Appendix A:

Figure A1: Ethical Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL

Human Research Ethics Committee (Medical)

Research Office Secretariat:
Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 301/2/4, 29 Princess of Wales Terrace, Parktown, 2193
Tel +27 (0)11-717-1252 /1234/2656/2700
Private Bag 3, Wits 2050
Office email: HREC-Medical.ResearchOffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CBP-180529-4 29/05/2018

TO WHOM IT MAY CONCERN:

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

Investigator: Dr Siham Shaban (student no. 1764671) et al

Supervisor: Dr Aijaz Ahmad

Department: Clinical Microbiology and Infectious Diseases

Project title: Identification of virulence factors in *Candida auris* and antifungal activity of monoterpene phenols against this pathogen.

Reason: Laboratory analysis. Will use fungal stock culture of *Candida auris* isolates along with a control laboratory *Candida albicans* SC5314 strain. No human participants will be involved in the study.

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

UNIVERSITY OF THE WITWATERSRAND
OFFICE OF THE DEPUTY VICE CHANCELLOR RESEARCH
29 MAY 2018
RESEARCH OFFICE HREC (Medical)
JOHANNESBURG

Copy – HREC (Medical) Secretariat: Rhulani Mkansi and Zanele Ndlovu.

Appendix B:

Composition and preparation of Media, Buffers and Stains

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

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

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

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

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

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

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

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

- **Gram's Safranin**
    - 10g safranin dye
    - 1000ml distilled water

Appendix C:

Table C1: Clinical isolates used in this study.

No.	Strains	Species
CAU 1	MRL 6326	*C. auris*
CAU 2	MRL 6334	*C. auris*
CAU 3	MRL 2397	*C. auris*
CAU 4	MRL 2921	*C. auris*
CAU 5	MRL 3499	*C. auris*
CAU 6	MRL 3785	*C. auris*
CAU 7	MRL 4587	*C. auris*
CAU 8	MRL 4000	*C. auris*
CAU 9	MRL 4888	*C. auris*
CAU 10	MRL 6333	*C. auris*
CAU 11	MRL 6277	*C. auris*
CAU 12	MRL 6339	*C. auris*
CAU 13	MRL 6125	*C. auris*
CAU 14	MRL 6194	*C. auris*
CAU 15	MRL 6338	*C. auris*
CAU 16	MRL 6183	*C. auris*
CAU 17	MRL 5765	*C. auris*
CAU 18	MRL 5762	*C. auris*
CAU 19	MRL 5418	*C. auris*
CAU 20	MRL 6005	*C. auris*
CAU 21	MRL 6015	*C. auris*
CAU 22	MRL 6059	*C. auris*
CAU 23	MRL 6065	*C. auris*
CAU 24	MRL 6057	*C. auris*
CAU 25	MRL 6173	*C. auris*

Appendix D:

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Detailed Status Information

Manuscript #	SREP-19-32958-T
Current Revision #	0
Submission Date	16th August 19
Current Stage	Manuscript Assigned to Peer-Reviewer/s
Title	Improved efficacy of antifungal drugs in combination with monoterpene phenols against Candida auris
Manuscript Type	Original Research
Collection	N/A
Manuscript Comment	
Corresponding Author	Dr. Aijaz Ahmad (aijaz.ahmad@wits.ac.za) (University of the Witwatersrand)
Contributing Authors	Dr. Siham Shaban , m patel
Authorship	Yes
Abstract	Purpose: Emergence of Candida auris has been described as a global health threat due to its ability to cause invasive infections with high mortality rate and multidrug resistance. Novel drugs and therapies are required to target this organism and its pathogenicity. Combination therapy and pathogenicity, as a drug target, have been proposed in recent years. This study evaluated the virulence factors in C. auris, combination antifungal activity of phenolic compounds with antifungal drugs and determined effect of the most active compound on positive pathogenicity markers of C. auris. Methods: Antifungal susceptibility profile of 25 clinical isolates of C. auris against antifungal agents as well as against phenolic compounds was obtained using CLSI guidelines. Combination of the most active phenolic compound with antifungal drugs was determined. Effect of carvacrol on the virulence factors was also studied. Results: Carvacrol was the most active phenol with median MIC of 125 µg/ml and its combination with fluconazole, amphotericin B, nystatin and caspofungin resulted synergistic and additive effects in 68%, 64%, 96% and 28%, respectively. Combination also reduced the MIC values of the drugs. All test strains showed adherence ability to epithelial cells and 96% of strains produced proteinase. None of the strains produced hyphae and phospholipase. At low concentrations, carvacrol significantly inhibited the adherence ability and proteinase production (both $p<0.01$). Conclusion: Carvacrol has antifungal and anti-virulence activity against C. auris. It also showed an enhanced antifungal activity in combination with antifungal agents. Therefore it has potential to be developed into a novel antifungal agent.
Techniques	Life sciences techniques, Cellular imaging [Microscopy]; Life sciences techniques, Signal transduction techniques [Small molecule library];
Subject Terms	Health sciences/Pathogenesis/Infection Biological sciences/Microbiology/Fungi/Fungal pathogenesis
Competing Interests Policy	There is **NO** Competing Interest.
Applicable Funding Source	National Research Foundation (NRF) - RDYR180418322304; Grant No: 116339 [Ahmad]

Stage	Start Date	End Date	Approximate Duration
Manuscript Assigned to Peer-Reviewer/s	1st October 19		
Manuscript Assigned to Editor	17th September 19		
Manuscript Assigned to Peer-Reviewer/s	16th September 19		

Appendix E:

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ORIGINALITY REPORT

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2	www.esp.org Internet Source	1%
3	Emily Larkin, Christopher Hager, Jyotsna Chandra, Pranab K. Mukherjee et al. "The Emerging Pathogen Candida auris: Growth Phenotype, Virulence Factors, Activity of Antifungals, and Effect of SCY-078, a Novel Glucan Synthesis Inhibitor, on Growth Morphology and Biofilm Formation", Antimicrobial Agents and Chemotherapy, 2017 Publication	1%
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