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MODULATION OF CELL DEATH AND PATHOGENICITY BY EUGENOL TOSYLATE CONGENERS IN *CANDIDA ALBICANS*

Shabir Ahmad Lone



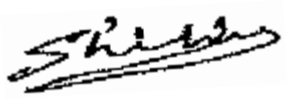
A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand

In fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg 2020

DECLARATION

I, Shabir Ahmad Lone declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in University of the Witwatersrand, Johannesburg. It was not submitted before for any degree or examination at this or any other University.



…… (Signature of Candidate)

8th day of June, 2020

DEDICATION

This work is dedicated to my parents

Ghulam Mohi Ud Din Lone

Taja Begum

And

To rest of my family!!!

LIST OF PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS:

1. **Lone SA**, Wani MY, Fru P, Ahmad A. Cellular apoptosis and necrosis as therapeutic targets for novel Eugenol Tosylate Congeners against *Candida albicans*. Scientific Reports, 2020;10(1):1191. doi: 10.1038/s41598-020-58256-4.
2. **Lone SA,** Ahmad A, Khan S. Inhibition of ergosterol synthesis in *Candida albicans* by novel Eugenol Tosylate Congeners targeting sterol 14α-demethylase (CYP51) enzyme. Archives of Microbiology, 2020;202(4):711-726. doi: 10.1007/s00203-019-01781-2.
3. **Lone SA**, Ahmad A. Inhibitory Effect of Novel Eugenol Tosylate Congeners on Pathogenicity of *Candida albicans*. BMC Complementary Medicine and Therapies, 2020;20(1):131. doi: 10.1186/s12906-020-02929-0.
4. **Lone SA**, Ahmad A. *Candida auris* – the growing menace to global health. Mycoses, 2019; 62(8):620-637. doi: 10.1111/myc.12904.
5. Malik MA, **Lone SA,** Wani MY, Talukdar IA, Dar OA, Ahmad A, Hashmi AA. S-benzyldithiocarbazate imine coordinated metal complexes kill *Candida albicans* by causing cellular apoptosis and necrosis. Bioorganic Chemistry, 2020;98:103771. doi: 10.1016/j.bioorg.2020.103771.
6. Dar OA, **Lone SA**, Malik MA, Aqlan FM, Wani MY, Hashmi AA, Ahmad A. Synthesis and synergistic studies of isatin based mixed ligand complexes as potential antifungal therapeutic agents. Heliyon, 2019;5(7):e02055. doi: 10.1016/j.heliyon.2019.e02055.
7. Dar OA, **Lone SA,** Malik MA, Wani MY, Hashmi AA, Ahmad A. Heteroleptic transition metal complexes of Schiff-base derived ligands exert their antifungal activity by disrupting membrane integrity. Applied Organometallic Chemistry, 2019; 33: e5128. doi.org/10.1002/aoc.5128.
8. Dar OA, **Lone SA,** Malik MA, Wani MY, Ahmad A, Hashmi AA. New transition metal complexes with a pendent indole ring: insights into the antifungal activity and mode of action. RSC Advances, 2019; 9, 15151-15157. doi.org/10.1039/C9RA02600B.
9. Malik MA, **Lone SA**, Gull P, Dar OA, Wani MY, Ahmad A, Hashmi AA. Efficacy of novel Schiff base derivatives as antifungal compounds in combination with approved drugs against *Candida albicans*. Medicinal Chemistry, 2019;15(6):648-658. doi: 10.2174/1573406415666181203115957.

PRESENTATIONS:

1. Presentation entitled "**Novel semi-synthetic compounds Eugenol Tosylate Congeners exhibited potent antifungal activity** (oral)" presented at Department of Clinical Microbiology and Infectious Diseases Research Day 6th November 2019 at the University of Witwatersrand, Johannesburg, South Africa.
2. Presentation entitled "**Potent In vitro antifungal activity of a modified monoterpene phenol Eugenol against *Candida albicans*** (poster)'' presented at 9th Cross-Faculty Postgraduate Symposium, 29th and 30th October 2018 at the University of Witwatersrand Johannesburg South Africa.
3. Presentation entitled "**Induction of apoptosis in *Candida albicans* by novel eugenol tosylate congeners** (poster)" presented at Faculty of Health Sciences Research Day, 6th September 2018 at the University of Witwatersrand Johannesburg South Africa.
4. Presentation entitled "**Potent In vitro activity of a modified monoterpene phenol Eugenol against *Candida albicans*** (poster)" presented at 48th Scientific Meeting of International Association for Dental Research (IADR) - South African Division. 30th and 31st August 2018 at Glenburn Lodge, Muldersdrift Johannesburg South Africa.
5. Presentation entitled "**Novel semi-synthetic eugenol analogs as efficient combinative antifungal agents** (poster)" presented at the Molecular Biosciences Research Trust (MBRT) postgraduate research day 30th November 2017 at the University of Witwatersrand Johannesburg South Africa.

ABSTRACT

The global prevalence of serious fungal diseases is increasing rapidly, which affects more than a billion people every year with a significant mortality rate. On the other hand, the development of new drugs to treat these fungal infections is slow, while the current antifungal therapy is insufficient and bound with limitations. Such circumstances are contributing to the development of drug resistance and also to the provision of a platform for new emerging multi-drug resistant species such as *Candida auris*. Thus, development of novel antifungal drugs with minimum or no toxicity and multi-target mechanisms of action can resolve these issues. In this regard, we synthesized seven novel eugenol tosylate congeners (ETC-1 – ETC-7) from a natural compound eugenol, which is well known and has been extensively studied for its antimicrobial and other pharmacological properties. These ETCs were tested for their antifungal activity against various fluconazole susceptible and resistant strains of *Candida albicans*. In this study, we selected *C. albicans* as a target organism due to the fact that this opportunistic pathogen is the most predominant species of genus *Candida* that can cause infections in humans and is widely used as a model organism in fungal research for its various unique characteristics.

Antifungal susceptibility testing was done by determining Minimum Inhibitory Concentrations (MIC) and Minimum Fungicidal Concentrations (MFC) and the results revealed the enhanced and potent antifungal effect of these compounds in comparison to their parent compound eugenol against both FLC susceptible and resistant isolates of *C. albicans*. The order of antifungal potency based on susceptibility results was ETC-5>ETC-6>ETC-7>ETC-1>ETC-4>ETC-2>ETC-3. To determine the multi-target mechanisms of antifungal action of these compounds, effect on both established and emerging drug targets was studied. The effect on the ergosterol biosynthesis pathway was evaluated by applying both *in silico* and *in vitro* approaches. The *in silico* studies, which includes molecular docking, confirmed

that these ETCs target sterol 14α-demethylase (CYP51), an essential enzyme in the ergosterol biosynthesis pathway, and thereby impaired ergosterol biosynthesis in both susceptible and resistant isolates. Test compound ETC-5 showed best docking score and is efficiently involved in its high affinity with the active site of CYP51 protein, followed by ETC-6 and ETC-7 respectively. Therefore, based on antifungal susceptibility and molecular docking results, only three most active compounds (ETC-5, ETC-6 and ETC-7) were selected for further studies. *In silico* findings were further corroborated by *in vitro* studies, where the test compounds significantly reduced ergosterol biosynthesis and downregulated the expression of sterol 14 α–demethylase encoding gene '*ERG11*'. The concentration dependent fungicidal effect was detected in a cell viability assay by using MUSE Cell Analyzer, where complete cell death was observed when cells were treated with higher concentrations of the test compounds. To study the effect of the most active ETCs (ETC-5, ETC-6 and ETC-7) on major virulence factors of *C. albicans*, the effect of these compounds on adherence (alamarBlue-based assay), morphogenesis (microscopy), secretion of hydrolytic enzymes (plate assay methods) and biofilm formation (XTT reduction assay) was studied. Furthermore, to study the in depth effect at molecular level, expression of genes related to pathogenicity of *C. albicans* such as *ALS1, ALS2, ALS3, ALS9, CPH1, HWP1, SAP1, SAP2, SAP3* and *PLB1* was tested by using quantitative reverse transcription PCR (RT-qPCR). From these results, it was evident that all the tested ETCs significantly reduced the virulence factors of *C. albicans* and down regulated the expression of their related genes.

To study the in depth mechanism of antifungal action of the most active ETCs (ETC-5, ETC-6 and ETC-7), the mode of cell death was determined. External induction of apoptosis in yeast cells is considered as an ideal model for the development of novel antifungal drugs. Therefore, we studied apoptotic effect of ETC-5, ETC-6 and ETC-7 in *C. albicans* cells by analysing major markers of yeast apoptosis, which include phosphatidylserine externalization, DNA damage, mitochondrial depolarization and decrease in cytochrome c oxidase activity.

FITC annexin V/PI double staining and TUNEL assay results revealed significant apoptotic effect through phosphatidylserine externalization and DNA damage, however necrotic effects were also observed at higher concentrations of these ETCs. Mitochondrial membrane potential and cytochrome c oxidase activity was also markedly decreased in *C. albicans* cells after being exposed to these test entities, which suggested the possible involved apoptotic pathway activated by these ETCs could reside in the metacaspase dependent pathway.

Based on the above results it is evident that these test compounds have potent antifungal activity, however it is very important to determine their cytotoxic effect. Therefore, cytotoxicity of these test compounds (ETC-5, ETC-6 and ETC-7) was determined by *in vitro* haemolytic assay using horse red blood cells (RBCs). All these compounds were observed to have very low toxicity on RBCs, with only 1.98% to 15.18% cell haemolysis at varying concentrations ranging from sub-MIC to MFC values. These *in vitro* results advocate that ETC-5, ETC-6 and ETC-7 are safe to use for *in vivo* studies using animal models.

Collectively, the findings of this study indicated that newly synthesized ETCs possessed potent antifungal activity and had multi-target mechanisms of action. The antifungal nature of these compounds is related to their ability to inhibit ergosterol biosynthesis and to subsequently result in apoptosis and necrosis. At sub-inhibitory concentrations, these test entities significantly inhibit the virulence factors and drastically reduced the biofilm formation. The overall results indicated that ETC-5 was the most active compound followed by ETC-6 and ETC-7, against both fluconazole susceptible and resistant isolates of *C. albicans*. Thus, these eugenol derivatives have the ability to be developed as new antifungal agents with multi-target mechanisms of action.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

ABC	ATP-binding cassette transporters
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis Inducing Factor
ALS	Agglutinin-like sequence
AmB	Amphotericin B
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCG	Bromocresol green agar
Bp	Base pair
BSA	Bovine serum albumin
BSI	Bloodstream infection
C. albicans	*Candida albicans*
C. glabrata	*Candida glabrata*
C. krusei	*Candida krusei*
C. parapsilosis	*Candida parapsilosis*
C. tropicalis	*Candida tropicalis*
$CaCl_2$	Calcium chloride

CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CDR	Complementarity-determining regions
Cfu	Colony forming unit
CH_3COOH	Acetic acid
CLSI	Clinical and Laboratory Standards Institute
Cm	Centimetre
CMC	Chronic mucocutaneous candidiasis
CNS	Central nervous system
CPO	Ciclopirox olamine
Cq	Quantitation cycle
CSF	Cerebrospinal fluid
Cu/Zn	Copper-zinc
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPI	Dots Per Inch
EDTA	Ethylenediaminetetracetic acid
ETC	Eugenol tosylate congeners
EUG	Eugenol

FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLC	Fluconazole
g	Gram
GIT	Gastrointestinal tract
h	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDC	Hematogenously disseminated candidiasis
HIV	Human immunodeficiency virus
HOCl	Hypochlorous acid
HWP1	Hyphal wall protein 1
ICU	Intensive care unit
Kb	Kilobase
KOH	Potassium hydroxide
L	Litre
LIP	Lipase
M	Molar
MD	Molecular dynamics

MDR	Multi-Drug Resistance
MFC	Minimum fungicidal concentration
MFS	Major facilitator superfamily
mg	Milligram
mg/ml	Milligrams per milliliter
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
mM	Millimolar
Mn	Manganese
MOMP	Mitochondrial outer membrane permeabilization
Mol	Mole
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAC	Non-*albicans Candida*
NCBI	National Center for Biotechnology Information
ng	Nanogram

NHLS	National Health Laboratory Service
nm	Nanometre
NMR	Nuclear Magnetic Resonance
OC	Oral candidiasis
OD	Optical density
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PDA	Potato Dextrose Agar
PDB	Potato Dextrose broth
pH	Potential hydrogen
PI	Propidium iodide
PLB	Phospholipase B
PS	Phosphatidylserine
PTP	Permeability transition pore
RBCs	Red blood cells
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute

RPMI	Roswell Park Memorial Institute
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
RVVC	Recurrent vulvovaginal candidiasis
S. cerevisiae	*Saccharomyces cerevisiae*
SAP	Secreted aspartyl proteinase
SD	Standard deviation
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
sec	Seconds
TLC	Thin-layer chromatography
Tris-HCl	Tris hydrochloride
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
U	Units
USA	United States of America
UV	Ultraviolet
VVC	Vulvovaginal candidiasis
w/v	Weight per volume
WHO	World Health Organisation
YNB	Yeast Nitrogen Base

YPD	Yeast Potato Dextrose
5-FC	5-flucytosine
5-FU	5-fluorouracil
°C	degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
%	Percentage
<	Less than
±	Plus-minus
≤	Less than or equal
≥	Greater than or equal

CHAPTER: 1 Introduction and literature review

Introduction

With an estimated 1.5 million existing fungal species, only few are pathogenic which can cause ailments ranging from simple superficial to life-threatening invasive infections. The opportunistic *Candida* species are the second most common causative agents of fungal infections worldwide and ranked fifth among hospital-acquired pathogens (Brown *et al.*, 2012; Sievert *et al.*, 2013). Infections caused by different *Candida* species continue to be life threatening and difficult to eradicate resulting in failure of clinical treatment. Among different *Candida* species, *Candida albicans* remains the most predominant and most isolated species.

With present clinical settings, there is a limited number of antifungal drugs available to treat fungal infections. Due to the overuse and ineffectiveness of these limited antifungals, there is enough evidence of emerging multidrug resistance against these antifungals by *C. albicans*. In addition, narrow antifungal spectrum and adverse side effects associated with the existing classes of antifungals amplifies the clear need to explore and identify new antifungal molecules with alternative and multiple mechanisms of action. It is of prior importance to understand the pathogenicity mechanism of this opportunistic pathogen and thereby unravel the particular virulence factors, which can be used as emerging targets for antifungal drugs development. To further unlock the powerful therapeutic strategies needed against *C. albicans*, drugs which can target established as well as emerging drug targets need to be explored.

Natural products have long been known to possess antimicrobial activities and are a source of new drugs (Dias *et al.*, 2012; Khameneh *et al.*, 2019). However, most of these natural products exhibit adverse physicochemical characteristics and have often been overlooked. Currently, modifying natural products to synthesize derivatives/analogues are of great scientific focus for discovering novel drugs with improved potency and safety.

Eugenol (4-allyl-1-hydroxy-2-methoxybenzene), the major component of essential oil of clove (*Syzygium aromaticum*), is one of the most extensively studied natural product for its pharmacological actions. However, due to its adverse physicochemical characteristics such as low solubility, pungent odour and sublimation, eugenol is rarely used in clinical applications. Modification in its structure can resolve these issues and can even improve its biological properties. Various studies have already been reported with regard to the antifungal activity of eugenol derivatives (Ahmad *et al.,* 2015; Carrasco *et al.,* 2012; Hipólito *et al.,* 2018; da Silva *et al.,* 2018).

In this study, we synthesized seven new eugenol tosylate congeners (ETC-1, ETC-2, ETC-3, ETC-4, ETC-5, ETC-6 and ETC-7) with different substituents on the pendent sulfonyl group and studied their antifungal activity against different fluconazole susceptible and resistant *C. albicans* strains. Based on the *in silico* and *in vitro* antifungal susceptibility results, we selected the three most active eugenol tosylate congeners (ETC-5, ETC-6 and ETC-7) for in-depth studies of their antifungal action. After determining the minimum inhibitory concentrations and minimum fungicidal concentrations, effect on cell viability by these compounds was evaluated using MUSE count and viability kit. We further investigated their effect on major virulence factors of *C. albicans*, which includes adherence, dimorphic switching, hydrolytic enzymes secretion, biofilm formation and also their effect on expression of genes (*ALS1, ALS2, ALS3, ALS9, CPH1, HWP1, SAP1, SAP2, SAP3* and *PLB1*) related to these virulence factors. Adherence and biofilm formation were studied by alamar blue dye and XTT reduction assays respectively, and hydrolytic enzyme secretion was evaluated by plate assays. The morphological transition was monitored microscopically and the effect of these test compounds on the expression of pathogenicity related genes was assessed by RT-qPCR. Targeting these virulence factors can be utilized as emerging drug targets to develop novel antifungal drugs.

Most widely used drugs to treat fungal infections target ergosterol biosynthesis pathway or its end product ergosterol. As eugenol has been reported to target the ergosterol biosynthesis pathway and knowing the importance of this pathway in antifungal drug development, we hereby determined the *in silico* and *in vitro* effect of ETC-5, ETC-6 and ETC-7 on the ergosterol biosynthesis pathway by studying their mechanism of binding to the Cytochrome P450 14α-sterol demethylase (CYP51) enzyme and quantifying the total intracellular sterol content. We also tested the effect on expression of the *ERG11* gene, which is related to the ergosterol biosynthesis pathway, by RT-qPCR. Molecular docking, simulations and ergosterol biosynthesis assay results collectively support the inhibition of ergosterol biosynthesis by these compounds in *C. albicans*.

Furthermore, to understand the in-depth mechanisms behind the antifungal activity of these compounds, we evaluated physiology and mode of cell death in response to these compounds by analyzing characteristic apoptotic markers including phosphatidylserine externalization (FITC Annexin V/PI-staining), DNA fragmentation (TUNEL assays), mitochondrial depolarization (JC-10 dye) and the decrease in cytochrome c oxidase activity. Induction of apoptosis in yeast cells is considered to be a powerful model for the screening of new antifungal agents and could provide a basis for future therapies. It has been shown that eugenol can induce apoptosis after decreasing ergosterol biosynthesis in *Candida* cells (Khan *et al.*, 2013). Lastly, knowing the importance of cytotoxicity experiments in modern pharmaceutical drug development processes, we determined the cytotoxicity of test compounds by haemolytic assay using horse red blood cells. The cytotoxicity assay results confirmed the safe use of these compounds even at higher concentrations.

Literature Review

1. Candidiasis

Candidiasis, a fungal infection caused by a variety of yeast species that belong to the genus *Candida*, accounts for approximately 700,000 deaths worldwide annually and is the most identified cause of hospital acquired infections (Bongomin *et al.*, 2017; Pfaller *et al.*, 2012, Wenzel and Gennings, 2005). *Candida* normally lives inside the body at different places such as mouth, throat, gut, vagina and on skin as commensals. It can however become pathogenic if it starts to grow abnormally. The abnormal growth of *Candida* is mostly due to the disturbance in host environment and immunity. The disease spectrum of candidiasis ranges from superficial mucosal ailments to life-threatening systemic infections.

1.1 Classification of candidiasis

Based on the area affected *Candida* infections may take on different forms:

1.1.1 Cutaneous candidiasis

Cutaneous candidiasis is an infection of the skin and is one of the most common forms of candidiasis (Figure 1). The infection is generally caused by the yeast *C. albicans* but may also be caused by other *Candida* species. The nature of cutaneous infection can be either acute or chronic. The most common forms of cutaneous candidiasis include:

Candidal folliculitis: A condition in which one or more hair follicles become infected and inflamed (**Figure 1A**). The rash may appear as white-headed pimples on head, face, upper and lower limbs, chest, back or buttocks.

Candidal intertrigo: An infection between intertriginous folds of adjacent skin caused by *C. albicans* (**Figure 1B**).

Candidal paronychia: An infection of soft tissue around fingernails or toenails (**Figure 1C**). The infection can either be acute or chronic.

Perianal candidiasis: An infection of skin around the anus (**Figure 1D**).

Chronic mucocutaneous candidiasis: Chronic mucocutaneous candidiasis (CMC) is an immune disorder of T cell lymphocytes characterized by recurrent or persistent infections of skin, nails and mucous membranes caused by *Candida*, mainly *C. albicans* (**Figure 1E and F**).

Congenital cutaneous candidiasis: A rare infection of skin in neonates, either present at birth or caused within 6 days of life (**Figure 1G**). It is caused by intrauterine candidal infection, but the exact cause is unknown. The disease can be localized skin infection to systemic involvement as respiratory distress, sepsis and even death.

Diaper candidiasis: An infection in diaper area of infants (**Figure 1H**).

Erosio interdigitalis blastomycetica: An infection of the web spaces of the fingers and toes caused by *C. albicans* (**Figure 1I**). The condition is characterized by oval shaped white macerated lesions, may extend onto the sides of the digits.

Candidal onychomycosis: An infection of the nail also known as tinea unguium caused by *Candida*. The discolouration (white or yellow) and thickening of the nail are the common symptoms of this infection that affects toenails more often than fingernails (**Figure 1J**).



Figure 1: Most common types of cutaneous candidiasis: **(A)** *Candida* folliculitis, **(B)** *Candida* intertrigo, **(C)** acute and chronic paronychia, **(D)** perianal and intergluteal candidal intertrio, **(E** and **F)** chronic mucocutaneous candidiasis, **(G)** congenital cutaneous candidiasis rashes, **(H)** Diaper candidal infection, **(I)** Erosio-interdigitalis blastomycetica, **(J)** candidal onychomycosis (Mansur *et al.*, 2012; Metin *et al.*, 2018; Rigopoulos *et al.*, 2008; Vazquez and Sobel, 2011; Kaufman *et al.*, 2017; Kutlubay *et al.*, 2017).

1.1.2 Mucosal candidiasis

Mucosal candidiasis is one of the most common forms of superficial candidiasis, and it affects various mucosal surfaces mostly oral, gastrointestinal and vaginal mucosa (**Figure 2**). Infection is caused predominantly by *C. albicans* and less frequently by other *Candida* species. The most common forms of mucosa candidiasis are:

1.1.2.1 Oral candidiasis: Oral candidiasis (OC), also called thrush or oropharyngeal candidiasis is an infection on mucous membrane of mouth (**Figure 2**). OC is primarily caused by *C. albicans* and less frequently by other species such as *C. tropicalis, C. glabrata, C. parapsilosis, C. krusei, C. stellatoidea, C. pseudotropicalis, C. famata, C. rugosa, C. geotrichium, C. dubliniensis* and *C. guilliermondii.* Around 50% of OC cases are caused by *C. albicans* alone and over 80% cases together with *C. tropicalis and C. glabrata* (Williams and Lewis, 2011). The infection is common in old aged people, newborns, patients on head and neck radiotherapy, exposure to broad spectrum antibiotics, inhaled corticosteroids, poorly tended dentures and people with compromised immune system particularly in HIV/AIDS patients. Recurrent episodes of OC in around 80% of HIV patients during HIV progression has been estimated, however, the antiretroviral therapy reduced that percentage significantly (Fidel, 2011; Garcia-Cuesta *et al.,* 2014). Based on clinical presentation, OC is further classified into different categories, which include:

Pseudomembranous candidiasis: Pseudomembranous candidiasis is the most common form of oral candidiasis that accounts for about 35% of oral candidiasis cases, frequently seen in neonates and patients with HIV, cancer, patients exposed to corticosteroids or broad-spectrum antibiotics and individuals with deteriorated immune system (Rhodus, 2012). The buccal mucosa, palate, tongue and oropharynx are common sites. Thrush develops into soft white curd-like lesions that when removed by wiping, leaving a red, bleeding and painful base (**Figure 2A**).

Erythematous candidiasis: Erythematous candidiasis is characterized by red raw lesions that usually appear on the palate or dorsal surface of the tongue (**Figure 2B**). Erythematous candidiasis accounts for 60% cases of oral candidiasis (Rhodus, 2012). Patients with suppressed immunity and denture wearing are more susceptible to chronic erythematous candidiasis.

Hyperplastic candidiasis: Chronic hyperplastic candidiasis also known as candidal leukoplakia is the least common form of OC and only accounts for 5% of total cases (Rhodus, 2012). The infection may present as persistent white plaque or speckled lesions commonly appearing on the commissural region of the buccal mucosa followed by the palate and tongue (**Figure 2C**). The infection is more common in men who are smokers and patients with HIV.

Denture related stomatitis: Denture related stomatitis is one of the common form of OC, usually seen in people who wear denture and in elderly people. About 65% of denture wearers are experience in this infection and in 90% of cases *Candida* species are involved (Salerno *et al.,* 2011). The condition symptoms are inflammation and erythema of the denture-bearing tissues (**Figure 2D**). An important risk factor for denture related stomatitis is poor oral hygiene (Williams and Lewis, 2011; Jabra-Rizk *et al.,* 2016).

Angular Cheilitis: Angular cheilitis also known as angular stomatitis and perleche is an inflammatory condition of one or both corners of the mouth, characterized by red fissuring patches in the corners (**Figure 2E**). It can be caused by infection, allergies or irritation and can affect all age groups. *Candida* species alone accounts for about 20% of such cases.

Median rhomboid glossitis: Median rhomboid glossitis is also known as glossal central papillary atrophy and it affects about 1% of the population. It is characterized by symmetrical lesions in the central dorsum of the tongue with erythema and loss of papillae (**Figure 2F**). Associated risk factors of this infection include smoking, HIV infection, corticosteroid sprays or inhalers and denture wearing.

1.1.2.2 Gastrointestinal candidiasis: The common sites for gastrointestinal candidiasis are the esophagus, stomach and small intestine. Patients with neoplastic disease are most commonly susceptible to this infection.

1.1.2.3 Esophageal candidiasis: Esophageal candidiasis, also known as candidal esophagitis, is a condition in which the esophagus is infected by *Candida* species usually by *C. albicans* **(Figure 2G)**. Esophageal candidiasis usually occurs in patients with compromised immune systems such as HIV/AIDS and cancer patients. People with this condition usually experience pain and difficulty in swallowing, weight loss, dry mouth, vomiting and chest pain.

1.1.2.4 Vaginal candidiasis: Candidiasis in the vagina is the second most common cause of vaginal infection usually due to excessive growth of *Candida* (Ilkit and Guzel, 2011) (**Figure 2H)**. The other terms commonly used for this infection are vulvovaginal candidiasis, vaginal candidiasis, candida vaginitis or vaginal thrush. The common symptoms are vaginal itching, irritation, abnormal vaginal discharge, pain during sexual intercourse and burning, discomfort when urinating and inflammation. Risk factors for vaginal candidiasis include pregnancy, HIV/AIDS, diabetes, chemotherapy, oral contraceptive and broad-spectrum antibiotic use. Around 75% of women population experienced vulvovaginal candidiasis once in their lifetime and around 20% of women suffer recurrent vulvovaginal candidiasis (Kabir *et al.*, 2012; Zeng *et al.*, 2018). *C. albicans* accounts for majority of vulvovaginal candidiasis cases followed by *C. glabrata* (Bitew and Abebaw, 2018).

1.1.2.5 Candidal balanitis: Balanitis is an inflammation of the glans penis. The symptoms include erythema of glans, foreskin and penis, with severe pain in the penis and foreskin. This infection is common affecting approximately up to 3% adults globally and is more common in uncircumcised males.

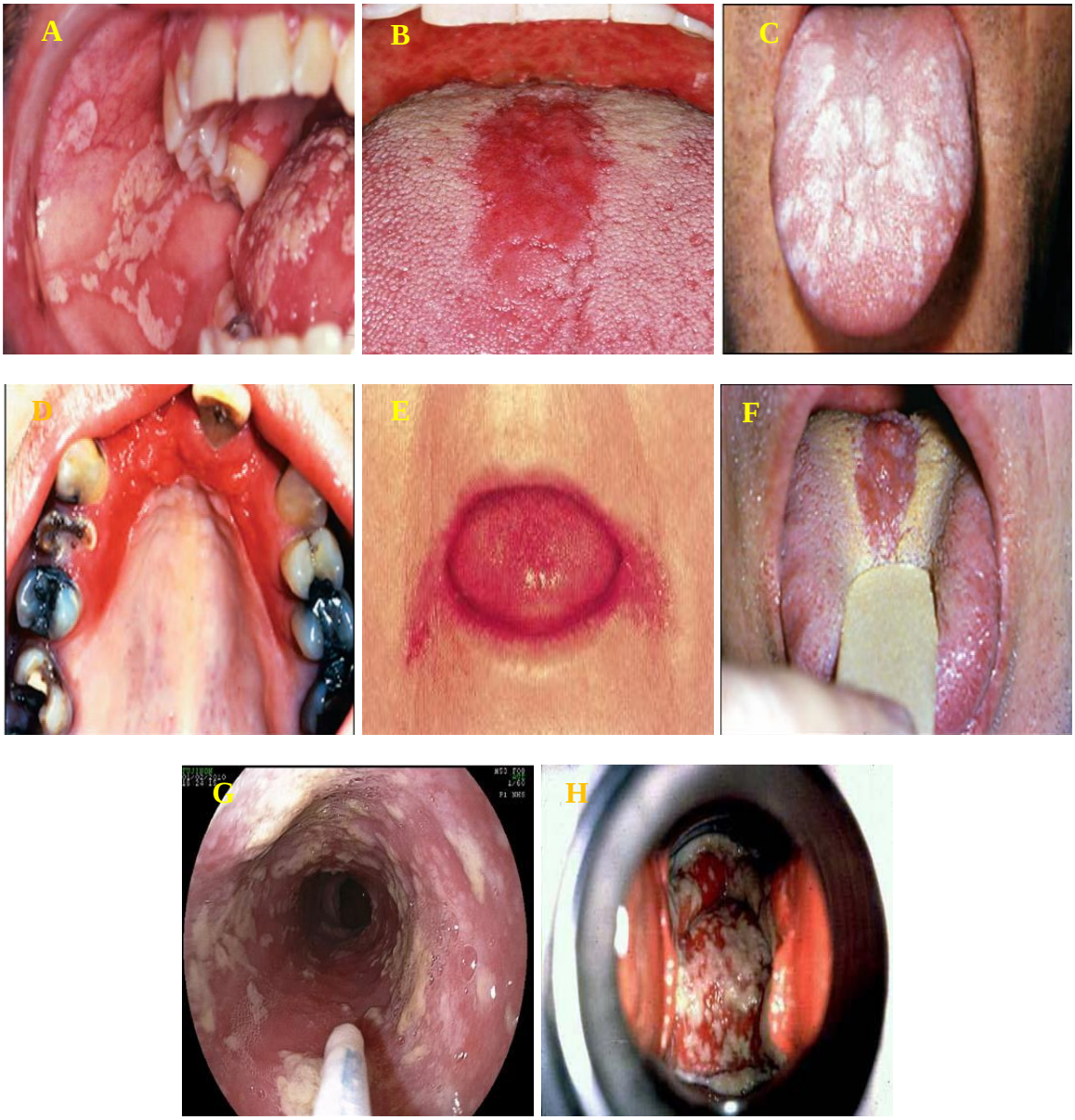


Figure 2: Most common forms of mucosal candidiasis: **(A)** Pseudomembranous candidiasis, **(B)** Erythematous candidiasis, **(C)** Hyperplastic candidiasis of the buccal mucosa and tongue, **(D)** Candida-associated denture stomatitis, **(E)** Angular cheilitis, **(F)** Median rhomboid glossitis, **(G)** Esophageal candidiasis, **(H)** Vulvovaginal candidiasis showing typical cottage cheese appearance of white clumpy vaginal discharge. (Jabra-Rizk *et al.*, 2016; Terezhalmy and Huber, 2011; Akpan and Morgan, 2010; Patricia BG *et al.*, 2016; Vazquez and Sobel, 2011).

1.1.3 Systemic or invasive candidiasis: Invasive candidiasis is an infection caused by different *Candida* species and is potentially serious and fatal infection that can affect the blood, heart, brain, eyes, bones, and other parts of the body (**Figure 3**). There are 15 *Candida* species known to cause invasive candidiasis, however more than 90% infections are caused

by five species; *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis, and C. krusei* (Turner and Butler, 2014). Among them *C. albicans* remains the most frequently isolated species. More recently *C. auris*, a newly emerged multidrug resistant *Candida* species, has also been reported to cause invasive candidiasis (Lone and Ahmad, 2019). There are two primary forms of invasive candidiasis:

1.1.3.1 Candidemia: Presence of *Candida* species in the bloodstream is called candidemia and is one of the most common form of invasive candidiasis (**Figure 3**). The symptoms may range from mild to extreme including fever and chills that do not improve even after antibiotic treatment. In severe conditions it may even cause septic shock with fast heart rate, rapid breathing and low blood pressure. The most common risk factors of candidemia are suppressed immune system, central venous catheter, use of steroids and broad-spectrum antibiotics, dialysis, diabetes, abdominal surgeries, neutropenia, ICU patients and an active candidiasis. Candidemia is emerging as one of the most common nosocomial bloodstream infections and is fourth most common bloodstream infection of ICU patients globally, with a prevalence rate of 6.9 per 1000 patients (Calandra *et al.,* 2016; Kett *et al.,* 2011). The mortality rate associated with candidemia has been reported around 30% – 60% (Kett *et al.,* 2011; Bougnoux *et al.,* 2008; Leroy *et al.,* 2009; Marriott *et al.,* 2009; Bassetti *et al.,* 2011). Mostly, *C. albicans* is responsible for the majority of infections but non-albicans species such as *C. glabrata, C. tropicalis, C. krusei, C. parapsilosis* and *C. auris* are also being frequently isolated.

1.1.3.2 Disseminated candidiasis: Disseminated candidiasis is an infection caused by *Candida* species that may involve multiple internal organs or single organ infections (**Figure 3**). This infection can affect every important organ including the liver, spleen, lungs, heart, kidneys, brain, eyes and even bones (Kullberg and Arendrup, 2015).

Hepatosplenic candidiasis: Hepatosplenic candidiasis is a frequent form of chronic disseminated candidiasis that involves the liver and spleen. *C. albicans* is the common

causative pathogen of this type of candidiasis. It usually occurs in patients with neutropenia, hematologic malignancy, patients undergoing chemotherapy, patients receiving corticosteroid therapy, chronic granulomatous diseases and diabetes. The common symptoms are persistent fever, abdominal pain, diarrhoea, nausea, vomiting and disturbed liver biochemistry.

***Candida* endophthalmitis:** Endophthalmitis is a purulent inflammation of the intraocular fluids. There are two different forms of *Candida* endophthalmitis; exogenous and endogenous form. Exogenous form is usually secondary to trauma or surgery or direct inoculation of fungi from the environment, while as endogenous form represents intraocular dissemination of systemic fungal infection. *Candida* species are the major causative agents of this infection. Compared to exogenous *Candida* endophthalmitis, the endogenous form is less common.

Renal candidiasis: Renal candidiasis is *a Candida* infection in which kidneys are involved. Risk factors associated with this type of infection include diabetes, HIV/AIDS, chemotherapy, patients with compromised or suppressed immune system, indwelling intravascular catheters and renal transplantation. The common symptoms and signs of renal candidiasis are antibiotic-resistant fever, candiduria, hematuria, hypertension, formation of fungus balls in ureters and renal pelvis followed by urinary obstruction. Severe renal candidiasis even may cause renal failure.

***Candida* endocarditis:** *Candida* endocarditis is the most common cause of fungal endocarditis that accounts for 2% of all cases of endocarditis. The risk factors are same as for invasive candidiasis, but intravenous drug use was previously reported as the most frequent risk factor.

***Candida* meningitis:** The infection of the central nervous system (CNS) due to *Candida* species is rare and can be secondary to disseminated candidiasis or direct inoculation by neurosurgical procedures. The risk factors include weakened immune system, neurosurgery, premature babies, broad spectrum antibiotics and corticosteroids. Fever, photophobia,

elevated CSF pressures, stiff neck, nausea and vomiting are common symptoms of *Candida* meningitis.

***Candida* arthritis and osteomyelitis:** These infections are rare but have increased with the increased frequency of candidemia and disseminated candidiasis. The infections occur following hematogenous dissemination or direct inoculation. The risk factors are almost same as for candidemia. Common manifestations include pain, erythema, edema, fever and difficulty in movement. The common sites involved are the spine, femora, sternum, and synovial joints.

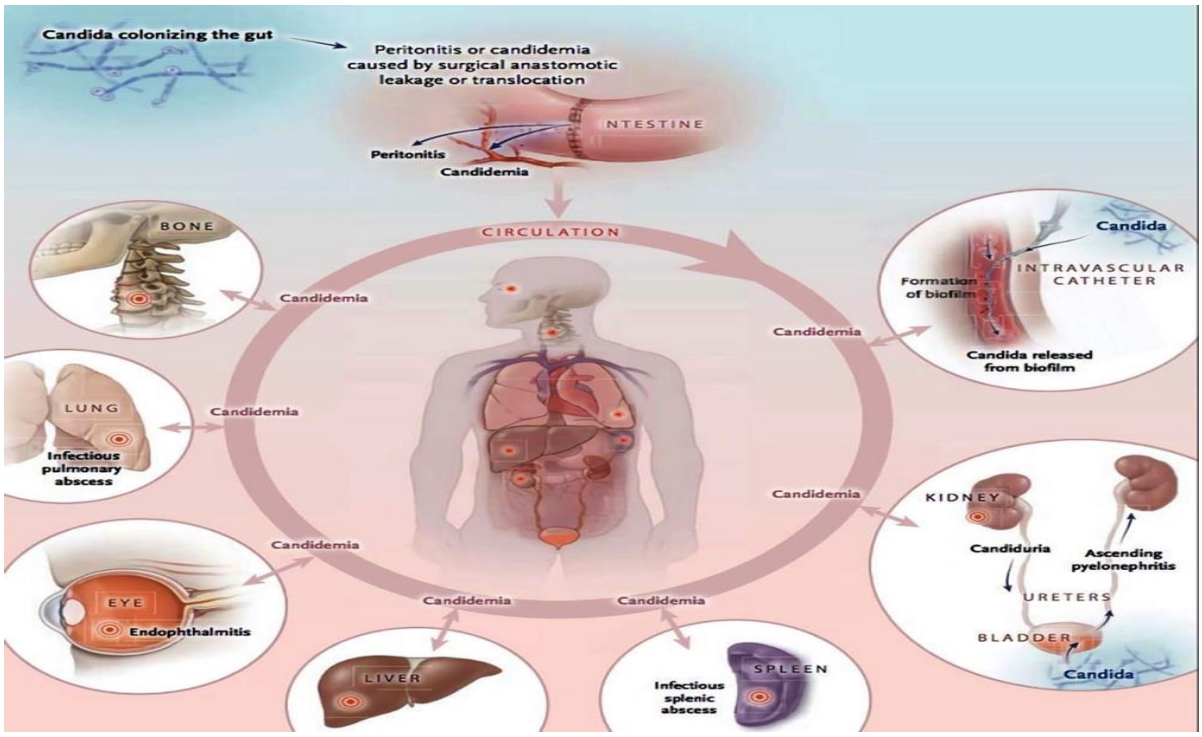


Figure 3: The figure represents colonization, dissemination and development of the two primary forms of invasive candidiasis; candidemia and disseminated candidiasis. Both these forms are serious and potentially fatal that can affect the blood, heart, brain, eyes, bones, and other parts of the body (Kullberg and Arendrup, 2015).

1.2 Candidiasis risk factors

Factors that increase the risk of developing candidiasis include:

diabetes, cancer, HIV/AIDS, smoking, the wearing of dentures, broad spectrum antibiotics, corticosteroids, burns, pregnancy, hormonal contraceptives, chemotherapy, prolonged

hospitalization, patients with implanted catheters, organ transplantation, bone marrow transplantation, leukopenia, recent surgery, total parenteral nutrition, kidney failure or haemodialysis, premature birth, nutrient deficiency, weakened or underdeveloped immune system, hematologic malignancies, severe trauma, solid neoplasms.

1.3 *Candida* species causing infections

There are approximately 1.5 million different fungal species, of which around 600 species, including over 20 *Candida* species, are known to be human pathogens (Hawksworth, 2001; Piccione *et al;* 2019). Among the *Candida* species *C. albicans, C. tropicalis, C. glabrata, C. krusei* and *C. parapsilosis* have been found to cause more than 90% of invasive infections (Pfaller and Diekema, 2007). Recently identified *C. auris* can also cause invasive infections with high mortality rate (Lone and Ahmad, 2019). Other important *Candida* species known to cause diseases in humans include *C. guilliermondii*, *C. kefyr, C. rugose*, *C. dublieniensis, C. famata, C. lusitaniae, C. norvegensis*, *C. incospicua*, *C. lipolytica*, and *C. pelliculosa*. However, *C. albicans* remains the most predominant and most isolated pathogenic species (Abegg *et al.,* 2012; Sardi *et al.*, 2013; Yapar, 2014).

2. Prevalence of *Candida* species in South Africa

South Africa is known for the huge population of immunocompromised individuals due to the rise in frequency of infectious diseases including HIV/AIDS, tuberculosis and various other diseases. Candidiasis, as described in the above section, mostly target immunocompromised individuals and therefore becomes a secondary infection in these patients. Despite much that has been studied about the prevalence of *Candida* species globally, there are only a limited number of studies on prevalence of *Candida* species and their infections in South Africa (Kreusch and Karstaedt, 2013; Makhado *et al*., 2014).

In a recent study, improper antifungal therapy, lack of documentation, limited surveillance programmes and research on fungi and fungal infections have been reported as important

reasons that make it difficult to find the actual prevalence of *Candida* species in South Africa (Africa and Abrantes, 2016). A surveillance report on invasive fungal infections from both public and private hospitals showed that *C. albicans* alone has caused 98% of infections (Naicker *et al.,* 2016). In another report from Dr. George Mukhari Academic Hospital, Pretoria, *C. albicans* and *C. krusei* were identified as the prevalent organisms in neonates (Makhado *et al.,* 2014). A public tertiary teaching hospital in Eastern Cape reported *C. albicans* (45.5%) as most frequent isolated fungal species followed by *C. glabrata* (31.1%), *C. tropicalis* (12.4%) and *C. dubliniensis* (11.0%) (Mnge *et al*., 2017). A study conducted on 128 *Candida* isolates collected from HIV-positive patients in South Africa, reported the prevalence of *Candida* species as *C. albicans* (82.2%), *C. glabrata* (9.4%), *C. dubliniensis* (7.8%) (Abrantes *et al.,* 2014). In 1990, *C. albicans* caused 62% of candidemia cases in South African hospitals both private and public. The prevalence of this *Candida* species decreased to 46% in 2005 – 2007, due to an increase in candidemia cases caused by *C. parapsilosis* (25%) and *C. glabrata* (23%) (Kreusch and Karstaedt, 2013). According to a national survey conducted during 2009 – 2010 in South Africa, *C. parapsilosis* was reported as the dominant species causing candidemia (Govender *et al.,* 2016).

The newly emerged multidrug resistant pathogen *C. auris* was also reported from South Africa, and in 2016 this pathogen caused nosocomial outbreaks in many hospitals mostly in Johannesburg and Pretoria. In the same year, a report from South African private and public sector hospitals declared *C. auris* as the 2^{nd} and 4^{th} most common cause of candidemia respectively. By now, *C. auris* accounts for ≈ 1 of every 10 cases of candidemia in South Africa and has now been isolated from ≥ 94 hospitals across the country (Govender *et al.,* 2018). All the above reports indicated a shift in prevalence of *Candida* species from *C. albicans* to non albicans *Candida* species. However, *C. albicans* persist as the most frequently isolated *Candida* species in South Africa.

3. *Candida albicans*

Among the *Candida* species, *C. albicans* is the most prevalent and predominant opportunistic fungal pathogen that can cause candidiasis in humans. Normally it is present as a commensal organism, but it becomes pathogenic under certain conditions. It is detected as a commensal organism in the gastrointestinal and genitourinary tract of around 70% of healthy population (Kabir *et al.,* 2012). Due to its disease-causing ability and various unique characteristics, it is mostly used as a model organism in research for studying fungal pathogenesis.

C. albicans is one of the first fungi in which the whole genome has been completely sequenced. The genome size is almost 14Mb (diploid stage) and consists of eight sets of chromosomes (Wang *et al.,* 2018). The whole genome contains around 6000 open reading frames (ORFs) of which more than fifty percent have not been characterized yet, and of the characterized approximately 774 ORFs that are specific to *C. albicans* are not present in any other closely related species (Jones *et al.,* 2004; Kabir *et al.,* 2012). *C. albicans* SC5314 is the most commonly used strain to study *C. albicans*.

3.1 Discovery of *Candida albicans*

It was Hippocrates around 400 B.C. who first mentioned oral candidiasis (Calderone, 2002). However, Pepys in 1665 termed this infection as thrush, and suggested that oral thrush arises in the host (Winner and Hurley, 1954; Wilson *et al.,* 2002). Later in 1839, Langenbeck identified the cause of thrush as a fungus (Langenbeck, 1839). In different observations, this fungus was reported to have various growth forms which can cause different types of infections (mucocutaneous, cutaneous and systemic infections) (Wilkinson, 1849; Mayer, 1862). Mycologist Charles Philippe Robin in 1847, named this fungus as *Oidium albicans*, using *albicans* from a Latin word "albus" meaning "to whiten" (Robin, 1853). Berkhout in 1923 reclassified it and gave the generic name *Candida*, derived from the Latin "toga *Candida*" that was a white robe worn by Roman Senators (Calderone, 2002).

Finally in 1954, the 8th Botanical Congress officially approved *Candida albicans* as the taxonomic name (Calderone, 2002). Currently, more than 160 species have been adapted within the genus of *Candida* (Turner and Butler, 2014).

3.2 Growth and morphology

C. albicans can grow on various culture media with different culture characteristics. The choice of the media depends on purpose of the growth. The basic media commonly used are Sabouraud Dextrose Agar (SDA) or broth (SDB), Yeast Nitrogen Base (YNB), blood agar, Sabouraud brain heart infusion agar, Yeast Potato Dextrose (YPD) agar or broth, Potato Dextrose Agar (PDA) or broth (PDB), Lee's synthetic medium and other selective or differential media such as CHROMagar *Candida*, *Candida* Bromocresol Green Agar (BCG Agar), and Bird-seed agar. On SDA the colonies appear smooth, white to cream colour with a sour beer aroma when grown at 25 – 37 °C for 24 – 48 h. The colonies on blood agar and PDA appear as white to creamy colonies with foot-like extensions arising from the margin and soft creamy colonies respectively. *C. albicans* on CHROMagar (differential media) develops green colour colonies.

C. albicans is a polymorphic fungus that can grow in different morphological forms (Sudbery *et al.,* 2004). The three main fungal cellular morphologies are yeast, pseudohyphae and hyphae (Noble *et al;* 2017), and the switching between the morphologies is considered to be essential for pathogenicity of *C. albicans* (Ahmad *et al.*, 2016) (**Figure 4)**. The unicellular ovoid-shaped budding yeast form is believed to be an important form involved in dissemination in the bloodstream (Saville *et al.,* 2003). The cell size in the yeast form ranges from 10 to 12 microns. Pseudohyphal cells are elongated ellipsoidal with constrictions at septal junctions. However, the role of pseudohyphae is unclear. In contrast, hyphal cells are parallel walled with uniform width and have true septa without constrictions. The cells in hyphal form are invasive than the yeast form and is essential for tissue invasion and

colonization of organs (Berman and Sudbery, 2002). Both pseudohyphae and hyphae morphologies are collectively called filamentous.

Other cellular morphologies that occur during phenotypic switching include white and opaque cells (Sudbery *et al.*, 2004). *C. albicans* can also form chlamydospores which survive under unfavourable conditions, but chlamydospores have not been detected or reported in patient samples (Staib and Morschhauser, 2007). There are various environmental cues that can affect morphology of *C. albicans*, such as temperature, pH, CO_2, nutrients, and quorum sensing (Mayer *et al.*, 2013).

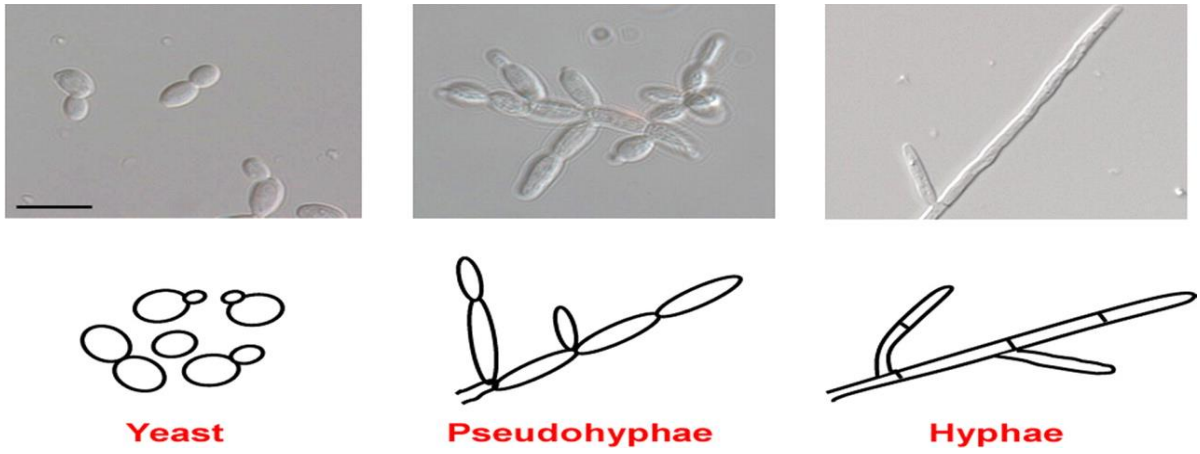


Figure 4: The three main cellular morphologies of *Candida albicans* are yeast, pseudohyphae and hyphae. (Thompson *et al.*, 2011).

The cell wall of *C. albicans* is a well-established dynamic structure that plays an essential role in morphogenesis, pathogenesis and cell viability (Hernandez-Chavez *et al.*, 2017). The structure, composition and biological properties of the cell wall are affected during the process of morphogenesis. The cell wall is composed of carbohydrates (90%) and proteins (10%) (Gow and Hube, 2012) (**Figure 5**). The polysaccharides are mainly present in three forms: (i) β-glucans (β-1,3 and β-1,6); (ii) chitin (linear polymer of *N*-acetyl-D-glucosamine); (iii) mannans (polymers of mannose) covalently associated with glycol (manno) proteins. The β-glucans and chitin are structural components in the inner cell wall layer and are responsible for shape and strength of the cell wall. The β-glucans represent 47 to 60% of dry weight of the

cell wall and chitin only accounts for 0.6 to 9% (Chaffin *et al.,* 1998). In contrast, mannose polymers are localized in the outer cell wall and represent about 40% of the cell wall content (Chaffin *et al.,* 1998). However, due to low permeability and porosity of the mannan layer it affects the permeability of cell wall to antifungals and also provides resistance of the cell wall against host response but does not influence shape of the cell wall (Gow and Hube, 2012).

The carbohydrates and proteins in the cell wall have various functions such as immune recognition by carbohydrates and adhesion with host cell surface mostly by proteins. Comparing human and fungal cells, the cell wall is the main difference and can be used as a target for antifungal drugs.

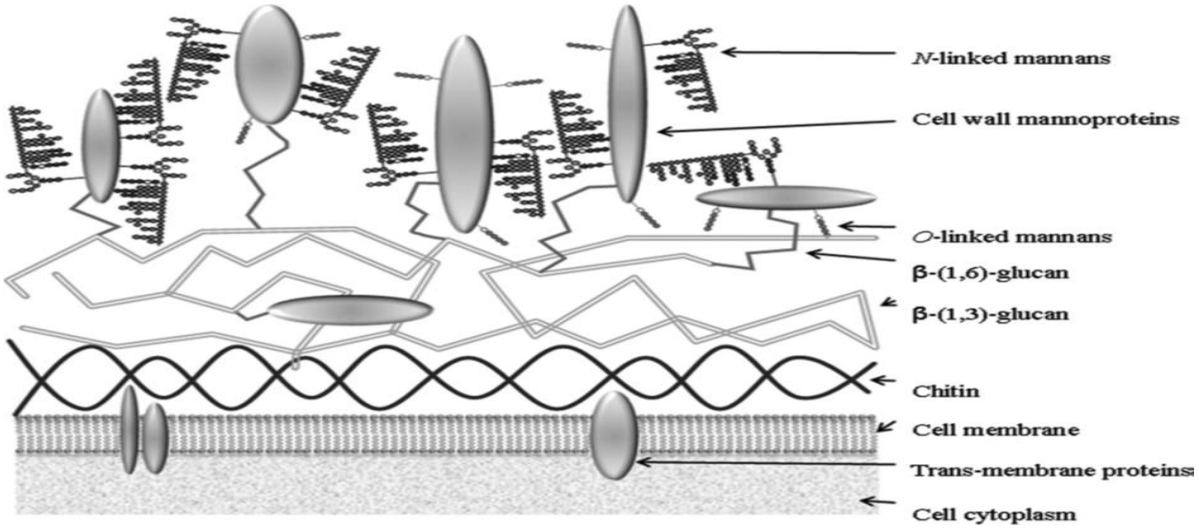


Figure 5: Structural composition of *Candida albicans* cell wall. Displaying arrangement of main components that are involved in immune activation and suppression thus making it an ideal drug target (Grubb *et al.,* 2008*).*

4. Antifungal drugs: Mode of action and resistance

In the field of drug development, novel discoveries have been achieved to control and treat the various life threating infectious diseases, however little progress has been attained in the development of effective antifungal agents. Due to the rise in fungal infections, drug resistance and the emergence of new multidrug resistant fungal species such as *Candida auris*, there is an urgent requirement for the development of new effective antifungal agents

with multiple or new mode of actions. The current major antifungal drug classes either target the cell envelop or nucleic acids as their drug targets (**Figure 6).**

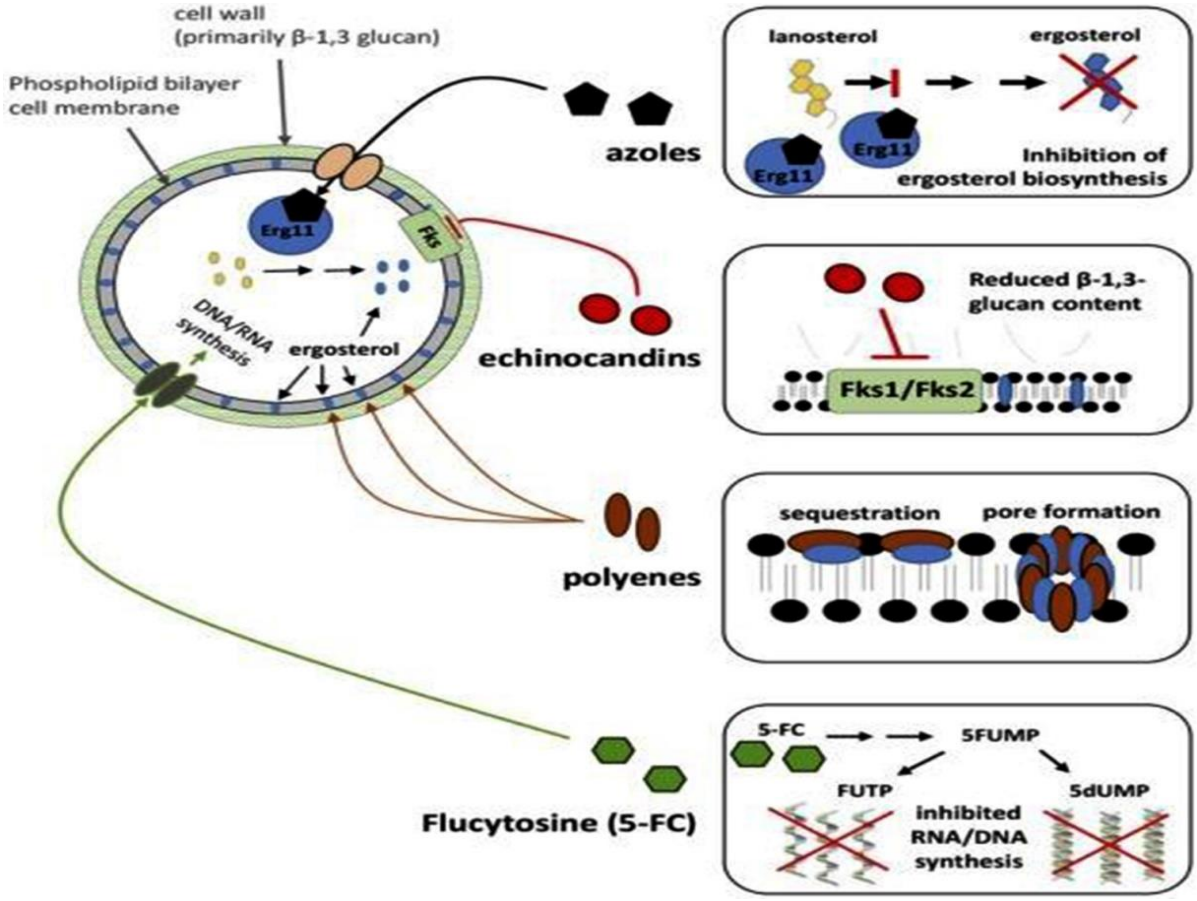


Figure 6: Mode of action of major antifungal drug classes (Morio *et al.*, 2017).

Lack of development of antifungal drugs is mainly due to the fact that fungi are eukaryotic and share a phylogenetic origin with human hosts (Roemer and Krysan, 2014). This in turn complicates the therapeutic challenge by limiting the number of drug targets that can be selectively exploited to target the pathogen. The current antifungal therapy is not adequate because of the availability of the limited number of antifungal drugs, and their overuse leads to the development of resistance. These existing antifungals also show host toxicity and other adverse side effects that limit their use in clinical practice (Chang *et al.*, 2017). The current antifungal drugs based on their targets in pathogenic fungi are classified in the following major groups:

4.1 Azoles

The azole class of antifungals are the most commonly used antifungal drugs to treat both systemic and mucosal fungal infections. They are less toxic and available in both oral and intravenous formulations. Azoles have been classified into two groups based on the nitrogen atoms in an azole ring. The first group imidazoles contain two nitrogen atoms in a ring and consist of miconazole, clotrimazole, ketoconazole, oxiconazole, econazole and tioconazole, whereas, second group triazoles such as fluconazole, itraconazole, voriconazole, posaconazole and terconazole have three nitrogen atoms in a ring (Maertens, 2004; Vandeputte *et al.,* 2012). Triazoles can be used for both mucosal and systemic infections and are safer with broad spectrum antifungal activity when compared to imidazoles which are mainly used for mucosal infections and have high toxicity and severe side effects. Though, due to the fungistatic nature of azoles treatment is prolonged, contributing in the development of drug resistance. Several studies have reported patterns of resistance to azoles in HIV and cancer patients with candidiasis (Marr *et al.,* 1997).

Azoles target the ergosterol biosynthesis pathway by inhibiting lanosterol 14α-demethylase, a cytochrome P450 (CYP)-dependent enzyme encoded by the *ERG11* gene, which converts lanosterol to ergosterol (Figure 6). Ergosterol is an important sterol of the fungal cell membrane that functions as a bioregulator of membrane permeability, fluidity and integrity. Azoles bind directly to an iron atom in the active site of the target lanosterol 14α-demethylase. The inhibition of 14α-demethylase resulting in depletion of ergosterol and accumulation of 14α-methylated sterol precursors (lanosterol, 4, 14-dimethylzymosterol, and 24-methylenedihydrolano sterol), which consequently affects the permeability of the cell membrane, inhibits growth and replication of fungal cell (Nigam, 2015).

A triazole, fluconazole is one of the most extensively used azole for the treatment of superficial and invasive candidiasis. Due to its lesser toxicity, low affinity for plasma proteins

and metabolic stability, it is suitable for use in both normal and immune-suppressed patients. However, increasing resistance of pathogenic yeasts against azole class of antifungals is becoming a challenge for clinicians to treat and provide an appropriate antifungal therapy in fungal infections. There are several known mechanisms involved in azole resistance, including efflux pump activation, modification of drug target and overexpression of the *ERG11* gene (Figure 7).

The overexpression of efflux pumps, which results in decreased accumulation of incoming drugs inside the cell, is the most common mechanism of resistance to azole antifungals (Prasad *et al.,* 1995; Prasad and Kapoor, 2005). There are two main classes of efflux proteins known to be involved in this resistance mechanism; the ATP-binding cassette (ABC) superfamily and major facilitator superfamily (MFS) which are encoded by *CDR1/CDR2* and *MDR1* genes respectively (**Figure 7)**. Overexpression of these genes has been observed in isolates of *C. albicans* resistant to azole antifungals. It has been assumed that overexpression of *CDR* genes in *C. albicans* can expel different azoles, whereas *MDR1* genes showed specificity to fluconazole (Kontoyiannis and Lewis, 2002).

The other mechanisms of resistance to azoles are modification of target protein (*Erg11p*) through chromosomal mutations that results in substitution of intrinsic amino acids. This is supported by the fact that some point mutations in the *ERG11* gene encodes for *Erg11p* that has been detected in drug resistant isolates of *Candida*. These point mutations affect the normal binding of drug to target protein via decreasing the affinity of the drug towards *Erg11p* (Wang *et al.,* 2009; Morio *et al.,* 2010). An over-expression of *ERG11* gene results in increased concentrations of the enzyme 14α-demethylase, therefore high concentrations of antifungals are required to inhibit the enzyme, which eventually leads to the development of resistance towards antifungals (Morschhäuser, 2002). Azole resistant isolates showed

overexpression of *ERG11* gene because of mutations in the transcription factor Upc2 and also by the formation of isochromosome (Dunkel *et al.*, 2008; Selmecki, 2006).

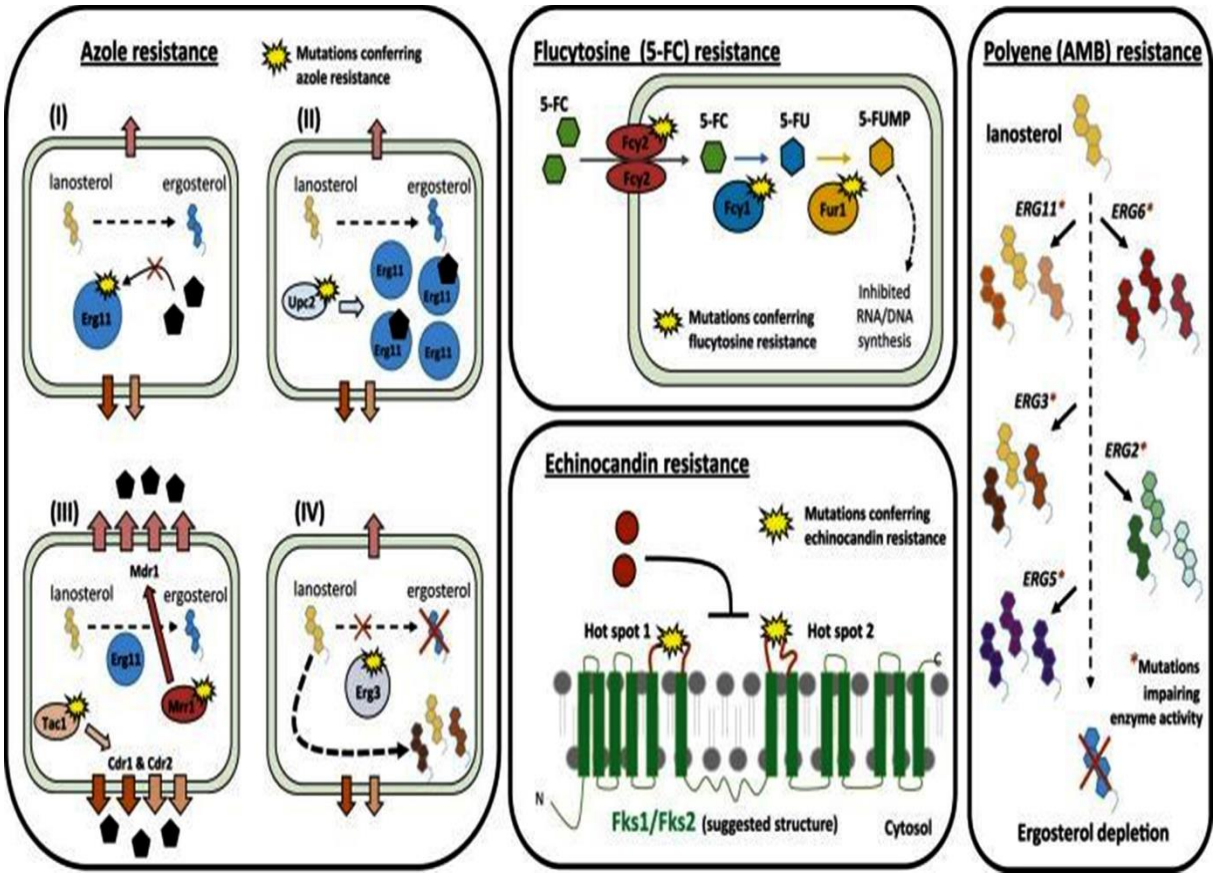


Figure 7: Mechanisms of resistance to major antifungal drug classes (Morio *et al.*, 2017).

4.2 Polyenes

Polyenes are more than five decade old antifungal drug class used for the treatment of life threatening invasive fungal infections. These are amphipathic organic molecules called macrolides synthesized from *Streptomyces* bacteria and containing hydrophobic polyene hydrocarbon and hydrophilic polyhydroxyl chains (Vandeputte *et al.*, 2012). Polyenes are fungicidal in action with broad spectrum of antifungal activity, though only amphotericin B, natamycin and nystatin are used in antifungal therapy.

Amphotericin B is administered intravenously and shows a broad spectrum of activity against various invasive fungal infections such as candidaemia, aspergillosis, blastomycosis, cryptococcosis, mucormycosis, coccidioidomycosis and histoplasmosis. Nevertheless, it is

associated with several adverse side effects including renal toxicity, hepatoxicity, neurotoxicity, infusion related toxicity and also affects blood potassium levels. However, currently lipid formulations of amphotericin B are available which exhibit low toxicity and are relatively better against a wide range of fungal infections but their high cost hampers their use and availability (Hamill, 2013). Nystatin and natamycin are effective against numerous fungal infections such as vaginal candidiasis, oropharyngeal candidiasis, esophageal candidiasis, fungal keratosis and corneal infections (Zotchev, 2003). However, due to high toxicity and low absorption in the gut these polyenes are generally used as topical agents.

For decades, it was believed that polyene drugs act on fungi by binding directly to ergosterol in the plasma membrane leading to the formation of pores in the membrane. Formation of pores results in leakage of ions and other cellular components, which ultimately leads to cell death (Ostrosky-Zeichner *et al.*, 2010). However, recently it has been revealed that polyenes act more like an "ergosterol-sponge" forming large extra-membranous aggregates that extract ergosterol from phospholipid in the membrane, reducing ergosterol in the cell **Figure 6** (Anderson *et al.*, 2014).

The development of resistance to polyene antifungals is rare, although cases of acquired resistance to amphotericin B have been reported for *C. lusitaniae, C. glabrata, C. albicans, C. tropicalis, C. rugosa* and *C. guilliermondii* (Perlin *et al.*, 2017). In addition, cases of treatment failure associated with amphotericin B have been reported in *C. lusitaniae, C. neoformans* and in multidrug resistant *C. auris* (Perlin *et al.*, 2017). The mechanisms involved in resistance to amphotericin B mainly lead to reduced levels of ergosterol in the membrane that can occur either by targeting the enzyme contributing to ergosterol biosynthesis or by mutations in the genes affecting sterol biosynthesis such *ERG1, ERG2, ERG3, ERG4, ERG6* and *ERG11* **(Figure 7)** (Perlin *et al.*, 2017). It has also been reported that increased activity of catalases leads to reduction in oxidative damage; and probably may be another mechanism of resistance

to amphotericin B (Sokol-Anderson *et al.,* 1986; Kanafani and Perfect, 2008). Nonetheless, in current antifungal therapy polyenes, particularly amphotericin B is still a drug of choice for clinicians.

4.3 Echinocandins

Echinocandins are the most recent class of antifungal drugs, approved for the treatment of fungal infections. These are amphiphilic lipopeptide compounds which have been produced during the fermentation of several fungi such as *Zalerion arboricola*, *Aspergillus nidulans var. echinulatus* and *Papularia sphaerosperma* (Vandeputte *et al.,* 2012; Bondaryk *et al.,* 2013). Currently, there are three echinocandins available for clinical use which includes caspofungin, micafungin and anidulafungin.

The echinocandins exhibit potent activity against numerous *Candida* species but are less active or completely inactive against *C. parapsilosis* (*C. parapsilosis sensu stricto, C. metapsilosis*, and *C. orthopsilosis*), *C. guilliermondii* and *Cryptococcus, Trichosporon, Rhodotorula* species (Pfaller *et al.,* 2013; Denning, 2003). Echinocandins are generally safe, effective and non-toxic to humans. It has recently been shown that around 60% of patients having candidaemia receive echinocandins as antifungal therapy (Cleveland *et al.,* 2012). However, the echinocandins are poorly absorbed in the GIT and have a short half-life, and therefore are only administered once daily intravenously.

The echinocandin drugs target the 1,3-β-glucan synthase enzyme, which is essential in the biosynthesis of β-1,3-glucan, the crucial component of the fungal cell wall, which provides structural integrity to fungal cells (**Figure 6**) (Bondaryk *et al.,* 2013). Inhibition of β-1,3-glucan synthesis results in osmotic instability, pseudohyphae formation, thickened cell walls, cell separation defects and even cell death (Spampinato and Leonardi, 2013; Ghannoum and Rice, 1999). The changes of other cellular contents have also been reported due to β-1,3-

glucan inhibition such as decrease in lanosterol and ergosterol content and an increase of chitin content in the cell wall (Ghannoum and Rice, 1999).

The mechanism of resistance in *Candida* species against echinocandin antifungal drugs involves point mutations in the *FKS* genes which encode 1,3-β-glucan synthase (**Figure 7**) (Perlin, 2007). These mutations in *FKS* genes generally occur in two hot spot regions of *FKS1* for all *Candida* species and *FKS2* in *C. glabrata* (Perlin, 2011). Mutations in *FKS1* and *FKS2* regions are associated with high minimum inhibitory concentrations (MICs), decrease in glucan synthase activity and chances of treatment failure. Echinocandins exhibited minimal drug interactions and are broadly used in current antifungal therapy.

4.4 Pyrimidine analogues

A pyrimidine analogue, flucytosine, was first approved in 1960s for using in antifungal therapy, however its use as monotherapy is rare due to the development of drug resistance. Therefore this drug is generally used in combination with other antifungal drugs such as amphotericin B or fluconazole to treat various invasive fungal infections (Perfect, 2017; Morace *et al.,* 2014). Flucytosine is available in both oral and intravenous formulations.

5-flucytosine (5-FC) showed antifungal activity only after being converted into 5-fluorouracil (5-FU) by an enzyme cytosine deaminase that is not present in humans. After the conversion of 5-FC into 5-FU, it is then converted into 5-fluorouridylic acid (FUMP) by UMP pyrophosphorylase. Further phosphorylation of FUMP inhibits protein synthesis by incorporating into RNA (Polak and Scholer, 1975). 5-FU is also converted to 5-fluorodeoxyuridine monophosphate that inhibits thymidylate synthase, an enzyme required for DNA synthesis in fungal cells (**Figure 6**). However thymidylate synthase is absent in most of the filamentous fungi and therefore use of flucytosine is restricted to pathogenic fungi only (Diasio *et al.,* 1978).

Resistance to 5-FC is associated with decrease in activity of enzymes of the pyrimidine pathway mainly due to mutations of genes encoding these enzymes (**Figure 7**) (Spampinato and Leonardi, 2013). In general, resistance to 5-FC may occur by loss of *FCY2* gene activity which encodes cytosine permease enzyme, responsible for conversion to FUMP. Furthermore, impaired activity of this enzyme results in decreased 5-FC uptake (Vermes, 2000; Morace *et al.,* 2014). The other mechanism of resistance is related to the loss of activity of the cytosine deaminase and uracil phosphoribosyl transferase encoded by *FCY1* and *FUR1* genes respectively. Both these genes (*FCY1* and *FUR1*) affect the conversion of 5-FC to 5-FU, while point mutation in *FUR1* gene is the most frequent type of acquired 5-FC resistance of fungal isolates (Edlind and Katiyar, 2010; Spampinato and Leonardi, 2013). It has been reported that around 30% of clinical isolates develop resistance against flucytosine during treatment (Espinel-Ingroff, 2008; Bondaryk *et al.,* 2013). Studies also revealed that approximately 10% of *C. albicans* isolates are intrinsically resistant to 5-FC.

4.5 Allylamines

Allylamines are a new class of antifungals with a broad spectrum of antifungal activity against fungal pathogens. This class includes terbinafine and naftifine which are effective against dermatophyte infections and are commonly used for the management of these infections. The underlying reason for this is due to the higher accumulation of these antifungal agents in skin and nail beds than in blood (Ngo *et al.,* 2016). These agents are active against various fungi such as *Aspergillus, Trichophyton, Fusarium, Microsporum, Epidermophyton, C. albicans, C. parapsilosis, Cryptococcus neoformans* and other *Candida* species (Campoy and Adrio, 2017). Allylamines exhibit both fungistatic and fungicidal effects depending on target species.

Allylamines target the ergosterol biosynthesis pathway by inhibiting squalene epoxidase enzyme. This is a key enzyme of the ergosterol biosynthetic pathway in fungi which is

involved in the conversion of squalene into 2,3-squalene epoxide. The inhibition of squalene epoxidase activity leads to intracellular accumulation of squalene that may interfere with the membrane permeability which subsequently results in disruption of cellular organization (Ghannoum and Rice, 1999). Reports also suggested that fungal cell death is mainly due to squalene accumulation rather than ergosterol deficiency (Ghannoum and Rice, 1999).

It has been reported that the mechanism of resistance to terbinafine is due to a single amino acid substitution in *Erg1p* (Osborne *et al.,* 2006; Cannon *et al.,* 2009). In *C. albicans,* terbinafine resistance might be also due to the upregulation of genes which encode for membrane transport proteins (*CDR1, AGP2* and *HOL3*). Upregulation of these genes leads to the expulsion of accumulated intracellular antifungal drugs (Zeng *et al.,* 2007). In another study, it has been suggested that resistance to allylamines is mediated by a mechanism which is similar to efflux pump mediating azole resistance (Odds, 2009). The other mechanisms such as induction of detoxification and stress tolerance may also contribute to resistance to allylamines (Cannon *et al.,* 2009).

5. Virulence factors

Pathogenicity is defined as the ability of an organism/pathogen to cause disease/damage in a host. Commensal microorganisms and even opportunistic pathogens lack this intrinsic ability to cause disease, however, they become pathogenic under certain predisposing conditions. *C. albicans*, typically present in humans as a commensal organism, becomes pathogenic in various immunocompromised conditions and can cause diseases ranging from localized superficial to life threating infections. This transition from the commensal form to the pathogenic form involves several biochemical and morphological changes, which are known as virulence factors.

Some of the major virulence factors of *C. albicans* include host recognition, adherence, morphological switching, secretion of tissue damaging hydrolytic enzymes and biofilm

formation (Mayer *et al.*, 2013). These virulence factors in *C. albicans* are vital for colonization, evade host defence, and invasion and damage to host tissue (Gow and Hube, 2012). The virulence factors can be utilized as emerging drug targets to develop new and effective antifungal drugs (Ahmad *et al.*, 2016). This approach of targeting virulence traits in *C. albicans* have many advantages which include; reduced survival pressure on the pathogen resulting in decrease in antifungal drug resistance, development of novel antifungal drugs with multiple drug targets, minimizing effect on natural microbiota of the host (Cegelski *et al.*, 2008; Karkowska-Kuleta *et al.*, 2009; Pierce and Lopez-Ribot, 2013). The major virulence factors of *C. albicans* are represented in **Figure 8** and detailed below:

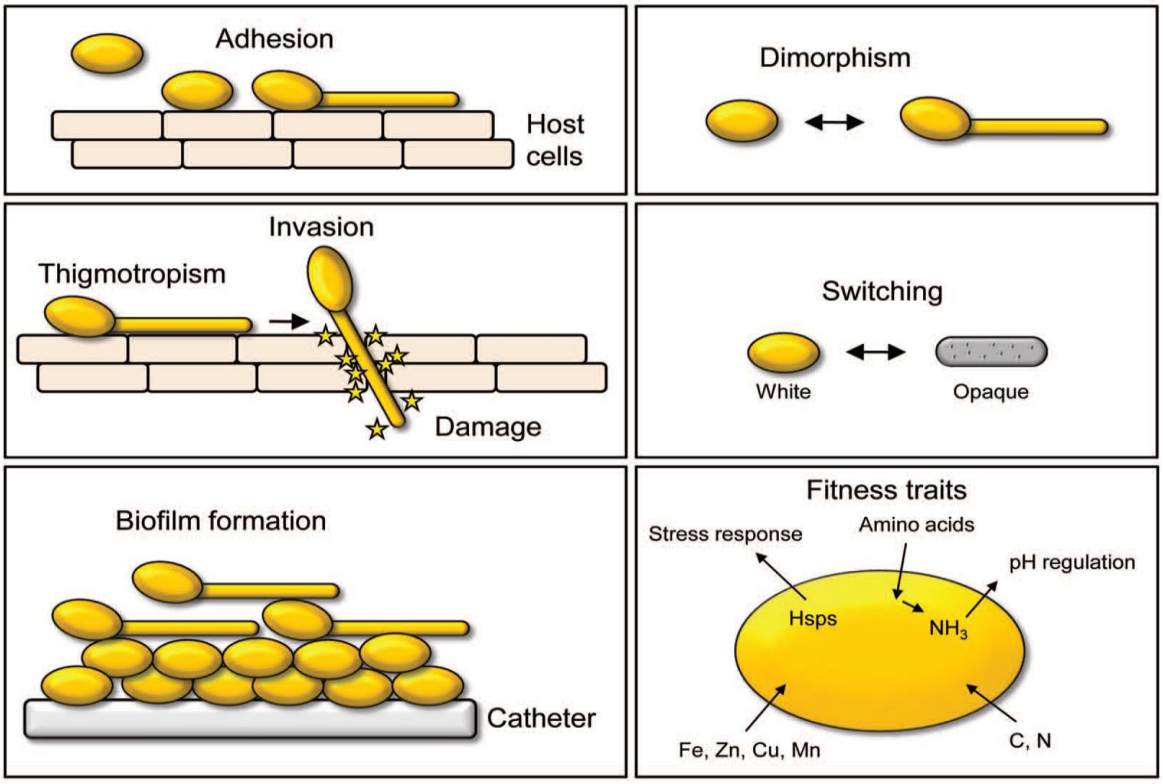


Figure 8: A brief representation of the mechanisms of some major virulence factors in the pathogenicity of *C. albicans*, including several fitness traits which also influence pathogenicity (Mayer *et al.*, 2013).

5.1 Adhesion

Adhesion of *C. albicans* to host cells involves a complex mechanism and is one of the crucial steps in establishment of *C. albicans* colonization and infection. In *C. albicans*, specialized

proteins called adhesins are responsible for adherence to host cells, abiotic surfaces, other microorganism cells and extracellular matrix proteins (Kabir *et al.,* 2012; Lim *et al.,* 2012). The agglutinin-like sequence (ALS) protein family consisting of eight members (Als1–7 and Als9) is considered the best studied adhesins in *C. albicans* (Tronchin *et al.,* 2008; Mayer *et al.,* 2013). The ALS genes encode glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins. Despite the fact that all eight ALS proteins are involved in the adherence of *C. albicans*, ALS3 (hypha-associated adhesin) is particularly essential for adhesion (Zordan and Cormack, 2012; Phan *et al.,* 2007; Murciano *et al.,* 2012). During infections of oral epithelial cells and the vagina, the upregulation in expression of the *ALS3* gene has been reported (Liu and Filler, 2011; Naglik *et al.,* 2008; Mayer *et al.,* 2013).

The other important adhesin of *C. albicans* is the GPI-linked hyphal wall protein (Hwp1) (Zordan and Cormack, 2012; Sundstrom, 2002). This adhesin functions as a substrate for mammalian transglutaminases and may create a covalent link between *C. albicans* and host cells. Both Als3 and Hwp1 were also shown to support biofilm formation by acting as complementary adhesins (Nobile *et al.,* 2008). Morphology independent proteins, which include GPI-associated proteins (Eap1, Iff4 and Ecm33), cell surface related proteases (Sap 9 and 10), non-covalent wall linked proteins (Mp65, a putative β-glucanase, and Phr1, a β-1,3 glucanosyl transferase) and integrin-like surface protein Int1 can also contribute to adhesion of *C. albicans* (Naglik *et al.,* 2011; Zhu and Filler, 2010).

5.2 Morphogenesis: transition from yeast to hyphae

The yeast to hyphae transition, termed commonly as dimorphism or dimorphic switch, is a highly regulated process which is controlled by several transcriptional factors and involves many biochemical pathways. In *C. albicans* this transition is considered as one of the most important virulence factor that has been studied significantly and is a focus in research for the identification and development of novel antifungal drug targets (Khan *et al.,* 2010; Lim *et al.,*

2012; Tyc *et al.,* 2014). It has been reported that both these growth forms (yeast and hyphae) are essential for pathogenicity and play different roles in the establishment of an infection (Gow *et al.,* 2002; Vila *et al.,* 2017).

The studies showed, that the development of infection hyphal growth form is necessary for the penetration of host barriers, invasion and tissue damage, whereas the yeast form is mainly involved in dissemination (Saville *et al.,* 2003; Shapiro *et al.,* 2011). A study by Jacobsen and colleagues showed that adhesion of the yeast cells is vital for the hyphal formation (Jacobsen *et al.,* 2012). The hyphae formation is also linked to the expression of genes encoding virulence factors that are not involved intrinsically in hyphal formation. These genes include agglutinin-like sequence protein (Als3), hyphal wall protein (Hwp1), secreted aspartic proteases (Sap4, Sap5 and Sap6) and hypha related proteins (Ece1 and Hyr1). There are numerous studies that reported semi-synthetic, synthetic or natural compounds targeting yeast to hyphae transition in *C. albicans* (Watanabe *et al.,* 2012; Vediyappan *et al.,* 2013; Shareck and Belhumeur, 2011). However, most of these compounds have cytotoxic effects; therefore further research is required in the development of safer molecules, which can target yeast to hyphae transition in *C. albicans* at lower concentrations with minimum or no side effects.

5.3 Hydrolytic enzymes

Candida albicans also secretes different types of extracellular hydrolytic enzymes which contribute to pathogenicity. These hydrolytic enzymes play an important role in adherence and destruction of host cells, which help *Candida* cells to invade and penetrate into the host cells (Silva *et al.,* 2011). In addition, these enzymes are also believed to increase the efficiency of extracellular nutrient acquisition. They are also thought to help the *Candida* cells to overwhelm the host immune system and make it ineffective (Karkowska-Kuleta *et al.,* 2009; Staniszewska *et al.,* 2012; Tsai *et al.,* 2013). It has also been reported that hydrolytic enzymes are secreted from the hyphal tip of the fungal cell (Pawar *et al.,* 2014). The most

common classes of hydrolytic enzymes that have been extensively studied include secreted aspartyl proteinases (SAPs), phospholipases and lipases (Sachin *et al.,* 2012; Mayer *et al.,* 2013; Pawar *et al.,* 2014).

5.3.1 Secreted aspartyl proteinases

Secreted aspartyl proteinases *(SAPs)* are extracellular hydrolytic enzymes secreted by *C. albicans* and other *Candida* species. They are important because of their contribution to pathogenesis of *C. albicans* during adhesion, tissue damage and evasion of antimicrobial attack (Deepa *et al.,* 2015). *SAPs* damage many human proteins by hydrolysis such as immunoglobin A, mucin, albumin, haemoglobin, complement component 3 and keratin that results in defects in the innate immune system of the host (Silva *et al.,* 2011; Aoki *et al.,* 2011; Pawar *et al.,* 2014; Hube *et al.,* 1998). *SAPs* have also been reported to contribute to phenotypic switching and hyphae formation of *C. albicans.* Additionally, due to their attribute of resisting phagocytosis and intracellular killing, they are involved in countering the host immune response (Naglik *et al.,* 2003; Khan *et al.,* 2010; Mayer *et al.,* 2013; Pawar *et al.,* 2014).

The *SAP* gene family comprises ten different members, *SAP1* to *SAP10*, encoding for enzymes with similar functions and characteristics. However, these enzymes have distinct molecular properties such as pH, molecular weight and isoelectric point, and also play many roles during the different stages of infection (Karkowska-Kuleta *et al.,* 2009; Pawar *et al.,* 2014). *SAP1*–8 are secreted and released into the surrounding medium, but *SAP9* and *SAP10* remain bound to the cell surface (Taylor *et al.,* 2005; Albrecht *et al.,* 2006). *In vitro* studies have shown that *SAP1–SAP3* is highly expressed in the yeast form and *SAP4–SAP6* are expressed in hyphal form, whereas *SAP9* and *SAP10* are expressed in both yeast and hyphal forms of growth. The roles of *SAP7* and *SAP8* are not yet completely understood. The exact role of these *SAPs* at individual level in the pathogenesis of *C. albicans* is not decisive

because of varying levels of expression during the course of infection (Naglik *et al.,* 2008; Mayer *et al.,* 2013; Kumar *et al.,* 2015). Numerous studies have reported expression of *SAP1–SAP3* in cutaneous candidiasis based on reconstituted human epidermis. However, several studies have shown that expression of *SAP1–SAP3* is essential for early stages of mucosal infections and for virulence in hematogenously disseminated candidiasis (HDC) (Correia *et al.,* 2010; Staniszewska *et al.,* 2012; Pawar *et al.,* 2014). The expressions of SAP4–SAP6 have been confirmed in both mucosal and systemic infections and contribute to organ invasion.

5.3.2 Phospholipases

Phospholipases are a group of enzymes secreted by *C. albicans* and many other fungal pathogens and their secretion is essential for the establishment of various infections including candidiasis. The extracellular phospholipase secretion from *C. albicans* was first detected by growing the *Candida* cells on solid media supplemented with egg yolk or lecithin and analyzing the products from lipid breakdown (Price *et al.,* 1982). Phospholipases hydrolyze one or more ester linkages of glycophospholipids of the host cell membranes, resulting in damage of the cell membrane followed by adherence and invasion into the host tissue (Karkowska-Kuleta *et al.,* 2009; Sardi *et al.,* 2013). Studies also reported higher levels of phospholipase production in clinical isolates of *C. albicans* (blood isolates) obtained from patients with disseminated candidiasis as compared to commensal isolates recovered from the oral cavities of healthy volunteers (Leidich *et al.,* 1998; Khan *et al.,* 2010).

Based on the ability of these enzymes to cleave a different and specific ester bond, these phospholipases have been classified into four different classes A, B, C and D. The five members of Class B (*PLB1–5*) are the only extracellular phospholipases that may contribute to the pathogenicity of *C. albicans* by disruption of host cell membranes and have both hydrolase and lysophospholipase-transacylase activities (Mavor *et al.,* 2005; Ghannoum,

2000). Both plb1Δ/Δ and plb5Δ/Δ mutants have been shown to be attenuated in virulence in a mouse model of systemic infection (Leidich *et al.,* 1998; Theiss *et al.,* 2006). The attenuation in virulence was due to the disruption of genes encoding phospholipase enzyme which resulted in inhibition of phospholipase secretion and subsequently reduced the ability of *C. albicans* to penetrate host cells and showed that the level of phospholipase activity was directly correlated with the pathogenicity of *C. albicans*.

5.3.3 Lipases

In *C. albicans*, the extracellular lipase activity was first reported in 1965 and the first lipase gene (*LIP1*) was identified in 1997 (Schaller *et al.,* 2005; Stehr *et al.,* 2003). Later in 2000, a full lipase gene family was identified and characterized, consisting of at least 10 members, *LIP1* to *LIP*10. *In vitro* expression studies have reported that these genes express differently based on the environmental conditions. For instance, *LIP3* to *LIP6* were expressed in all media and at all-time points of growth, whereas *LIP2* and *LIP9* were only expressed in media without lipids. Transcripts of most lipase genes were detected during the yeast to hyphal transition (Hube *et al.,* 2000).

Moreover, a study reported that the lipase gene family of *C. albicans* is expressed during systemic mouse infection, artificial skin infection, and human oral candidosis. It has been shown that extracellular lipases are also involved in both cutaneous and systemic infections of *C. albicans,* and the expression levels of these genes depends on the severity and type of infection (Stehr *et al.,* 2004). All these studies revealed the role of lipases in the pathogenicity of *C. albicans,* however very little is known due to the negligence of lipases in comparison to other hydrolytic enzymes such as proteinases and phospholipases.

6. Ergosterol biosynthesis

Ergosterol is the main sterol of fungal cell membranes which is primarily synthesized in the endoplasmic reticulum, and plays an essential role in regulating membrane permeability,

rigidity and fluidity (Douglas and Konopka, 2014; Dhingra and Cramer, 2017). Due to its various crucial functions, any depletion or inhibition in ergosterol synthesis can disturb the normal growth of the cell. The majority of currently available antifungals which includes azoles, polyenes, allylamines, thiocarbamates and morpholines target the ergosterol biosynthetic pathway or its end product ergosterol (Sanglard *et al.,* 2003).

The azole class of antifungals target ergosterol biosynthesis pathway by inhibiting the lanosterol 14-alpha demethylase (*ERG11* gene) which converts lanosterol to ergosterol. Similarly, allylamines and morpholines inhibit squalene epoxidase (*ERG1* gene), and sterol C-14 reductase (*ERG24* gene)/sterol C-8 isomerase (*ERG2* gene), respectively. On the contrary, polyenes bind directly to ergosterol within the membrane and form pores on the membrane, resulting in leakage of ions and other cellular contents which eventually leads to cell death. Recently, Anderson and co-workers exhibited a novel antifungal mechanism related to polyenes in which they mentioned that polyenes act more like an "ergosterol-sponge" that extracts ergosterol from phospholipid in the membrane, reducing the amount of ergosterol in the cell (**Figure 6)** (Anderson *et al.,* 2014).

Awareness of the importance of the ergosterol biosynthesis pathway has resulted in the enzymes and their genes controlling different steps in this pathway being well studied. The essential genes reported for ergosterol biosynthesis include *ERG1*, *ERG7*, *ERG9*, *ERG25*, *ERG26*, and *ERG27*, however genes such as *ERG2*, *ERG3*, *ERG5*, *ERG6*, and *ERG24* are considered to be non-essential (Lv *et al.,* 2016). *ERG* gene mutations or disruptions thereof have been reported to lead to increased or decreased sensitivity to antifungal drugs. In *C. albicans*, the key transcription factor Upc2 is vital to the ergosterol biosynthesis pathway (Silver *et al.,* 2004). This Upc2 senses the intracellular sterols levels, which leads to the activation of genes obligatory for sterol uptake and biosynthesis (Silver *et al.,* 2004).

For the discovery and development of novel antifungal molecules ergosterol biosynthetic pathway is still the most important cellular pathway as an established drug target. There are numerous studies which reported natural compounds and their derivatives that target the ergosterol biosynthesis pathway in *C. albicans* via different modes of action. Recently, Ahmad and colleagues reported that eugenol and its derivatives target the ergosterol biosynthesis pathway in *C. albicans* by inhibiting the lanosterol 14-α demethylase enzyme and down regulating the expression of its related gene *ERG11* (Ahmad *et al.,* 2015). In another study, novel aminopiperidine derivatives inhibit ergosterol synthesis in *C. albicans* by targeting C-14 reductase (Hata *et al.,* 2010). Natural molecules such as eugenol, methyl eugenol and anisaldehyde have also been reported to exhibit an antifungal effect against *C. albicans* by inhibiting ergosterol synthesis (Ahmad *et al.,* 2010; Shreaz *et al.,* 2011). Earlier reports have shown that carvacrol and thymol showed fungicidal activity by disrupting ergosterol biosynthesis (Ahmad *et al.,* 2011). Thus, the ergosterol biosynthesis pathway in pathogenic yeast could still be a site of target to discover novel drugs with improved and broad-spectrum antifungal activity.

7. Biofilm formation

One of the important characteristics of *C. albicans* is its ability to form biofilms on biotic and abiotic surfaces. A biofilm is a structural community of microbial cells that adhere to a surface covered by an extracellular polymeric matrix. Microbial cells in biofilm form exhibit properties that are different from their planktonic form which include reduced growth rates, enhanced resistance to antifungal therapeutics and the host immune factors (Donlan, 2001; Nobile and Johnson, 2015).

In a study, six master transcriptional factors have been reported to control biofilm formation in *C. albicans* which include Efg1, Tec1, Bcr1, Ndt80, Brg1, and Rob1 (Nobile *et al.,* 2012). Recently, it has been shown that heat shock protein (Hsp90) is required for biofilm antifungal

drug resistance as well as for biofilm dispersal (Robbins *et al.,* 2011). With the increasing use of biomaterials, implants and use of medical catheters, *Candida* biofilm formation has drastically increased, which in turn has increased the concerns of drug resistance. Biofilm formation on indwelling medical devices is associated with high mortality rates in hospitalized patients (Römling and Balsalobre, 2012; Nobile and Johnson, 2015). There are several studies which have already shown that biofilms drastically increased tolerance of *Candida* species to the conventional antifungal drugs (Ramage *et al.,* 2001). Therefore, there is an urgent need of new drugs which can either inhibit biofilm formation or physically penetrate through biofilms to reach the pathogenic cells.

8. Apoptosis in yeast

Apoptosis is a well-controlled cellular suicide program essential for metazoan homeostasis and maintenance; however deregulation in this program can lead to multiple diseases. Similar to metazoan cells yeast cells can undergo apoptosis, which was first discovered in 1997 by using the budding yeast *Saccharomyces cerevisiae* as an experimental organism (Madeo *et al.,* 1997). In this study, it was revealed that the apoptotic cells have a mutation in the *CDC48* gene, which was confirmed by using mutant strains showing key markers of apoptosis as mainly observed in mammalian apoptotic cells (Madeo *et al.,* 1997). It has also been reported that *CDC48* mutant (cdc48S565G) induced apoptosis may involve mitochondria (Braun *et al.,* 2006; Zischka *et al.,* 2006). Since then a number of orthologs, pathways and regulators of apoptosis in yeast has been identified similar to mammalian apoptosis. Therefore, yeasts are considered to be an ideal model for efficiently investigating the various mechanisms of apoptosis and for the development of programmed cell death (PCD)-directed antifungal drugs, which can specifically target fungal apoptotic-regulators and do not have major consequences for human cells.

Yeast cells undergoing PCD display many key markers of apoptosis, such as phosphatidylserine externalization (PS), mitochondrial depolarization, decrease in cytochrome c oxidase activity, reactive oxygen species (ROS) accumulation, the margination of chromatin in nuclei, nuclear fragmentation and DNA degradation (**Figure 9**).

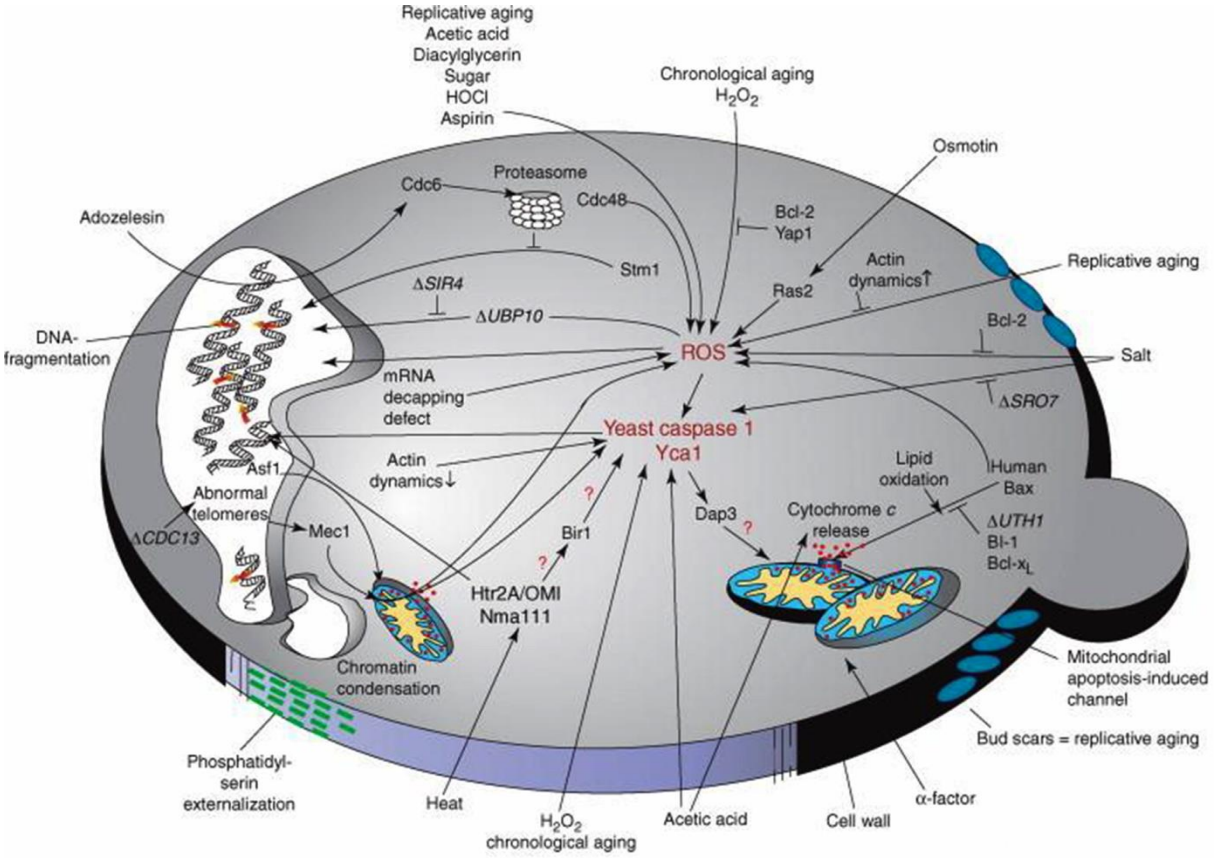


Figure 9: Apoptosis in the yeast cell. An overview on extrinsic and intrinsic stimuli that can induce apoptosis in yeast cells by activating conserved apoptotic key players that are interdependent on each other. Red question marks indicate pathways that are known in mammals but not in yeast thus far (Madeo *et al.*, 2004).

Apoptosis involves highly complex mechanisms and operates through two major pathways; extrinsic and intrinsic (**Figure 9**). It has however been reported that the two pathways are linked, and that their molecules can influence each other (Igney and Krammer, 2002). There are various stimuli that can induce apoptosis in yeast cells and these can be either extrinsic or intrinsic.

Extrinsic apoptotic pathway can be triggered by various stress conditions and chemicals such as hydrogen peroxide (H_2O_2), acetic acid (CH3COOH), hypochlorous acid (HOCl), ethanol, valproic acid, UV irradiation, salt stress, hyperosmotic stress, oxidative stress, aspirin, mating pheromone exposure and many heavy metal ions (**Figure 9**) (Phillips *et al.*, 2003; Carmona-Gutierrez *et al.*, 2010). H_2O_2 and CH3COOH at low concentrations are known to induce apoptosis, however strains lacking the *YCA1* gene (yeast caspase-1) can survive better than the wild type when exposed to H_2O_2 and CH3COOH (Mazzoni and Falcone, 2008). On the other hand, overexpression of *YCA1* gene augments apoptosis-like cell death that is stimulated by H_2O_2 or CH3COOH. Salt stress has been reported to induce cell death in yeast with the exhibition of nuclear markers of apoptosis but can be rescued by expression of *BCL2* or deletion of *YCA1* (Huh *et al.*, 2002; Wadskog *et al.*, 2004). HOCl induces apoptosis in the yeast cell by generating oxygen radicals (King *et al.*, 2004). It has been reported that hyperosmotic stress induced apoptosis-like cell death in yeast, mediated by a caspase-dependent mitochondrial pathway partially dependent on cytochrome c (Silva *et al.*, 2005). Yeast cells exposure to mating pheromone can undergo apoptosis-like cell death and shown markers of apoptosis such as ROS accumulation, increase in intracellular Ca^{2+}, permeability transition pore formation, mitochondrial fragmentation and the release of cytochrome c (Gourlay *et al.*, 2006).

The intrinsic pathway of apoptosis involves a diverse range of non-receptor mediated stimuli that trigger intracellular signals and are mitochondrial mediated (**Figure 9**). The significance of mitochondrial involvement in yeast apoptosis is well known. During apoptosis, oxidative stress results in the opening of mitochondrial permeability transition pore (PTP), which activates mitochondrial outer membrane permeabilization (MOMP) followed by the release of cytochrome c and other pro-apoptotic factors such as Bax, Yca1p, Aif1p leading to the activation of the apoptotic cascade (**Figure 9**) (Simon *et al.*, 2000). Cytochrome c oxidase is located on the outer face of the inner mitochondrial membrane and is the terminal enzyme of

the respiratory electron transport chain. Several studies have reported that release of cytochrome c is associated with yeast apoptosis (Carmona-Gutierrez *et al.,* 2010).

In yeasts, mitochondria are a site for both the origin and target of ROS, which are one of the known triggers of apoptosis and necrosis and play a crucial regulatory role in many apoptotic pathways in yeast (Jia *et al.,* 2019). A study reported ROS as a promoter and not a by-product in yeast apoptosis (Madeo *et al.,* 1999). ROS accumulation results in oxidative damage of proteins, nucleic acids and lipids and can induce apoptosis or necrosis depending on the concentration. Some other main apoptosis inducing factors such as AIF1, YCA1 are also involved in ROS mediated cell death. It has been shown that ROS can directly activate the metacaspase-dependent apoptosis pathway (Jia *et al.,* 2019).

A pro-apoptotic gene Bax (from the Bcl-2 gene family) in humans is also expressed in yeast and can induce apoptotic cell death accompanied by cytochrome c release from mitochondria by altering mitochondrial membrane permeability, which results in decrease in cytochrome c oxidase (Madeo *et al.,* 2002). However, mutant forms of Bcl-xL (anti-apoptotic Bcl-2 family member) can prevent Bax induced apoptotic cell death in yeasts (Madeo *et al.,* 2002). The only caspase which play an essential role in yeast apoptosis is known as metacaspase Yca1p, an ortholog of mammalian caspases (Carmona-Gutierrez *et al.,* 2010). Under conditions of oxygen stress, disruption of *YCA1* results in decreased cell death and the formation of apoptotic markers. The existence of an apoptosis inducing factor (AIF) homolog in yeast proved the conservation of apoptotic essentials from yeast to man (Modjtahedi *et al.,* 2006). The yeast Aif1p functions very similar to its mammalian equivalent AIF upon inducing apoptosis. Induction of apoptosis by H_2O_2, acetate and aging, results in translocation of Aif1p from the mitochondria to nucleus. Besides its role in apoptosis, AIF in both mammals and yeast play a vital role during optimal oxidative phosphorylation (Modjtahedi *et al.,* 2006).

Drug-induced apoptosis in fungi, particularly in yeasts, is of paramount value. Several currently used antifungal drugs are known to induce apoptosis in yeast and these include amphotericin B, ciclopirox olamine (CPO), osmotin, dermaseptin, pradimicin and histatin (Almeida *et al.,* 2008). However, most of these drugs showed high human toxicity. To overcome the adverse side effects of these antifungals, there is an urgent need to find novel antifungal molecules that can induce programmed cell death in yeast and other filamentous fungi with minimum or no human toxicity.

There are numerous studies which reported induction of apoptosis in yeasts by various natural products and their derivatives such as lycopene, Coumarin, nerol, eugenol, limonene, plagiochin E, *Ocimum sanctum* essential oil and its two major constituents methyl chavicol and linalool (Jia *et al.,* 2019; Khan *et al.,* 2013; Wu *et al.,* 2010; Khan *et al.,* 2014). These compounds induce apoptosis in yeast via different pathways, however many of them exhibit adverse physicochemical characteristics and have been overlooked, which resulted in decline of research interest and investment in this area. Therefore, further in depth research is required to modify natural compounds, increase their potency and safety and also know their actual mechanism behind their antifungal activity, which may result in the development of novel drugs that can induce apoptotic cell death in yeast.

9. Eugenol derivatives as future potential antifungal drugs

Nature is an endless source of products of medicinal importance, and since ancient times these products has been used as therapeutic agents for the treatment of several diseases. It has been reported that 80% of the world's population is committed to herbal/traditional medicine of plant origin for some part of primary healthcare (Ekor, 2014). Recently, it has been shown that around 25% of all medicines have plant origin (Gurnani *et al.,* 2014). However, most of these natural products exhibit adverse physicochemical characteristics and have often been overlooked, despite the fact that these natural products are sources of new drugs.

The advancement in drug discovery techniques overwhelms this issue through molecular modification of natural products which leads to the development of secondary metabolites with improved biological properties as well as minimum or no side effects. These secondary metabolites are sometimes referred to semi-synthetic drugs or derivatives/analogues which have already been reported to be effective against various infectious diseases (Butler *et al.*, 2014; Stratton *et al.*, 2015; Ahmad *et al.*, 2015). In 1998, a report based on a pharmaceutical industry perspective stated that an approximately 60% of the anti-infective and antitumor drugs in the market or under clinical trial are derived from natural products through structural modifications (Shu, 1998). Recently in 2014, it has been shown that out of the 237 anti-infection drugs (antifungal, antibacterial, antiviral and antiparasitic) excluding vaccines, 138 (approximately 58.30%) are natural products or their derivatives (da Silva *et al.*, 2018). Thus, research in this area is promising for the development of new antifungal drugs (Newman and Cragg, 2016).

Eugenol (4-allyl-2-methoxyphenol) is one of the comprehensively studied natural products for its various biological actions. It is a phenolic compound from the phenylpropanoids class of chemical compounds with a molecular weight of 164.2 g/mol. It is a weak acid which is slightly soluble in water and is soluble in organic solvents. Eugenol is a colourless to pale yellow oily liquid extracted from several essential oils which include clove oil, nutmeg, cinnamon, basil, and bay leaf. However, clove oil is the main source of eugenol, containing 45-90% of the total oil, and even the name is derived from the scientific term for clove *Eugenia caryophyllata* or *Syzygium aromaticum* (Zhang *et al.*, 2013). It was first isolated in 1929 and its commercial production began in the 1940s in the USA. Currently, eugenol can also be produced synthetically by the allylation of guaiacol with allylchloride.

In recent years, eugenol gained much attention in the field of drug development due to its broad range of biological and pharmacological activities such as analgesic, anti-inflammatory,

antioxidant, antiallergic, anticarcinogenic, antimutagenic, anaesthetic, antiparasitic, antiviral, antibacterial, and antifungal (Olea *et al.,* 2019). However due to its hypersensitivity reactions and cytotoxic effects, its clinical testing has been halted. Therefore, modifications of its chemical structure is sought out to be an important strategy to obtain molecules with desirable physicochemical and improved biological properties. The enhanced antimicrobial activity of eugenol by its derivatisation has already been reported (Carrasco *et al.,* 2012; Hipolito *et al.,* 2018; da Silva *et al.,* 2018)

Because of the insufficiency, inefficacy and adverse side effects associated with the current antifungal therapy, eugenol has been studied broadly for its antifungal activities. However, little is known about the targets and mechanisms of action behind the antifungal activity of eugenol, thus in-depth study and further modification at molecular level of eugenol may lead to the development of new antifungal agents with known mechanisms of action and improved antifungal activity.

Several studies have already reported the mechanisms of antifungal activity of eugenol and its derivatives (Ahmad *et al.,* 2015; Carrasco *et al.,* 2012; Hipolito *et al.,* 2018; da Silva *et al.,* 2018). Ahmad and colleagues reported that eugenol tosylate derivatives target ergosterol biosynthesis and showed synergistic interactions with fluconazole against different isolates of *C. albicans* (Ahmad *et al.,* 2015). In a recent study, new Mannich base-type derivatives of eugenol were synthesized and shown to possess potent antifungal activity even equivalent to fluconazole (Abrão *et al.,* 2015). It has been shown that structural modification of eugenol with the insertion of a nitro group results in a derivative with improved antifungal effect (Carrasco *et al.,* 2012). Hipolito and colleagues recently reported that eugenol-based glucoside derivatives demonstrated enhanced antifungal activity compared to eugenol against different *Candida* species, and has also shown high affinity for squalene epoxidase, an enzyme involved in fungal ergosterol biosynthesis (Hipolito *et al.,* 2018).

Using virulence factors of *C. albicans* as drug targets could be a new paradigm to discover novel antifungal molecules. Few studies revealed that eugenol significantly decreased the pathogenicity of *C. albicans* by targeting its virulence factors such as adherence, biofilm formation and morphogenesis (De Paula *et al.,* 2014). Eugenol has also been shown to induce apoptosis after decreasing ergosterol biosynthesis in *Candida* cells (Khan *et al.,* 2013). Fungicidal activity of eugenol through envelop damage in *C. albicans* has also been reported (Chami *et al.,* 2005). Moreover, eugenol has also been tested as antifungal agent in animal models (Ahmad *et al.,* 2005; Chami *et al.,* 2004). There are numerous studies which suggested that the antifungal mechanism of eugenol is related to its ability of targeting the cell membrane and consequently inhibiting ergosterol biosynthesis resulting in the disruption of integrity and functionality of the cell membrane (de Oliveira *et al.,* 2013; Pinto *et al.,* 2009).

Based on these characteristics of eugenol, we hypothesized that the tosylate derivatisation of this molecule will lead us to discover new derivatives which can act on all the established as well as emerging drug targets without losing their antifungal property. In addition we also hypothesized that this derivatisation will improve the physiochemical properties as well as reduce the cytotoxicity when compared to the parent compound.

Aim of the study

With the advent of HIV/AIDS, modern day life style diseases and advanced surgeries, the rate of fungal infections increased dramatically over the past few decades. On the other hand, the rate of clinically approved antifungal drugs has decreased, which in turn put additional pressure on the available antifungal drugs leading to drug resistance. Consequently there is a clear demand to either improve the current therapy options or to develop new drugs with different and multiple drug targets. The aim of this study was to investigate the antifungal activity of newly synthesized Eugenol Tosylate Congeners (ETCs) against different fluconazole susceptible and resistant *Candida albicans* isolates, by evaluating the effect of these ETCs on the ergosterol biosynthesis pathway and virulence factors at phenotypic and molecular levels. In addition, we also determined the physiology and mode of cell death in response to these compounds by studying apoptosis.

To achieve the aim, following objectives have been pursued:

Objective 1: To evaluate minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of eugenol and seven newly synthesized ETCs against different fluconazole susceptible and resistant *C. albicans* isolates.

Outcome: The detailed methodology, results and discussion are described in **Chapter 2** (Publication 1).

Short summary: Basic screening of the seven newly synthesized ETCs for their antifungal effect has been done by *in vitro* antifungal susceptibility testing. Broth microdilution assay was used to determine MIC and MFC as per approved guidelines described by Clinical and Laboratory Standards Institute (CLSI) guidelines M27-A. From the results, it was observed that all the newly synthesized ETCs showed improved and potent antifungal activity in comparison to their parent compound eugenol (MIC 500 µg/ml; MFC 1000 µg/mL) against different FLC susceptible and resistant isolates of *C. albicans*. These derivatives exhibited a

drastic decrease in MIC and MFC values when compared to their parent compound, eugenol (MIC 500 µg/mL; MFC 1000 µg/mL). For FLC susceptible isolates the MIC and MFC values decreased down to 0.125 µg/mL and 0.5 µg/mL respectively and for FLC resistant isolates these values were 0.25 µg/mL and 1.0 µg/mL respectively. The order of antifungal potency based on susceptibility results is ETC-5>ETC-6>ETC-7>ETC-1>ETC-4>ETC-2>ETC-3. These results revealed that all the test compounds (ETC-1 to ETC-7) possess potent antifungal effect at varying degrees.

Objective 2: To study the effect of novel ETCs on the ergosterol biosynthesis pathway and cell viability of *C. albicans* by applying a combined approach of *in silico* and *in vitro* methodologies.

Outcome: The detailed methodology, results and discussion are described in **Chapter 3** (Publication 2).

Short summary: In this objective, we selected the most active compounds (ETC-5, ETC-6 and ETC-7) based on their *in silico* total binding energy and *in vitro* susceptibility testing results. *In silico* studies were performed using molecular docking and dynamic simulations. *In vitro* studies included ergosterol biosynthesis assays in which total intracellular sterol content was quantified spectrophotometrically. The gene expression of *ERG11* (the gene encoding 14-α demethylase enzyme) was determined by using a quantitative reverse transcription PCR (RT-qPCR) technique. A cell viability assay by a MUSE Cell Analyzer was used to detect the effect of these selected ETCs on cellular viability.

From the *in silico* studies, it has been observed that these ETCs have a potential to bind the heme group in CYP51 and inhibit 14-α demethylase enzyme. Following up on this, *in vitro* results confirmed that these ETCs (ETC-5, ETC-6 and ETC-7) significantly decreased ergosterol synthesis by targeting the lanosterol 14-α demethylase enzyme, down regulated expression of *ERG11* and also drastically reduced cellular viability in *C. albicans*. The

antifungal effect was dose dependent and therefore at higher concentrations these ETCs become fungicidal in activity as seen in the cell viability assay. For FLC susceptible isolates test compounds exhibited cell death in the range of 32.0 to 43.9%, 39.2 to 60%, and 76.3 to 84.2% at 0.25×MIC, 0.5×MIC and 1×MIC values of test compounds respectively. The figures for FLC resistant isolates was 22.1 to 37.6%, 31.4 to 47.2%, and 63.5 to 79.7%.The decrease in total ergosterol content at MIC and 0.5 × MIC values of these test entities for FLC susceptible strain was 78 to 90% and 52 to 65% respectively, and for FLC resistant strain these values were 68 to 79% and 36 to 51% respectively. The down-regulation in expression of *ERG11* gene in FLC susceptible and resistant strains was detected in the range 2.44 to 4 and 1.85 to 2.9 folds respectively at MIC value of these test compounds. In all these findings, ETC-5 was the most active compound followed by ETC-6 and ETC-7 against both FLC susceptible and resistant strains of *C. albicans*.

Based on these *in silico* and *in vitro* susceptibility results, only ETC-5, ETC-6 and ETC-7 were selected for further studies.

Objective 3: To determine the effect of ETCs on major virulence factors of *C. albicans*, such as adherence, morphogenesis, hydrolytic enzymes secretion and biofilm formation.

Outcome: The detailed methodology, results and discussion are described in **Chapter 4** (Publication 3).

Short summary: In this chapter, we tested the effect of the most active ETCs (ETC-5, ETC-6 and ETC-7) on the key virulence factors of *C. albicans*. The methods such as alamarBlue-based assay, microscopy, plate assay methods and XTT reduction assay were performed for adherence, morphogenesis, hydrolytic enzymes secretion and biofilm formation respectively. The ETCs significantly inhibited adherence in *C. albicans* with an inhibition range of 23% to 66% and 16% to 57% for FLC susceptible and resistant isolates respectively. Yeast to hyphae transition in *C. albicans* cells were visualized microscopically and it was observed that ETCs

could completely inhibit the hyphal growth at MIC value in both FLC susceptible and resistant isolates. Furthermore, ETCs were also able to arrest the hyphal growth at sub-inhibitory concentrations. These congeners reduced the secretion of hydrolytic enzymes (proteinases and phospholipases) in *C. albicans* cells to varying extents. Inhibition of proteinase activity was in the range of 15% to 48% and 2% to 37% for FLC susceptible and resistant isolates respectively. The values for phospholipase activity were 15% to 33% and 8% to 34% for FLC susceptible and resistant isolates respectively. Moreover, test compounds (ETC-5, ETC-6 and ETC-7) significantly inhibited biofilm formation in *C. albicans*. The percentage inhibition of biofilm formation was in the region of 10% to 77% and 7% to 58% for FLC susceptible and resistant strains respectively. Moreover, the rate of inhibition in biofilm formation was dependent on concentration and treatment time.

From all the assay results, it was observed that the activity of these test compounds was dose dependent, even though all the congeners were significantly effective at their sub-inhibitory concentrations. Our results also demonstrated that these test entities target virulence factors of *C. albicans* that converted the commensal microbe into a pathogen. Consequently the targeting of virulence factors could be a new approach to designing and exploring novel antifungal agents with multiple drug targets.

Objective 4: To evaluate the effect of these compounds on the expression profile of genes involved in pathogenicity of *C. albicans*, such as adherence genes *(ALS1, ALS2, ALS3 and ALS9)*, morphogenesis genes (*CPH1, HWP1*) and genes encoding for the production of hydrolytic enzymes (*SAP1, SAP2, SAP3 and PLB1*) by using RT-qPCR.

Outcome: The detailed methodology, results and discussion are described in **Chapter 4** (Publication 3).

Short summary: In the previous objective it had been confirmed that these tosylates significantly diminished *C. albicans* pathogenicity via the targeting virulence factors.

Subsequently, in pursuing this objective we evaluated the effect of these ETCs on the expression of genes associated with pathogenicity of *C. albicans* which include (*ALS1*, *ALS2*, *ALS3*, *ALS9*, *CPH1*, *HWP1*, *SAP1*, *SAP2*, *SAP3* and *PLB1*). RT-qPCR was used for gene expression analysis. Results indicated a significant down-regulation in expression levels of genes linked to adherence (*ALS1*, *ALS2*, *ALS3* and *ALS9*), morphogenesis (*CPH1*, *HWP1*), proteinases (*SAP1*, *SAP2*, and *SAP3*) and phospholipases (*PLB1*) in comparison to the control (untreated cells) that was set to 1.0 with a fold range between 1.8 to 2.5, 2.3 to 3.2, 2.4 to 3.0, 2.0 to 2.6, 2.4 to 2.9 and 1.6 to 2.2, 2.2 to 2.9, 2.0 to 2.6, 1.7 to 2.3, 2.0 to 2.6 folds for FLC susceptible and resistant strains respectively. These findings further confirm our corollary for ETCs of targeting major virulence factors in *C. albicans*, and now it has been also revealed that these compounds not only target biochemical pathways but also influence molecular pathways of pathogenicity in *C. albicans*.

Objective 5: To determine the physiology and mode of cell death in response to these ETCs by analyzing major apoptotic markers in yeast such as phosphatidylserine externalization, DNA fragmentation, mitochondrial depolarization and decrease in cytochrome c oxidase activity.

Outcomes: The detailed methodology, results and discussion are described in **Chapter 2** (Publication 1).

Short summary: The aim here was to determine the mode of cell death by detecting the apoptotic and necrotic effects of the three most active ETCs (ETC-5, ETC-6 and ETC-7) against *C. albicans* isolates. The characteristic apoptotic markers which include phosphatidylserine externalization, DNA fragmentation, mitochondrial depolarization and decrease in cytochrome c oxidase activity were analyzed by flow cytometry using FITC Annexin V/PI-staining, fluorescence microscopy (TUNEL assay), JC-10 mitochondrial membrane potential assay by monitoring fluorescence intensity and cytochrome c oxidase

assay by using a spectrophotometer respectively. From the flow cytometry results it was revealed that these compounds can significantly induce apoptosis and necrosis in both FLC susceptible and resistant isolates in the range; early apoptosis 5% to 54%, late apoptosis 2% to 48% and necrosis 1% to 51%. In the TUNEL assay DNA damage, a hallmark of late apoptosis, was observed that corroborate the results observed with FITC Annexin V/PI-staining. In both these assays the apoptotic effect was dose dependent as the number of late apoptotic and necrotic cells increased with the increasing concentration of these compounds. *Candida* cells after being exposed to varying concentrations of test compounds showed significant reduction in mitochondrial membrane potential (MMP) in comparison to untreated control cells. The ETCs decreased MMP by 1.04 to 2.96, 1.56 to 3.44 and 3.55 to 7.57 folds at concentrations of 0.5 × MIC, 1 × MIC and 2 × MIC respectively. A decrease in MMP leads to release of cytochrome c from the mitochondria into the cytosol which directly correlates with a decrease in cytochrome c oxidase activity. This statement supports and confirms our results where these ETCs after mitochondrial depolarization considerably reduced the cytochrome c oxidase activity up to 6.20% to 18.07%, 4.42% to 13.92% and 2.39% to 9.88% at 0.5 × MIC, 1 × MIC and 2 × MIC values of test compounds respectively. These results advocated that the most active derivatives (ETC-5, ETC-6 and ETC-7) induced cell death in *C. albicans* in a dose dependent manner by activating apoptotic and necrotic pathways. Induction of apoptosis in yeast cells is considered as powerful model for the screening of new antifungal agents and could provide a basis for future therapies.

Objective 6: To determine the cytotoxic effect of these newly synthesized ETCs, by using *in vitro* haemolytic assay.

Outcomes: The detailed methodology, results and discussion are described in **Chapter 2** (Publication 1).

Short summary: Newly synthesized ETCs showed potent antifungal activity and also induced apoptosis in *C. albicans*, therefore it was important to test their cytotoxic effect before subjecting them to *in vivo* studies. The haemolytic assay was performed using horse red blood cells to check the cytotoxicity of the most potent ETCs (ETC-5, ETC-6 and ETC-7) at 0.5 × MIC, 1 × MIC and 2 × MIC values. ETCs showed cell haemolysis in the range of 1.98% to 15.8%, hence confirming their low cytotoxicity effect and declaring them as possibly safe to use. This *in vitro* haemolytic assay is a possible screening tool for gauging *in vivo* toxicity to host cells and warrants the use of these compounds in animal models which will be the next stage of drug development with these compounds.