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Modulation of antifungal drug resistance and combination activity of Eugenol Tosylate Congeners with antifungal drugs in *Candida albicans*

Windy Sekgele

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

Johannesburg November

DECLARATION

I, Windy Sekgele declare that this Dissertation is my own work. It is being submitted in fulfillment for the degree of Masters of Sciences in Medicine in the faculty of Health Sciences, University of the Witwatersrand, Johannesburg. This degree has not been submitted before for examination at this university or any university.

_____ _____

Windy Sekgele Date

MSc. candidate

DEDICATION

To the creator of the heaven and Earth, I wouldn't have done it without with you. Thank you Jehovah.

To my sister Mabatho Sekgele, my brothers Kabelo Sekgele and Kgothatso Sekgele, my nephew Kamogelo Sekgele and my son Neo Sekgele thank you all for your continuous support and encouragement through challenging phases of my studies.

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CONFERENCE PRESENTATIONS

- **Oral Presentation**: Inhibition of the proton *ATPase* by Eugenol Tosylate Congeners and their combination activity with fluconazole in *Candida albicans*. 48th scientific meeting of the South African Division of the International Association of Dental Research. Glenburn Lodge, Muldersdrift, Johannesburg. 31 August 2018.

- **Poster Presentation**: Combination activity of Eugenol Tosylate Congeners with fluconazole against *Candida albicans.* Faculty of Health Sciences Research Day, University of the Witwatersrand, 6 September 2018.

- **Flash talk and Poster Presentation**: Inhibition of the proton *ATPase* by Eugenol Tosylate Congeners and their combination activity with fluconazole in *Candida albicans.* 9th Cross Faculty Post Graduate Symposium, University of the Witwatersrand, 29 and 30 October 2018.

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ABSTRACT

Introduction

The increased incidence of antifungal drug resistance in *Candida albicans (C. albicans)* species has been reported world-wide. Semi synthetic products have been investigated as potential treatment to treat *Candida* infections. To alleviate the burden of disease, new treatment options have been looked at such as combination therapy using natural products and synthetic drugs and also to develop new drug targets in *C. albicans* species. The aim of this study was to evaluate the modulation of antifungal drug resistance and combination activity of eugenol tosylate congeners with antifungal drugs in *Candida albicans*.

Materials and methods

Fifty *C. albicans* isolates collected from HIV and cancer patients who attended Charlotte Maxeke Academic Hospital were included in the study. Drug susceptibility testing was performed using fluconazole (FLC), nystatin (NYS) and caspofungin (CAS) antifungal drugs against *C. albicans* strains. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of six eugenol tosylate congeners (ETCs; C1-C6) and eugenol (EUG) were determined against drug resistant and susceptible *C. albicans* strains. The antifungal activity of the combination of ETCs with antifungal drugs (FLC, NYS and CAS) in a 1:1 combination ratio was investigated by determining the fractional inhibitory concentration index. The effect of ETCs on the activity of the proton *ATPase* (H^+ *ATPase*) was evaluated by determining the rate of proton efflux in *C. albicans* cells treated with ETCs. The effect of ETCs on intracellular pH of *C. albicans* cells was investigated to evaluate the effect of a dysfunctional H^+ *ATPase* enzyme on the intracellular pH of *C. albicans* in cells treated with ETCs and in untreated cells. In addition, the effect of ETCs on activity of drug efflux pumps was evaluated. The haemolytic effect of ETCs on horse red blood cells was investigated. Analysis of variance was used to investigate the significant differences between independent groups using graph pad prism 5.

Results

Of the 50 isolates tested for drug susceptibility, 78% were susceptible to FLC, 14% were resistant and 8% were susceptible dose dependent. Ten isolates (20%) were susceptible to CAS, 5 (10%) were resistant and 35 (70%) were categorized as intermediate. Forty four (88%) isolates were susceptible to NYS and 6 (12%) were resistant. Cross resistance was observed in one isolate which was resistant to both NYS and FLC. Multidrug resistance was also observed in one isolate which was resistant to both CAS and FLC. The MICs obtained for ETCs (C1-C6) ranged from 0.13 to 2 mg/ml. For EUG the MICs ranged from 0.33 and 0.5 mg/ml. Congener 1 was the most active compound with decreased MICs ranging between 0.13 and 0.250 mg/ml against both drug susceptible and resistant *C. albicans* strains. The combination activity of NYS with ETCs exhibited the most synergistic interactions (43%) compared to the combinations of ETCs with CAS (14%) and FLC (0%). All ETCs successfully reduced the rate of H^+ efflux in both FLC susceptible and resistant strains at previously determined MICs, however congener 6 showed the greatest effect on reducing the rate of H^+ efflux by 75% in glucose starved cells and by 38% in cells supplied with glucose in both FLC resistant and susceptible strains. Intracellular pH of FLC resistant and susceptible *C. albicans* cells treated with ETCs was less acidic (pH 6.6-6.8) than intracellular pH of untreated *C. albicans* cells with functional H^+ *ATPase* (pH 6.40). ETCs showed no effect on activity of drug efflux pumps in FLC susceptible strains. However in FLC resistant strains C2, C4 and EUG exhibited inhibitory effects on activity of drug efflux pumps. ETCs induced hemolysis of RBC in a concentration dependent manner however the percentage hemolysis was below 15% for all ETCs in all tested concentrations.

Conclusions

Eugenol tosylate congeners exhibited antifungal activity against both drug resistant and susceptible *C. albicans* strains at acceptable concentrations for modified compounds. The synergistic effect exhibited by ETCs in combination with NYS is a promising therapeutic strategy to combat *C. albicans* infections. Congener 2, C4 and EUG significantly inhibited activity of drug efflux in FLC resistant strains indicating that the compounds can be successfully used for the reversal of FLC drug resistance in *C. albicans* strains. In addition the hemolysis assay indicated that ETCs have great potential as antifungal drugs and may be used as safe and effective treatment in clinical applications.

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LIST OF ABBREVIATIONS

ABC	: ATP-binding cassettes
ALS	: Agglutinin-like sequence
Amp B	: Amphotericin B
ATP	: Adenosine triphosphate
ATCC	: American type culture collection
Ca^{2+}	: Calcium ion
C. albicans	: *Candida albicans*
C. auris	: *Candida auris*
C. glabrata	: *Candida glabrata*
C. krusei	: *Candida krusei*
C. parapsilosis	: *Candida parapsilosis*
C. tropicalis	: *Candida tropicalis*
$CaCl_2$	: Calcium Chloride
CAS	: Caspofungin
CDC	: Centers for Disease Control and Prevention
CLSI	: Clinical Laboratory Standard Institute
DMSO	: Dimethyl-sulfoxide
EUCAST	: European Committee on Antibiotic Susceptibility Testing
EUG	: Eugenol
et al	: and others
ETCs	: Eugenol tosylate congeners
FICI	: Fractional inhibitory concentration index

FLC	: Fluconazole
H^+ *ATPase*	*:* Proton *ATPase*
H^+	: Hydrogen ion
HAART	: Highly active antiretroviral therapy
HCl	: Hydrochloric acid
HIV	: Human immuno-deficiency virus
HREC	: Human Research Ethics Committee
KCl	: Potassium chloride
K^+	: Potassium ion
MDR	: Multi drug resistance
MFC	Minimum fungicidal concentration
MFS	: Major facilitator superfamily
MIC	: Minimum inhibitory concentration
MW	: Molecular weight
Na^+	: Sodium ion
NaCl	: Sodium chloride
NaOH	: Sodium hydroxide
NYS	: Nystatin
OPC	: Oropharyngeal candidiasis
PBS	: Phosphate buffered saline
pH	: Hydrogen ion concentration
P-type *ATPase*	: Proton *ATPase*
R6G	: Rhodamine 6G

RBC	: Red blood cells
rpm	: Revolutions per minute
SBA	: Sabouraud broth
SDA	: Sabouraud dextrose agar
SEM	: Standard error of the mean
UTI	: Urinary tract infection
Vanadate	: Sodium orthovanadate
VF	: Virulence factors
V-type *ATPase*	: Vacuolar *ATPase*

LIST OF SYMBOLS AND UNITS

α	: Alpha
β	: Beta
%	: Percentage
°C	: Degrees centigrade
CFU	: Colony forming units
X	: Times
M	: Molar
mM	: Millimolar
µM	: Micromolar
nm	: Nanometer
g	: Relative centrifugal force
g	: Grams
mg	: Milligrams
µg	: Micrograms
ml	: Millilitre
µl	: Microlitre
=	: Equals
±	: Plus-minus
>	: Greater than
<	: Lesser than
≥	: Greater than or equal to
≤	: Lesser than or equal to

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Candida albicans (C. albicans) forms part of the mucosal micro biota in 40-60% of the human population (Sardi *et al*., 2013b). However, the overgrowth of this species results in the development of various infections ranging from cutaneous to invasive and disseminated *Candida* infections. The ability of *C. albicans* to initiate infection and become invasive is associated with its dimorphic phenotype, being able to switch from yeast to hyphae morphology in response to environmental conditions (Cullen and Sprague, 2012). In addition, *C. albicans* secretes adhesins and produces extracellular hydrolytic enzymes which play a role in the colonization of host tissues, nutrition acquisition and invasion and damaging of host tissues (Sardi *et al*., 2013a). *Candida* infections are opportunistic infections and are more frequent in immune compromised individuals and account for more than 30% of world mortality (Kaplan *et al.*, 2000; Pfaller and Diekema, 2007). In human immunodeficiency virus (HIV) infected patients, oral-oesophagal candidiasis remains among the most frequent infections even with the introduction of highly active antiretroviral therapy (HAART) and the subsequent decline of some opportunistic infections (Kaplan *et al.*, 2000; Pfaller and Diekema, 2007).

Like all other infections, candidiasis have a treatment plan for clinical use which includes the use of three major classes of antifungal drugs which are the polyenes (amphotericin B, nystatin, and natamycin), azoles (imadazoles and triazoles) and echinocandins (caspofungin, micafungin and anidulafungin) (Pappas *et al*., 2016). These classes of antifungal drugs have a distinct mode of action and specific target sites in *C. albicans*. Recently there has been an increase in the incidence of antifungal drug resistance in some *C. albicans* species (Robbins *et al*., 2016). Antifungal drug resistance to fluconazole (FLC), nystatin (NYS) and caspofungin (CAS) have been documented in *C. albicans* strains (Owotade *et al*., 2016; Nenoff *et al*., 2016). There are various mechanisms used by *C. albicans* to confer resistance to the used antifungal drugs such as drug target alterations and overexpression of certain genes (*ERG, FKS1, CDR1P, CDR2P* and *MDR)* (Sanglard *et al*., 1997; Marichal *et al*., 1999; Perlin, 2007). In order to combat *C. albicans* infections, new therapeutic strategies need to be developed such as using natural compounds and combination therapy. More research should be conducted on identifying new drug targets in *C. albicans.*

1.2 LITERATURE REVIEW

1.2.1 Epidemiology

Candida infections affect at least 250000 individuals per year and are responsible for more than 50000 deaths world-wide (Sardi *et al*., 2013b). The incidence and prevalence of *Candida* infections have increased since the last four decades especially in immune compromised individuals and patients admitted in intensive care units with life threatening diseases (Arendrup *et al*., 2005). Furthermore reports have shown that these infections have become the fourth leading nosocomial infection world-wide (Martins *et al.*, 2014).

Candida species are commensal to humans, they can either grow aerobically or anaerobically depending on area of the body they colonize. When growing in oral cavity they grow aerobically and in the gut they grow anaerobically (Evans *et al*., 1974; Shepherd and Sullivan, 1976). They are Gram positive species with the ability to grow in two different morphologies. They can either grow as yeasts (unicellular), pseudohyphae or in hyphal (multicellular) form depending on the temperature and pH of their environment (Sudbery *et al*., 2004). Yeasts are small single cells, with an average size of 10-12 µm in diameter and an oval shape. The pseudohyphae form also known as filamentous morphology is formed by yeast buds joined to one another with their width larger at the center than the ends (Sudbery *et al*., 2004). Hyphae cells grow in a polarized manner, are elongated with uniform width, possess true septa and are attached end to end (Odds, 1988). Another characteristic feature of hyphae cells is that they have pores in their septa which are important for cell to cell communication (**Figure 1.1**) (Gow, 1994).

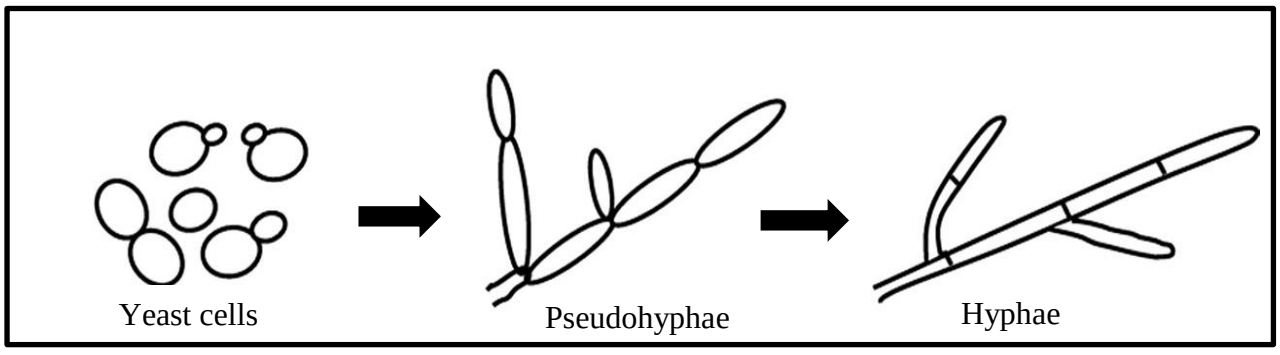


Figure 1.1: Three morphological forms of *Candida albicans* (Thompson *et al*., 2011).

Candidiasis is a fungal infection caused by different species that belong to the genus *Candida* (Odds, 1988). This genus is made up of more than 200 different species, with more than 17 species that are known to be the causative agents of human infections. However it has been reported that more than 90% of these infections are due to *C. albicans, Candida glabrata (C. glabrata), Candida krusei (C. krusei), Candida tropicalis (C. tropicalis) and Candida parapsilosis (C. parapsilosis)* (Pfaller and Diekema, 2007; Chakrabarti *et al.*, 2009). Furthermore there are studies reporting on the emergence of *Candida* infections due to *Candida auris* (*C. auris*) (Clancy and Nguyen, 2017).

A study conducted in North America at a medical centre with patients infected with fungal infections revealed an increased incidence of *Candida* infections due to non-*albicans* species with *C. glabrata* the most frequently isolated species (Sardi *et al*., 2013a). Recently, *C. parapsilosis* has been identified as a significant nosocomial pathogen with clinical implications that includes septic arthritis and fungaemia which are usually due to prosthetic devices and invasive clinical procedures (Canto' *et al*., 2011). *Candida tropicalis* was previously reported to be prevalent in cases of candidemia in Brazil and worldwide (Sardi *et al*., 2013a). Statistical reports show that about 60% of patients infected with this species develop systemic infections and it is highly observed in patients suffering from haematological disorders, cancer and bone marrow recipients (Kontoyiannis *et al*., 2001; Leung *et al*., 2002; Colombo and Guimarães, 2003).

According to the Centers for Disease Control and Prevention (CDC), *C. albicans* account for the majority of fungal infections in humans (CDC, 2017). A study by Xie *et al.* (2008) showed that *C. albicans* is the most frequent isolated species (58%) in surgical patients with invasive fungal infection followed by *C. tropicalis* (17%) and *C. glabrata* (15%). The characteristic feature that makes *C. albicans* to be more infectious than other *Candida* species is its ability to form biofilms (Donlan, 2002). Biofilms are described as structural forms where microorganisms stick to each other in a specific and organized manner within a slimy extracellular matrix composed of extracellular polymeric substances (Costerton *et al*., 1995). Studies have shown that *C. albicans* does not exist in a planktonic free form in host tissues but is commonly present in a group forming a multicellular community on tissues, catheters and prostheses (Donlan, 2002; Ramage and Lo' per-Ribot, 2005; Soll, 2008).

The ability of *C. albicans* to form biofilms encourages the symbiotic relationships with hosts and also allows for its survival in hostile environments (Davey and O'toole, 2000). In the study by

Hawser and Douglas. (1994) isolates of *C. glabrata* and those of *C. parapsilosis* were significantly less likely to form biofilms than the more pathogenic *C. albicans*. Biofilms may also assist in maintaining the role of *C .albicans* as pathogenic by invading the host immune system, withstanding competitive pressure from other microorganisms and becoming resistant to antifungal treatment (Nobile and Johnson, 2015).

1.2.2 Pathogenicity of *Candida albicans*

Candida species have recently been identified as a significant cause of lethal infections (Vincent *et al*., 2009). Studies have shown that the immune response to *Candida* infections is site and species specific (Vidigal and Svidzinski, 2009; Sardi *et al*., 2013a). For an example vaginal and skin infections stimulate a phagocytic response involving mononuclear phagocytes and neutrophilis (Vidigal and Svidzinski, 2009). For the past decades yeasts were associated with the pathogenesis of fungal infections and an immune compromised host was thought to be a contributing factor to the successful establishment of a fungal infection (Finlay and Falkov, 1989). Recently researchers discovered that *Candida* species have evolved new strategies that allows for the successful development of an infection and invasion of the host immune (Sardi *et al.*, 2013a). These strategies are scientifically defined as virulence factors (VF) (**Figure 1.2**) and they vary from one *Candida* species to another. In addition VF expression also varies with the type of infection, area of the body involved and stage of the infection (Sardi *et al*., 2013a).

The pathogenicity of *Candida* infections is facilitated by the expression of VF which plays a role in adherence to human tissues and medical devices, secretion of hydrolytic enzymes (proteases, haemolysins, lipases and phospholipases) and formation of biofilms (Sardi *et al*., 2013b). Host recognition is an important VF in *C. albicans* as it allows *C. albicans* to bind to the host cells proteins. Invasion of the host tissues is facilitated by the ability of *C. albicans* to switch from yeast cells to filamentous growth (Cullen and Sprague, 2012).

For successful adherence of *C. albicans* cells to host tissues and other abiotic surfaces, specialized proteins known as adhesins are secreted (Verstrepen and Klis, 2006; Garcia *et al*., 2011). Agglutinin-like sequence (ALS) proteins are the most studied adhesins in *C. albicans* and are very crucial in cell adhesion. In addition studies showed that ALS3 is upregulated during the infection of oral epithelial cells and vaginal infection (Cheng *et al*., 2005; Zakikhany *et al*., 2007; Naglik *et al*., 2011; Wächtler *et al*., 2011). Other important adhesins are the Hwp1 which are the hyphal associated proteins (Staab *et al*., 1999). These proteins are the substrates for the mammalian

transglutaminases that covalently links *C. albicans* hyphae to host cells. Staab *et al*. (1999) showed that Hwp1 mutants exhibited decreased adherence to buccal epithelial cells.

Following adherence of *C. albicans* to host tissues and hyphal growth, *C. albicans* secretes specialized enzymes known as hydrolases which facilitates penetration into host cells (Wächtler *et al*., 2011) and acquisition of extracellular nutrients (Naglik *et al*., 2003). There are three different classes of hydrolases which are phospholipases, proteases and lipases. Aspartic proteases are secreted to the surrounding medium and have been shown to be important in the damage of host tissues (Schaller, 1999). Phospholipases disrupt the host membrane and allow for invasion of host cells. Lipases are involved in acquisition of nutrients through digestion of lipids, adhesion to host tissues, initiation of inflammatory process through infecting immune cells and self-defence by lysing other microbes (Stehr *et al*., 2004; Gacser *et al*., 2007). A study by Gacser *et al*. (2007) showed that when secretion of lipases are inhibited, there is minimum tissue damage during infection of human tissues and also that lipase-negative mutants were significantly less virulent in mouse models. The last class of hydrolases is haemolysins which are very important in *C. albicans* for cell survival and iron acquisition (Vaughn and Weinberg, 1978). Moreover, haemolysins are involved in attacking and killing of host red blood cells and are essential for the successful establishment of infection (Manns *et al*., 1994).

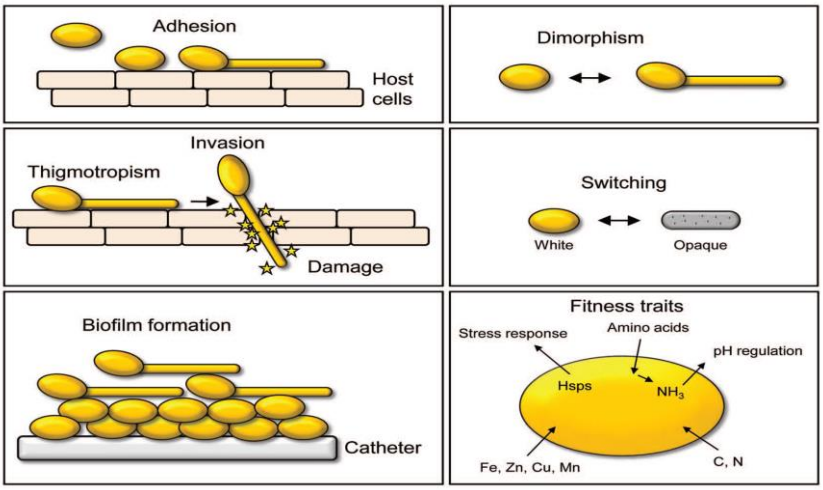


Figure 1.2: *Candida albicans* pathogenicity mechanisms (Mayer *et al*., 2013)

1.2.3 Infections caused by *Candida albicans*

Candida albicans forms part of the normal flora of an individual's mucosal oral cavity, vagina and gastrointestinal tract (Shao *et al*., 2007; Al-Dwairi *et al*., 2014). However, it becomes a major public health problem with increased medical and economic importance when it overgrows and become infectious (Almirate *et al*., 2005). Candidiasis is characterised based on the area of the body it colonises. When the infection is present in the mouth or throat it is normally identified as oral thrush and in the vagina it is identified as a genital yeast infection. Other types of *C. albicans* infections include urinary tract infections and mucocutaneous candidiasis. Some *Candida* infections are difficult to treat, and may lead to systemic infections associated with high mortality rates (Almirate *et al.*, 2005).

Oral thrush is highly common in elderly, new-born babies and immune-compromised individuals such as cancer patients, individuals infected with HIV and organ transplant recipients (Pankhurst, 2013). The symptoms of oral thrush include soreness of the mouth and throat, yellow or white patches on the tongue, gums and lips (**Figure 1.3B**). Oral thrush is treated with antifungal drugs in the form of liquids, lozenges and pills. If the infection is not treated, it may spread to the oesophagus (Pankhurst, 2013).

Genital yeast infections are more common in females than in males (Sobel *et al*., 1998). Three out of four women experience yeast infection once during their life time (Sobel *et al*., 1998). Vaginal infections occur when the balance in the vagina changes, such as during pregnancy, when using certain medication, when levels of *Lactobacillus* in the vagina are disrupted or less likely due to diabetes (Goncalves *et al*., 2016). Yeast infections can be passed from one person to the next during sexual intercourse. Everyone is at risk of contracting vaginal yeast infections but immune comprised individuals, pregnant women and those who had just taken antibiotics are at greater risk. Symptoms of genital yeast infections include; genital burning, itching and white cheese like discharge from the vagina (**Figure 1.3A**). Acute yeast infection can be treated using over-the-counter antifungal medication including pills and topical antifungal creams (Goncalves *et al*., 2016).

Urinary tract infection (UTI) is one of the infections that are most common in women rather than in men. Urinary tract infection involves infections of the bladder, urethra and kidneys in a severe case. *Candida albicans* is one of the microbes responsible for UTI. Individuals who are at increased risk of contracting UTI are those who are taking antibiotics, immune compromised

patients and patients who had a urinary catheter inserted on them. Symptoms of UTI include increased frequency to urinate, pelvic pains and presence of blood in the urine. Treatment of this type of infection includes the use of FLC antifungal drugs (Pappas *et al*., 2016).

Cutaneous or superficial candidiasis is a type of *Candida* infection that affects skin and mucous membranes (Armstrong *et al.*, 2016). This infection occurs mostly in warm, moist and sweaty areas of the body which supports growth and survival of yeast cells. The common areas of the body which are highly affected are skin between toes and fingers, armpits, area below breast and corners of the mouth (**Figure 1.3C**). Poor hygiene, diabetes, wearing one diaper for longer period and a weak immune system increases the risk of developing skin *Candida* infections. The most common symptoms include occurrence of a red rash around affected areas, appearance of blisters and lesions with pus. For skin infections due to *Candida*, antifungal creams are normally prescribed such as miconazole and clotrimazole (Armstrong *et al.*, 2016).



A. Genital yeast infection due to *C. albicans*

B. Tongue showing symptoms of *Candida* infections

C. Superficial skin infection caused by *C. albicans*

Figure 1.3: Infections caused by *C. albicans* affecting different areas of the body (Darmstadt *et al*., 2000; James and Berger, 2006).

1.2.4 Treatment and management of candidiasis

Candidiasis is one of the most common infections that are acquired during hospital stay or surgical procedures (Hidron *et al*., 2008). This affects health of most patients and becomes the reason for increasing length of hospital stay and mortality rate. Therefore hospitals have made efforts to manage and prevent the spread of such infections to patients. Different types of disinfectants are used in hospital wards for sterilization of hospital equipment to eliminate microorganisms and prevent possible infections. Formaldehyde is one of the disinfectants used

against bacteria, fungi and viruses**.** Glutaraldehyde is used for sterilization and disinfection of critical equipment such as dialysers, surgical tools and endoscopes due to its excellent microbicidal activity (Herruzo-Cabrera *et al*., 1999). Hydrogen peroxide is widely used as an environmental friendly surface disinfectant due to its excellent stability and broad-spectrum activity against bacteria, fungi and viruses (Rutala and Weber, 2008). The combination of hydrogen peroxide with peracetic acid was found to be more effective especially against glutaraldehyde-resistant mycobacteria. This combination is mostly used when disinfecting haemodialysers but it is not as effective when used in gastrointestinal endoscopes as claimed by the leading United States of America manufacturers (Guideline for isolation precaution. 2014). For disinfection of hard surfaces and spillages of clinical samples such as blood and other body fluids that may contain bacteria or viruses, sodium hypochlorite and iodophors solutions are commonly used. Low-level disinfectants such as phenols are rarely used in hospitals as they are not effective against most problematic spore-forming bacteria such as *Clostridium difficile* (Rutala and Weber, 2008). The multipurpose quaternary ammonium compound disinfectants are used as pre-operative disinfectants and are also used for hard surface cleaning and disinfection of hospital instruments. Ammonium compounds have broad antimicrobial spectrum including bacteria, fungi and most virus (Wenzel, 1995; Plowman *et al.,* 1999).

Treatment for *Candida* infections differs substantially and is based on area of the body which is infected, the patient's medical history and immune status, the specific species of *Candida* responsible for the infection and the susceptibility status of the responsible species to the specific antifungal drugs (Vincent *et al*., 1998). In the past few years, there have been significant changes in management of *Candida* infections, in terms of the use of appropriate antifungal drugs for candidiasis, mucosal candidiasis and other forms of invasive candidiasis (Pappas *et al*., 2016).

Antifungal prophylaxes have been employed as a strategy to manage candidiasis especially for patients admitted in intensive care unit and those who are at risk for the infection (Eggimann *et al*., 1999). Fluconazole is the most common antifungal agent used for prophylaxis and has shown to be the most effective (Eggimann *et al*., 1999). The use of antifungal drugs as prophylaxes have shown to be effective in reducing the incidence of candidiasis by at least 50% although the challenge with this strategy is that there has not been an increase on the survival rate of patients (Pelz *et al*., 2001).

In clinical practices, candidiasis is commonly controlled by use of three different classes of antifungal drugs which are; azoles, polyenes and echinocandins (Pappas *et al*., 2016). However there are two more antifungal agents that are used to treat candidiasis which is pyrimidine and allylamine but their use is very limited due to adverse effects (Georgopapadakou and Walsh, 1996). The pyrimidine class targets the fungal deoxyribonucleic acid and the ribonucleic acid pathways whereas allylamines drugs act by inhibiting squalene epoxidase enzyme which plays a role in the synthesis of ergosterol in yeast microbes (Birnbaun, 1990). Polyene antibiotics target the fungal cell membrane, azoles target the fungal ergosterol biosynthesis pathway and echinocandins inhibit glucan synthesis in the fungal cell (**Figure 1.5**) (Holz, 1974; Georgopapadakou and Walsh, 1996; Douglas, 2001).

1.2.4.1 Polyenes

Polyenes are a class of antifungal drugs which includes nystatin, natamycin and amphotericin B. They are large macrolide structures containing 26-28 carbon molecules with many hydroxyl groups that give them the amphipathic nature (**Figure 1.4**). Polyenes are fungicidal drugs and act against a variety of fungal species compared to other antifungal drugs (Shapiro *et al*., 2011). The molecular mechanism of polyenes involves binding to the membrane sterols, causing increased membrane permeability leading to leakage of ions (potassium, protons) and other cytoplasmic molecules resulting in cell death (Anderson *et al*., 2014). Polyenes were also found to interfere with the function of the membrane-associated oxidative enzymes which also lead to cell death (Gubbins and Anaissie, 2009).

Natamycin is used to treat eye infections of the conjunctiva, cornea and the eye lid. Natamycin is formulated as a topically cream, a lozenge or as an eye drop, and results in fungal death through the disruption of the fungal cell membrane. Natamycin was first discovered in 1955 from the fermentation of bacterium *Streptomyces natalensis* (Ellepola and Samaranayake, 2000). Natamycin is used to treat fungal infections that are due to *Candida, Cephalosporium, Penicillium and Aspergillus* species and was approved in 1978 in the United States as the most effective and safe medicine (WHO, 2017). However, when natamycin is administered orally, only small amounts of the drug or nothing is absorbed from the gastrointestinal tract making it to have poor oral bioavailability (Ellepola and Samaranayake, 2000).

Amphotericin B is an amphiphilic compound with greater affinity to bind to ergosterol in the fungal plasma membrane (Readio and Bittman, 1982). Amphotericin B is produced by the

bacterium *Streptomyces nodosus* and is one of the oldest and widely used antifungal drug. Previous studies have reported the *in vivo* and *in vitro* antifungal activity of amp B against various strains of *Candida* species, *Blastomyces dermatitidis, Coccidioides* and *Cryptococcus neoformans (*Parrillo and Dellinger, 2008; Shapiro *et al*., 2011). Amphotericin B was previously the antifungal drug of choice however there were health problems associated with its use which includes the development of nephron and hepato-toxicity (Wong-Beringer *et al.,* 1998; Fanos and Cataldi, 2001). This led to the use of a lipid formulation of amp B which is less toxic but more expensive (Herbrecht *et al.,* 2003; Groll and Kolve, 2004).

Nystatin is a slightly water-soluble compound produced from the biosynthesis of a bacterial strain *Streptomyces noursei* (Sekurova *et al.,* 2004). The structure of NYS have both hydrophilic and hydrophobic sides that enable it to intercalate into fungal cell membrane thereby forming channels through which intracellular leakage takes place (Fjaervik *et al*., 2005). Nystatin is widely used as a topical agent due to systemic toxicity (Ellepola and Samaranayake, 2000). Topical administration of NYS is effective in treating oropharyngeal candidosis (OPC) in immune-competent patients. However, the clinical cure rate of candidiasis in immune-compromised HIV patients is only 52% compared with 87% for FLC (Pons *et al*., 1997). *Candida albicans* strains resistant to NYS have been reported (Doughari and Peter, 2009; Mukasa *et al*., 2015). A study conducted in 2009 in Nigeria showed that 78.6% of all *C. albicans* isolates tested were completely resistant to NYS and other antifungal drugs (Doughari and Peter, 2009). However, NYS resistance in *C. albicans* isolates from a woman diagnosed with vulvovaginal infection was only observed in 0.61% of the screened *C. albicans* strains (Mukasa *et al*., 2015). In another study performed from HIV positive patient, NYS resistance was observed in 2.8% of the collected *C. albicans* strains, 61.1% of the isolates were susceptible while 36.1% were dose dependent susceptible to NYS (Dar *et al*., 2015).

1.2.4.2 Echinocandins

Echinocandins were the first class of antifungal drugs developed to target the fungal cell wall (Debono and Gordee, 1994). Echinocandins are lipopeptide molecules consisting of a large cyclic peptoid with N- linked acyl fatty acid chains that anchor on the fungal cell membrane to facilitate its antifungal activity (**Figure 1.4**) (Denning, 2003). Echinocandins are produced as fermentation metabolites with reduced antifungal activity; however, echinocandins are modified to improve their antifungal spectrum, solubility and pharmacokinetic characteristics (Debono and Gordee, 1994). Echinocandins are generally large in size resulting in poor oral absorption; hence they are

only administered intravenously (Grover, 2010). Three semisynthetic echinocandin derivatives were developed for clinical use namely CAS, anidulafungin and micafungin.

Echinocandins have both fungistatic and fungicidal activity depending on the target fungal species (Pfaller *et al.,* 2006). These antifungal agents are fungicidal against most *Candida* species and fungistatic against *Aspergillus* species (Sucher *et al*., 2009). Their mode of action involves inhibition of glucan synthesis via none competitive inhibition of 1, 3 - β-glucan synthase enzyme which is responsible for formation of beta-glucans in the fungal cell wall (Douglas, 2001). Destruction of glucan synthesis pathway prevents the fungi from resistance to osmotic forces leading to cell death (Adams, 2004). Echinocandins have limited fungal activity against *Candida* biofilms and have a few drug interactions compared to azoles. They have shown synergistic interaction with amp B and additive activity with FLC (Espino-Ingroff, 1998). Antifungal drug resistance in echinocandins is very low, although some studies have reported resistance in *C. parapsilosis, C. tropicalis, C. lusitanie, C. glabrata* and *C. albicans* (Bodey, 1992; Hitchcock *et al*., 1995). Antifungal drug resistance to echinocandins involves alterations in the enzyme (Fks1-Fks2 complex) responsible for glucan synthesis (Garcia-effron, 2009).

Micafungin and anidulafungin are both echinocandin derivatives commonly used as prophylaxis against invasive fungal infections. They have decreased anti-fungal activity against *C. parapsilosis* and little or no activity against *Cryptococcus neoformans.* They have shown increased fungistatic and fungicidal activity against most *Candida* species (Espinel-Ingroff, 2003). Caspofungin is a semisynthetic echinocandin derivative and the first licensed echinocandin antifungal drug in the United States (Abruzzo *et al.,* 2000). Caspofungin is commonly used in clinical practices due to its antifungal spectrum and pharmacokinetic properties (Letscher-Bru and Herbrecht, 2003). Although CAS possesses antifungal activity, it has poor oral bioavailability and poor intestinal absorption, therefore it is delivered intravenously (Bellmann and Smuszkiewicz, 2017). In a study by Abruzzo *et al*. (1997), the administration of CAS by oral route showed reduced efficacy. Resistance to CAS was reported to be less than 3% in *C. albicans* strains in the year 2005 (Castanheira *et al*., 2010). Increased CAS resistance is often reported in *C. glabrata* than any other *Candida* species (Matsumoto *et al*., 2014).

1.2.4.3 Azoles

The first azole antifungal drug was introduced in 1944 and this changed the approach to treatment of fungal infections (Saag and Dismukes, 1988). Azoles are fungistatic antifungal drugs and

contain two or three nitrogen molecules in the azole ring. They are classified as imidazoles (miconazole, clotrimazole and ketoconazole) or triazoles (itraconazole, FLC) respectively (**Figure 1.4**) (Kumari *et al.*, 2011). Imidazoles are used for superficial mycoses whereas triazoles are used for both superficial and systemic infections and have greater affinity for fungal cytochrome P450 enzyme (Zonios and Bennet, 2008). Azoles function through binding to the enzyme 14-alpha-demethylase / CPY51 which is involved in the conversion of lanesterol into ergosterol which is an essential component of the fungal plasma membrane (Georgopapadakou and Walsh, 1996). The binding of azole to this enzyme alters the structural conformation of the active site to prevent binding of lanesterol thus inhibiting the synthesis of ergosterol (Georgopapadakou and Walsh, 1996). Inhibition of ergosterol synthesis does not only disrupt the structure of the plasma membrane but also other functions such a nutrients transport and chitin synthesis (Georgopapadakou and Walsh, 1996). Azoles are administered orally with increased bioavailability making them the antifungal agents of choice in the treatment and/or prophylaxis of *Candida* infections due to their good safety profile (Zonios and Bennet, 2008).

Fluconazole is a water-soluble triazole antifungal drug that is extensively used for systematic *Candida* infections due to its safety profile and limited side effects (Saag and Dismukes, 1988; Brammer *et al*., 1990). Fluconazole is active against Dermatophytes, *Cryptococcus neoformans,* dimorphic fungi and most *Candida* species excluding *C. krusei* and *C. glabrata* (Mcllroy, 1991; Roder *et al*., 1991). However, continuous and prolonged use of FLC has led to several *Candida* species developing drug resistance against this antifungal drug (Becher and Wirsel, 2012). Bitew and Abebaw. (2018) reported FLC and flucytosine drug resistance in *C. albicans* isolates. The study of Owotade *et al*. (2016) reported that 1.94% of *C. albicans* isolated from HIV patients was resistant to FLC.

The development of new triazoles brought greater advances to the management of candidiasis due to their safety profile and increased bioavailability (Roemer and Krysan, 2014). Previously used azole (imidazoles, itraconazole) drugs have some limitations including their fungistatic nature, heterogeneous activity and their interactions with other drugs that are metabolized by the cytochrome P450 enzyme (Bodey, 1992). New triazole derivatives that have been developed recently include posaconazole, voriconazole and ravuconazole. These drugs have increased spectrum of antifungal activity and are more potent as compared to their precursors (Bodey, 1992). Voriconazole is the most recommended second generation synthetic traizole and a derivative of FLC (Hitchcock *et al*., 1995). Voriconazole have increased antifungal spectrum

compared to FLC, have greater pharmacokinetic properties than those of itraconazole and is highly absorbed orally and not affected by gastric pH (Hitchcock *et al*., 1995; Patterson and Coates, 1995).

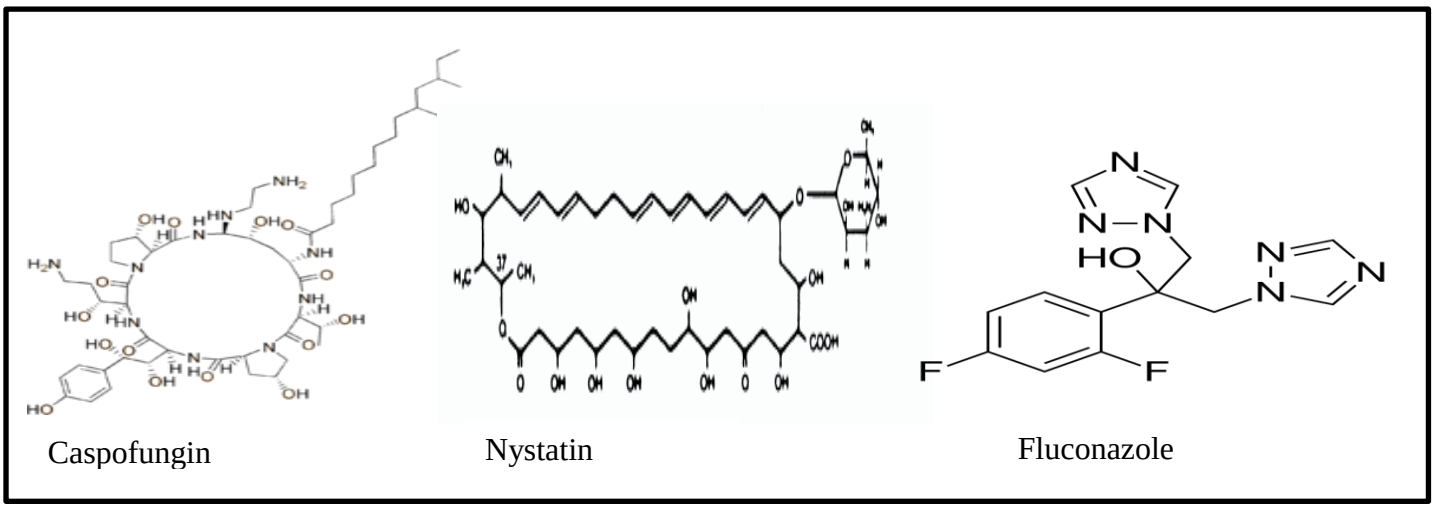


Figure 1.4: Chemical structure of three major antifungal drugs used to treat *Candida* infections (Hollier and Cox, 1995; Deresinski and Stevens, 2003; Jaervik and Zotchev, 2005).

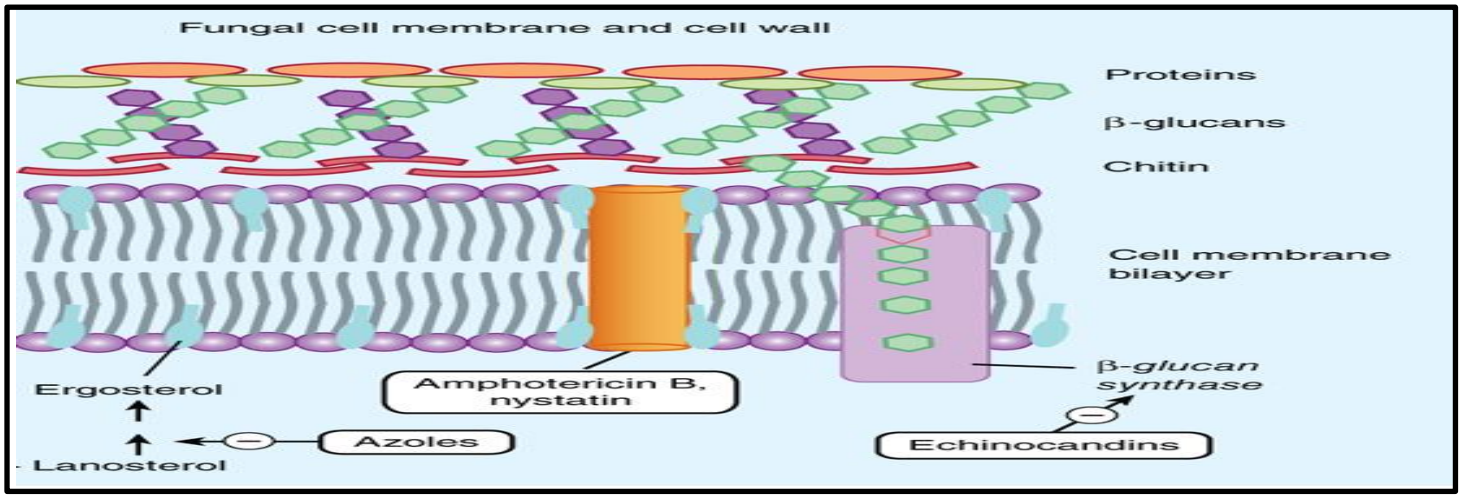


Figure 1.5: Schematic diagram indicating the mechanism of action of echinocandins, azoles and polyenes antifungal drugs on the fungal plasma membrane and cell wall (Katzung *et al*., 2012).

1.2.5 Antifungal drug resistance

The development of drug resistance in *Candida* has become a major public health problem and is a major cause of treatment failure in patients with compromised immunity (Arendrup and Patterson, 2017). Resistance is defined as a relative insensitivity of a microorganism to an antimicrobial agent tested *in vitro* (Rex *et al*., 1997). Resistance to drugs is classified into three groups which are intrinsic or primary resistances, acquired resistance and clinical resistance

(Arendrup and Patterson, 2017). Intrinsic resistance can occur naturally without previous exposure to antifungal drugs whereas acquired resistance develops in the process of antifungal treatment. Clinical resistance is referred to as a failure of antifungal treatment which depends on different factors such as the pharmacokinetics of the drugs, the infectious species and the immune status of the host (Cowen and Steinbach, 2008; Robbins *et al*., 2016). The rate at which drug resistance is attained differs greatly with the different class of antifungal drugs (Cowen and Steinbach, 2008). Azole resistance is more prevalent whereas polyene resistance is very rare and this is due to their pharmacological actions (Ellis, 2002). The most concerning issue is that most *Candida* species are becoming resistant to the first and second line antifungal treatment which is FLC and echinocandins (Perlin, 2011; CDC, 2013).

1.2.5.1 Mechanism of drug resistance

There are various mechanisms that are deployed by *Candida* species to overcome the effect of antifungal drugs. Each class of antifungal drug has a distinct mode of action against the target pathogen hence the mechanism of resistance is associated with the characteristic of the pathogen that interferes with the antifungal activity of the drugs. Antifungal drug resistance in *Candida* species is associated with drug target alteration, overexpression of genes and reduction of intracellular accumulation of the drug (Venkateswarlu *et al.,* 1995; Sanglard *et al.,* 1995).

1.2.5.2 Drug target alteration and overexpression of genes

The most common drug resistance mechanism employed by most microorganisms includes evolving mutations in the target genes which reduce drug efficacy and binding (Robbins *et al.*, 2017). This mechanism is prevalent in *Candida* species against azole antifungal drugs. Previous studies have reported various mutations in the azole target gene *ERG11* which encodes for 14-alpha-demethylase enzyme in *C. albicans* isolates. These mutations therefore were reported to be localised in the hot spot regions by the enzyme binding site (Vanden *et al*., 1990; Franz *et al*., 1998; Marichal *et al*., 1999). Therefore these mutations reduce the affinity of azoles to bind the 14-alpha-demethylase enzyme and prevent ergosterol production. However, this mechanism is only effective when there is upregulation of the *ERG11* gene (Morio *et al.*, 2010). The drug target alteration mechanism has also been observed in *C. albicans* and non *C. albican* species against echinocandins (Michael and Pfaller, 2012). Mutations in the gene *FKS1* which encodes for the major and presumed catalytic subunit of 1, 3-β-D-glucan synthase enzyme are responsible for the resistant phenotype against echinocandins through reducing the binding affinity of the drugs to the enzyme (Park *et al*., 2005). These mutations were reported to be clustered in two different hot spot

regions of the complex which are the *HS1* and the *HS2* of the *FKS1* gene (Park *et al.,* 2005; Perlin, 2007).

1.2.5.3 Reduction of intracellular drug accumulation

Microorganisms have evolved strategies to avoid the accumulation of toxic drugs intracellularly. Low amounts of drug reaches the target site leading to the survival of the microbe. This resistance mechanism involves activation of the drug efflux pumps located on the cells plasma membrane to pump the toxic drugs and antibiotics out of the cells (Cannon *et al*., 2009). There are two main classes of efflux pumps which are the ATP-binding cassette (ABC) proteins and the major facilitator superfamily (MFS). The ABC proteins uses the hydrolysis of adenosine triphosphate (ATP) as a source of energy to transport compounds across the membrane whereas the MFS pumps use proton-motive force to transport compounds across the membrane (Prasad and Rawal, 2014). The three most important efflux pumps in *C. albicans* are cdr1p, cdr2p, mdr which are encoded by *CDR1, CDR2, MDR1* genes respectively (Coste *et al*., 2006; Morschhauser *et al*., 2007). Overexpression of these genes was found to be the main mechanism of azole resistance in *Candida* species (Chen *et al.,* 2010). Several studies have reported that azole resistant isolates display transcriptional activation of genes encoding cdr1p, cdr2p and mdr proteins (**Figure 1.6**) (Sanglard *et al.,* 1997; Cannon *et al*., 2009). Furthermore, *Candida* isolates reported to have increased expression of efflux pump genes have also been found to show increased efflux activity of antifungal drugs (Cannon *et al*., 2009).

Ahmad *et al.* (2013) reported on the reversal of FLC drug resistance by two monoterpene phenols, thymol and carvacrol that blocked the drug transport pumps. A study done by Yaojun *et al*. (2016) showed that beauvericin counteracted multi-drug resistant *C. albicans* by blocking the ABC transporters. In their study, they investigated the effect of beauvericin on ABC transporters using rhodamine 6G dye (R6G) as an indicator to study the efflux function of *C. albicans* ABC transporters. It was found that beauvericin inhibited the efflux of R6G dye by ABC transporters even in the presence of glucose.

Another study conducted by Bhattacharya *et al*. (2016) reported on the combination fluorescent assay which demonstrated increased efflux pump activity as a resistance mechanism in azole-resistant vaginal *Candida albicans*. The authors have determined minimum inhibitory concentrations (MICs) against FLC, itraconazole and clotrimazole for 38 *C. albicans* isolates. Minimum inhibitory concentration values indicated that 22, 18 and three isolates were resistant to

FLC, clotrimazole and itraconazole respectively. The efflux pump activity of the isolates was also assessed by using two fluorescence dyes, alanine- β- napthylamide for the detection of the ABC and MFS transporters and R6G for detection of ABC transporters. Their results indicated that the two efflux assays have strong agreement between high efflux rates and azole drug resistance. They also performed gene expression studies where they measured the messenger ribonucleic acid expression levels of drug resistant genes. The real time polymerase chain reaction (PCR) was used to measure the level of gene expression of four genes that are associated with antifungal drug resistance: *CDR1*, *CDR2*, *MDR1* and *ERG11*. Of the 22 FLC resistant isolates, 12 have shown overexpression of *CDR1* gene, 10 have shown overexpressed of *CDR2* gene, 11 have shown upregulation of MDR1 expression and 14 have shown overexpression of *ERG11* gene compared to the control strain. Collectively, overexpression of efflux pumps was observed in 72% of FLC resistant isolates.

Szczepaniak *et al.* (2017) reported on blocking and dislocation of the *Candida albicans cdr1p* transporter by styrylquinolines. The authors performed drug susceptibility testing of styrylquinolines against the *C. albicans* wild-type strains and mutant strains lacking cdr1p, cdr2p or both transporters. All strains were sensitive to styrylquinolines with MICs less than 0.4mM. The rhodamine 6G assay was carried out to determine the effect of styrylquinolines on ABC transporters. Two compounds of styrylquinolines effectively reduced the efflux of R6G dye in wild-type strains by at least 2 fold compared to control.

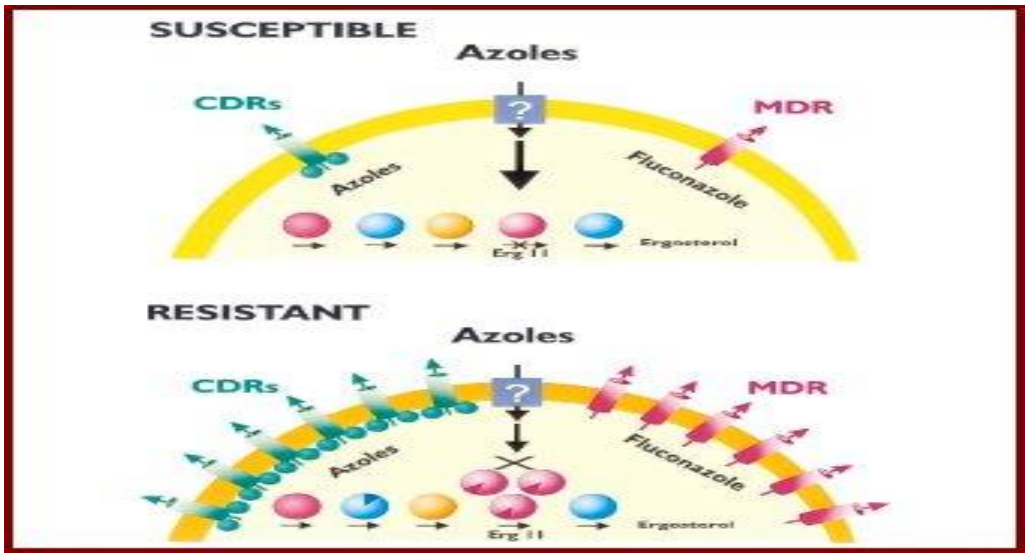


Figure 1.6: Schematic diagram indicating the role of drug efflux pumps in conferring antifungal drug resistant in *C. albicans*. Overexpression of drug efflux pumps (CDRs and MDR) in azole resistant strains is responsible for the resistant phenotype (White *et al.*, 1998).

1.2.6 Alternative antifungal treatment

The incidence of infectious diseases continues to rise worldwide and drug resistance has become a major threat in public health. Hence the development of new antimicrobials is critical. Back in the olden days certain plants were believed to have some healing properties such as anti-inflammatory, anti-oxidant and antimicrobial properties. These plants were greatly used to heal common infections and certain diseases. Today some of those plants are still used and are part of the habitual therapy such as the use of cranberry juice from the plant *Vaccinium macrocarpon* to treat infections associated with the urinary tract (Heinrich *et al*., 2004). In addition garlic from the plant *Allium sativum*, lemon balm from the plant *Melissa officinalis* and the tee tree from the plant *Melaleuca alternifolia* have also been reported in the manuals of phytotherapy as antimicrobial agents with increased antimicrobial spectrum (Heinrich *et al*., 2004). The actual active components of these plants were found to be the essential oils rather than the plant extracts that had the greatest use in the treatment of infectious diseases (Chouhan *et al.,* 2017).

Essential oils can be used as alternative antifungal therapy as they have been reported as safe and effective antifungal agents against *Candida* infections (Serra *et al*., 2018). The majority of essential oils that have been investigated for their antifungal activity are found on natural plants making them less expensive and with decreased side effects including toxicity. Wilkins and Board. (1989) reported that there are more than 1340 plants which are potential sources of antimicrobial compounds but only a few have been studied. Moreover, essential oils can be investigated for their anti-microbial activity in combination with clinically used antifungal drugs. The resulting combination interaction may be synergistically resulting in safe and cost-effective treatment (Khan *et al*., 2012).

Eugenol (4-allyl-1-hydroxy-2-methoxybenzene) (EUG), a major phenolic compound found in clove oil from the plant *Eugenia caryophyllata thunb* **(Figure 1.7)**, is a natural compound that has been extensively studied for its pharmacological actions such as antimicrobial, anti-oxidant, anti-inflammatory and local anaesthetic effects (Koch *et al.,* 1971; Pramod *et al*., 2010). Over the past years, EUG was greatly used in dentistry as the main ingredient in different dental materials such as in filling and impression materials and also in dental cement (Sarrami *et al*., 2002). However, EUG was later reported to be toxic to human soft tissues (Sarrami *et al*., 2002). Many adverse dental cases have been reported following the use of EUG in mouth rinses, surgical and periodontal packs and in impression pastes (Koch *et al*., 1971; Vilaplana *et al*., 1991). As a result,

the use of EUG in dental treatment was phased out due to toxicity issues and other unfavourable physicochemical properties such as low solubility, pungent odour and sublimation (Zhang *et al*., 2013).

There are various methods that have been studied to improve the physicochemical and biological properties of EUG and making it an effective and safe drug. These methods include modification of EUG through the process of tosylation or glycosylation to produce EUG analogues with favourable physicochemical properties (Zhang *et al*., 2013; Ahmad *et al*., 2015). Ahmad *et al*. (2015) reported on synthesis of ETCs which are analogues of EUG synthesized through the process of tosylation. This process involves treatment of the hydroxyl group of EUG with p-toluene-sulfonyl chlorides and different functional groups under suitable conditions resulting in synthesis of different compounds which are referred to as ETCs (**Table 1.1**). Eugenol tosylate congeners are classified as semi-synthetic drugs and were first synthesized to investigate their mode of action against *C. albicans* through targeting cytochrome P450 14α-sterol demethylase enzyme (Ahmad *et al*., 2015).

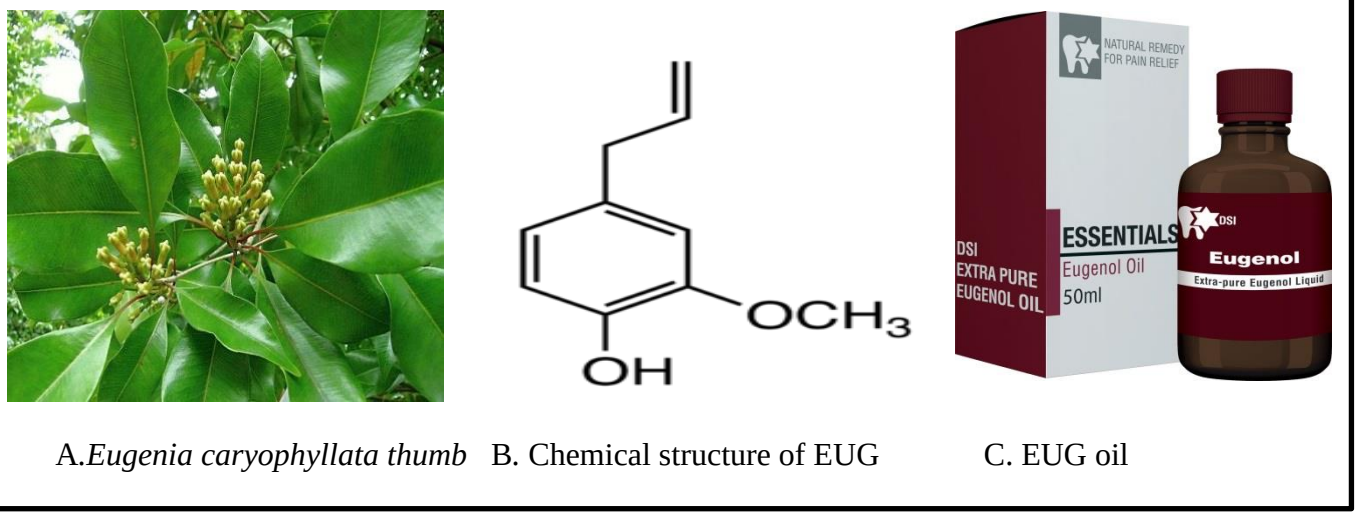
Eugenol was reported to perform better as an antimicrobial agent in combination with known antifungal drugs hence it can be used in combination therapy against multiple drug resistant *C. albicans* (Ahmad *et al*., 2015). There are various studies reporting on the antimicrobial activity of EUG and its derivatives. The study by de Souza *et al.* (1996) reported on the potent antimicrobial activity of bromo EUG derivatives. Ahmad *et al*. (2015) studied the synergistic interaction of ETCs with FLC against *C. albicans*. They found that EUG had the highest MIC of 500µg/ml compared to MICs of ETCs which ranged between 1µg/ml and 62µg/ml and MICs of FLC ranged between 4µg/ml and 25µg/ml for both clinical susceptible and resistant *C. albicans* isolates.

A study by Sajjad *et al.* (2012) reported on the anti-candidal activity of essential oils alone and in combination with amp B or FLC against multi-drug resistant isolates of *Candida albicans*. In their study, they performed drug susceptibility testing using three azole drugs; FLC, itraconazole and ketoconazole and amp B against *C. albicans* strains. Their results showed that all *C. albicans* strains were resistant to three azole drugs with MICs ranging from 128– 256µg/ml for FLC, 4 – 128µg/ml for itraconazole and 2-256µg/ml for ketoconazole. However, 13 isolates were resistant to amp B with MICs ranging from 32–128µg/ml and the sensitive isolates had MICs of 0.25 – 1.0µg/ml.

In the same study, they investigated the antimicrobial activity of 17 plant essential oils and four active compounds. Only four essential oils and four active compounds (*Cinnamomum verum*, EUG, cinnamaldehyde, *Cymbopogon citratus*, *Cymbopogon martini*, citral, geraniol and *Syzygium aromaticum*) were active against *C. albicans* strain. *Cymbopogon martini*, citral and cinnamaldehyde were the most active compounds in inhibiting the growth of *Candida* with MICs ranging from 90 – 100μg/ml (Sajjad *et al.*, 2012).

The authors have also determined the fractional inhibitory concentration index (FICI) by investigating the combination activity of the four active essential oils and active compounds with FLC and amp B against four *C. albicans* strains. A FICI of ≤ 0.5 was recorded as synergistic, >0.5-4.0 as indifferent (no interaction) and >4.0 as antagonistic. All compounds combined with FLC and amp B revealed different levels of drug interactions; however, EUG exhibited synergistic effect when combined with both FLC and amp B with FICI of 0.187 for both combinations. Citral showed increased synergy in combination with FLC whereas cinnamaldehyde showed increased synergy in combination with amp B. Above all there was no drug combination that exhibited an antagonistic effect against the tested strains.

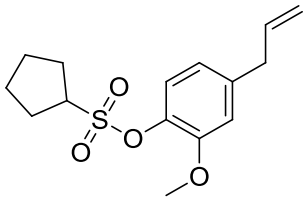
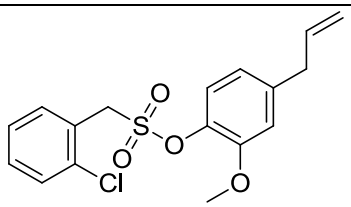
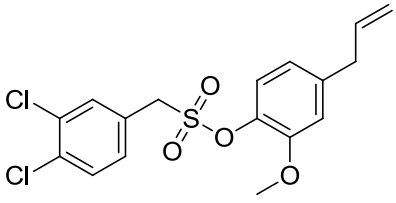
The toxicity effects associated with the use of EUG as an antifungal agent have been extensively studied over the past years (Hemaiswarya and Doble. 2009; Khan *et al*., 2011; Gündel *et al*., 2019). Different eukaryotic cell types have been used to illustrate the toxicity effects of EUG. In a study by Khan *et al*. (2011) the haemolytic effect of essential oils and active compounds including EUG on sheep red blood cells (RBC) were reported. Their results revealed that EUG showed decreased haemolysis of sheep RBC at their fungicidal concentration against fungi. At a concentration of 1152μg/ml, the percentage haemolysis caused by EUG on sheep RBC was 5.6%. In another study by Gündel *et al*. (2019) EUG showed moderate toxicity to human mononuclear cells of peripheral blood at a concentration of 500μg/ml and reduced cell viability by 30%. A study reported by Hemaiswarya and Doble. (2009) EUG caused 5.2% haemolysis on human RBC at a concentration of 2.5mM (0.41mg/ml).

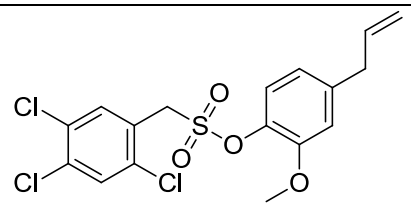
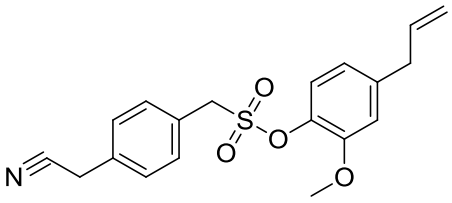
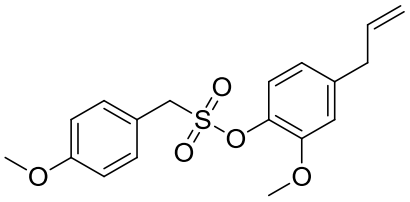


A.*Eugenia caryophyllata thumb* B. Chemical structure of EUG C. EUG oil

Figure 1.7: Shows the plant *Eugenia caryophyllata thumb* (A), the chemical structure of EUG (B), the commercial oil of EUG (C).

Table 1.1: Different structures of ETCs with their molecular weight and chemical name.

ETCs	Structure of compound	Chemical name
Congener 1 M.W=296.38		4-allyl-2-methoxyphenyl cyclopentanesulfonate
Congener 2 M.W=352.83		4-allyl-2-methoxyphenyl (2-chlorophenyl)methanesulfonate
Congener 3 M.W=387.28		4-allyl-2-methoxyphenyl (3,4-dichlorophenyl)methanesulfonate

Congener 4 M.W=421.72		4-allyl-2-methoxyphenyl (2,4,5-trichlorophenyl)methanesulfonate
Congener 5 M.W=357.42		4-allyl-2-methoxyphenyl (4-(cyanomethyl)phenyl)methanesulfonate
Congener 6 M.W=348.41		4-allyl-2-methoxyphenyl (4-methoxyphenyl)methanesulfonate

1.2.7 Combination therapy

The increased emergence of drug resistance to the three major classes of antifungal agents (azoles, polyenes and echinocandins) and the limitations associated with them such as toxicity and increased cost has led to the need for the development of new strategies to increase the efficacy of available antifungals and to reduce the adverse effects associated with them. To overcome these challenges, combination therapy which is the use of two or more drugs simultaneously with a synergistic effect was introduced over the past two decades as an alternative therapy to treat complex infectious diseases. This type of therapy has shown to have greater potential over monotherapy in terms of alleviating the emergence of drug resistance and decreasing dose-related toxicity which is a major problem with the currently used drugs (FLC and amp B) (Khan *et al*., 2012). In addition, combination of two agents can produce an increased killing activity resulting into a decrease in the pathogen population size and reducing the period of treatment (Hill and Cowen, 2015; Spitzer *et al*., 2017).

With all the above-mentioned advantages that come with the use of combination therapy, the overall goal for employing it is to broaden the antimicrobial spectrum of the interacting drugs through obtaining a synergistic or additive effect. The relation of the interacting drugs can either be additive, synergistic, indifferent or antagonistic (Levin and Harris, 1975). An additive interaction is observed when the combined activity of the two drugs is lesser than the sum of the activity of the drugs used alone. Synergy is obtained when the combined activity of the drugs is greater than the sum of the activity of the drugs alone and indifference is observed when the combination of the two drugs does not show increased activity compared to the most active antifungal drug used alone (Levin and Harris, 1975). The most unfavourable interaction is antagonism which is obtained when the combined activity of the drugs is lesser than the activity of the least active drug (Levin and Harris, 1975).

There are several studies reporting on the combination activity of natural compounds with known antifungal drugs. The study of Ahmad *et al*. (2013) investigated the synergistic activity of two monoterpenes (carvacrol and thymol) with FLC in the reversal of efflux-mediated antifungal resistance. Combination interaction studies were performed between FLC and the two monoterpenes. Synergy was observed in 84% of the combinations between FLC and thymol against susceptible isolates and 72% of combinations showed synergy in resistant *Candida* isolates. Combinations between FLC and carvacrol showed 89% of synergy in susceptible strains and 90% of combinations showed synergy in resistant strains.

The study of Shrestha *et al.* (2015) studied a combination approach to treating fungal infections. In their study, they reported antifungal activity of an amphiphilic Tobramycin analogues C_{12} and C_{14} alone and in combination with four azoles; FLC, itraconazole, posaconazole and voriconazole against seven *C. albicans* strains. Their results illustrated that all strains were synergistically inhibited by C_{12} when combined with all azoles with the exception of two strains in the combination of C_{12} with FLC and voriconazole. Fractional inhibitory concentration index values obtained ranged from 0.12-1 for FLC, 0.07-0.5 for itraconazole and 0.07-0.75 for posaconazole and voriconazole. The combination of C_{14} with azoles synergistically inhibited growth in some strains. Most synergistic interactions were observed when C_{14} was combined with posaconazole and itraconazole whereas a few synergies were observed in combination with FLC and voriconazole. The FICI values obtained ranged from 0.28-1 for FLC with C_{14}, 0.18-0.62 itraconazole with C_{14}, 0.18-0.49 for posaconazole with C_{14} and 0.14-1 for voriconazole with C_{14}. The MICs of C_{12} and C_{14} against *C. albicans* strains ranged from 16-32µg/ml and 8µg/ml

respectively. Minimum inhibitory concentration values ranged from 12.5-25µg/ml for itraconazole, 25µg/ml for FLC, 10-20µg/ml for posaconazole and 10µg/ml for voriconazole.

The study of Liu *et al.* (2017) reported on the synergistic antifungal effect of FLC combined with licofelone against resistant *C. albicans*. In this study, they determined the MICs of licofelone and FLC in all isolates. The MIC of licofelone was 128µg/ml in all isolates and that of FLC ranged from 1-512µg/ml. The combination of licofelone with FLC exhibited strong synergistic antifungal effects against resistant *C. albicans* strains. In FLC resistant strains, MIC of FLC was 512µg/ml but when combined with licofelone MIC was reduced to 1 and 0.5µg/ml in each strain. The FICI for resistant strains was 0.127 and 0.250 indicating a synergistic interaction. In the same study, they also determined the effect of licofelone on activity of the drug efflux pumps in FLC resistant *C. albicans* strains. Rhodamine 6G assay was used to study the role of licofelone on the drug efflux pumps. Their results showed that licofelone treated isolates and the control group (untreated) have the same declining trend. Therefore there was no significant difference observed between the two groups.

The study of De Castro *et al*. (2015) investigated the antifungal activity and mode of action of thymol and its synergism with nystatin against *Candida* species. In their study, they determined MICs and Minimum Fungicidal Concentrations (MFC) of thymol and nystatin against *C. albicans, C. tropicalis* and *C. krusei*. Minimum inhibitory concentration and MFC values for thymol were 39µg/ml against *C. albicans* and *C. krusei* and 78µg/ml for *C. tropicalis*. For NYS, the MIC and MFC values were 1.9µg/ml for all three *Candida* species. In the same study, they also determined the combination activity of thymol and NYS in a 1:1 combination ratio and calculated FICI for all combinations. All combinations were synergistic with a FICI value of 0.25 for all three *Candida* species.

The study of da Silva *et al*. (2017) investigated the antifungal activity of EUG and its association with nystatin in *C. albicans*. In their study, they determined MIC and MFC of EUG, NYS and CAS against *C. albicans* ATCC90028 strain. Minimum inhibitory concentration and MFC values were 625µg/ml and 25µg/ml for EUG and NYS respectively. They also investigated the combination activity of EUG and NYS against *C. albicans* strain. Their results showed an indifferent effect between the two compounds (EUG and NYS) with a FICI value of 1.125. However, when the two compounds were combined, the MIC of NYS was reduced from 25µg/ml to 3.125µg/ml but that of EUG remained the same at 625µg/ml.

In another study by Canturk. (2018), the synergistic anticandidal effect of ferulic acid and CAS against *C. albicans and C. glabrata* was investigated. An antimicrobial assay followed by a checkerboard microdilution assay for ferulic acid and CAS was performed. The MIC of ferulic acid and CAS against *C. albicans* was 40µg/ml and 2µg/ml respectively and for *C. glabrata* 20µg/ml and 4µg/ml respectively. The combination activity of the two compounds (ferulic acid and CAS) showed a synergistic interaction with FICI values of 0.0375 and 0.1875 for *C. albicans* and *C. glabrata* respectively.

1.2.8 Proton pumps as new drug target

There are several studies that have reported on the importance of fungal proton *ATPase* (H^+ *ATPase*) as an emerging drug target. Previous studies have indicated that the fungal H^+ *ATPase* is important for fungal cell growth, maintenance of intracellular pH and plays a role in the pathogenicity of fungal species (Monk, 1994; Manzoor *et al.*, 2004; Ahmad *et al*., 2010; Liu and Kohler, 2015). All these characteristic features make this enzyme a good target for drug discovery. *ATPases* are membrane-bound enzymes that couple ion movement through a membrane using the hydrolysis and synthesis of ATP **(Figure 1.8)** (Tsang *et al*., 2014). There are different types of *ATPases* and are classified based on their functional properties such as F-, A-, P-, E-, and V- type *ATPases*. The V- type and P-type *ATPases* are more important in regulating pH homeostasis in *C. albicans* (Tsang *et al*., 2014).

The P-type *ATPases* are located on the plasma membrane of several eukaryotic species. P-type *ATPase* functions to translocate various compounds across the plasma membrane including phospholipids and ions using the hydrolysis of ATP as a source of energy (Perlin *et al.*, 1997). There are several *ATPase* enzymes that belong to the P-type class and transport different ions such as; potassium (K^+), sodium (Na^+) *ATPase* of animal cells, calcium (Ca^{2+}) *ATPases* of red blood cells and H^+ *ATPases* of some eukaryotic cells. The regulation of P-type *ATPase* is complex and little work has been done on it. The enzyme is known to be autoregulated through the production of the membrane potential (Seto-Young and Perlin, 1991). The fungal P-type *ATPase* is constantly phosphorylated during biogenesis and also when glucose is metabolised (Chang and Slayman, 1991). In lower eukaryotes, P-type *ATPase* is essential for maintaining the organisms' intracellular pH and the electrochemical proton gradient which is important for nutrient uptake (Manzoor *et al*., 2004).

The vacuolar proton *ATPase* is known as the V-type *ATPase* and plays a significant role in generating and sustaining pH gradient in organelles (Dechant and Peter, 2011). Vacuolar *ATPase* contributes to the regulation of cytosolic pH in fungi (Martinez-Munoz and Kane, 2008). Inactivation of the V-type pumps changes the intracellular and extracellular pH of fungal cells and this disturbs various cellular processes including processing and sorting of proteins, receptor-mediated endocytosis and autophagy (Kane, 2006). The extracellular pH of fungi plays a vital role in establishing its morphology and in acidic environment it appears in yeast form (Sudbery, 2011). When pH increase it triggers hyphal formation and it becomes virulent. *Candida albicans* V-type *ATPase* is essential for many physiological and virulent activities (Naglik *et al*., 2003). Furthermore, the secretion and the activity of lipases and proteinases virulence attributes requires an optimal vacuolar pH (Naglik *et al*., 2003). *Candida albicans* strains that have altered vacuolar activity have shown decreased filamentation and are less virulent (Eck *et al*., 2005; Palanisamy *et al*., 2010; Zhang *et al*., 2010).

The study of Ahmad *et al*. (2010) reported the proton translocating *ATPase*-mediated fungicidal activity of EUG and thymol. The authors investigated the ability of *Candida* isolates to pump out intracellular protons (H^+) to the external medium and reported that when cells were treated with EUG and thymol at MIC values the retention of H^+ efflux was 36% and 54% respectively. The study also revealed that glucose-stimulated H^+ efflux in both drug susceptible and resistant strains was reduced. Moreover, they investigated the intracellular pH of *Candida* isolates to check if cells with normal H^+ *ATPase* activity maintain the constant internal pH as cells with dysfunctional *ATPase* activity. They reported that cells with normal H^+ *ATPase* activity maintained intracellular pH while cells exposed to EUG and thymol had decreased intracellular pH.

In another study by Manzoor *et al*. (2004), they reported the inhibition of H^+ efflux by phosphocreatine in *C. albicans*. Their study showed that cells treated with increasing concentration (1mM, 2mM, and 5mM) of vanadate showed decreased acidification of pH 6.85 and 6.95. However untreated cells showed strong extracellular acidification. Phosphocreatine also showed decreased acidification similar to the trend obtained in cells treated with vanadate. In the same study, they measured the rate of H^+ efflux in the presence and absence of different concentration of vanadate in *C. albicans* cells. Cells treated with 1mM, 2mM and 5mM vanadate showed 66%, 77%, and 88% reductions respectively in the rate of H^+ efflux compared to untreated cells. Phosphocreatine at the concentration of 25mM showed 83% reduction in the rate of H^+ efflux.

The combination of the two compounds, vanadate (5mM) and phosphocreatine (25mM) showed a decreased rate of H^+ efflux with a percentage reduction of 99%. Manzoor *et al*. (2004) suggested that vanadate and phosphocreatine interact with H^+ *ATPase* in almost the same manner hence their effect on the rate of H^+ efflux is more or less equal. Phosphocreatine was also thought to bind to the H^+ *ATPase* enzyme through the phosphate moiety to bring about conformational change whereas vanadate which is an inhibitor of the plasma membrane H^+ *ATPase* is an analogue to phosphate, it was therefore suggested that the binding of vanadate to the H^+ *ATPase* occurs where ATP binds through its phosphate group.

The study of Tsang *et al*. (2014) reported that the sub MIC levels of purpurin inhibit membrane *ATPase*-mediated H^+ efflux activity in human fungal pathogen *C. albicans*. They investigated the effect of purpurin at different concentrations (0.1-0.5µg/ml) on the activity of H^+ efflux by measuring the pH of the external environment. They found that the effect of purpurin on the pH of the external medium is significant compared to the control and it is concentration dependent. In addition, decreased concentrations of purpurin were required to reduce glucose-dependent H^+ efflux activity. This altered the pH homeostasis of *C. albicans* cells and resulted in a reduction in acidification of external medium.

A study by Liu and Kohler. (2015) reported on the antagonism of FLC and a proton pump inhibitor in *C. albicans*. In their study, they investigated the effect of blocking H^+ efflux from fungal cytosol to the extracellular environment using omeprazole while disturbing transportation of H^+ from the cytosol to the vacuole with FLC suggesting that this may lead to toxic acidification of the cytosol and antifungal synergy. The combination of omeprazole with FLC exhibited an antagonistic interaction whereby omeprazole prevented cell growth inhibition by FLC. They also determined the effect of the two drugs on the cytosolic pH. Their results indicated that cytosolic pH of *C. albicans* cells became acidic following treatment with omeprazole and the results were consistent with the concept that omeprazole inhibits the activity of the plasma membrane H^+ *ATPase*. The cytosol of cells exposed to FLC was not acidified. Omeprazole in combination with FLC neutralised pH of the cytosol antagonizing the effect of omeprazole. Micafungin was used as a control at low concentrations and neutralized pH of the cytosol. In addition, the effect of FLC and omeprazole were investigated on the acidification of the fungal vacuole. The vacuole of cells exposed to FLC showed a neutral pH as well as the vacuole of cells exposed to omeprazole and both drugs. However, cells exposed to Micafungin showed acidification of the vacuole.

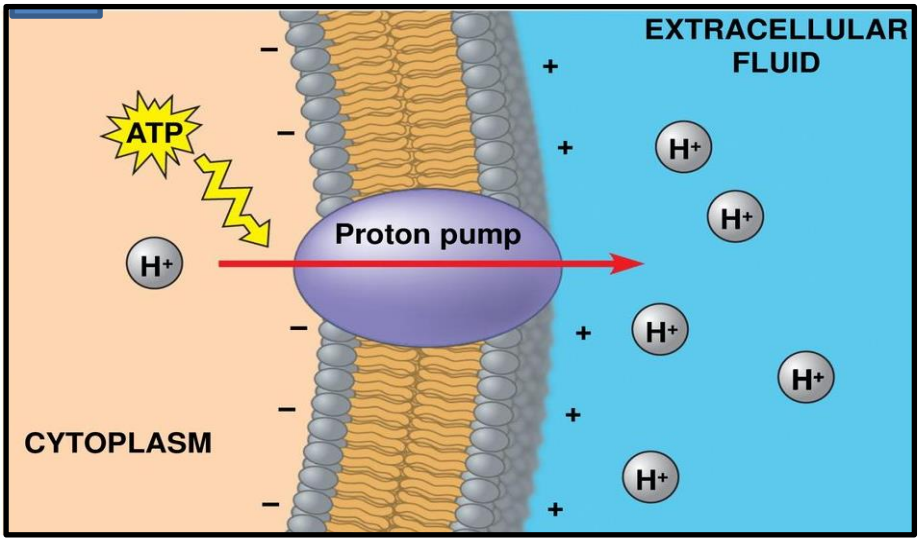


Figure 1.8: Schematic diagram indicating the Proton *ATPase* on the fungal plasma membrane responsible for the efflux of protons to the extracellular environment (Fitzpatrick and Tunbridge, 2016).

1.3 STUDY RATIONALE

The continuous use of antifungal drugs has resulted in drug resistance in some *C. albicans* strains. In order to circumvent this increase in antifungal drug resistance, new therapeutic approaches need to be developed and more studies on the mechanisms of antifungal drug resistance need to be conducted. Although baseline information on the resistance status of *C. albicans* strains is available and combination therapy has already been studied as an alternative antifungal treatment, there are no reported studies on the use of ETCs in combination with NYS and CAS against *C. albicans.* Therefore, there is a need for more knowledge on the combination activity of NYS and CAS with ETCs as an alternative treatment for resistant *C. albicans* strains. In addition, new drug target in *C. albicans* have been identified such as targeting the H^+ *ATPase* pump and the drug efflux pumps. The effect of ETCs on inhibiting the activity of H^+ *ATPase* and drug efflux pumps has not been reported before and is therefore of significance to be studied.

1.4 AIM OF THE STUDY

To evaluate the modulation of antifungal drug resistance and combination activity of eugenol tosylate congeners with antifungal drugs in *Candida albicans.*

1.5 OBJECTIVES OF THE STUDY

1. To determine drug susceptibility of different clinical *C. albicans* isolates against FLC, NYS and CAS.
2. To investigate the antifungal activity of ETCs (C1-C6) against drug susceptible and drug-resistant *C. albicans* strains.
3. To determine the antifungal activity of the combination of ETCs (C1-C6) with antifungal drugs in a 1:1 ratio against drug susceptible and resistant *C. albicans* strains by calculating the FICI values.
4. a. To evaluate the effect of ETCs (C1-C6) on the activity of H^+ *ATPase* by determining the rate of H^+ efflux in FLC susceptible and resistant *C. albicans* strains.

   b. To evaluate the effect of ETCs (C1-C6) on intracellular pH of FLC resistant and susceptible *C. albicans* strains.
5. To evaluate the effect of ETCs (C1-C6) on the activity of drug efflux pumps in FLC susceptible and resistant *C. albicans* strains.
6. To investigate the haemolytic effect of ETCs (C1-C6) on horse RBC.

CHAPTER 2: MATERIALS AND METHODS

2.1 *Candida albicans* strains and antifungal drug susceptibility testing

The drug susceptibility test was performed to determine the susceptibility status of *C. albicans* isolates to FLC, NYS and CAS antifungal drugs. Minimum inhibitory concentrations defined as the lowest drug concentration that inhibits microbial growth were determined using the broth microdilution method as described by the Clinical Laboratory Standard Institute (CLSI, 2008).

2.1.1 Collection of *Candida albicans* isolates

Fifty *C. albicans* isolates were used in this study. Twenty-two isolates were collected from cancer (head and neck) and 28 were from HIV positive patients with oral candidiasis from Charlotte Maxeke Johannesburg Academic hospital. The isolates were collected in 2015 by the principal investigator, Dr Julitha Molepo from the saliva of cancer and HIV positive patients. Patients were given sterile distilled water to rinse their mouth and spit the water into the sputum jar. *Candida albicans* isolates were identified using API® ID 32 kit following the manufacturer's instructions (BioMérieux, Marcy-l'Étoile, France)**.** These isolates were stored in sterile distilled water at -20°C in the Department of Oral Biological Sciences at the University of the Witwatersrand, Johannesburg. The reference strain *C. albicans* ATCC90028 was purchased from the National Health Laboratory Services (Johannesburg, South Africa) and was used as a quality control strain. The study was approved by the Human Research Ethics Committee (HREC) (Wits University), waiver number W-CJ-170607-1 (Appendix 2).

2.1.2 Antifungal drugs

The antifungal drugs tested in this study included FLC, NYS and CAS and were purchased in powder form from Sigma Aldrich (St. Louis, Missouri, United States). The antifungal drugs were prepared according to the manufacturer's instructions. Briefly, FLC and NYS were dissolved in 100% dimethyl-sulfoxide (DMSO) to yield a concentration of 50mg/ml and 25mg/ml stock solutions respectively. For working solutions, the drugs were further diluted with sterile distilled water to yield a concentration of 1mg/ml for FLC and 0.625mg/ml for NYS. Caspofungin was dissolved in sterile distilled water to yield a stock concentration of 15.24mg/ml and further diluted to a working concentration of 0.625mg/ml.

2.1.3 Preparing the standardized inoculum for broth microdilution

Candida albicans isolates were sub-cultured on Sabouraud Dextrose Agar (SDA) plates and incubated at 37°C for 24 hours. A standardised inoculum (0.5 McFarland standards) of *C.*

albicans (1.5 x10^6 CFU/ml) was prepared by suspending five to six colonies from a 24 hours culture in 5ml of 0.9% normal saline.

2.1.4 Broth Microdilution method

The antifungal drug susceptibility test was performed on fifty *C. albicans* isolates using the broth microdilution method according to the Clinical and Laboratory Standard Institute M27-A2 (CLSI, 2008). The assay was carried out on a 96 microtiter plate. Sabouraud Dextrose Broth (SAB) was added to each well of the plate at a volume of 100 µl. Hundred microliters of the antifungal drugs: FLC, NYS and CAS were added to the first wells of the first row of a 96 microtiter plate followed by a two-fold serial dilution. A hundred microliters of the standardised inoculum were then added to all dilutions. For growth control, wells containing 100µl of SAB medium with no drugs and 100µl of the inoculum suspension were included. A negative control (solvent control) well containing 1% DMSO and the inoculum suspension was also included. Another negative control well containing SAB medium only was included to confirm the viability and sterility of the experiments. Experiments were performed in triplicate to validate the results. The microtiter plates were incubated at 37°C for 24 hours. The presence or absence of *C. albicans* growth was determined by measuring the optical density of each well using aiMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, California, United States) at 595 nm. The percentage growth for each well was correlated with the relative optical density estimated by using the following equation:

$$\left(\frac{\text{Optical Density of each well containing the drug}}{\text{Optical Density of the drug-free well}}\right) \times 100$$

Growth inhibition for FLC was measured at 50% inhibition and at 90% inhibition for NYS and CAS (Alastruey-Izquierdo *et al*., 2015).

2.2 Investigation of the Antifungal activity of Eugenol Tosylate Congeners

To investigate the antifungal activity of ETCs, MIC defined as the lowest drug concentration required to inhibit microorganism growth and MFC which is defined as the lowest drug concentration required to kill the microorganism were determined.

2.2.1 Eugenol Tosylate Congeners and Eugenol

Eugenol solution was purchased from Sigma Aldrich (St. Louis, Missouri, United States) and was diluted using 1% DMSO to yield a working concentration of 8mg/ml. Eugenol tosylate congeners

(C1-C6) were synthesized by our collaborators at the University of Texas, United States of America and were also diluted in 1% DMSO to a working concentration of 8mg/ml.

2.2.2 *Candida albicans* isolates

A total of eleven drug resistant and susceptible isolates (3 FLC resistant, 3 NYS resistant, 2 CAS resistant and 3 isolates susceptible to all antifungal drugs) were used in this experiment. The reference strain *C. albicans* ATCC90028 was used for quality control. A standardised inoculum for each isolate was prepared according to the procedure described in section **2.1.3** above.

2.2.3 Determination of the Minimum inhibitory concentration

Minimum inhibitory concentration of EUG and ETCs (C1-C6) was determined using the broth microdilution assay according to the Clinical and Laboratory Standard Institute (CLSI, 2008) guidelines and the same procedure as described in section **2.1.4** was followed. Antifungals were replaced with EUG and ETCs (C1-C6). All experiments were performed in triplicate to validate the results.

2.2.4 Determination of the Minimum Fungicidal Concentration

Minimum fungicidal concentration of EUG and ETCs (C1-C6) was determined from the MIC broth microdilution test wells in which growth was inhibited. From each of these wells, 100 µl was spread on SDA plates. The plates were incubated at 37ºC for 24 hours or until growth was observed on the growth control plates. Minimum fungicidal concentration was recorded as the minimum drug concentration that killed *C. albicans* cells with no colonies observed on the agar plates.

2.3 Determination of the antifungal activity of Eugenol Tosylate Congeners in combination with antifungal drugs

The combination activity of ETCs (C1-C6) with FLC, NYS and CAS were evaluated against drug-resistant and susceptible *C. albicans* strains. The method described by Van Vuuren and Viljoen. (2011) was followed.

2.3.1 Eugenol Tosylate Congeners and Antifungal drugs

Eugenol tosylate congeners (C1-C6) and antifungal drugs (FLC, NYS and CAS) were prepared as mentioned above in section **2.2.1**. Eugenol tosylate congeners (C1-C6) were combined with the antifungal drugs in a 1:1 volume ratio. The assay was carried out on a 96 microtiter plate. A hundred microliters of SAB was added to each well of the plate followed by the addition of ETCs

with antifungal drugs at specified ratios followed by a two-fold serial dilution. Three FLC resistant, three NYS resistant and two CAS resistant strains and three strains susceptible to all antifungal drugs were used. Hundred microliters of the inoculum suspension (1.5 x10^6 CFU/ml) was added directly to the wells. Growth control wells were included which contained SAB medium and inoculum suspension. Negative control wells were also included and contained SAB medium only to confirm the sterility and viability of the experiment. The microtiter plate was incubated at 37°C for 24 hours. The experiment was performed in triplicate to validate the results. The presence or absence of *C. albicans* growth was determined by measuring the optical density of each well using aiMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, California, United States) at 595 nm. The percentage growth in each well was correlated with the relative optical density estimated by the following equation adapted from Alastruey-Izquierdo *et al*. (2015).

$$\left(\frac{\text{Optical Density of each well containing the drug}}{\text{Optical Density of the drug−free well}}\right) \times 100$$

The combination activity of ETCs (C1-C6) with antifungal drugs in a 1:1 ratio was calculated by determining the FICI based on the zero interaction theory of Loewe additivity (Loewe and Muischnek, 1926). The FICI values were calculated using the equation below:

$$FICI = FIC_a + FIC_b = \frac{MICa\ IN\ COMBINATION}{MICa\ ALONE} + \frac{MICb\ IN\ COMBINATION}{MICb\ ALONE}$$ (Loewe and Muischnek, 1926)

Where MIC_a is the MIC of the ETCs and MIC_b is the MIC of the antifungal drugs. FIC_a is the fractional inhibitory concentration of ETCs and FIC_b is the fractional inhibitory concentration of antifungal drugs. Interpretations of FICI values: synergy when FICI is ≤0.5, additive between 0.5 and 1.0, indifferent between 1.0 and 4.0 and antagonistic when FICI values are>4.0.

2.4 Evaluation of the effect of Eugenol Tosylate Congeners on activity of proton *ATPase*

The effect of ETCs on activity of H^+ *ATPase* was investigated by determining the rate of H^+ efflux in both FLC resistant and susceptible *C. albicans* strains following the method described by Manzoor *et al.* (2002).

2.4.1 Culture preparation

Three FLC resistant and three FLC susceptible strains were used. The isolates were grown on SDA plates and incubated at 37°C for 24 hours. After incubation, single colonies were picked from the SDA plates and inoculated into 100ml SAB in an Erlenmeyer flask. The culture was then incubated in a shaking incubator at 37°C for 8 hours at 200 rpm. Following incubation, the cell culture was transferred into 50ml Falcon tubes to harvest the cells. The tubes were centrifuged at 4500 rpm for 2 minutes and the supernatant was discarded. The cell pellet was washed twice with 1x phosphate buffered saline (PBS). The collected cell pellet was weighed and 100mg was suspended in 0.1M Potassium Chloride (KCl) and 0.1mM Calcium Chloride ($CaCl_2$) solution for all experiments.

2.4.2 Measurements of proton efflux

The procedure described by Manzoor *et al*. (2002) was followed. For each strain (three FLC resistant and three susceptible), cells were treated with ETCs (C1-C6), EUG, FLC, CAS and 5mM sodium orthovanadate (vanadate), which was used as a positive control. These compounds were added to cell suspension described in section **2.4.1** at their previous determined MICs. The cell suspension was kept in a double-jacketed glass container connected to a water circulator at 25°C for temperature regulation with constant stirring. For each experiment, initial pH of the suspension was set at pH 7.0 using either 0.01M sodium hydroxide (NaOH) or hydrochloric acid (HCl). A benchtop pH Meter (Thermo Scientific™ Orion Star™ A211) was used to record pH of cell suspension every 60 seconds for a period of ten minutes. Sodium hydroxide was added to the cell suspension every 60 seconds to neutralise the pH of cell suspension to pH 7.00. The effect of energy stimulation on activity of H^+ *ATPase* was also investigated. Glucose was added to the cell suspension at a concentration of 5mM and the pH of cell suspension was measured. An untreated control was included which contained *C. albicans* cells suspended in 0.1M KCl and 0.1mM $CaCl_2$ solution (without any of the test compounds). The experiment was performed in duplicate to validate the results. The rate of H^+ efflux was calculated from the volume of 0.01NaOH added to cell suspension every 60 seconds over a period of 10 minutes to neutralise the pH. The equation below was used to calculate the rate of H^+ efflux:

$$\textbf{Rate } \{P\ [H^+]\ out\} = \frac{\boldsymbol{Volume\ of\ NaOH\ (ml) \times 2 \times 10^{-2}\ nmoles}}{\boldsymbol{Weight\ of\ cells\ (mg) \times Time(minutes)}}$$

The amount of NaOH consumed is proportional to the rate of proton efflux.

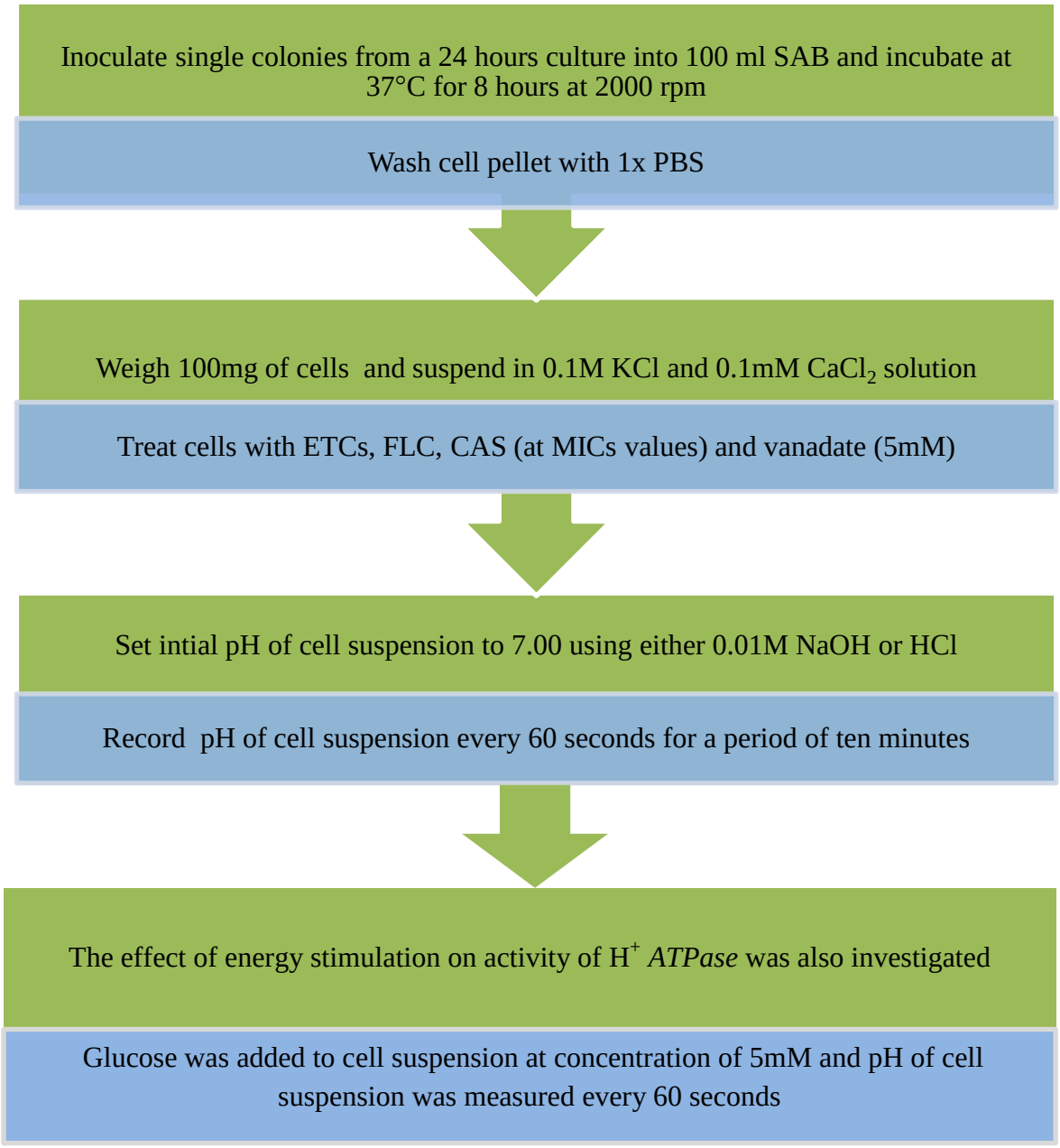


Figure 2.1: Representation of the methodology for investigating the effect of ETCs on activity of proton *ATPase* in *C. albicans* cells.

2.4.3 Evaluation of the effect of Eugenol Tosylate Congeners on intracellular pH of *Candida albicans* cells

The effect of ETCs (C1-C6) on intracellular pH of FLC resistant and susceptible *C. albicans* strains was investigated (Manzoor *et al*., 2002). This assay was performed to evaluate the effect of a dysfunctional H^+ *ATPase* on intracellular pH of *C. albicans* cells treated with ETCs (C1-C6) and in untreated cells.

2.4.3.1Culture preparation

Three FLC resistant and three FLC susceptible strains were used. The same procedure used in section **2.4.1** above was followed to culture the strains.

2.4.3.2 Nystatin preparation

NYS was purchased in powder form from Sigma Aldrich (St. Louis, Missouri, United States) and it was dissolved in DMSO to yield a stock concentration of 10mM. The working solution of 1mM was prepared by diluting lml of the stock concentration in 9ml of sterile distilled water. Nystatin solution was used for the disruption of the *Candida* plasma membrane in the procedure below.

2.4.3.3 Measurements of intracellular pH

For each strain (three FLC resistant and three susceptible), cells were treated with ETCs (C1-C6) and EUG at previously determined MICs. The initial pH of cell suspension was set at pH 7.0 following addition of the test compounds using either 0.01M NaOH or HCl. The cell suspension was then incubated in a shaking incubator at 37°C for 30 minutes at 200 rpm. Nystatin at a concentration of 20μM was added to cell suspension and the pH was neutralised to pH 7.0. The cell suspension was incubated at 37°C for 1 hour in a shaking incubator at 200 rpm to allow for disruption of the *Candida* plasma membrane by NYS. After 1 hour of incubation, intracellular pH of the cells was measured directly from the cell suspension using a benchtop pH Meter (Thermo Scientific™ Orion Star™ A211). The pH of the external medium at which NYS induced no further shift on the pH was taken as an estimate of the intracellular pH (Manzoor *et al.*, 2002). An untreated control was included and it contained *C. albicans* cells suspended in 0.1M KCl and 0.1 mM $CaCl_2$ solution. The experiment was performed in triplicate to validate the results.

2.5 Evaluation of the effect of Eugenol Tosylate Congeners on activity of drug efflux pumps

The effect of ETCs (C1-C6) on activity of drug efflux pumps was determined using R6G dye as described by Ahmad *et al.* (2013) with modifications described by Yaojun *et al.* (2016).

2.5.1 Culture preparation

Two FLC resistant and two FLC susceptible strains were used. The isolates were grown on SDA plates and incubated at 37°C for 24 hours. After incubation, single colonies were picked from SDA plates and inoculated into 100ml SAB in an Erlenmeyer flask. The culture was incubated in a shaking incubator at 37°C for 8 hours at 200 rpm. Following incubation, the cell culture was transferred into 50ml falcon tubes to harvest the cells. The tubes were centrifuged at 4500 rpm for

2 minutes and the supernatant was discarded. The cell pellet was washed twice with 1x PBS. The collected cell pellet was weighed and 2g was suspended in 100ml of 1x PBS solution for all experiments.

2.5.2 Effect of Eugenol Tosylate Congeners on activity of drug efflux pumps

For each strain (two FLC resistant and two FLC susceptible strains), cells were de-energised by adding 5mM of 2, 4 dinitrophenol (to inhibit the production of ATP in the mitochondria of cells) and 2-Deoxy-D-Glucose (to inhibit glycolysis) to the cell suspension. The cell suspension was incubated at 37°C for 60 minutes at 200 rpm. Following incubation, cells were centrifuged at 4500 rpm for 2 minutes and washed twice with 1x PBS. Cells were then treated with ETCs (C1-C6) and EUG at their previously determined MICs and incubated at 37°C for 30 minutes at 200 rpm. After incubation, cells were washed twice with 1x PBS and 10µM of R6G dye was added to the cell suspension and the cells were incubated at 37°C for 45 minutes at 200 rpm. Following incubation, cells were washed twice and re-suspended in 1x PBS. Five-milliliter aliquots were withdrawn at 5 minutes intervals for a period of 25 minutes and centrifuged at 9000 rpm for 2 minutes. Supernatants were collected and absorbance was measured at 527 nm. After 25 minutes, energy-dependent efflux was determined by adding 0.1M of glucose to cells re-suspended in glucose-free PBS. The cell suspension was incubated at 37°C for 40 minutes. Following incubation, 5ml aliquots were withdrawn at 5 minutes intervals for 35 minutes and centrifuged at 9000 rpm for 2 minutes. Supernatants were collected and absorbance was measured at 527 nm. An untreated control containing *C. albicans* cell suspended in PBS was also included. The experiment was done in triplicate to validate the results **(Figure 2.2)**.

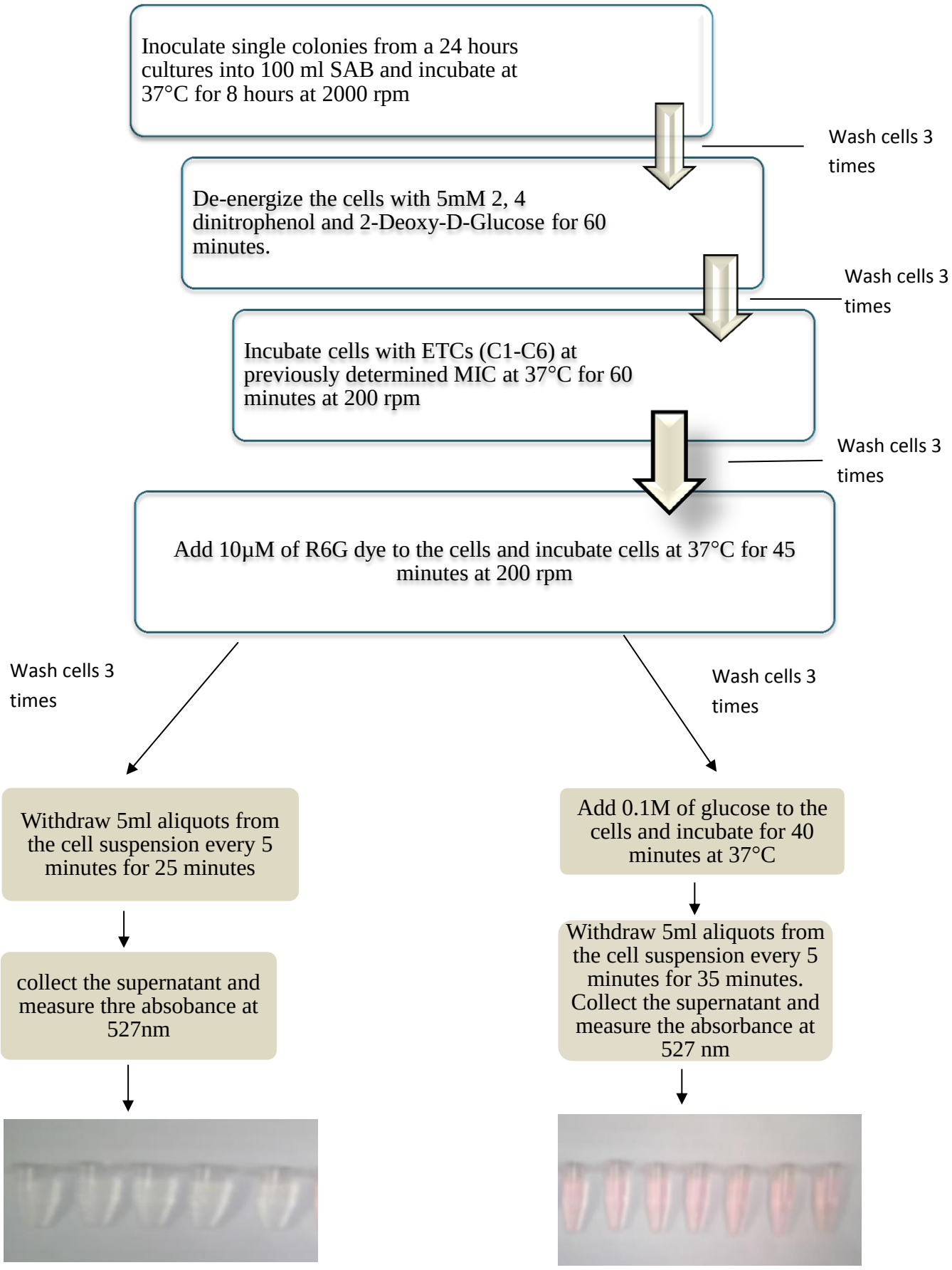


Figure 2.2: Flow diagram indicating the methodology for investigating the effect of ETCs on the activity of the drug efflux pumps in *C. albicans* cells.

2.6. Investigation of the haemolytic effects of Eugenol Tosylate Congeners on horse red blood cells

The hemolytic effect of ETCs (C1-C6) on horse RBC was investigated following the method described by Riaz *et al*. (2012) with modification by Mohammedi and Atik. (2014).

2.6.1 Extraction of red blood cells

Horse blood was purchased from the National Health Laboratory Services (Johannesburg, South Africa). Five millilitres of horse blood (5ml) was centrifuged at 500 **g** for 5 minutes and the supernatant containing the blood plasma, leukocytes and platelets was discarded. The remaining cell pellet consisting of RBCs was washed three times with 150mM sodium chloride (NaCl) solution and centrifuged at 500 **g** for 5 minutes for each wash. A 2% erythrocyte cell suspension was prepared by weighing 2g of the RBC pellet and suspending in 100ml of sterile 1× PBS (pH 7.4).

2.6.2 Hemolysis assay

The assay was carried out on clear 2ml Eppendorf tubes. Twenty microliters of ETCs (C1-C6) and EUG were added to each tube in graded concentrations (2mg, 1mg, 0.5mg, 0.25mg and 0.125mg). The prepared RBC suspension (180µl) was added to each tube containing the test compounds. The tubes were incubated for 45 minutes at 37°C. Following incubation, the tubes were placed on ice for 5 minutes and were then centrifuged at 1310 **g** for 5 minutes. After centrifugation 100µl of the supernatant was collected and diluted with 900µl of PBS. Two hundred microliters of the mixture were transferred to a 96 microtiter plate and the absorbance of the liberated haemoglobin was measured using the aiMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, California, United States) at 540nm. Two controls were prepared, negative control containing RBC suspension with PBS and another negative control (solvent control) containing RBC suspension with 1% DMSO. The positive control consisted of RBC suspension with 0.1% Triton x-100 and it was used for complete lysis of RBC (100% hemolysis). The experiment was performed in triplicate to validate the results. The percentage hemolysis for each sample was calculated by the following equation Riaz *et al*. (2012):

$$\% \text{ haemolysis} = \frac{[Absorbance\ of\ RBC\ treated\ with\ test\ compounds - Absorbance\ of\ blank]}{[Absorbance\ of\ RBC\ treated\ with\ 0.1\%\ tritonx{-}100 - Absorbance\ of\ blank]} \times 100$$

2.7 Statistical Analysis

The data obtained were presented as mean values. Analysis of variance was used to investigate the significant differences between independent groups using graph pad prism 5. The difference in rate of H^+ efflux in *C. albicans* cells treated with different test compounds compared to untreated cells was analyzed using Dunnett's test, a multiple comparison procedure. Furthermore, the difference in efflux of R6G dye in *C. albicans* cells treated with ETCs compared to untreated cells was also analyzed using Dunnett's test. Statistical significance was considered at $P< 0.05$.

CHAPTER 3: RESULTS

3.1 *Candida albicans* strains and drug susceptibility testing

Antifungal drug susceptibility (MICs) testing of 50 *C. albicans* isolates against FLC, NYS and CAS was performed following the broth microdilution method as described by the CLSI, 2008. From the 50 tested isolates, MIC values obtained ranged from 0.122µg/ml to 250µg/ml for FLC, 0.122µg/ml to 1.95µg/ml for NYS and from 0.122µg/ml to 3.9µg/ml for CAS **(Table 3.1)**. One percent DMSO was used as a negative control and it showed no inhibitory activity on the growth of *C. albicans* strains. According to the breakpoints set by the CLSI in 2017, 39 isolates were susceptible to FLC (MIC ≤2µg/ml) and seven were resistant to FLC (MIC ≥8µg/ml). The susceptibility status of four isolates was not categorized because their MICs does not fall under any of the categories set by the CLSI and their MIC was 3.9µg/ml. For isolates that were treated with CAS, 10 were susceptible (MIC ≤0.25µg/ml), five were resistant (MIC ≥1 µg/ml) and 35 were categorized as intermediate (MIC 0.5µg/ml). Currently, there are no standard breakpoints set by the CLSI for NYS, therefore the breakpoints used by other authors who have performed similar studies were used in this study to categorize the isolates in terms of their susceptibility to NYS (Nenoff *et al*., 2016). Forty-four isolates were susceptible to NYS (MIC <1µg/ml) and six were resistant to NYS (MIC ≥1µg/ml). The quality control strain ATCC90028 was susceptible to all tested antifungal drugs with MICs of 0.49µg/ml for FLC, 0.24µg/ml for NYS and MICs below 0.122µg/ml for CAS. These results are shown in **Table 3.2**. Resistance to more than one antifungal drug was observed in two isolates which were both isolated from HIV positive patients. Cross-resistance was observed in isolate 004 (resistant to both FLC and NYS) and isolate 002B (resistant to both CAS and FLC).

Table 3.1: Mean Minimum inhibitory concentration of FLC, NYS and CAS against 50 clinical *C. albicans* isolates.

***C. albicans* isolates**	**FLC MIC (µg/ml)**	**NYS MIC (µg/ml)**	**CAS MIC (µg/ml)**
1(002 B)	>250	0.49	1.95
2(003)	3.9	0.24	0.98
3(004)	62.5	1.95	0.98
4(005)	0.98	1.95	0.98
5(008)	1.95	0.49	0.98
6(015)	0.24	0.49	<0.122
7(020)	0.98	0.122	<0.122
8(024)	0.98	1.95	0.49
9(025)	0.98	0.98	<0.122
10(027)	0.98	1.95	< 0.122
11(034)	1.95	0.49	< 0.122
12(037)	1.95	0.49	0.98
13(042)	0.49	0.24	0.49
14(050)	0.98	0.49	0.24
15(052)	1.95	0.24	0.98
16(053)	0.24	0.49	3.9
17(091)	1.95	0.98	0.98
18(097)	0.49	0.24	0.98
19(0100)	0.24	0.24	0.49
20(167-1)	<0.122	1.95	0.98
21(167-2)	0.122	0.98	0.49
22(C-87)	0.49	0.98	1.95
23(C-89)	0.98	0.98	3.9
24(CR02)	125	0.12	0.98
25(CR04)	0.98	0.49	< 0.122
26 (CR05)	0.24	0.12	0.49
27(CR08)	0.49	0.12	0.49
28(CR10)	0.98	0.24	0.24

C. albicans isolates	FLC MIC (µg/ml)	NYS MIC (µg/ml)	CAS MIC (µg/ml)
29(CR13)	0.98	0.49	0.49
30(CR14)	0.98	0.24	0.98
31(CR16)	3.9	0.98	0.98
32(CR17)	0.98	0.49	0.24
33(CR19)	1.95	0.49	0.49
34(CR21)	0.98	0.49	0.98
35(CR22)	0.49	0.98	0.98
36(CR23)	31.5	0.49	0.98
37(CR24)	62.5	0.24	0.98
38(CR33)	0.98	0.49	<0.122
39(CR34)	0.98	0.24	0.49
40(CR34B)	3.9	0.122	0.98
41(CR38)	0.122	0.24	0.49
42(CR44)	0.98	0.49	0.49
43(CR54)	0.49	0.49	0.98
44(CR58)	0.49	0.49	0.98
45(CR61)	125	0.24	0.98
46(CR63)	0.98	1.95	0.49
47(CR66)	0.98	0.24	0.98
48(N27)	3.9	0.12	1.95
49(A71)	125	0.12	0.49
50(180-1)	1.95	0.24	0.49
ATCC90028	0.49	0.24	<0.122

MIC- Minimum inhibitory concentration, **FLC**-fluconazole, **NYS**- nystatin, **CAS**- caspofungin. **ATCC90028-** quality control strain.

Table 3.2: Antifungal drug susceptibility status of 50 clinical *C. albicans* isolates.

Antifungal agents	MIC interpretive categories (n; %)			
	Susceptible	**Intermediate**	**Susceptible dose dependent**	**Resistance**
Fluconazole	39 (78%)	-	4 (8%)	7 (14%)
Nystatin	44 (88%)	-	-	6 (12%)
Caspofungin	10 (20%)	35 (70%)	-	5 (10%)

(-) category not specified by the CLSI

3.2 Antifungal activity of Eugenol Tosylate Congeners

The antifungal activity of ETCs (C1-C6) against NYS, FLC and CAS drug resistant and susceptible *C. albicans* strains was investigated by determining MIC and MFC of these compounds following the CLSI guidelines, 2008. Chlorhexidine was used as a positive control. Minimum inhibitory concentration and MFC of chlorhexidine against both drug susceptible and resistant *C. albicans* strains were the same ranging from 2.25 to 5.2 $\times 10^{-3}$%. All ETCs exhibited antifungal activity against both drug resistant and susceptible *C. albicans* strains (**Table 3.3**). Minimum inhibitory concentrations obtained for ETCs ranged from 0.13 to 2mg/ml. For EUG, MICs ranged from 0.33 to 0.5mg/ml against both drug susceptible and resistant *C. albicans* strains. Congener 1 was the most active compound with decreased MICs compared to other compounds, and the MICs obtained ranged between 0.13 and 0.250mg/ml against both drug susceptible and resistant strains. Congener 2 and C3 showed consistent inhibitory activity with MICs greater than 2mg/ml and 0.5mg/ml respectively against both drug resistant and susceptible *C. albicans* strains. Minimum inhibitory concentration for C4 ranged from 0.21 to 0.5mg/ml in all strains. Minimum inhibitory concentrations for C5 and C6 were greater than 2mg/ml against FLC resistant strains and 1mg/ml against CAS resistant strains.

The fungicidal activity of ETCs was also investigated and MFC was determined. **Table 3.3** also indicates MFCs of ETCs obtained against both drug resistant and susceptible isolates. Minimum fungicidal concentrations of ETCs ranged between 1 and greater than 2mg/ml and MFC of EUG ranged between 0.67 and 1mg/ml. Minimum fungicidal concentration of C1, C2 and C6 was greater than 2mg/ml in all tested strains. In addition, MFCs of C3, C4 and C5 were greater than 2mg/ml in FLC and CAS resistant strains but in NYS resistant strains, MFC of these compounds was 1mg/ml.

Table 3.3: Mean Minimum inhibitory concentration and mean Minimum fungicidal concentrations of Eugenol Tosylate Congeners against drug resistant and drug susceptible *C. albicans* strains.

Compounds		**FLC resistant Strains (n=3)**	**NYS resistant strains (n=3)**	**CAS resistant Strains (n=2)**	**Strains susceptible to FLC, NYS and CAS (n=3)**
EUG (mg/ml)	MIC	0.5	0.25	0.25	0.33
	MFC	1	1	1	0.67
C1 (mg/ml)	MIC	0.21	0.13	0.25	0.21
	MFC	>2	>2	>2	>2
C2 (mg/ml)	MIC	>2	>2	>2	>2
	MFC	>2	>2	>2	>2
C3 (mg/ml)	MIC	0.5	0.5	0.5	0.5
	MFC	>2	1	>2	>2
C4 (mg/ml)	MIC	0.21	0.5	0.19	0.5
	MFC	>2	1	>2	>2
C5 (mg/ml)	MIC	>2	0.33	1	0.25
	MFC	>2	1	>2	>2
C6 (mg/ml)	MIC	>2	1	1	1
	MFC	>2	>2	>2	>2
Chlorhexidine ($\times 10^{-3}$) (%)	MIC	3	2.5	2.25	5.2
	MFC	3	2.5	2.25	5.2

3.3 Antifungal activity of Eugenol Tosylate Congeners in combination with antifungal drugs

The antifungal activity of ETCs with antifungal drugs (FLC, NYS and CAS) was investigated by combining the compounds in equal volumes (1:1 ratio). Eugenol tosylate congeners were combined with antifungal drugs in a 1:1 ratio and FICI was determined to interpret the drug interactions.

3.3.1 Combination activity of FLC with ETCs in a 1:1 ratio

The individual MICs of FLC in combination with ETCs are shown in **Table 3.4**. The drug interactions of the combined compounds are also indicated as FICI in **Figure 3.1**. All ETCs in combination with FLC exhibited additive effect with FICI values ranging between 0.5 and 1.0 against FLC drug resistant strains (**Table 3.4**). The combination of FLC with EUG, C1 and C2 showed indifferent effect whereas C3, C4, C5 and C6 exhibited additive effect against FLC susceptible strains. No synergy (FICI $\leq$0.5) or antagonistic (FICI>4.0) effect was observed following the combination of ETCs with FLC in both FLC drug resistant and susceptible strains. The mean MIC of FLC alone against FLC resistant strains was 104µg/ml and 500µg/ml for EUG, however when the two compounds were combined (FLC and EUG) the mean MIC was reduced to 86µg/ml. The FICI value of 1.00 was obtained indicating additive effect following the FICI classification system described by Van Vuuren and Viljoen, (2011) (**Table 3.4**). In susceptible strains, MIC of FLC alone was 1.46µg/ml and 330µg/ml for EUG. However the combination of the two compounds had MIC of 1.95µg/ml which is greater than MIC of FLC alone. Therefore the FICI value of 1.35 was obtained indicating an indifferent drug interaction. Furthermore, the combination of C3 with FLC exhibited additive effect against both FLC resistant and susceptible strains with FICI value of 0.73 and 0.67 respectively. The FICI of FLC in combination with C1, C2, C4, C5 and C6 were 0.76, 0.68, 0.97, 0.95 and 0.88 respectively in FLC drug resistant strains. In FLC susceptible strains, the FICI obtained were 1.83, 1.16, 0.67, 0.90 and 0.56 for combinations between FLC with C1, C2, C4, C5 and C6 respectively.

Table 3.4: Antifungal activity of ETCs in combination with FLC in a 1:1 ratio in terms of MICs against FLC drug resistant and susceptible *C. albicans* strains.

***C. albicans* phenotype**	**Compounds**	**Mean MIC_A**	**Mean MIC_B**	**Mean MIC_{A+B}**	**FIC_A**	**FIC_B**	**FICI**	**FICI Interpretation**
FLC Resistant strains(n=3) Repeated 3x	FLC+ EUG	104	500	86	0.83	0.17	1.00	Additive
	FLC+ C1	104	201	52	0.5	0.26	0.76	Additive
	FLC+ C2	104	2000	68	0.65	0.03	0.68	Additive
	FLC+ C3	104	500	62.5	0.6	0.125	0.73	Additive
	FLC+ C4	104	500	83	0.8	0.17	0.97	Additive
	FLC+ C5	104	2000	94	0.9	0.047	0.95	Additive
	FLC+ C6	104	1000	83	0.8	0.08	0.88	Additive
FLC Susceptible strains(n=3) Repeated 3x	FLC+ EUG	1.46	330	1.95	1.34	0.006	1.35	Indifferent
	FLC+ C1	1.46	201	2.65	1.82	0.013	1.83	Indifferent
	FLC+ C2	1.46	2000	1.7	1.16	0.0009	1.16	Indifferent
	FLC+ C3	1.46	500	0.98	0.67	0.002	0.67	Additive
	FLC+ C4	1.46	500	0.98	0.67	0.002	0.67	Additive
	FLC+ C5	1.46	250	1.3	0.89	0.005	0.90	Additive
	FLC+ C6	1.46	1000	0.82	0.56	8.2×10^{-5}	0.56	Additive

MICA- Minimum inhibitory concentration of FLC alone, MICB- Minimum inhibitory concentration of ETCs alone, MIC A+B- Minimum inhibitory concentration of the combined compounds (FLC and ETCs). $FIC_A = \frac{MIC\ (A+B)}{MICA}$, $FIC_B = \frac{MIC\ (A+B)}{MICB}$, $FICI = FIC_A + FIC_B$

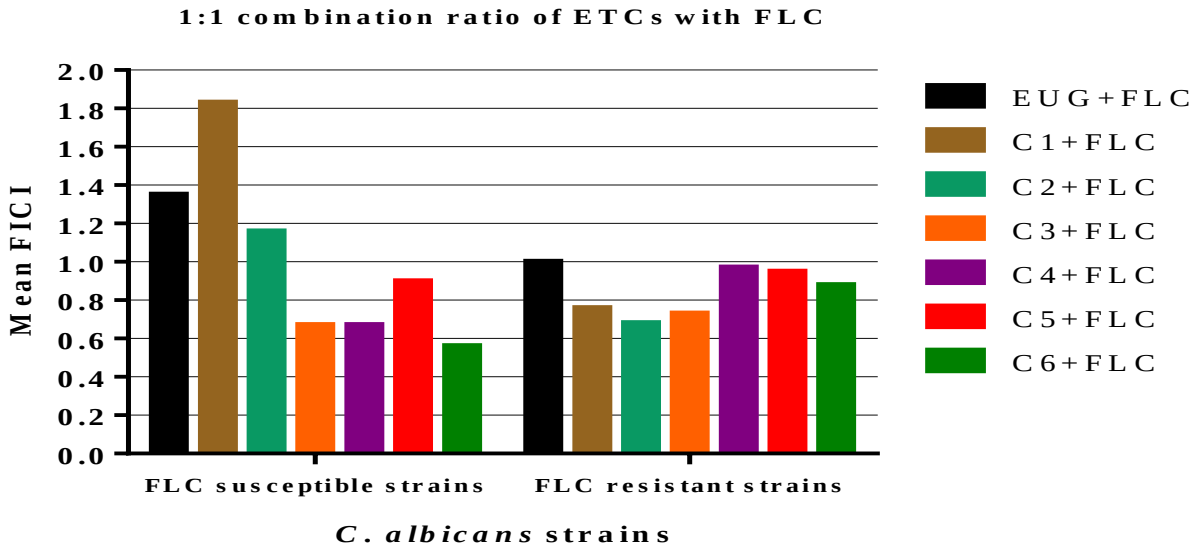


Figure 3.1: Antifungal activity of ETCs in Combination with FLC in a 1:1 Ratio against FLC susceptible and resistant *C. albicans* strains. The drug interactions of the combined compounds are indicated as FICI.

3.3.2 Combination activity of NYS with ETCs in a 1:1 ratio

Minimum inhibitory concentrations of NYS in combination with ETCs are shown in **Table 3.5**. The combination interaction studies were also performed between NYS and ETCs against NYS resistant and susceptible strains in a 1:1 ratio. All ETCs in combination with NYS against NYS resistant strains exhibited synergistic effect with FICI values which are lesser than 0.5, except for C4 which exhibited an additive effect with FICI value of 1.0 (**Figure 3.2**). In NYS susceptible strains, the combination activity of EUG, C1, C3, C4 and C6 with NYS exhibited indifferent effect with FICI values ranging between 1.0 and 4.0, whereas C2 and C5 exhibited additive effect with FICI values ranging from 0.5 to 1.0. No antagonistic effect was observed between the combination of ETCs with NYS against both NYS resistant and susceptible strains.

In NYS resistant strains, MIC of NYS alone was 1.95µg/ml and for C1 alone was 130µg/ml, however when the two compounds (NYS and C1) were combined the MIC was reduced to 0.98µg/ml. The FICI value obtained was 0.51 indicating that the combination of the two compounds exhibits a synergistic effect against NYS resistant strains. In addition, the MIC for the combination of NYS and C4 was 1.95µg/ml against NYS resistant strains and the FICI was 1.00 indicating an additive effect. The FICI for the combination of ETCs with NYS against NYS resistant strains were 0.25, 0.50, 0.50, 0.50 and 0.50 for EUG, C2, C3, C5, and C6 respectively (**Table 3.5**).

In NYS susceptible strains, the MIC for the combination of NYS and C1 was 0.98µg/ml and the FICI was 1.63 indicating an indifferent drug interaction which is different to the drug interaction obtained in NYS resistant strains (synergy). Another different drug interaction was observed when NYS was combined with C4 against NYS susceptible and resistant strain. In susceptible strains the FICI value was 1.35 indicating an indifferent effect whereas in resistant strains an additive effect was observed (FICI= 1). The FICI of EUG, C2, C3, C5 and C6 in combination with NYS were 1.37, 0.80, 3.81, 0.55 and 1.35 respectively (**Figure 3.2**).

Table 3.5: Antifungal activity of ETCs in combination with NYS in a 1:1 ratio in terms of MICs against NYS drug resistant and susceptible *C. albicans* strains.

***C. albicans* phenotype**	**Compounds**	**Mean MIC_A**	**Mean MIC_B**	**Mean MIC_{A+B}**	**FIC_A**	**FIC_B**	**FICI**	**FICI Interpretation**
NYS Resistant strains(n=3) Repeated 3x	NYS+ EUG	1.95	250	0.49	0.25	0.002	0.25	Synergy
	NYS+ C1	1.95	130	0.98	0.5	0.008	0.51	Synergy
	NYS+ C2	1.95	2000	0.98	0.5	0.005	0.50	Synergy
	NYS+ C3	1.95	500	0.98	0.5	0.002	0.50	Synergy
	NYS+ C4	1.95	500	1.95	1	0.004	1.00	Additive
	NYS+ C5	1.95	330	0.98	0.5	0.003	0.50	Synergy
	NYS+ C6	1.95	1000	0.98	0.5	0.001	0.50	Synergy
NYS Susceptible strains (n=3) Repeated 3x	NYS+ EUG	0.6	330	0.82	1.37	0.002	1.37	Indifferent
	NYS+ C1	0.6	201	0.98	1.63	0.005	1.63	Indifferent
	NYS+ C2	0.6	2000	0.49	0.8	0.0002	0.80	Additive
	NYS+ C3	0.6	500	2.28	3.8	0.005	3.81	Indifferent
	NYS+ C4	0.6	500	0.81	1.35	0.002	1.35	Indifferent
	NYS+ C5	0.6	250	0.33	0.55	0.001	0.55	Additive
	NYS+ C6	0.6	1000	0.81	1.35	0.0008	1.35	Indifferent

MICA- Minimum inhibitory concentration of NYS alone, MICB- Minimum inhibitory concentration of ETCs alone, MIC A+B- Minimum inhibitory concentration of the combined compounds (NYS and ETCs). $FIC_A = \frac{MIC\ (A+B)}{MICA}$, $FIC_B = \frac{MIC\ (A+B)}{MICB}$, $FICI = FIC_A + FIC_B$

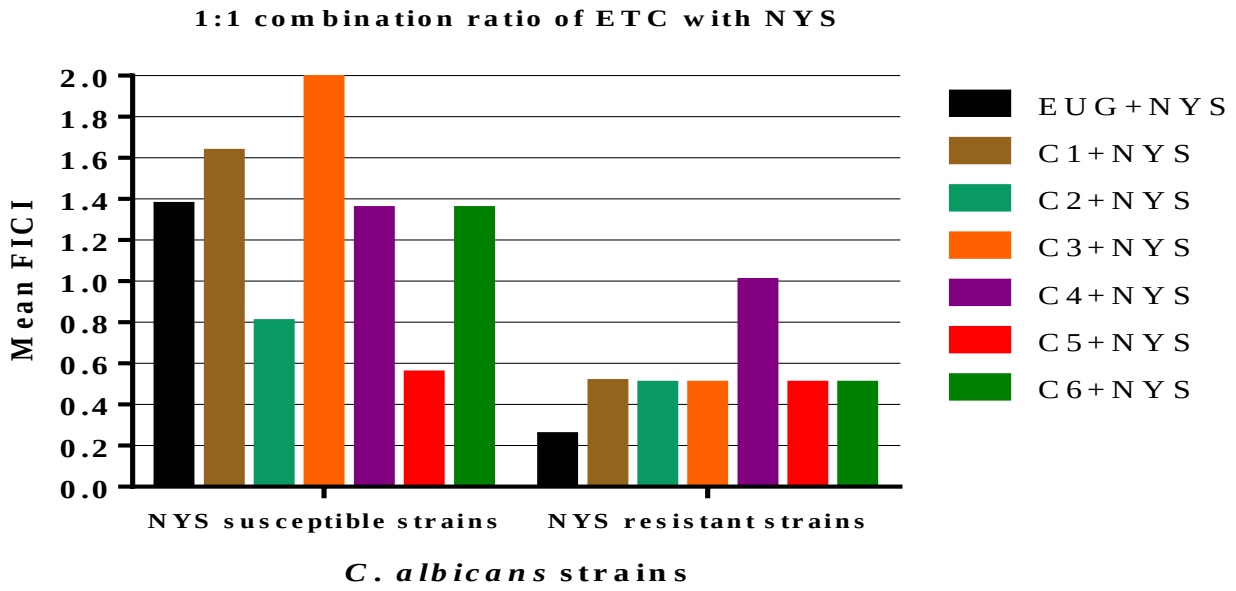


Figure 3.2: Antifungal activity of ETCs in Combination with NYS in a 1:1 Ratio against NYS susceptible and resistant *C. albicans* strains. The drug interactions of the combined compounds are indicated as FICI.

3.3.3 Combination activity of CAS with ETCs in a 1:1 ratio

The individual MICs of CAS in combination with ETCs are shown in **Table 3.6**. The drug interactions of the combined compounds are also indicated as FICI in **Figure 3.3**. The combination activity of ETCs with CAS was carried out in a 1:1 ratio against *C. albicans* isolates that are resistant and susceptible to CAS. In CAS resistant strains, synergy was observed between the combination of CAS with C2 and C3 with FICI values of 0.25 and 0.5 respectively. Additive effect was observed between the combination of CAS with EUG, C4, C5 and C6 with FICI value of 1.0. Indifferent effect was observed between the combination of CAS with C1 with a FICI value greater than 1.0 but less than 4.0. In CAS susceptible strains all ETCs and EUG in combination with CAS exhibited indifferent effect except for C6 which exhibited additive effect (**Table 3.6**). The combination activity of C1 with CAS in both CAS resistant and susceptible strains exhibited an indifferent effect with FICI values of 2.03 and 1.50 for CAS resistant and susceptible strains respectively.

In CAS resistant strains, the combination activity of C3 with CAS had MIC of 1.95µg/ml whereas the individual MICs were 3.9µg/ml and 500µg/ml for CAS and C3 respectively. The FICI value obtained was 0.50 indicating that the combination of the two compounds exhibits a synergistic

effect. However a different drug effect was observed when C3 was combined with CAS against CAS susceptible strains. An indifferent effect was observed with a FICI value of 1.50.

The combination of C6 and CAS exhibited consistent effect in both CAS resistant and susceptible strains exhibiting additive effect. In CAS resistant strains, the obtained FICI was 1.00 and in susceptible strains the FICI was 0.76. In CAS resistant strains, the FICI of EUG, C2, C4 and C5 in combination with CAS were 1.02, 0.25, 1.02 and 1.00 respectively **(Figure 3.3)**. In CAS susceptible strains the FICI values of EUG, C1, C2, C3, C4 and C5 in combination with CAS was 1.50 for all combinations.

Table 3.6: Antifungal activity of ETCs in combination with CAS in a 1:1 Ratio in terms of MICs against CAS drug resistant and susceptible *C. albicans* strains.

***C. albicans* phenotype**	**Compounds**	**Mean MIC_A**	**Mean MIC_B**	**Mean MIC_{A+B}**	**FIC_A**	**FIC_B**	**FICI**	**FICI Interpretation**
CAS Resistant strains (n=2) Repeated 3x	CAS+ EUG	3.9	250	3.9	1	0.02	1.02	Additive
	CAS+ C1	3.9	250	7.8	2	0.03	2.03	Indifferent
	CAS+ C2	3.9	2000	0.98	0.25	0	0.25	Synergy
	CAS+ C3	3.9	500	1.95	0.5	0.004	0.5	Synergy
	CAS+ C4	3.9	190	3.9	1	0.02	1.02	Additive
	CAS+ C5	3.9	1000	3.9	1	0.004	1.00	Additive
	CAS+ C6	3.9	1000	3.9	1	0.004	1.00	Additive
CAS Susceptible strains (n=2) Repeated 3x	CAS+ EUG	0.65	330	0.98	1.5	0.003	1.50	Indifferent
	CAS+ C1	0.65	201	0.98	1.5	0.005	1.50	Indifferent
	CAS+ C2	0.65	2000	0.98	1.5	0.0005	1.50	Indifferent
	CAS+ C3	0.65	500	0.98	1.5	0.002	1.5	Indifferent
	CAS+ C4	0.65	500	0.98	1.5	0.002	1.5	Indifferent
	CAS+ C5	0.65	250	0.98	1.5	0.004	1.5	Indifferent
	CAS+ C6	0.65	1000	0.49	0.75	0.005	0.76	Additive

MICA- Minimum inhibitory concentration of CAS alone, MICB- Minimum inhibitory concentration of ETCs alone, MIC A+B- Minimum inhibitory concentration of the combined compounds (CAS and ETCs). $FIC_A = \frac{MIC\ (A+B}{MICA}$, $FIC_B = \frac{MIC\ (A+B)}{MICB}$, $FICI = FIC_A + FIC_B$

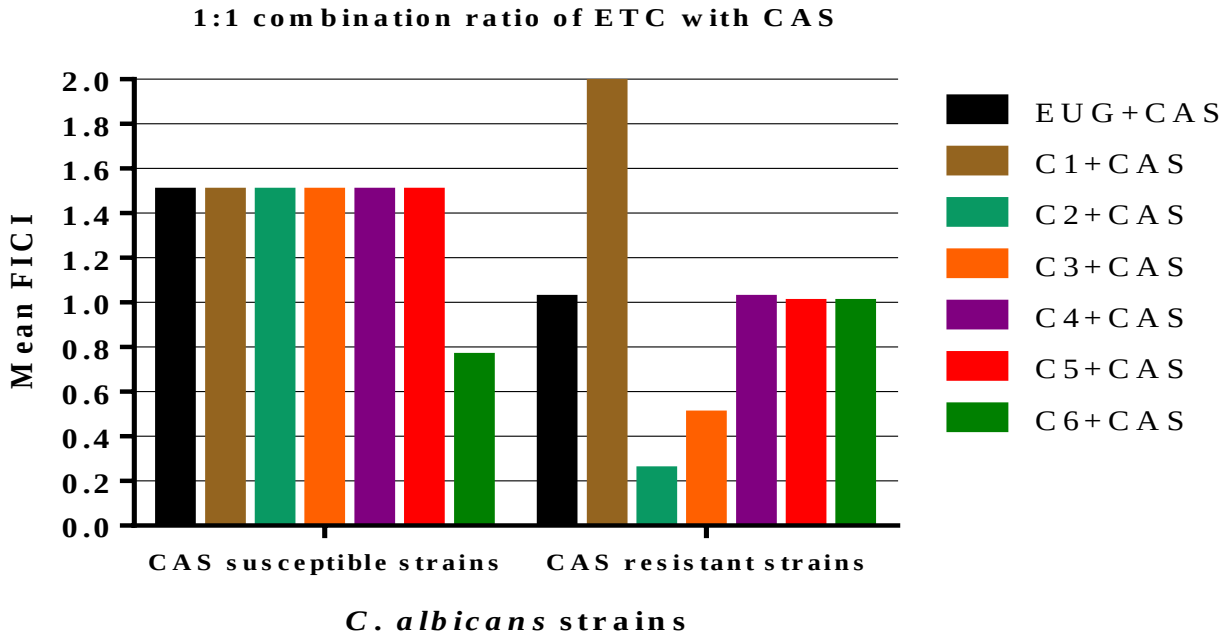


Figure 3.3: Antifungal activity of ETCs in combination with CAS in a 1:1 ratio against CAS susceptible and resistant *C. albicans* strains. The drug interactions of the combined compounds are indicated as FICI.

3.3.4 Summary of the combination activity of ETCs with all antifungal drugs

The overall combination studies revealed that the antifungal activity of the combined drugs have increased antifungal activity compared to when the drugs are used individually. The summary of all combination interaction between ETCs and the antifungal drugs is illustrated in **Figure 3.4**. Nystatin in combination with ETCs exhibited most synergistic interactions compared to the combinations of ETCs with CAS (14%) and FLC (0) (**Figure 3.4A** and **Figure 3.4C**). Out of the total combinations between NYS and ETCs, 43% of the combinations were synergistic, 21% showed additive and 36% showed indifferent effect. Fluconazole in combination with ETCs exhibited additive effect in 79% of the combinations and indifferent effect in 21% of the combinations (**Figure 3.4A**). The combination activity of CAS with ETCs exhibited less synergistic interactions (14%) with 50% of combinations being indifferent. Additive interactions were observed in 36% of the combinations (**Figure 3.4B**).

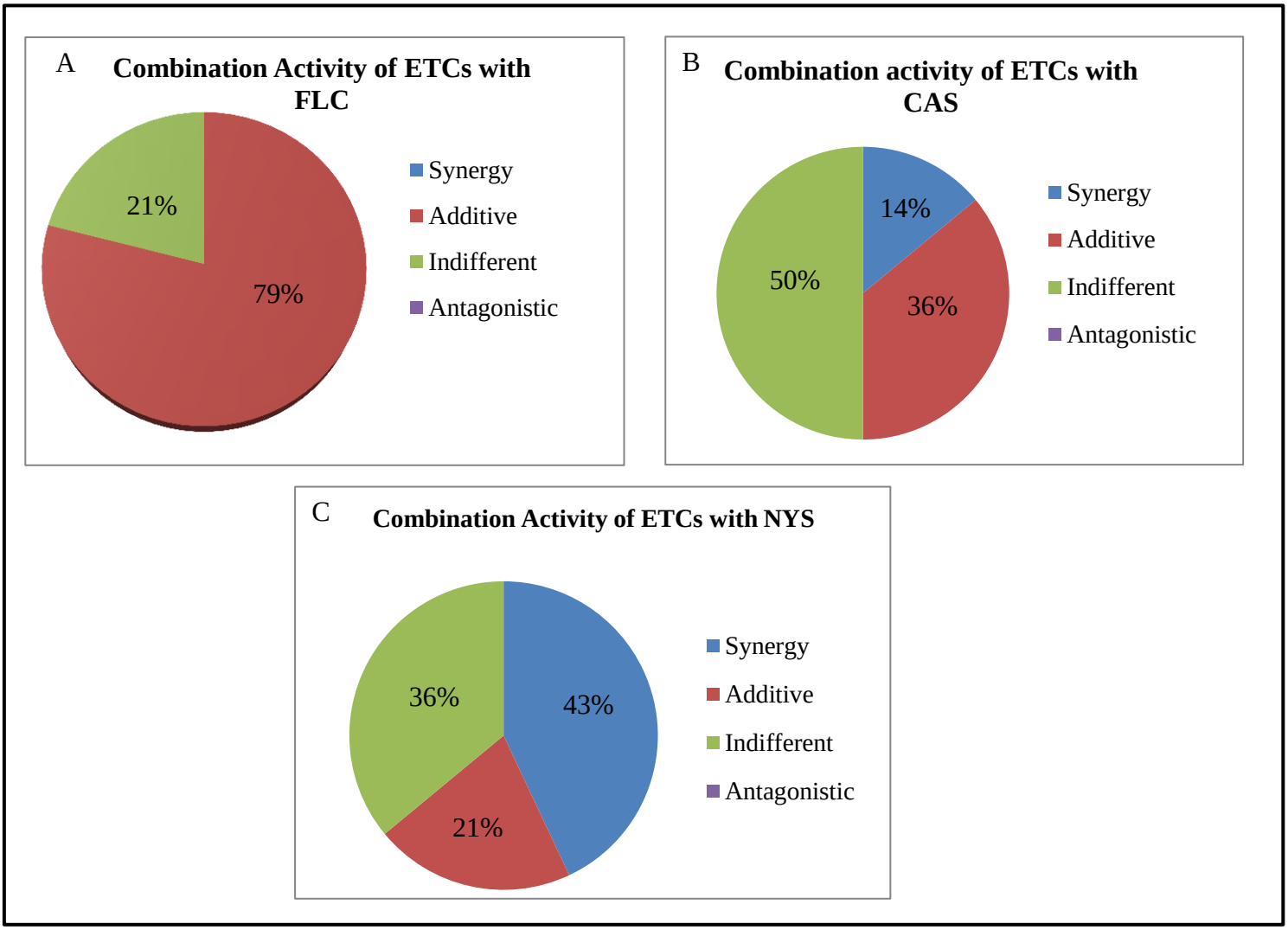


Figure 3.4: Drug interactions of ETCs with three antifungal drugs against both drug susceptible and resistant *C. albicans* strains.

3.4 The effect of Eugenol Tosylate Congeners on activity of proton *ATPase* in *Candida albicans* strains

The activity of the H^+ *ATPase* in FLC drug resistant and susceptible strains was investigated by determining the rate of H^+ efflux following the treatment of *C. albicans* cells with ETCs (C1-C6) using previously determined MICs. The effect of energy stimulation on activity of the H^+ *ATPase* was also investigated. An untreated control was included in the study where cells were not treated with any compounds and a positive control included cells treated with vanadate. **Table 3.7** to **Table 3.10** shows the pH of cell suspensions in both FLC resistant and susceptible strains in glucose-starved cells and in cells supplied with glucose recorded in a period of 10 minutes and the total volume of NaOH consumed. The amount of NaOH consumed was considered to be proportional to the rate of H^+ efflux. The calculated rate of H^+ efflux in glucose-starved and in cells supplied with glucose is shown in **Figure 3.5** and **Figure 3.6** respectively.

3.4.1 H^+ efflux in glucose-starved cells

The efflux of H^+ from the cell decreased the pH of the cell suspension. In FLC susceptible strains the pH of untreated cell suspension ranged between 6.85 and 6.95, and the pH of cell suspension treated with ETCs ranged between 6.96 and 7.00. The pH of cell suspensions treated with FLC and CAS ranged between 6.85 and 6.96 and between 6.82 and 6.97 respectively. The pH of cell suspensions treated with EUG and vanadate ranged between 6.96 and 7.00 and between 6.98 and 7.00 respectively (**Table 3.9**). In FLC resistant strains the pH of untreated cell suspension ranged between 6.86 and 6.94 while the pH of cell suspension treated with ETCs ranged between 6.89 and 7.00. The pH of cell suspensions treated with FLC and CAS ranged between 6.87 and 6.98 and between 6.86 and 6.97 respectively. The pH of cell suspensions treated with EUG and vanadate ranged between 6.87 and 6.98 and between 6.97 and 7.00 respectively (**Table 3.7**). In addition, the amount of sodium hydroxide added to the cell suspensions to neutralise the pH following the efflux of H^+ to the cell suspension was higher in untreated glucose-starved cells as compared to ETCs and vanadate treated cells. The total volume of sodium hydroxide added to untreated cells was between 170µl and 175µl for both resistant and susceptible strains and in ETCs treated cells the volume ranged between 95µl and 40µl. For vanadate, the total volume of NaOH added was 25µl and 35µl for FLC susceptible and resistant strains respectively. These results indicated that more NaOH was used to neutralise the pH of the cell suspension in untreated cells compared to cells treated with ETCs and vanadate.

3.4.2 H^+ efflux in cells supplied with glucose

In FLC susceptible strains, the pH of untreated cell suspension supplied with glucose ranged between 6.73 and 6.90 (**Table 3.10**) while the pH of cell suspension treated with ETCs and supplied with glucose ranged between 6.84 and 6.98 showing an increase in pH compared to untreated cells supplied with glucose. The addition of glucose in cells treated with FLC and CAS showed a similar trend to that observed in untreated cells supplied with glucose and the pH of cell suspension ranged between 6.80 and 6.91 for cells treated with FLC and between 6.79 and 6.95 for cells treated with CAS. The pH of cell suspension treated with EUG and vanadate supplied with glucose ranged between 6.86 and 6.97 and between 6.95 and 6.97 respectively. The total volume of NaOH added to untreated cells supplied with glucose was 415 µl and in ETCs treated cells the volume ranged between 260 µl and 200 µl. For vanadate, the total volume of NaOH added was 170 µl.

In FLC resistant strains the pH of untreated cell suspension supplied with glucose ranged between 6.70 and 6.87 (**Table 3.8**) while the pH of cell suspension treated with ETCs and supplied with glucose ranged between 6.83 and 6.98 showing an increase in pH compared to untreated cells. The addition of glucose in FLC and CAS treated cell suspension showed a similar trend to that observed in untreated cells supplied with glucose and the pH of the cell suspension ranged between 6.71 and 6.89 for FLC and between 6.75 and 6.91 for CAS. The pH of cell suspension treated with EUG and vanadate supplied with glucose ranged between 6.82 and 6.97 and between 6.89 and 7.00 respectively. These results show that the pH of the cell suspension supplied with glucose is more acidic compared to the pH in glucose-starved cells. In addition, the total volume of NaOH added to the cell suspension in untreated cells supplied with glucose was 475 µl and in ETCs treated cells the volume ranged between 290 µl and 205 µl. For vanadate, the total volume of NaOH added was 145 µl.

The observations made in terms of the pH of the cell suspension in untreated, ETCs treated and antifungal treated cells with and without glucose show that the addition of glucose to the cell suspension as a source of energy stimulates the efflux of H^+ resulting in decreasing pH of the cell suspension. This resulted in an increased volume of sodium hydroxide added to the cell suspension compared to glucose-starved cells. The activity of ETCs on the efflux of H^+ in both FLC susceptible and resistant strains exhibited a similar effect as there was a small difference recorded on the pH of the cell suspension treated with the different ETCs. Furthermore, the activity of ETCs showed a similar trend to that observed in cells treated with vanadate which was used as a positive control.

3.4.3 Rate of H^+ efflux in glucose-starved cells

The rate of H^+ efflux was calculated from the volume of 0.01M NaOH added to the cell suspension every 60 seconds over a period of 10 minutes following a decrease in the pH of the cell suspension. The formula given in section **2.4.2** was used to calculate the rate of H^+ efflux. In untreated cells, the rate of H^+ efflux was threefold greater than the rate of H^+ efflux in cells treated with ETCs and in cells treated with vanadate in both FLC resistant and susceptible strains. The antifungal drugs showed no effect on the rate of H^+ efflux as there was a very small difference on the calculated rate of H^+ efflux in untreated cells and in cells treated with FLC and CAS (**Figure 3.5**).

3.4.4 Rate of H^+ efflux in cells supplied with glucose

The results showed that glucose stimulates the activity of the H^+ *ATPase* by increasing the rate of H^+ efflux in cells supplied with glucose as compared to glucose-starved cells. The rate of H^+ efflux was increased by 68% in untreated FLC resistant cells supplied with glucose compared to untreated glucose-starved cells. In untreated susceptible cells, glucose increased the rate of the H^+ efflux was by 65% compared to untreated glucose-starved cells. Untreated cells supplied with glucose showed an increased rate of H^+ efflux compared to cells treated with ETCs. The same trend observed in glucose-starved cells treated with the antifungal drugs is also observed in cells supplied with glucose, there is a small difference in the rate of H^+ efflux in FLC and CAS treated cells compared with untreated cells (**Figure 3.6**).

All ETCs successfully reduced the rate of H^+ efflux in both FLC susceptible and resistant strains supplied with glucose and in glucose-starved cells compared to untreated cells. Congener 6 had the greatest effect on reducing the rate of H^+ efflux by 75% in glucose-starved cells and by 38% in cells supplied with glucose in both FLC drug resistant and susceptible strains. Eugenol also reduced the rate of H^+ efflux in both strains by 78 % in glucose-starved cells and by 36% in cells supplied with glucose. The antifungal drugs FLC and CAS reduced the rate of H^+ efflux by 11% and 7% respectively in cells supplied with glucose. In glucose-starved cells the rate of H^+ efflux was reduced by 6.3% only in cells treated with FLC. Vanadate reduced the rate of H^+ efflux by 41.5% in cells supplied with glucose and by 75.3% in glucose-starved cells.

Overall, the statistical analysis (P-value) (**Appendix 1A and 1B**) showed that there was a significant reduction on the rate of H^+ efflux in cells treated with ETCs compared to untreated cells in both FLC susceptible and resistant strains. However, there was no significant reduction on the rate of H^+ efflux in cells treated with CAS in the presence and absence of glucose with P-value

> 0.05 in both FLC susceptible and resistant strains. In FLC susceptible strains there was significant reduction on the rate of H^+ efflux in cells supplied with glucose and treated with FLC. However, in FLC resistant strains there was no significant reduction on the rate of H^+ efflux in cells treated with FLC (**Figures 3.5** and **Figure 3.6**).

Table 3 .7: pH of the cell suspension in glucose-starved cells following treatment of FLC resistant strains with ETCs and vanadate (positive control) for the period of 10 minutes.

Time (minutes)	**pH of the cell suspension**										
	Treatment of the cell suspension										
	Untreated control	EUG	C1	C2	C3	C4	C5	C6	FLC	CAS	Vanadate
0	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00
1	6.86	6.87	6.89	6.92	6.94	6.95	6.94	6.95	6.87	6.86	6.97
2	6.89	6.88	6.93	6.99	7.00	6.97	6.95	7.00	6.88	6.88	6.97
3	6.89	6.90	6.94	6.98	6.98	6.96	6.96	6.99	6.90	6.88	6.97
4	6.89	6.92	6.95	6.99	7.00	6.97	6.98	6.98	6.91	6.90	6.98
5	6.91	6.94	6.95	6.99	6.99	6.98	6.98	6.99	6.91	6.90	6.99
6	6.92	6.94	6.96	6.99	6.97	6.98	7.00	6.98	6.93	6.92	6.97
7	6.92	6.95	6.97	6.98	6.98	6.99	6.99	6.99	6.95	6.94	700
8	6.92	6.97	6.98	7.000	7.00	6.98	6.99	6.98	6.96	6.95	7.00
9	6.93	6.98	6.98	7.00	6.99	6.99	6.99	7.00	6.97	6.95	6.99
10	6.94	6.98	6.98	7.000	7.00	6.98	6.98	7.00	6.98	6.97	6.98
Total volume of NaOH added to the cell suspension	**175µl**	**135 µl**	**95 µl**	**45 µl**	**40 µl**	**55 µl**	**60 µl**	**40 µl**	**150 µl**	**170 µl**	**35 µl**

Table 3.8: pH of the cell suspension supplied with glucose in FLC resistant strains treated with ETCs and vanadate (positive control) for the period of 10 minutes.

Time (minutes)	**pH of the cell suspension**										
	Treatment of the cell suspension										
	Untreated control + glucose	EUG + glucose	C1 + glucose	C2 + glucose	C3 + glucose	C4 + glucose	C5 + glucose	C6 + glucose	FLC + glucose	CAS + glucose	Vanadate + glucose
0	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00
1	6.7	6.82	6.84	6.87	6.85	6.88	6.89	6.83	6.71	6.75	6.89
2	6.77	6.90	6.85	6.90	6.88	6.90	6.91	6.87	6.78	6.80	6.90
3	6.81	6.90	6.87	6.92	6.90	6.92	6.91	6.90	6.82	6.82	6.92
4	6.82	6.92	6.89	6.92	6.93	6.93	6.92	6.90	6.87	6.83	6.95
5	6.83	6.93	6.91	6.93	6.95	6.93	6.95	6.94	6.87	6.84	6.97
6	6.84	6.96	6.92	6.93	6.95	6.94	6.96	6.95	6.87	6.88	6.97
7	6.86	6.95	6.93	6.94	6.97	6.94	6.95	6.96	6.88	6.87	6.99
8	6.85	6.97	6.93	6.95	6.98	6.95	6.96	6.96	6.88	6.90	6.98
9	6.85	6.95	6.93	6.95	6.97	6.96	6.97	6.96	6.88	6.92	7.00
10	6.87	6.96	6.94	6.95	6.97	6.96	6.96	6.95	6.89	6.91	6.99
Total volume of NaOH added to the cell suspension	**475µl**	**235 µl**	**290 µl**	**220 µl**	**205 µl**	**230 µl**	**205 µl**	**250 µl**	**395 µl**	**395 µl**	**145 µl**

Table 3.9: pH of cell suspension in glucose-starved cells following treatment of FLC susceptible cells with ETCs and vanadate (positive control) for the period of 10 minutes.

Time (minutes)	**pH of the cell suspension**										
	Treatment of the cell suspension										
	Untreated control	EUG	C1	C2	C3	C4	C5	C6	FLC	CAS	Vanadate
0	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00
1	6.85	6.96	6.98	6.98	6.98	6.96	6.97	6.97	6.85	6.82	6.98
2	6.89	6.99	7.00	6.98	6.99	6.99	7.00	6.98	6.87	6.85	6.98
3	6.88	7.00	6.99	6.98	6.97	6.99	6.99	6.98	6.90	6.88	6.98
4	6.90	6.99	6.99	6.98	6.98	6.99	6.99	6.99	6.91	6.89	6.99
5	6.93	7.00	6.99	6.98	6.99	6.99	6.99	6.99	6.90	6.92	7.00
6	6.93	6.98	6.99	6.99	6.99	6.99	6.98	6.99	6.91	6.93	7.00
7	6.94	7.00	6.99	6.99	6.99	6.99	6.98	6.99	6.93	6.95	6.99
8	6.93	6.99	6.99	6.99	7.00	6.99	7.00	7.00	6.95	6.96	7.00
9	6.93	6.99	6.99	6.99	6.99	7.00	6.99	6.99	6.96	6.95	7.00
10	6.95	6.99	7.00	6.98	6.97	6.99	6.98	6.98	6.96	6.97	7.00
Total volume of NaOH added to the cell suspension	**170 µl**	**35µl**	**40 µl**	**45 µl**	**40 µl**	**45 µl**	**35 µl**	**40 µl**	**170 µl**	**165 µl**	**25 µl**

Table 3.10: pH of the cell suspension supplied with glucose in FLC susceptible cells treated with ETCs and vanadate (positive control) for the period of 10 minutes.

Time (minutes)	**pH of the cell suspension**										
	Treatment of the cell suspension										
	Untreated control+ glucose	EUG + glucose	C1 + glucose	C2 + glucose	C3 + glucose	C4+ glucose	C5 + glucose	C6 + glucose	FLC + glucose	CAS + glucose	Vanadate + glucose
0	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00
1	6.73	6.86	6.87	6.84	6.88	6.86	6.85	6.87	6.80	6.79	6.95
2	6.80	6.92	6.89	6.86	6.89	6.89	6.91	6.91	6.81	6.80	6.96
3	6.83	6.92	6.87	6.90	6.92	6.91	6.93	6.92	6.84	6.82	6.95
4	6.83	6.93	6.91	6.90	6.93	6.91	6.93	6.95	6.86	6.83	6.97
5	6.84	6.94	6.91	6.94	6.96	6.93	6.94	6.95	6.87	6.84	6.96
6	6.86	6.95	6.92	6.94	6.97	6.96	6.95	6.98	6.87	6.87	6.96
7	6.87	6.95	6.92	6.95	6.95	6.93	6.96	6.96	6.89	6.90	6.95
8	6.89	6.96	6.92	6.95	6.96	6.95	6.95	6.96	6.90	6.93	6.96
9	6.89	6.97	6.92	6.97	6.96	6.95	6.95	6.95	6.91	6.93	6.96
10	6.90	6.97	6.93	6.97	6.98	6.95	6.95	6.95	6.91	6.95	6.96
Total volume of NaOH added to the cell suspension	**415 µl**	**215 µl**	**260 µl**	**255 µl**	**205 µl**	**245 µl**	**230 µl**	**200 µl**	**365 µl**	**375 µl**	**170 µl**

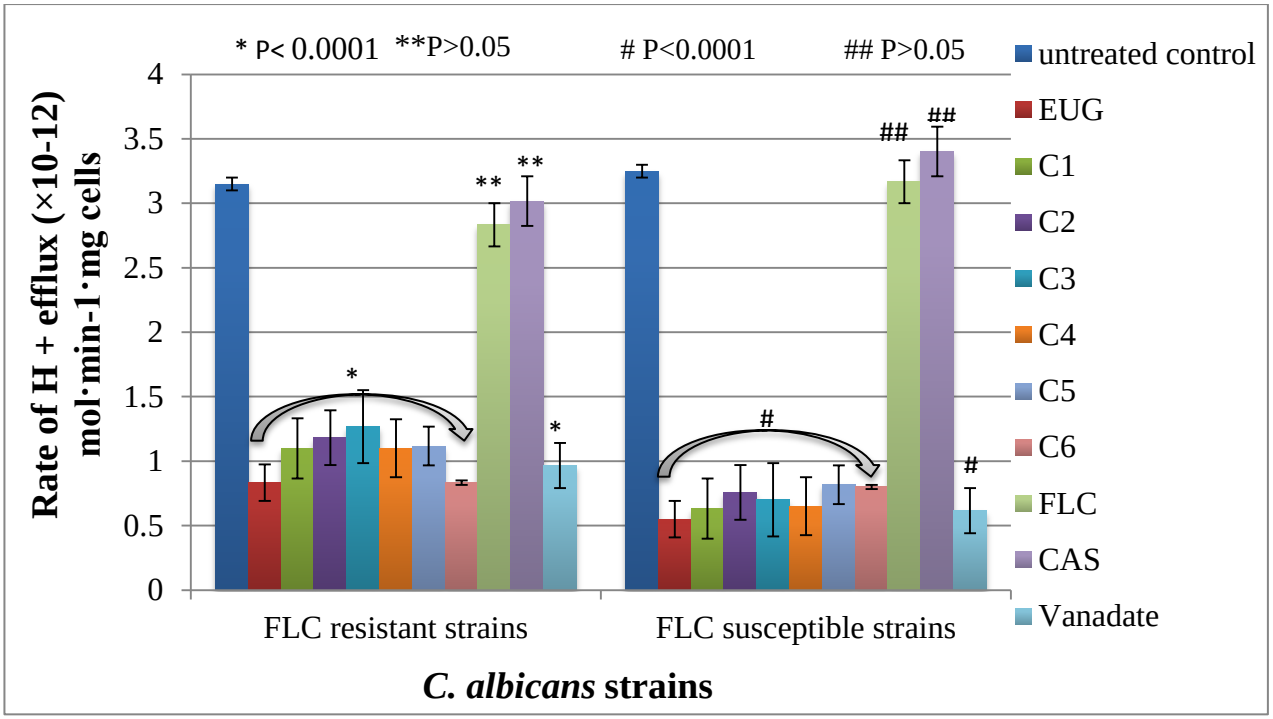


Figure 3.5: Mean rate of proton efflux in glucose starved FLC drug resistant and susceptible cells treated with ETCs at MIC. The level of significance was determined between cells treated with ETCs (C1-C6) and untreated cells (negative control), $P \leq 0.05$ indicates significance. ±SEM.

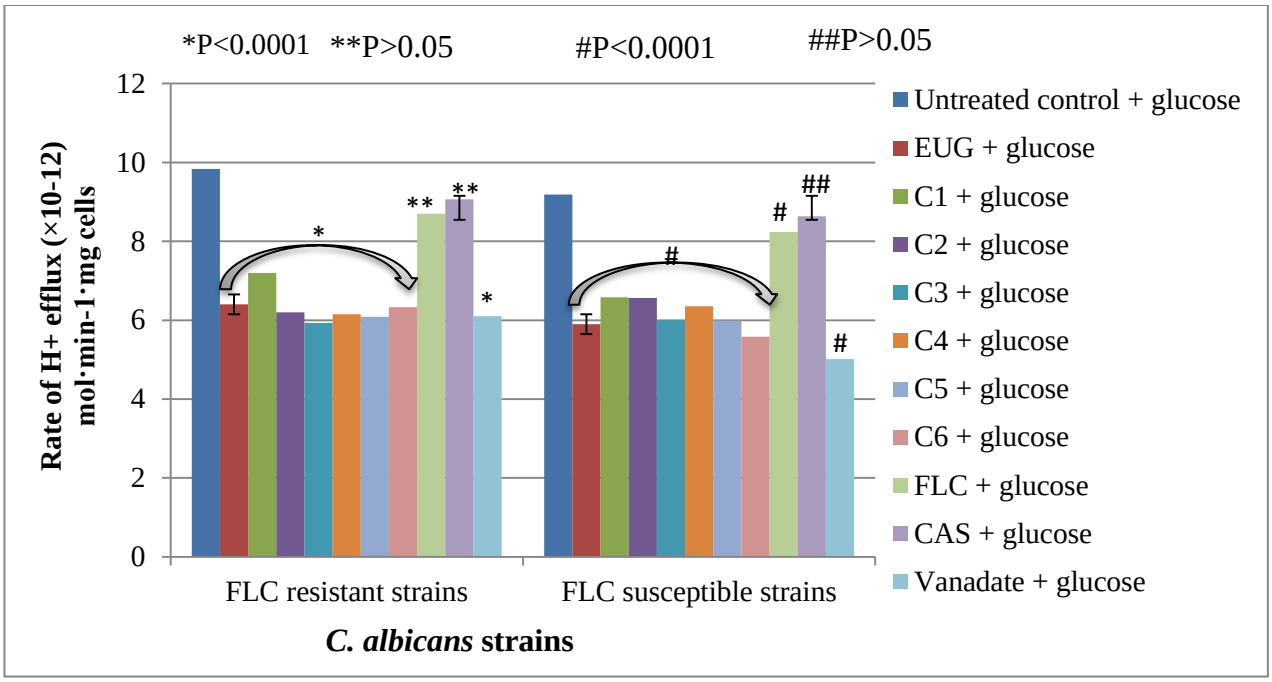


Figure 3.6: Mean rate of proton efflux in FLC drug resistant and susceptible cells supplied with glucose and treated with ETCs at MIC. The level of significance was determined between cells treated with ETCs (C1-C6) and untreated cells (negative control), $P \leq 0.05$ indicates significance. ±SEM.

3.4.2 The effect of Eugenol Tosylate Congeners on Intracellular pH of *Candida albicans* cells

The Intracellular pH of FLC resistant and susceptible strains was measured following treatment of *C. albicans* cells with ETCs. This assay was performed to evaluate the effect of a dysfunctional H^+ *ATPase* on intracellular pH of *C. albicans* cells. The H^+ *ATPase* pumps out H^+ from the cytosol of cells to the extracellular environment resulting in neutral intracellular pH. In this study, ETCs increased intracellular pH of *C. albicans* cells to be less acidic compared to the intracellular pH of untreated *C. albicans* cells.

In FLC resistant strains intracellular pH of untreated cells was 6.47 and the intracellular pH of cells treated with ETCs was 6.81, 6.78, 6.88, 6.81, 6.55 and 6.81 for C1, C2, C3, C4, C5 and C6 respectively (**Figure 3.7**). A similar trend was also observed in FLC susceptible strains were the intracellular pH of untreated cells was very low compared to the intracellular pH of cells treated with ETCs.

The intracellular pH of FLC susceptible strains was 6.37 for untreated cells and 6.62, 6.68, 6.60, 6.59, 6.63, 6.71 for cells treated with C1, C2, C3, C4, C5 and C6 respectively. The intracellular pH for cells treated with EUG varied widely between FLC resistant and susceptible strains with intracellular pH of 6.76 for resistant strains and 6.41 for susceptible strains. In FLC susceptible strains, there was a small difference in the intracellular pH of untreated cells and the intracellular pH of cells treated EUG. Furthermore, the intracellular pH of FLC resistant strains treated with EUG, C1, C2, C3, C4 and C6 was greater than the intracellular pH of FLC susceptible strains treated with the same compounds. However, C5 showed opposite results whereby the intracellular pH in FLC resistant strains is lower (pH= 6.55) than the intracellular pH (pH= 6.63) in FLC susceptible strains.

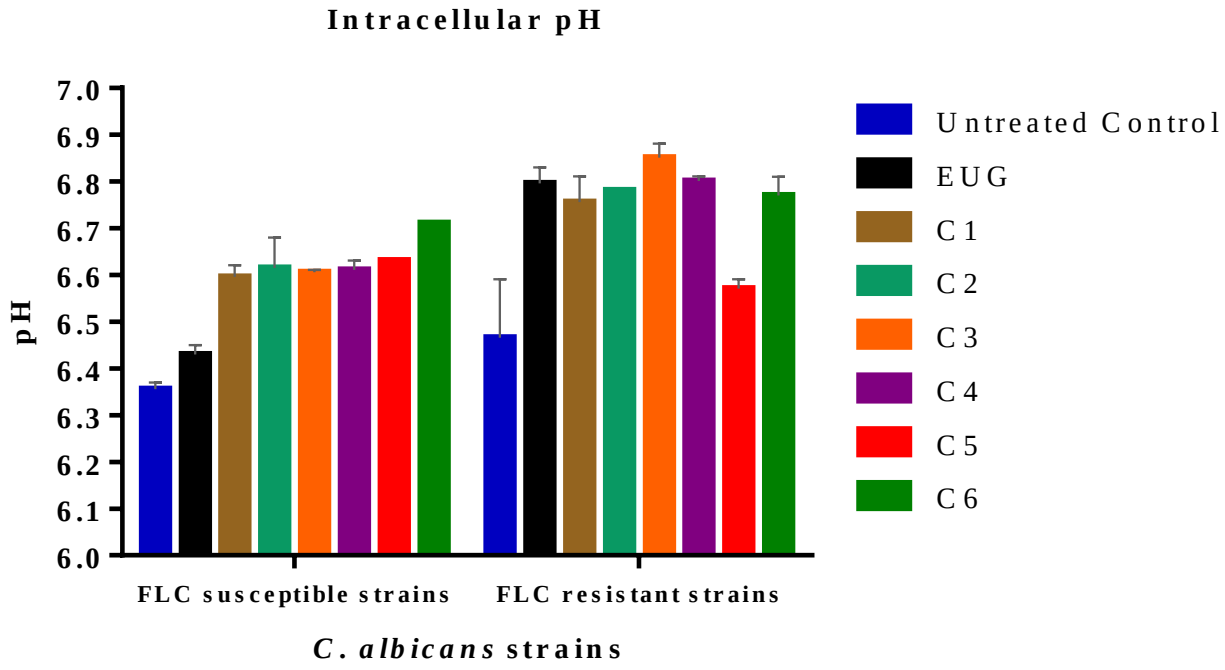


Figure 3.7: Intracellular pH of FLC susceptible and resistant cells treated with ETCs at MIC**.** ± SEM.

3.5 The effect of Eugenol Tosylate Congeners on activity of drug efflux pumps in *Candida albicans* cells

The effect of ETCs on inhibiting activity of drug efflux pumps in FLC resistant and susceptible *C. albicans* strains was investigated. The effect of energy stimulation was also investigated following the addition of glucose to cell suspension. A standard curve illustrated in **Figure 3.9** was constructed from known concentrations of R6G dye and their absorbance was measured at a wavelength of 527nm. The efflux of R6G dye from cells treated with ETCs was extrapolated from the standard curve.

The concentration of the dye out of the cells was determined by measuring the absorbance of the supernatant following incubation of cell suspension with ETCs and glucose after 60 minutes. **Figure 3.8** shows the supernatant collected from centrifuging the cell suspension of untreated FLC resistant strains. The supernatant was clear in the first 25 minutes before the addition of glucose to the cell suspension. Following the addition of glucose to the cell suspension, the collected supernatant was light pink in color indicating the presence of the dye out of the cells.

In FLC susceptible strains the efflux of R6G dye in untreated glucose-starved cells ranged from 0.15 to 0.25µM. The addition of glucose to the cell suspension increased the efflux of the dye to 0.4µM (**Figure 3.10**). Eugenol tosylate congeners at previously determined MICs showed no effect in reducing the efflux of the dye. The efflux of the dye in cells treated with C3 and EUG was equivalent to that of untreated cells. The addition of glucose to the cell suspension in cells treated with C1, C2, C4, C5 and C6 increased the efflux of the dye compared to untreated cells (**Figure 3.11 A-B**).

In FLC resistant strains (**Figure 3.11 C-D**) the efflux of R6G dye in untreated glucose-starved cells ranged from 0.22 to 0.30µM, the addition of glucose to the cell suspension increased the efflux of the dye to the concentration ranging from 0.4 to 0. 5µM. The difference in the efflux of R6G dye was observed after the addition of glucose to the cell suspension. The efflux of R6G dye in glucose-starved cells treated with C1, C3, C5 and C6 at MIC in cells showed a similar trend to that observed in untreated cells as there was a small difference in concentration of the dye out of the cells. In cells treated with C5 and C6 reduced efflux of the dye after 30 minutes when glucose was added to the cells was observed. In cells treated with EUG, C2 and C4 at MIC, significant efflux reduction of R6G dye was observed compared to untreated cells. However, C2 was the most active compound in reducing the efflux of the dye especially after the addition of glucose to

the cell suspension. The activity of C4 was consistent from the beginning (T=0) to the end (T=60) as there was reduced efflux of the dye compared to that of untreated cells and it was not affected by the addition of glucose to the cell suspension (**Figure 3.11C**).

Statistical analysis indicated in **Figure 3.10** shows that there was no significant difference in the efflux of R6G dye in the first 25 minutes in untreated FLC susceptible and resistant strains as the calculated P-values were greater than 0.06. However, after the addition of glucose to the cell suspension (t= 30-60 minutes), there was a significant difference in the efflux of the dye with a P-value lesser than 0.0001. In FLC susceptible strains, there was no significant difference in the efflux of the dye in cells treated with C2, C3, C4, C5, C6 and EUG compared to untreated cells (**Figure 3.11A-B**) (**Appendix 1C**). However, in cells treated with C1, there was a significant increase (P= 0.002) in the efflux of the dye compared to untreated cells. In FLC resistant strains, there was no significant difference in the efflux of the dye in cells treated with C3, C5 and C6 compared to untreated cells. However, cells treated with EUG, C2 and C4 showed a significant reduction in the efflux of the dye with P-values lesser than 0.05. (**Figure 3.11D**) (**Appendix 1D**).

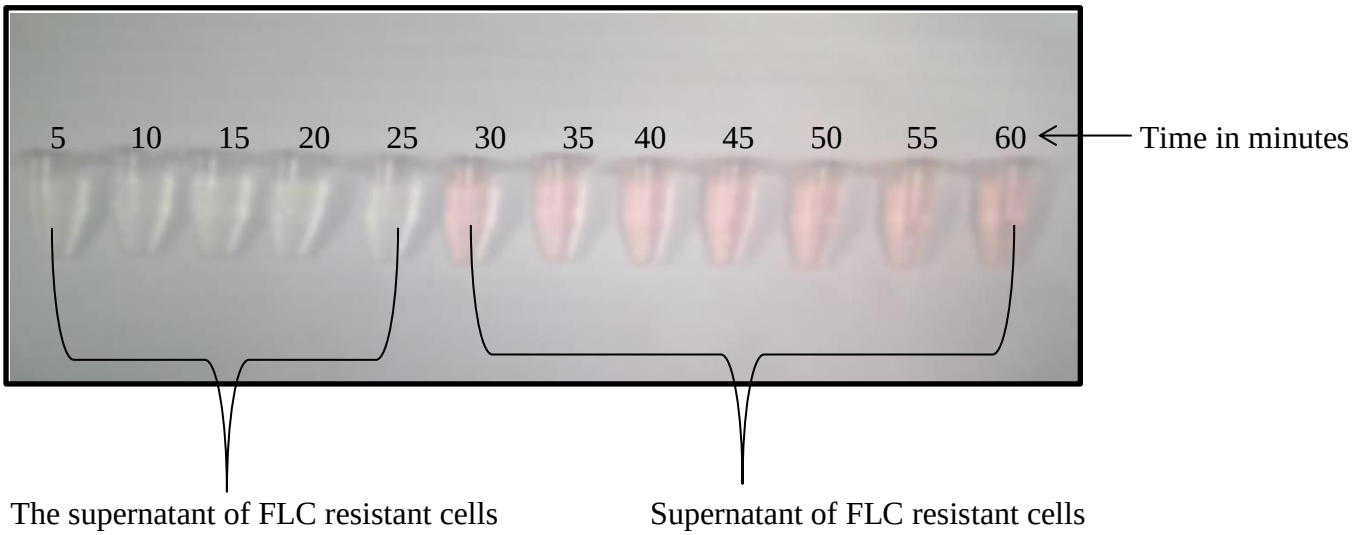


Figure 3.8: Collected supernatant following the efflux of R6G dye in untreated FLC resistant *C. albicans* cells.

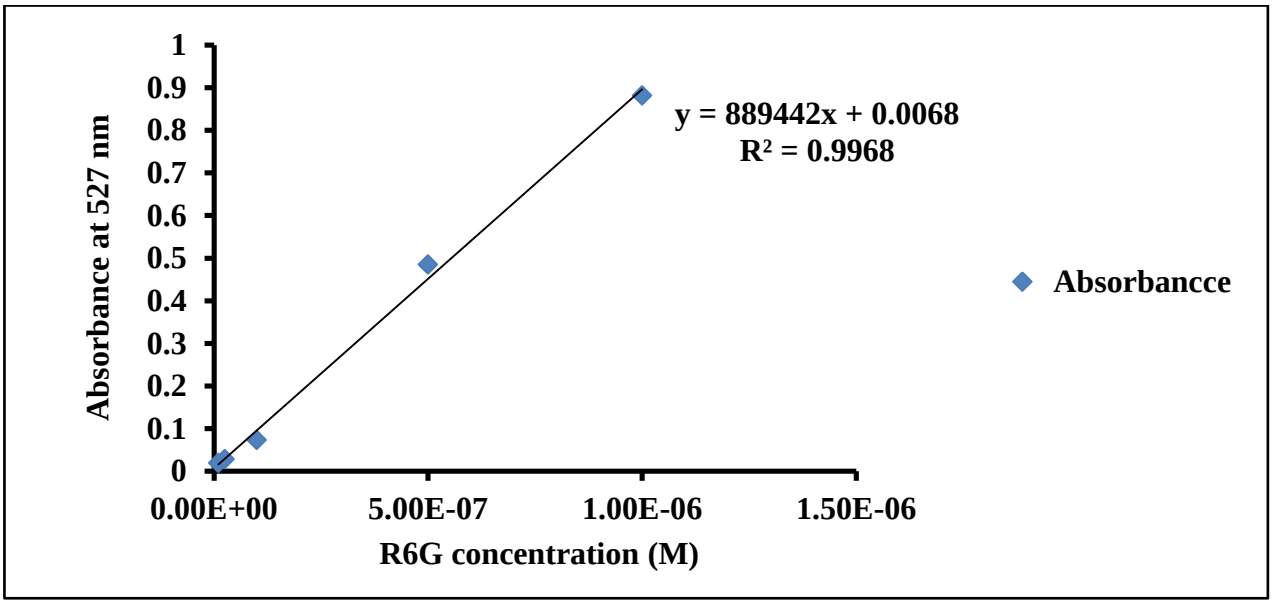


Figure 3.9: Standard curve indicating the concentration of R6G dye with corresponding absorbance values.

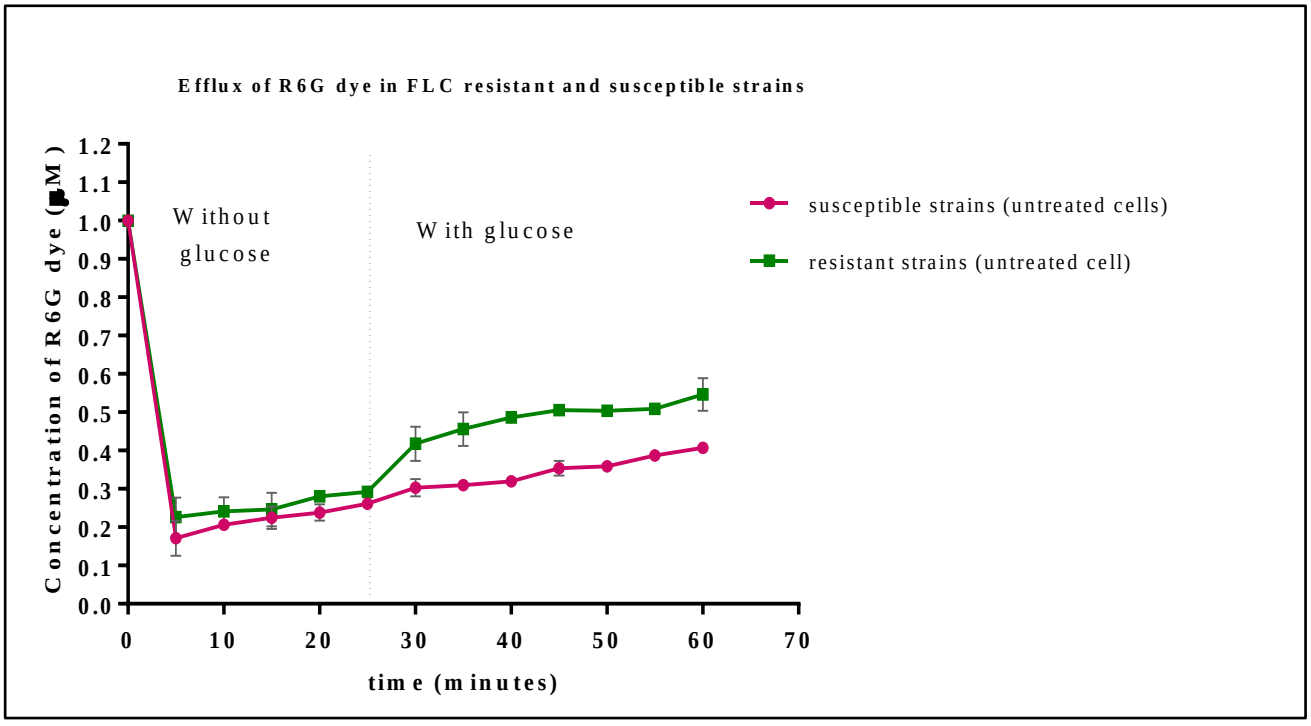


Figure 3.10: Concentration of R6G dye pumped out from FLC susceptible and resistant *C. albicans* cells. P (0-25 minutes) > 0.06 and P (30-60 minutes) = 0.0001. ± SEM.

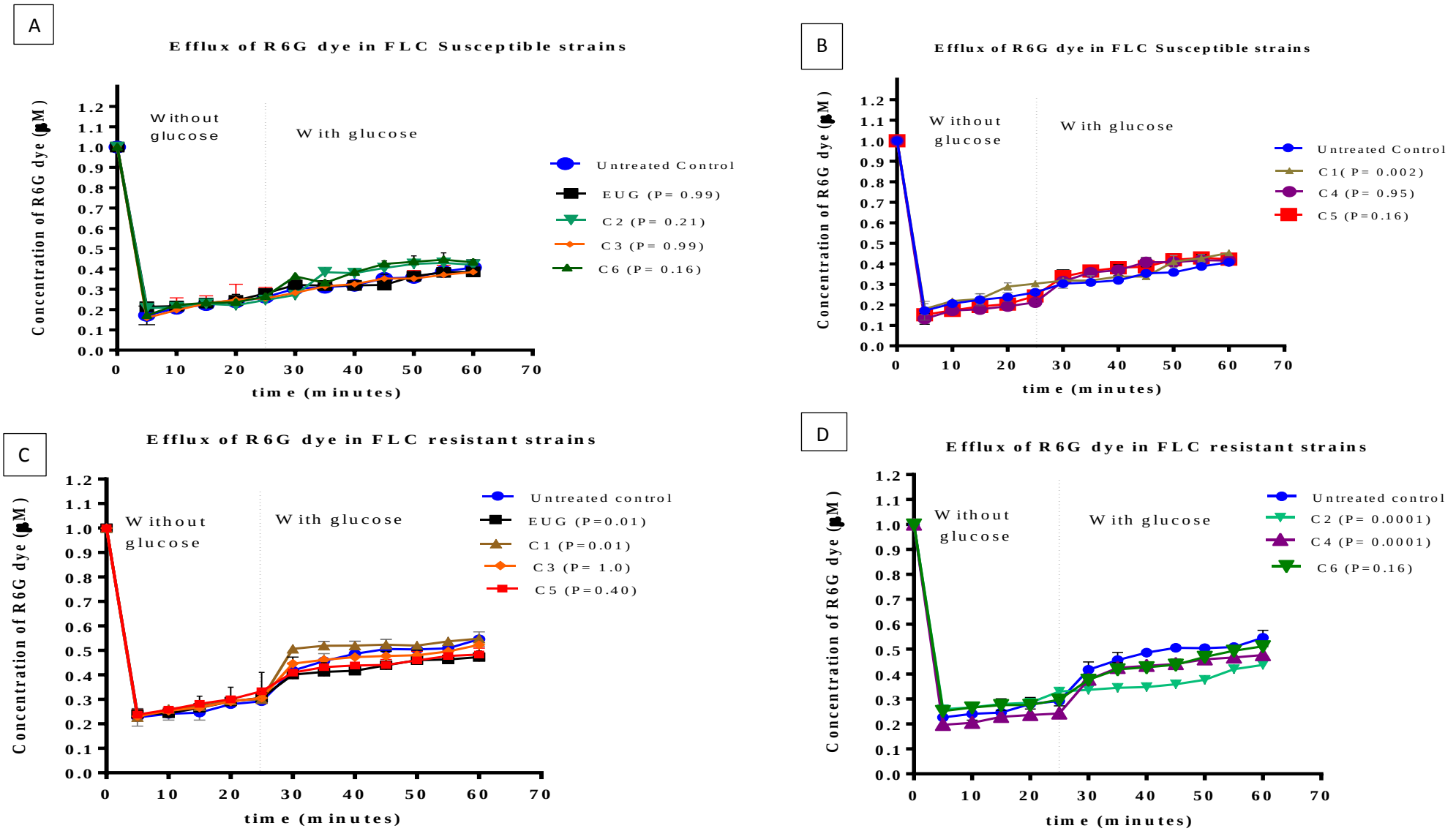


Figure 3.11: Concentration of R6G dye pumped out from FLC susceptible and resistant *C. albicans* cells treated with ETCs and EUG at MIC. ± SEM

3.6 Haemolytic activity of Eugenol Tosylate Congeners on horse red blood Cells

The haemolytic effect of ETCs was performed on horse RBCs to investigate their toxicity effect on RBC. Eugenol tosylate congeners were tested in five different concentrations (2, 1, 0.5, 0.25 and 0.125mg/ml) which include their previously determined MIC values against *C. albicans* **(Table 3.3)**. One percent DMSO and PBS were used as negative controls. Tritonx-100 was used as a positive control and the haemolysis of RBC is indicated in **Figure 3.12**. Incubation of horse RBCs with tritonx-100 induced haemolysis of the cells resulting in release of the constituents of the cell into the solution. After centrifugation, there was no clear separation of the cell pellet and supernatant in cells treated with tritonx-100 since the cells were lysed. However, in cells treated with PBS, there was a clear separation of the pellet and a clear supernatant was observed. This indicated that the RBCs was not lysed. A similar observation was made in RBCs treated with 1% DMSO and **Table 3.11** showed that there was less than 10% haemolysis of the RBC.

Eugenol tosylate congeners induced haemolysis of RBCs in a concentration-dependent manner indicated in **Table 3.11**. However, the haemolytic effects of ETCs were very low compared to the effects of tritonx-100. This is consistent with the observation made in **Figure 3.12** where there was a clear separation of the RBCs pellet and the supernatant showing that there was decreased haemolysis of the cells. Congener 3 showed a slight increase in the haemolysis of RBCs at higher concentrations (2mg/ml, 1mg/ml and 0.5mg/ml) compared to other congeners. Overall all ETCs showed less than 15% haemolysis on horse RBCs. The percentage haemolysis on RBCs caused by ETCs at previously determined MIC values against *C. albicans* are 5.1%, 2.7%, 2.6%, 5.6%, 1.2%, 3.4%, 3.4% for EUG,C1-C6 respectively. Therefore, the mean percentage haemolysis caused by ETCs (C1-C6) at MICs is 2.7%.

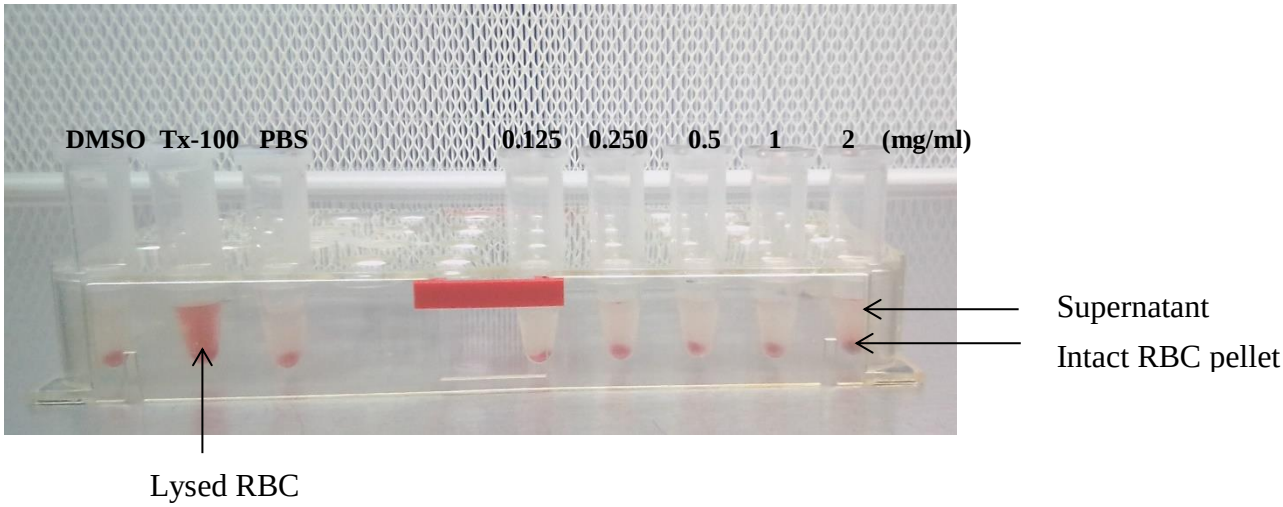


Figure 3.12: Hemolysis of red blood cells following treatment with eugenol tosylate congeners at various concentrations.

Table 3.11: Percentage hemolysis of red blood cells following treatment with eugenol tosylate congeners in various concentrations.

Concentration of the test compounds	Mean percentage haemolysis of horse red blood cells								
	DMSO	Tritonx-100	EUG	C1	C2	C3	C4	C5	C6
1%	3.5±0.23	-	-	-	-	-	-	-	-
2mg/ml	-	-	6.4±3.22	3.7±0.44	2.6±0.38	11.9±1.07	3.1±0.82	3.4±0.94	3.4±0.34
1mg/ml	-	-	5.3±0.26	3.6±0.35	2.1±0.73	5.9±0.64	2.2±0.47	2.4±0.25	3.2±0.27
0.5mg/ml	-	-	5.1±0.40	3.7±0.15	2.0±0.52	5.6±1.17	1.2±0.58	2.3±0.56	2.7±0.82
0.25mg/ml	-	-	2.6±0.44	2.7±0.77	1.5±0.06	4.2±1.27	1.5±1.09	2.2±1.35	1.8±0.94
0.125mg/ml	-	-	2.4±1.31	2.8±0.24	0.5±0.09	2.0±0.78	1.5±0.70	2.00±0.4	1.8±0.74
0.1%	-	100	-	-	-	-	-	-	-

1% DMSO- negative control, Triton X-100 –positive control. ± SEM

CHAPTER 4: DISCUSSION

Candidiasis is a major health problem in immune-compromised individuals such as in cancer and HIV patients or in individuals who had undergone invasive surgical procedures. In situations like this, candidiasis is commonly referred to as a secondary infection which further compromises the immune system of patient causing their health to deteriorate, resulting in death. *Candida albicans* form part of the mucosal microbiota in 40-60% of human population however when it overgrows it becomes infectious (Sardi *et al*., 2013b). *Candida albicans* infections are classified as a nosocomial infection indicating that they are mostly acquired in hospitals during the period patients are admitted or through invasive procedures as *Candida albicans* can form biofilms on catheters and other surgical equipment (Canto' *et al*., 2011; Martins *et al.*, 2014). Hence it is recommended that effective procedures should be employed for disinfection of hospital equipment. In addition, equipment used in hospital theatres should be properly sterilized to avoid growth of *Candida* as it can be life-threatening (Hidron *et al*., 2008).

There are different types of disinfectants that are used in hospitals to help reduce microbial contamination which includes formaldehyde, hydrogen peroxide, paracetic acid, sodium hypochlorite, iodophors, phenols and quaternary ammonium compounds (CDC, 2008). However, the number of patients acquiring *Candida* infections in hospitals is still high (CDC, 2008). Therefore effective antifungal drugs are needed to treat *Candida* infections in these patients. Azoles are the most prescribed antifungal drugs and are also used as prophylaxis for patients in intensive care unit (Eggimann *et al*., 1999). The drawback with azoles is that most of the drugs in this class are fungistatic in nature and this result in *Candida* species developing drug resistance to azole drugs (White *et al*., 1998)

Antifungal drug resistance is a major international health problem and a primary cause of mortality. In developing countries and undeveloped countries where there is limited or poor access to quality healthcare and medicines, infectious diseases such as candidiasis continue to be the main contributing cause of death (O'Donnell, 2007).

The Centers for Disease Control and Prevention, the Genetic and Rare Diseases information Centers and the United States of America National Cancer Institute released a statement on antimicrobial resistance as a global threat with *C. albicans* being the leading fungal resistant pathogen (Granier, 2000).

According to CDC, antifungal drug resistance in *C. albicans* has no pathological effect on the disease caused by this species and they also indicated that "there is no difference in severity of disease caused by susceptible strains and resistant ones" (CDC, 2018). However, drug resistance reduces the options available for therapy.

Over the past decades, there has been ongoing research on developing new therapeutic strategies to combat *C. albicans* infections and also to reverse antifungal drug resistance in this species. Intensive research has been done on using natural compounds such as plant extracts, essential oils and plant fractions as an alternative treatment for *C. albicans* infections (Arif *et al*., 2009; Ahmad *et al*., 2012; Freires *et al*., 2014). In addition, natural compounds have been modified to enhance their antifungal activity and also to improve their physicochemical properties such as solubility, drug absorbance and bioavailability (Newman *et al*., 2003; Ulrich-Merzenich, 2014).

Combination therapy has also been investigated as one of the promising therapeutic strategies against resistant *C. albicans* species. Most studies have shown that using natural compounds in combination with known antifungal drugs exhibits synergistic antifungal effect against *C. albicans* (Alves-Silva *et al*., 2013; De Castro *et al*., 2015; da Silva *et al*., 2017). Synergistic and additive interactions of synthetic drugs in combination have also been reported in other studies (Liu *et al.*, 2017; Canturk, 2018). The advantage of using combination therapy against drug-resistant *C. albicans* strains is the use of low drug concentration avoiding toxicity to human cells. Another aspect that has been looked at towards development of new antifungal drugs involves determining new drug targets in *C. albicans.* Some work has been done on targeting proton *ATPase*, both V-type and P-type. Alteration of the activity of these two H^+ pumps has been shown to affect intracellular pH of *C. albicans* cells leading to inhibition of fungal growth and reduced virulence (Liu and Kohler, 2015).

In this study ETCs which are semi-synthetic compounds derived from EUG, a natural product found in clove oil, were investigated. The antifungal activity of ETCs was determined against drug-resistant and susceptible *C. albicans* strains. The antifungal activity of the combination of ETCs with known antifungal drugs (FLC, NYS and CAS) was also investigated to determine the effect of drug interaction. From the results obtained further studies were conducted to understand the mechanism of action of these compounds. In this regard, the effect of ETCs on activity of the H^+ *ATPase* was evaluated by measuring extracellular pH of *C. albicans* cell suspension and intracellular pH of *C. albicans* cells. In addition, the effect of ETCs on inhibiting activity of drug efflux pumps (cdr1p, cdr2p and mdr) and reversing antifungal drug resistance in FLC resistant

strains was investigated. The toxicity effect of ETCs was determined to assure that these compounds only target *C. albicans* cell and not human cells.

4.1 Antifungal drug susceptibility against *Candida albicans*

Candida albicans isolates that were screened were collected from immune compromised patients with head and neck cancer and from HIV positive patients. Drug susceptibility testing was performed using FLC, NYS and CAS against *C. albicans* isolates. Fluconazole, NYS and CAS antifungal drugs are the three most prescribed antifungal drugs in patients with candidiasis. Most of the isolates were resistant to FLC (14%), followed by NYS (12%) and CAS (10%). Previous studies have also shown that FLC drug resistance is more common compared to NYS and CAS, especially in *C. albicans* species (Ellis, 2002). Bitew and Abebaw. (2018) revealed that *C. albicans* was the most isolated *Candida* species (58.6%) in patients with vulvovaginal candidiasis and was found to be resistant to FLC and flucytosine but not to echinocandins. Another study by Owotade *et al*. (2016) showed that *C. albicans* was the dominating *Candida* species isolated from HIV (73.16%), cancer (44.95%) and from healthy individuals (42.86%). In another study by Lacka *et al*. (2015) FLC drug resistance was observed in two *C. albicans* strains with MICs of 16µg/ml and 256µg/ml which are similar to those obtained in this study. In addition, Cataldi *et al*. (2017) reported FLC resistance in 4.54% *C. albicans* strains isolated from patients tested positive for *Candida* infections and presented clinical signs of *Candida* infections.

The increased prevalence of FLC drug resistance in *C. albicans* isolates was suggested to be due to overuse of FLC as prophylaxis and also as a treatment for *C. albicans* associated infections (Becher and Wirsel, 2012; Leonart *et al*., 2017). In this study, FLC resistant isolates had MICs ranging from 31.5µg/ml to 250µg/ml which are very high concentrations compared to MICs reported in other studies (Lacka *et al*., 2015; Owotade *et al*., 2016 Bitew and Abebaw. 2018). However, Sajjad *et al.* (2012) reported similar results on MICs of FLC against *C. albicans* strains. All tested *C. albicans* isolates had MICs ranging from 128– 256µg/ml for FLC (Sajjad *et al.*, 2012). Liu *et al.* (2017) have reported FLC MICs as high as 512µg/ml among FLC resistant strains. Another factor that contributes to increased prevalence of FLC drug resistance is the fungistatic property of FLC (Sanglard *et al.*, 2003; Uppuluri *et al.*, 2008). Antifungal drug resistance to FLC was suggested to be acquired resistance (Berkow and Lockhart, 2017). Fluconazole act by inhibiting the activity of 14-alpha-demethylase enzyme which converts lanosterol to ergosterol, an important component of fungal plasma membrane (Georgopapadakou

and Walsh, 1996). The inhibition of this enzyme results in accumulation of lanosterol on the cytosol of fungal cell leading to cell growth arrest, not death (Kelly *et al.,* 1997).

Nystatin drug resistance in *C. albicans* species is rare, however in this study resistance to NYS was observed in 12% of the tested isolates with MICs ranging from 0.12µg/ml to 1.95µg/ml. In Nigeria a study conducted by Doughari *et al*. (2009) showed that 78.6% of the screened *C. albicans* strains were completely resistant to NYS. Other studies have shown that *C. albicans* isolates are commonly susceptible to NYS (Nenoff *et al*., 2016). In a study conducted in Uganda, NYS resistance was observed in 0.61% of *C. albicans* strains isolated from women diagnosed with vulvovaginal infection (Mukasa *et al*., 2015). However, these results were criticised by Nenoff *et al*. (2016) because the agar diffusion test was used instead of using recommended methods such as EUCAST or CLSI. In another study done in Ethiopia, NYS resistance was observed in 1.3% of tested isolates which were collected from HIV positive individuals with oropharyngeal candidiasis (Moges *et* al., 2016). In 2015 antifungal drug susceptibility was performed in *C. albicans* strains isolated from HIV positive and negative patients with and without oropharyngeal candidiasis in India (Moges *et* al., 2016). In HIV positive patients 2.8% of isolates were resistant to NYS, 61.1% were susceptible while 36.1% were dose-dependent susceptible. In HIV negative patients no resistant strains were observed (Dar *et al*., 2015). The difference in percentages of resistant isolates reported by different authors is due to the fact that different breakpoints are used by different investigators since there are no standard breakpoints set for NYS susceptibility by CLSI and the EUCAST committees.

Nystatin drug resistance in *C. albicans* obtained in this study were very high compared to other studies. This could be due to different geographic locations and the medical history of the selected patients which might have included previous exposure to NYS. The low prevalence of NYS resistance might be due to its fungicidal activity against *C. albicans* and most fungal species. The use of NYS as antifungal agent is limited to topical creams and oral use against superficial and gastrointestinal infections due to poor water solubility and toxic side effects when administered intravenously (Semis *et al.*, 2013). To overcome this problem, NYS was reformulated as a lipid complex with increased antifungal activity and decreased toxicity effects both *in vivo* and *in vitro* (Semis *et al.*, 2013). Nystatin, like all other polyene antifungal drugs, act by binding to ergosterol in fungal plasma membrane and forming transmembrane pores that are selective to monovalent ions including potassium, sodium and phosphate ions leading to impairment of cellular respiration then cell death (Anderson *et al*., 2014; Mukasa *et al*., 2015). Resistance to NYS is due to the

inactivation of important proteins involved in biosynthesis of ergosterol resulting in depletion of ergosterol and less target molecules for NYS binding (Sanglard and Odds, 2002).

Drug resistance to CAS in *C. albicans* was also observed in this study. Among the total isolates that were tested, 10% were resistant to CAS with MICs ranging from 1µg/ml to 3.9µg/ml. Similarly to NYS, CAS resistance in *C.albicans* is very low (Castanheira *et al*., 2010). Castanheira *et al*. (2010) have reported prevalence of less than 3% CAS resistance for the year of 2005. However acquired CAS resistant has been reported in some *C. albicans* and other non-*albicans* species (Hitchcock *et al*., 1995; Desnos-Ollivier *et al*., 2007). Increased CAS resistance is more often reported in *C. glabrata* than any other *Candida* species; however *C. albicans* has been widely reported as causative agent of most invasive infections (Dannaoui *et al*., 2012; Matsumoto *et al*., 2014). This is caused by prolonged administration of CAS to patients with *C. glabrata* associated infections leading to acquired CAS resistance in this species (Arendrup and Perlin, 2014). This might also be the case with the results observed in this study with an increased number of isolates resistant to CAS. The isolates tested were collected from immune-compromised HIV positive patients and from patients with head and neck cancer. Their histories on previous exposure to antifungal drugs were unknown and the possibility might have been that CAS was administered as treatment for any form of *Candida* infections. The Infectious Disease Society of America reported on guidelines for management of different types of candidiasis: The recommendation in HIV infected patients was a loading dose of 70mg followed by a 50mg daily of CAS to minimise recurrent oropharyngeal and oesophagal candidiasis (Pappas *et al*., 2016).

Echinocandins are by far the only class of antifungal drugs that act to target fungal cell wall (Douglas, 2001). They do so by inhibiting glucan synthesis which is an essential component of fungal cell wall (Douglas, 2001). The increased MICs of CAS is due to mutations (stop codons, deletions or substitutions of amino acids) in *FSK1* gene which encodes for major catalytic subunit of 1, 3-β-D-glucan synthase enzyme (Park *et al.*, 2005; Perlin, 2007; Arendrup and Perlin, 2014). *Candida* species that have shown to be intrinsically resistant to echinocandins were found to contain mutations on *FSK1* gene and displayed increased MIC values ranging between 0.5 and 8µg/mL (Pfaller *et al*., 2011; Beyda *et al*., 2012).

An interesting observation was made where two isolates collected from HIV positive patients showed to be resistant to two different classes of antifungal drugs. Isolate 004 was resistant to both FLC and NYS and isolate 002B was resistant to FLC and CAS. Oral candidiasis is more

prevalent in HIV positive patients and these three antifungal drugs are mostly prescribed to patients with oropharyngeal and oesophagal candidiasis hence it is most likely for the isolates to acquire resistant to any, both or all prescribed antifungal drugs (Zaoutis *et al.*, 2005; Cowen, 2008). Azole and polyene cross-resistance is more common compared to echinocandin multidrug resistance (Parker *et al*., 2010). This is due to the fact that azole and polyene resistance is associated with ergosterol synthesis. *Candida albicans* isolates resistant to both azole and polyenes were reported to have mutations on the *ERG11* gene and either on the *ERG3* or *ERG5* genes (Martel *et al*., 2010; Parker *et al*., 2010). Another multidrug resistance case was reported as a stepwise development over a period of 5 years to three different antifungal drugs (azoles, polyenes and echinocandins) against *C. albicans* from a patient diagnosed with mucosal infections (Jensen *et al*., 2015).

4.2 Antifungal activity of Eugenol Tosylate Congeners

The antifungal activity of ETCs and EUG against drug-resistant and susceptible *C. albicans* strains was investigated. Eugenol tosylate congeners are derivatives of EUG which have been extensively studied as potential antimicrobial agents (Ahmad *et al*., 2010; Sajjad *et al.*, 2012; da Silva *et al*., 2017; Pavesi *et al*., 2018). Eugenol was previously used in dentistry however it was phased out due to toxicity reports to human soft tissues (Sarrami *et al*., 2002). In contrast, EUG was reported to have low cytotoxicity activity against human erythrocytes at low concentrations (Hemaiswarva *et al*., 2009). In addition, EUG nanoemulsion was reported to be the safest formulation to be used to avoid cytotoxicity in peripheral blood mononuclear cells (Sarrami *et al*., 2002; Miao *et al*., 2007; Gündel *et al*., 2019). In the current study, EUG was modified through the process of tosylation to enhance the antifungal activity and other physicochemical properties such as solubility, absorbance and bioavailability.

All six ETCs exhibited antifungal activity against tested *C. albicans* strains. The antifungal activity of these compounds varied from one compound to another as different MICs were obtained for each compound. Overall, C1 was the most active compound with a lower concentration (0.13 and 0.250mg/ml) required to inhibit *C. albicans* growth compared to other compounds and EUG. Congener 2 was the least active compound with increased concentrations (2mg/ml) required to inhibit *C. albicans* growth. The MIC obtained for EUG ranged from 0.25 to 0.5mg/ml against *C. albicans*. These results are consistent with data that have been reported on the MICs of EUG obtained against *C. albicans* isolates (Ahmad *et al*., 2015; da Silva *et al*., 2017).

Minimum fungicidal concentration of ETCs obtained shows that the compounds have fungicidal activity since their MIC values were obtained one well below MFC values.

The difference in antifungal activity of ETCs in terms of MICs against *C. albicans* strains is associated with their chemical structures. Different functional groups were used to modify EUG and produce ETCs. These functional groups contribute to the antifungal activity of ETCs. In this study, phenyl functional group in C1 improved the antifungal activity of the compound compared to the parental drug EUG. Cyclopentane functional groups are present in most of synthetic compounds and have greater therapeutic potential against most problematics microbes including *C. albicans, C. neoformans* and *Aspergillus fumigatus* (Babu *et al*., 2006). Congener 4 also showed improved antifungal activity compared to C2 and C3 which also consist of chlorine atoms in their phenyl ring. The study of Woo *et al*. (2013) indicated that the addition of the same halogen atom (chlorine) to the parental group resulting in dihalogenated (3, 4-dichlorophenyl) or trihalogenated (2, 4, 5-trichlorophenyl) compounds significantly improves the inhibitory activity of compounds. Moreover, they explained that increased lipophilicity of the compounds contributes significantly to their inhibitory abilities. Hence C4 (4-allyl-2-methoxyphenyl (2, 4, 5-trichlorophenyl) methane sulfonate) exhibited increased antifungal activity against *C. albicans* strains compared to other chlorinated compounds (C2 and C3). This is the first study to illustrate the antifungal activity of ETCs against drug-resistant and susceptible *C. albicans* strains hence results obtained cannot be compared to previous studies.

4.3 Antifungal activity of Eugenol Tosylate Congeners in combination with antifungal drugs

Combination therapy was introduced as a solution to solve complex infectious diseases. In this study, increased prevalence of antifungal drug resistance in *C. albicans* strains was observed in three of the most commonly used antifungal drug; FLC, NYS and CAS. The combination activity of ETCs with these antifungal drugs was determined.

4.3.1 Combination of ETCs with FLC

The FICI revealed that all the combinations between ETCs and FLC in a 1:1 ratio exhibited additive effect against FLC resistant strains. This indicated that the antifungal activity of all ETCs (C1, C2, C3, C4, and C6) and EUG with FLC are lesser than antifungal activity of the drugs used individually (Levin and Harris, 1975).

In FLC susceptible strains, EUG, C1 and C2 in combination with FLC in a 1:1 ratio exhibited indifferent interaction suggesting that combination of the two compounds does not exhibit an increased antifungal activity compared to most active compound used alone (FLC) (Levin and Harris, 1975). Additive effect was exhibited following the combination of C3, C4, C5, and C6 with FLC against FLC susceptible strains. Overall no synergistic effect was observed following combination of ETCs with FLC in both FLC susceptible and resistant strains. Previous studies have reported synergy in most of the combinations between their compounds of interest (licofelone, C12, C14, EUG,) with FLC in a 1:1 ratio against both FLC resistant and susceptible strains (Khan *et al*., 2012; Shrestha *et al*., 2015; Liu *et al*., 2017). Ahmad *et al*. (2015) reported on combination activity of FLC with the derivatives of EUG in a 1:1 ratio. In their study, they observed 36% of synergistic interactions, 41% of additive and only 23% of indifferent interactions in *C. albicans*.

4.3.2 Combination of ETCs with NYS

The FICI for combination activity of ETCs with NYS against NYS resistant strains showed that all combinations in a 1:1 ratio exhibited synergistic effect except for C5 which exhibited an additive effect. These results show that antifungal activity of ETCs combined with NYS is greater than antifungal activity of drugs used alone (Levin and Harris, 1975). No synergy was observed in NYS susceptible strains, however, EUG, C1, C3, C4 and C6 in combination with NYS exhibited indifferent effect whereas C2 and C5 exhibited additive effect. In the study of da Silva *et al.* (2017), combination activity of EUG with NYS exhibited an indifferent interaction and these results are similar to those observed in this study against NYS susceptible strains. However, the study of De Castro *et al*. (2015) showed that combination activity of NYS with thymol, a natural compound exhibit a synergistic effect against *C. albicans* strains.

Eugenol tosylate congeners have shown to have increased antifungal activity in combination with NYS in 1:1 ratio. Unlike with FLC, combination of NYS with ETCs is effective and exhibited greater antifungal activity even against susceptible strains and this is illustrated by most synergistic and additive effect obtained following treatment of *C. albicans* strains with ETCs in combination with NYS.

4.3.3 Combination of ETCs with CAS

The combination activity of ETCs with CAS in 1:1 ratio was also determined. Congener 2 and C3 in combination with CAS exhibited synergistic effect based on calculated FICI value of 0.25 and 0.50 respectively against CAS resistant strains. Congener 1 showed indifferent effect whereas

EUG, C4, C5 and C6 in combination with CAS exhibited additive effect against CAS resistant strains. Most of the drug interactions in a 1:1 combination ratio between CAS and ETCs were indifferent (50%) indicating that the combination of CAS with ETCs does not exhibit increased antifungal activity compared to the antifungal activity of the most active compound used alone (CAS). The combination of CAS with semi-synthetics compounds derived from the aminoglycoside tobramycin did not exhibit synergy against *C. albicans* strains (Shrestha *et al.,* 2015). However study of Canturk. (2018) showed that combination of CAS with ferulic acid exhibited a synergistic effect against both *C. albicans* and *C. glabrata*.

4.4. The effect of Eugenol Tosylate Congeners on activity of proton *ATPase* in fluconazole susceptible and resistant *Candida albicans* strains

The mechanism of action of ETCs on *C. albicans* was investigated by evaluating the effect of ETCs on the rate of H^+ efflux in *C. albicans* cells. The H^+ *ATPase* enzyme located on the fungal plasma membrane is responsible for the efflux of H^+ to external medium maintaining intracellular pH of the cells.

All ETCs were effective in reducing the rate of H^+ efflux in both glucose-starved cells and in cells supplied with glucose in FLC resistant and susceptible strains. Similar results were also reported by Ahmad *et al*. (2010). Eugenol reduced the rate of H^+ efflux in both FLC susceptible and resistant strains by 78% in glucose-starved cells and by 36% in cells supplied with glucose. These results are consistent with those reported by Ahmad *et al*. (2010). Eugenol tosylate congeners showed varying inhibitory activity of H^+ *ATPase* in both FLC resistant and susceptible strains. This was observed in **Figure 3.5** and **Figure 3.6** where the rate of H^+ efflux in cells treated with ETCs fluctuated. The trend observed for inhibitory activity of vanadate (positive control) on activity of H^+ *ATPase* was also observed in cells treated with ETCs indicating that ETCs and vanadate have same inhibitory effect on *C. albicans* cells, which suggest that they might act in the same manner. Manzoor *et al*. (2004) reported that treatment of *C. albicans* cells with 5mM of vanadate results in decreased extracellular pH and these results are consistent with the observation made in the current study, whereby cells treated with ETCs and 5mM vanadate showed decreased (neutral) extracellular pH ranging between 6.95 and 7.00. The antifungal drugs FLC and CAS showed no effect on activity of H^+ *ATPase.* There was no significant difference in the rate of H^+ efflux in cells treated with FLC or CAS and untreated cells.

The effect of energy stimulation on activity of the H^+ *ATPase* was also investigated. The rate of H^+ efflux in untreated cells supplied with glucose was stimulated by at least 68% compared to

untreated glucose-starved cells. This might be due to the fact that H^+ *ATPase* belongs to P-type class of *ATPases* which utilises the hydrolysis of ATP as a source of energy (Haruta *et al*., 2015). Therefore, in the current study, glucose was supplied to cell suspension as a source of energy and activity of H^+ *ATPase* increased. This was observed with increase in the rate of H^+ efflux in cells supplied with glucose compared to glucose-starved cells (**Figure 3.5** and **Figure 3.6**). This was also shown with the rapid decline in pH of cell suspension to pH 6.7 in cells supplied with glucose whereas in glucose-starved cells, pH of cell suspensions ranged between 6.95 and 7 (**Table 3.7** to **Table 3.10**).

The results obtained in **Figure 3.5** and **Figure 3.6** illustrated that there was no significant difference observed on the rate of H^+ efflux in untreated FLC resistant and susceptible strains. This suggests that the H^+ *ATPase* plays no role in conferring antifungal drug resistance in *C. albicans* but has a significant role in pathogenicity of *Candida* species (Liu and Kohler, 2015). Antifungal drug resistance has no impact on pathology of candidiasis indicating that there is no difference in severity of the disease caused by drug-susceptible or resistance strains (CDC, 2018).

4.4.1 The effect of Eugenol Tosylate Congeners on intracellular pH of *Candida albicans* cells

Intracellular pH of FLC resistant and susceptible *C. albicans* strains was measured following treatment of these cells with ETCs at previously determined MICs. Intracellular pH of FLC susceptible strains treated with ETCs was slightly lower compared to intracellular pH of FLC resistant cells treated with ETCs. In addition, intracellular pH of untreated cells was lower with pH of 6.34 and 6.37 in FLC resistant and susceptible strains respectively. These results suggest that H^+ production in cells that were treated with ETCs was reduced hence; the pH was increased (less acidic) compared to the pH of untreated *C. albicans* cells.

The study by Liu and Kohler. (2015) showed that when *C. albicans* cells were treated with omeprazole, a known inhibitor of H^+ *ATPase* enzyme, intracellular pH of cells decreased and the pH was more acidic. This was due to the fact that H^+ accumulated in the cytosol without being pumped out as the activity of enzyme (H^+ *ATPase*) responsible for pumping H^+ to extracellular environment was inhibited. Another study reported on intracellular pH of *C. albicans* following inhibition of activity of H^+ *ATPase* by EUG and thymol which resulted in acidic intracellular pH (Ahmad *et al*., 2010).

In this study, intracellular pH of *C. albicans* cells treated with ETCs was greater than intracellular pH of untreated cells with functional H^+ *ATPase* activity. These results are different from those reported by Liu and Kohler. (2015) and Ahmad *et al*. (2010), which showed that, inhibition of H^+ *ATPase* activity results in acidification of the cytosol of the cell. However, this study showed that treating *C. albicans* cells with ETCs reduced activity of H^+ *ATPase* leading to less acidic pH of the cytosol in *C.albicans* cells. These suggested that ETCs does not act to inhibit activity of H^+ *ATPase* but may target other processes in which H^+ are generated from. Therefore, blocking proton production from metabolic processes results in less acidic to neutral pH (6.6-6.89) in the cytosol and in neutral extracellular environment of cells (pH = 6.95-7).

The study of Scialò *et al*. (2017) indicated that during cellular respiration, electron transport chain transport H^+ to the cytosol from mitochondria resulting in an electrochemical gradient that stores energy to be used later by the cell. These H^+ are then transported back to mitochondrion through F-*ATPase* known as *ATP-synthase* as it generates ATP while pumping H^+ to the mitochondria. Some H^+ are transported to the vacuole and other organelles via V-*ATPase* while the P-*ATPase* (*H^+-ATPase*) efflux H^+ out of the cell to extracellular environment. Therefore, blocking electron transport chain prevents pumping of H^+ to the cytosol resulting in a less acidic pH (6.6-6.89) in the cytosol and fewer H^+ to be efflux out of the cell. Hence, activity of *H^+-ATPase* appeared to be reduced following treatment of cells with ETCs. Ultimately, inhibition of electron transport chain leads to decreased energy production by respiratory chain and increased production of reactive oxygen species then cell death (Scialò *et al*., 2017). The study by Grahl *et al*. (2015) indicated that treatment of *C. albicans* cells with methylene blue disrupted electron transport chain and in turn reduced proton gradient and ATP production.

4.5. The effect of Eugenol Tosylate Congeners on activity of drug efflux pumps in *Candida albicans*

The drug efflux pumps are important biological proteins in *C. albicans* as they play a vital role in the survival of the species. The drug efflux pumps act by removing toxic substances such as antifungal drugs out of *C. albicans* cells (Khandelwala *et al.,* 2018). There are two main efflux pumps that have been reported to be responsible for conferring FLC resistance in *C. albicans* species: ABC proteins and MFS pumps (Prasad and Rawal, 2014). In this study, activity of drug efflux pumps in FLC resistant and susceptible strains was investigated using R6G dye assay. The effect of ETCs on inhibiting activity of drug efflux pumps was determined. In FLC susceptible strains the efflux of R6G dye in untreated, glucose-starved cells ranged between 0.15µM and

0.22µM, while in untreated glucose-starved FLC resistant cells the efflux of the dye ranged between 0.22µM and 0.30µM (**Figure 3.10**). Statistical analysis indicated that there was no significant difference in the efflux of the dye in cells without glucose in both FLC resistant and susceptible cells. However, there was a significant difference in efflux of the dye in cells supplied with glucose between FLC resistant and susceptible cells in cells with glucose. Similar observations were reported by Ahmad *et al*. (2012) and Gbelska *et al*. (2017). This observation is due to lack of energy since cells were firstly de-energized by use of 2, 4-dinitrophenol which inhibit production of ATP in the mitochondria and 2-Deoxy-D-glucose was used to inhibit glycolysis, the metabolic pathway where glucose is broken down and ATP is produced.

The addition of glucose as a form of energy to cell suspension increased efflux of the dye in FLC susceptible strains to 0.38µM at 60 minutes whereas in FLC resistant strain the efflux of the dye in cells supplied with glucose increased to 0.57µM at 60 minutes. Glucose was shown to stimulate activity of drug efflux pumps as there was notable difference observed in the efflux of the dye following addition of glucose in both FLC resistant and susceptible strains. The ABC proteins utilize the hydrolysis of ATP for removal or transportation of compounds across plasma membrane and again it was reported that R6G dye is a substrate to ABC proteins (Yaojun *et al.,* 2016), therefore ABC proteins; cdr1 and cdr2 are responsible for increased efflux of R6G dye in FLC resistant cells.

The efflux of R6G dye in FLC susceptible cells treated with ETCs was more or less the same as the efflux of the dye in untreated FLC susceptible cells (**Figure 3.11A-B**). In this case, addition of glucose to cell suspension showed no effect on the efflux of the dye. These may indicate that in susceptible strains, drug efflux pumps are not overexpressed hence most of the dye remained inside the cells. These results correlate to those observed in combination studies whereby most of the combination ratios between FLC and ETCs in FLC susceptible strains exhibited an indifferent interaction indicating that antifungal activity of two combined drugs does not exhibit an increased activity.

However, in FLC resistant strains, there was a significant difference observed in the efflux of the dye in cells treated with C1, C2, C4 and EUG and in untreated cells. There was a decrease in the efflux of the dye in cells treated with C2, C4 and EUG compared to untreated cells, although the difference was observed following addition of glucose to cell suspension (**Figure 3.11C-D**). This suggested that C2, C4 and EUG inhibit the activity of drug efflux pumps and also proves that cdr1 and cdr2 are responsible efflux pumps for conferring FLC drug resistance in *C. albicans* strains.

Yaojun *et al*. (2016) indicated that *C. albicans* strains with knocked out *CDR1* and *CDR2* genes showed decreased efflux of R6G dye in multidrug-resistant strains, however, cells with only *CDR1* gene knocked out showed a greater decline on the efflux of the dye compared to cells with only *CDR2* knocked out. Furthermore, another study showed that FLC resistant *C. albicans* strain overexpresses *CDR1* gene and not *MDR1* gene (Ahmad *et al*., 2013). This suggests that C2, C4 and EUG act by targeting the drug efflux pumps, cdr1 and cdr2 to be more precise.

Eugenol, C2 and C4 may be used as potential therapeutic agents for reversal of FLC drug resistance in *C. albicans* strains. Combination studies showed that combination activity of C2, C4, EUG with FLC exhibit additive effect in 1:1 combination ratios. This could indicate that when these compounds are combined their antifungal activity is increased because C2, C4 and EUG will target the drug efflux pumps and inhibit their activity of removing FLC from cells. This will then allow FLC to enter *C. albicans* cells and reach its target which is P450 14alpha-demethylase enzyme. Fluconazole will interact with the enzyme resulting in altered conformation of the enzyme preventing conversion of lanosterol to ergosterol. This will then lead to an accumulation of lanosterol in the cell cytosol and altered structure of plasma membrane due to decreased levels of ergosterol and then cell death.

4.6 Hemolytic activity of Eugenol Tosylate Congeners on red blood cells

The haemolytic activity of ETCs on RBC was investigated. This was performed to check whether ETCs act to target *C. albicans* cells only and not human cells. Five different concentrations of ETCs and EUG ranging from 2mg/ml to 0.125mg/ml were tested against RBC. Tritonx-100 at 0.1% was used as a positive control and PBS was used a negative control. The effect of 1% DMSO on RBC was also determined. The percentage haemolysis of RBC was calculated from the total haemolysis caused by tritonx-100 and was considered as 100% haemolysis (Riaz *et al*., 2012; Mohammedi and Atik, 2014). All ETCs showed less than 15% haemolysis of RBC in all tested concentrations. In addition, EUG exhibited less than 7% haemolysis of RBC in all tested concentrations. The results obtained in this study for haemolysis of RBC using EUG are consistent with those reported by Hemaiswarva and Doble. (2009). Another study also reported similar results on haemolytic effect of EUG on sheep RBC (Khan *et al*., 2011). By far there are no studies which have reported on haemolytic activity or toxicity effects of ETCs.

The previous results indicated that ETCs have great antifungal activity against both drug resistant and susceptible *C. albicans* strains. It was also shown that ETCs in combination with antifungal drugs at specific ratios exhibit synergy. Therefore this study has shown that ETCs have great

potential to be used as safe and effective antifungal drugs especially since it has been illustrated that they have no haemolytic activity against RBC at concentrations that were determined to inhibit *C. albicans* growth.

Horse red blood cells were used in this study instead of human red blood cells, however, this may not have a significant effect on the results obtained because horse RBC have similar structural properties to human RBC (Khan *et al*., 2011). Both human and horse RBC have a diameter of 6-8.2µm, are both thicker around the edges (2–2.5µm) and thinner towards the centre (0.8–1µm) (Turgeon, 2004). Both human and horse RBC have a biconcave disk like shape (Khairy *et al*., 2010). In both species, RBC major function is to transport oxygen throughout the body (Sackmann, 1995; Sellnow, 2006). Therefore since horse RBC resemble most anatomical and physiological characteristic to human RBC, the effect of ETCs on horse RBC cell will not vary greatly to the effect of ETCs on human RBC. Moreover, there are other studies that reported on the effect of EUG on RBC from other organisms and their results are consistent with those reported in this study. Thus horse RBCs were appropriate for use to investigate haemolytic effects of ETCs.

CHAPTER 5: CONCLUSIONS, LIMITATIONS AND FUTURE RESEARCH

5.1 Conclusions

Antifungal drug susceptibility test performed on isolates from HIV and cancer patients showed that antifungal drug resistance in *C. albicans* is indeed a serious public health problem. *C. albicans* is the most problematic *Candida* species and is responsible for majority of *Candida*-related infections.

- High resistance rates to FLC, NYS and CAS were observed in this study and this is a reflection of South African profile in terms of antifungal drug susceptibility in *C. albicans*. This raises concerns especially since SA is the leading country worldwide with increased rates of HIV positive immune-compromised individuals. Therefore it is of best interest and lifesaving to discover new therapeutic agents to alleviate the health burden caused by drug-resistant *C. albicans*.
- Eugenol tosylate congeners possess antifungal activity against *C. albicans* at acceptable concentrations for modified compounds and can be used as a potential treatment for *C. albicans* infections.
- The synergistic effect exhibited by ETCs in combination with NYS might be a promising strategy to combat *C. albicans* infections. The ideology of using combination therapy successfully is that combined agents should have different target sites on target species and this study revealed that ETCs may act to disrupt metabolic processes of cell to generate ATP and also by inhibiting *cdr1* and *cdr2* drug efflux pumps.
- This study also indicated that antifungal activity of ETC was improved when combined with the antifungal drugs and in turn, the concentration of antifungal drugs required to inhibit growth was decreased. This shows that combination therapy does not only inhibit the fungal growth but also solves toxicity problems associated with using increased drug concentrations of antifungal drugs. In addition cytotoxicity studies showed that ETCs have no toxic effects on horse RBC indicating that ETCs have great potential as antifungal drugs and can be used as a safe treatment in clinical applications.

5.2 Limitations of the study

- *Candida albicans* commonly occur in a biofilm instead of as planktonic cells, therefore it is essential to perform studies on biofilms rather than on single cells as biofilm may reveal interactions that may occur in real life situations.
- The present study is an *in vitro* study, observations made does not really reflect possible outcomes in real life situations, therefore *in vivo* studies should be performed which will involve internal human body and all biochemical processes that are involved.
- Gene expression studies would have given better insight on expression levels of drug efflux pumps in cells treated with ETCs and in untreated cells.

5.3 Future research

- Different functional groups were used to modify EUG and produce six derivatives with the aim of improving antifungal activity, solubility and other physicochemical properties of EUG. The antimicrobial assay performed to determine the inhibitory activity of ETCs against drug resistant and susceptible *C. albicans* strains showed that the different derivatives exhibit antifungal activity at different levels; hence the structural activity relationship of ETCs should be investigated to determine the biochemical mechanisms of ETCs against *C. albicans*.
- The current study hypothesized that ETCs may act to target H^+ *ATPase* enzyme located on the fungal plasma membrane. However results obtained in this study suggested that ETCs do not act to target H^+ *ATPase* since intracellular pH of cells treated with these compounds was neutral rather than being acidic as reported by previous studies. Thus, it is suggested that ETCs might act by interfering with metabolic processes of cell to prevent ATP production. Therefore more studies should be conducted to investigate mechanism of action of ETCs with regards to blocking H^+ production in *C. albicans* cells and the effects on ATP production.
- In addition, this study only looked at the effect of ETCs on ABC proteins which include cdr1 and cdr2 drug efflux pumps since R6G dye used in this study is the known substrate to CDR efflux pumps. Overexpression of MFS drug efflux pumps are other mechanisms used by *C. albicans* to confer FLC drug resistance. Therefore future studies should look at effect of ETCs on MFS drug efflux pump in *C. albicans* using fluorescence dyes that are known to be substrates of these pumps.
- Moreover, it is recommended that gene expression studies should be carried out to investigate expression levels of drug efflux pumps in FLC resistant strains compared to FLC susceptible strains and whether ETCs have effect on expression levels of drug efflux pumps.

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APPENDICES

Appendix 1: Statistical Analysis

1. A: Statistical analysis fore rate of H^+ efflux in FLC resistant strains**.**

Within each row, compare columns (simple effects within rows)

Number of families: 2

Number of comparisons per family: 10

Alpha: 0.05

Dunnett's multiple comparisons test

	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
glucose starved cells					
negative control vs. Eug	2.317	1.158 to 3.476	Yes	****	< 0.0001
negative control vs. C1	2.05	0.8910 to 3.209	Yes	****	< 0.0001
negative control vs. C2	1.967	0.8076 to 3.126	Yes	****	< 0.0001
negative control vs. C3	1.883	0.7243 to 3.042	Yes	***	0.0002
negative control vs. C4	2.05	0.8910 to 3.209	Yes	****	< 0.0001
negative control vs. C5	2.033	0.8743 to 3.192	Yes	****	< 0.0001
negative control vs. C6	2.317	1.158 to 3.476	Yes	****	< 0.0001
negative control vs. FLC	0.3167	-0.8424 to 1.476	No	ns	0.9875
negative control vs. CAS	0.1333	-1.026 to 1.292	No	ns	0.9996
negative control vs. Vanadate	2.183	1.024 to 3.342	Yes	****	< 0.0001

Cells supplied with glucose					
negative control vs. Eug	3.433	2.274 to 4.592	Yes	****	< 0.0001
negative control vs. C1	2.633	1.474 to 3.792	Yes	****	< 0.0001
negative control vs. C2	3.633	2.474 to 4.792	Yes	****	< 0.0001
negative control vs. C3	3.9	2.741 to 5.059	Yes	****	< 0.0001
negative control vs. C4	3.683	2.524 to 4.842	Yes	****	< 0.0001
negative control vs. C5	3.75	2.591 to 4.909	Yes	****	< 0.0001
negative control vs. C6	3.5	2.341 to 4.659	Yes	****	< 0.0001
negative control vs. FLC	1.133	-0.02571 to 2.292	No	ns	0.0586
negative control vs. CAS	0.7667	-0.3924 to 1.926	No	ns	0.3719
negative control vs. Vanadate	3.733	2.574 to 4.892	Yes	****	< 0.0001

1. B: Statistics analysis for rate of H^+ efflux in FLC susceptible strains.

Within each row compare columns (simple effects within rows)

Number of families: 2

Number of comparisons per family: 10

Alpha: 0.05

Dunnett's multiple comparisons tests

	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Cells supplied with glucose					
Negative control vs. Eug	3.28	2.41 to 4.16	Yes	****	< 0.0001
Negative control vs. C1	2.6	1.73 to 3.47	Yes	****	< 0.0001
Negative control vs. C2	2.62	1.74 to 3.49	Yes	****	< 0.0001
Negative control vs. C3	3.18	2.31 to 4.06	Yes	****	< 0.0001
Negative control vs. C4	2.83	1.96 to 3.71	Yes	****	< 0.0001
Negative control vs. C5	3.19	2.32 to 4.07	Yes	****	< 0.0001
Negative control vs. C6	3.6	2.73 to 4.47	Yes	****	< 0.0001
Negative control vs. FLC	0.95	0.0766 to 1.82	Yes	*	0.026
Negative control vs. CAS	0.55	-0.323 to 1.42	No	ns	0.4285
Negative control vs. Vanadate	4.17	3.29 to 5.04	Yes	****	< 0.0001

glucose starved cells					
Negative control vs. Eug	2.7	1.83 to 3.57	Yes	****	< 0.0001
Negative control vs. C1	2.62	1.74 to 3.49	Yes	****	< 0.0001
Negative control vs. C2	2.49	1.62 to 3.37	Yes	****	< 0.0001
Negative control vs. C3	2.55	1.68 to 3.42	Yes	****	< 0.0001
Negative control vs. C4	2.6	1.73 to 3.47	Yes	****	< 0.0001
Negative control vs. C5	2.43	1.56 to 3.31	Yes	****	< 0.0001
Negative control vs. C6	2.45	1.58 to 3.32	Yes	****	< 0.0001
Negative control vs. FLC	0.0833	-0.790 to 0.957	No	ns	0.9996
Negative control vs. CAS	-0.152	-1.03 to 0.722	No	ns	0.9993
Negative control vs. Vanadate	2.63	1.76 to 3.51	Yes	****	< 0.0001

1. C: Statistical analysis for the efflux of R6G dye in untreated FLC susceptible and resistant cells

Compare in each cell mean with the other cell mean in that row

Number of families: 1

Number of comparisons per family: 13

Alpha: 0.05

Sidaks multiple comparisons test

	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
susceptible strains (untreated cells) - resistant strains (untreated cell)					
Row 1 T= 0 MINUTES	0	-0.05667 to 0.05667	No	ns	> 0.9999
Row 2 T= 5 MINUTES	-0.055	-0.1117 to 0.001666	No	ns	0.0606
Row 3 T= 10 MINUTES	-0.0345	-0.09117 to 0.02217	No	ns	0.507
Row 4 T= 15 MINUTES	-0.0215	-0.07817 to 0.03517	No	ns	0.9513
Row 5 T= 20 MINUTES	-0.0425	-0.09917 to 0.01417	No	ns	0.2419
Row 6 T= 25 MINUTES	-0.031	-0.08767 to 0.02567	No	ns	0.6522
Row 7 T= 30 MINUTES	-0.1145	-0.1712 to -0.05783	Yes	***	0.0001
Row 8 T= 35 MINUTES	-0.1465	-0.2032 to -0.08983	Yes	****	< 0.0001
Row 9 T= 40 MINUTES	-0.1665	-0.2232 to -0.1098	Yes	****	< 0.0001
Row 10 T= 45 MINUTES	-0.152	-0.2087 to -0.09533	Yes	****	< 0.0001
Row 11 T= 50 MINUTES	-0.1455	-0.2022 to -0.08883	Yes	****	< 0.0001
Row 12 T= 55 MINUTES	-0.121	-0.1777 to -0.06433	Yes	****	< 0.0001
Row 13 T= 60 MINUTES	-0.139	-0.1957 to -0.08233	Yes	****	< 0.0001

1. D: Statistical analysis for efflux of R6G dye in FLC susceptible cells

Compare column means (main column effect)

Number of families: 1

Number of comparison per family: 7

Alpha: 0.05

Dunnett's multiple comparisons test

	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Negative Control vs. Eug	-0.004346	-0.03031 to 0.02162	No	ns	0.9974
Negative Control vs. C1	-0.03708	-0.06305 to -0.01111	Yes	**	0.0017
Negative Control vs. C2	-0.01969	-0.04566 to 0.006276	No	ns	0.2127
Negative Control vs. C3	0.005169	-0.02080 to 0.03114	No	ns	0.9937
Negative Control vs. C4	0.007731	-0.01824 to 0.03370	No	ns	0.948
Negative Control vs. C5	-0.002808	-0.02878 to 0.02316	No	ns	0.9996
Negative Control vs. C6	-0.021	-0.04697 to 0.004968	No	ns	0.1624

1. E: Statistical analysis for the efflux of R6G dye in FLC resistant cells

Compare column means (main column effect)

Number of families: 1

Number of comparison per family: 7

Alpha: 0.05

Dunnett's multiple comparisons test

	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
negative control vs. Eug	0.02296	0.003418 to 0.04251	Yes	*	0.0139
negative control vs. C1	-0.02458	-0.04412 to -0.005033	Yes	**	0.0071
negative control vs. C2	0.051	0.03146 to 0.07054	Yes	****	< 0.0001
negative control vs. C3	0.0003462	-0.01920 to 0.01989	No	ns	> 0.9999
negative control vs. C4	0.04012	0.02057 to 0.05966	Yes	****	< 0.0001
negative control vs. C5	0.01223	-0.007313 to 0.03177	No	ns	0.3989
negative control vs. C6	0.01585	-0.003697 to 0.03539	No	ns	0.1611

Appendix 2: Ethical Clearance

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

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Ref: W-CJ-170607-1 07/06/2017

TO WHOM IT MAY CONCERN:

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

Investigator: Windy Sekgele (student no. 669596), Dr J Molepo.

Project title: Modulation of antifungal drug resistance and combination activity of eugenol tosylate congeners with antifungal drugs in *Candida albicans*.

Reason: This study uses existing stock cultures of *Candida albicans* isolated under project M120423 and stored by Dr J Molepo. A control strain will be a commercial strain of *Candida albicans* (ATCC90028) There are no human participants.

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2017 -06- 07
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Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

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Appendix 3: Plagiarism report

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