

An *in-vitro* Study of Antifungal Activity of Gymnemic Acid



UNIVERSITY *of the*
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for the degree of MSc. Oral Medicine.**

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KEYWORDS

Gymnemic acid

Chlorhexidine gluconate

Nystatin

Candida Spp

Inhibition zone

Bud size

Hyphae length

Antifungal activity

ABSTRACT

Background:

Candida species are frequently isolated from oral mucosal surfaces of healthy individuals and is the most common genus responsible for up to 75% of all candidal infections. The most common problems associated management of oral candidiasis are antifungal drug resistance and side effects. Natural medicine is an emerging field and is being explored to overcome drug resistance and to reduce side effects. Gymnemagenin (will be known as Gymnemic acid; GA) is a purified extract from *Gymnema sylvestre*, a slow growing, perennial, medicinal plant found in Central and Western India, Tropical Africa and Australia is regarded as one of the plants with potent antimicrobial and antifungal activity.

Aim:

The aim of the study was to determine the effectiveness of Gymnemic acid to inhibit *Candida* growth and hyphae development.

Method:

Disc diffusion tests were carried out on the agar plates containing the prepared *Candida* (3 plates each week). Samples of *candida albicans* were transferred using a sterile loop from underneath and at the edge of each disc (NYS, CHX, GA and DH₂O), for each medicament, and transferred to bovine serum vials (n=24). This was repeated for week 2 and week 3 (n=72). These vials were incubated for 24 hours at 37°C. At 2, 4, 6 and 24 hours, 10 µl from each vial was inoculated onto a glass slide (n=16/week). Each half of the glass slide contained the smear from “under” and “edge”, from which bud surface area and hyphal length measurements were done after staining. Only 32 slides were sent for PAS staining (representing hour 6 and hour 24); 8 for each medicament.

Results:

There was no zone of inhibition around GA and DH₂O discs, while CHX and NYS discs exhibited significantly large inhibition zones.

Although GA did not show any inhibition at the edge of the paper disc, upon incubation of the smear taken from the edge of the paper discs, the initial bud surface area at hour 0 for GA was already smaller than the cell surface of CHX and NYS respectively. From 2-4 hours, CHX showed a progressive decrease in bud size; NYS increased at a faster rate than GA. The greatest decrease in bud size occurred from 4-6 hours for all medications. Only GA progressively decreased in bud size from 6 to 24 hours. NYS increased in bud size from 6-24 hours and CHX showed little change in bud surface area.

At hour 0, hyphae length base-line readings showed no hyphal growth across all treatments.

Over the 24 hour period for the three weeks, GA treatment significantly reduced the hyphal growth as compared to CHX and NYS treatments ($p=0.05$). There were no significant differences found in hyphal length of GA under for week 1, 2 and 3.

Conclusion:

The complete eradication of *candida albicans* may not be desirable as it could disrupt the balance of normal flora. Thus treatment with natural substances such as GA may be beneficial. Since *candida albicans* has the ability to switch between yeast, hyphal and pseudo hyphal morphologies is one of its virulence factors, targeting prevention of this transition will impact its pathogenesis and clinical presentation. GA reduced the hyphae length and bud size of *Candida*, but unlike NYS and CHX, did not inhibit the Candidal growth. The reduction in bud size and hyphae length indicated that GA reduced the pathogenic potential of *candida albicans*, rendering it less virulent.

DECLARATION

I, the undersigned, Sana Alhadi Asmyou, hereby declare that the work contained I this dissertation titled; “An *in-vitro* Study of Antifungal Activity of Gymnemic Acid” is my original work and has not been previously in its entirety or in any part submitted at any university for any degree or examination.

A handwritten signature in black ink, appearing to read 'Sana', with a long horizontal stroke extending to the right.

Dr. Sana Alhadi Asmyou

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DEFINITIONS

Mannan: a group of polysaccharides present in the walls of yeast cells and inhibitors cell-mediated immunity.

Gymnemic acid: Derivative of the slow growing plant *Gymnema sylvestre*.

Gymnemagenin analytical standard (Item: 52181 SIGMA-ALDRICH): Will be known in the thesis as Gymnemic acid. The rationale is: This Gymnemic acid A comprises of gymnemic acids A1, A2, A3, and A4 and named gymnemagenin.

ABBREVIATIONS

API	: Analytical Profile Index
BHI	: Brain heart infusion
BSA	: Bovine Serum Albumin
<i>Candida</i> spp	: <i>Candida</i> species
CHX	: Chlorhexidine gluconate
DH ₂ O	: De-ionised water
GA	: Gymnemic acid
GA edge	: <i>Candida</i> spp found at the edge of the inhibitory zone of the paper disc of Gymnemic acid on the agar plate.
GA under	: <i>Candida</i> spp found under the paper disc of Gymnemic acid on the agar plate.
H ₀ :	: Null hypothesis
H _A :	: Alternative hypothesis
NYS	: Nystatin
PAS	: Periodic acid–Schiff
PBS	: Phosphate buffered saline
SD	: Standard deviation
YPD	: Yeast Peptone Dextrose

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CHAPTER ONE

1.1. Introduction

Candida is frequently isolated from oral mucosal surfaces of healthy individuals (Sardi *et al.*, 2013) and is the most common genus responsible for up to 75% of all *Candidal* infections (Achkar *et al.*, 2010). Emerging non- *albicans* species, such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei*, have been found to colonize human mucocutaneous surfaces (Sobel *et al.*, 2006). These species are also isolated from microflora in patients with candidiasis, albeit at a lower frequency.

The interactions between *Candida* and the host are extremely complex. The host innate and acquired defense mechanisms allow resident microflora, including *Candida*, to survive as a commensal species. Any alteration in the host, micro-environment, can lead to the transformation of *C. albicans* into a pathogenic organism, causing infections that may be potentially lethal. These alterations include changes in the intraoral environmental, host defense, and an array of local, systemic and iatrogenic factors such as prolonged antibiotic use and introduction of medical devices (Mukherjee *et al.*, 2015).

Colonization by *Candida spp* starts at a young age and the organisms are usually acquired during parturition or nursing. The most common body sites with asymptomatic *Candida spp* colonization are the oral cavity, rectum, and vagina (Jenkinson *et al.*, 2002). In healthy adult subjects, Candidal carriage is about 40% in the mouth and between 20% to 25% in the vaginal mucosa of women (Jenkinson *et al.*, 2002).

Candidal infections may be treated with topical or systemic antifungal agents and topical antifungal agents are the first choice of treatment for candidiasis (Dupont *et al.*, 2006). Systemic antifungal agents are indicated in cases of poor patient compliance to topical agents; for prophylaxis of relapsing disease; in esophageal candidiasis and in *Candida* onychomycosis.

The most common problems associated with antifungal drugs are resistance and side effects. *Candida spp* has the ability to survive by utilizing available nutrients and adapts to new environmental conditions much quicker than other co-colonizers (Sherrington *et al.*, 2017). It develops drug resistance easily by transforming into different morphological forms, depending

on the environmental conditions. These morphological forms, such as germ tubes, pseudo-hyphae and mycelial formation, enhance its pathogenicity and survival. Pseudo-hyphal and mycelial forms often manifest in systemic infections and these forms are less susceptible to most antifungals (Ramage *et al.*, 2012). *Candida albicans* also forms germ tubes in serum within a short time, a characteristic that is thought to enhance penetration of tissues such as epithelium, liver and lungs, amongst others (Yang *et al.*, 2014). Thus treatments targeting the inhibition of these morphological adaptations are desired (Noble *et al.*, 2010). Side effects and resistance are more commonly associated with systemic antifungal use (Pappas *et al.*, 2015; Jensen *et al.*, 2016), such as amphotericin B and fluconazole. Renal toxicity, electrolyte abnormalities, infusion reactions, and hepatotoxicity are some of the reported side effects (Nett *et al.*, 2016).

Natural medicine is an emerging field and is being explored to overcome drug resistance and to reduce side effects (Nadkarni *et al.*, 1976; Komalavalli *et al.*, 2000). *Gymnema sylvestri* is a slow growing, perennial, medicinal plant found in Central and Western India, Tropical Africa and Australia (Komalavalli *et al.*, 2000; Vedyappan *et al.*, 2013). *In vitro* studies of its derivative, (Gymnemic acid) have shown a broad spectrum of antimicrobial and antifungal activity towards bacteria and fungi including *Candida albicans*. Its antifungal activity is attributed to the ability to inhibit yeast-to-hypha conversion in *Candida albicans* species (Vedyappan *et al.*, 2013).

The purpose of this study was to compare the antifungal activity of Gymnemic acid with two commonly used topical antifungal agents, namely; nystatin and chlorhexidine (digluconate).

The clinical benefit of this study would be to explore the use of medicinal plant extracts, such as Gymnemic acid, in the management of oral fungal diseases.

CHAPTER TWO: LITERATURE REVIEW

2.1 *Candida* species

Candida is a genus of yeast that is implicated in most fungal infections, many of which reside as commensals, when their population is within equilibrium (Fundyga *et al.*, 2002). *Candida* comprises of over 200 species that include *Candida albicans*, *Candida glabrata*, *Candida dubliniensis*, *Candida guilliermondii*, *Candida krusei*, *Candida lusitanae*, *Candida parapsilosis* and *Candida tropicalis* (Scully *et al.*, 1994). *Candida albicans* is the most prevalent species found in the oral cavity and comprises more than 80% of all oral yeast isolates (Coronado-Castellote *et al.*, 2013).

Along with *C. albicans*, an increasing number of non-*albicans* species have been recognized in human diseases. They include *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *C. krusei* species. The latter two are resistant to some antifungal agents (Coleman *et al.*, 2010; Sanguinetti *et al.*, 2015). *C. dubliniensis* was isolated along with *C. albicans*, from cases of oral candidiasis in individuals with HIV infection (Marsh *et al.*, 2009).

2.2 *Candida* carriage

The normal carriage of *candida albicans* in health is around 800 organisms/ml of saliva. This value could increase to counts in excess of 20 000 organisms/ml in unstimulated saliva, in the presence of oral candidiasis (Challacombe *et al.*, 1994). An increase in *candida albicans* may results in symptomatic disease, when local and or systemic predisposing factors alter the host's microenvironment (Patil *et al.*, 2015).

The carriage patterns of *candida albicans* are affected by oral habits, various methods used for sample analysis and geographical locations (Cannon *et al.*, 1995 and Kleinegger *et al.*, 1996). Carriage rates of 7.7% have been reported in Asian children, compared to 70% in children from the West (Samaranayake *et al.*, 2009). Carriage of *candida albicans* and incidence of candidiasis is not well documented in the literature, but recently resistance to commonly used antifungals have been reported (Dos Santos Abrantes *et al.* 2014; Mnge *et al.*, 2017). This is a cause for

concern, as antifungal resistance is an emerging problem, not limited to South Africa but prevalent across Africa (Dos Santos Abrantes *et al.*, 2014).

2.3 Clinical Variants of Oral Candidiasis

Oral candidiasis, oropharyngeal candidiasis, or thrush is used to describe the mouth infection while the term yeast infection is reserved for the genital area. Oral candidiasis is the most common human *Candida species* infection and the incidence is currently on the increase due to an increase in aging population and the number of immunocompromised patients (Pires *et al.*, 2002. Pinto *et al.*, 2008). Generally, the infection is more common amongst infants, elderly, people taking chemotherapy and those suffering from compromised diseases like AIDS. The clinical variants include acute pseudomembranous, acute atrophic, chronic hyperplastic, chronic atrophic, median rhomboid glossitis, and angular cheilitis (Akpan *et al.*, 2002). Oral candidiasis has a varied clinical presentation. It can present with features such as white spots inside the mouth and tongue which rub off to reveal a bleeding surface, a sore throat with difficulty in swallowing and cracks at the corners of the of mouth (Joseph *et al.*, 2016).

2.4 Predisposing factors to *Candida species* infection

Fungi are part of the normal oral, vaginal and gastro-intestinal flora, but its importance to the human body remains undefined (Garcia-Cuesta *et al.*, 2014). Nevertheless, some fungal species are known to cause disease when homeostasis is disturbed or when they find their way to other organs (Martins *et al.*, 2014). Ghannoum and colleagues (2010) analyzed differences in the fungal microbiome at different places in the body. (Ghannoum *et al.*, 2010) and found the fungal species distribution pattern to vary greatly between different people. About 20% of the study participants had at least one of the following four most common genera of pathogenic fungi: *Aspergillus*, *Candida*, *Cryptococcus*, and *Fusarium spp* (Ghannoum *et al.*, 2010).

Candida species have the ability to grow under varied environmental conditions and overgrowth of this opportunistic organism initiates inflammation of the mucosal tissues (Wan Harun *et al.*, 2013). Therefore, *Candida* has the ability to infect numerous body organs. *Candida* infections vary from superficial to systemic infections. These infections include genital and non-genital mucocutaneous candidiasis, *Candidal meningitis*, endophthamitis, *Candidal endocarditis*, and hepatosplenic candidiasis, amongst others.

Candida infections have gained attention due to the emergence of HIV infection and the increased use of immunosuppressive chemotherapy (Epstein *et al.*, 1986; Pomerantz *et al.*, 1992). Identification of the specific *Candida* strains causing the infection is important because the *Candida species* isolated from the sites of infection differ widely, both in their ability to cause infection (Allen *et al.*, 1994) and in their susceptibility to antifungal drugs (McIlroy *et al.*, 1991).

The increase in immuno-compromised patients and patients hospitalized with serious medical conditions, such as cancer, organ transplants and non-transplant surgery, have resulted in an increase in opportunistic fungal infections (Pfaller *et al.*, 2007, 2010).

Ill-fitting dentures and changes in the oral microenvironment may also contribute to *Candida* proliferation, which may escalate to systemic infections. Systemic infections may also be caused by prolonged use of certain drugs, hormonal influences, stress or other disease conditions (Kinloch-de Loës *et al.*, 1993) such as diabetes, xerostomia, prolonged antibiotic use, old age, poor nutrition and HIV infection (Garcia-Cuesta *et al.*, 2014). Other predisposing factors include pregnancy, nutrient deficiencies, other dietary factors and poor oral hygiene.

This diversity of *Candida* infections requires a broad range of diagnostic and therapeutic strategies (Pappas *et al.*, 2009).

2.5 Pathogenesis and diagnosis of oral candidiasis

Candida must colonize, invade and multiply in order to cause disease (Coronado-Castellote, 2013). The pathogenesis of candidiasis requires three factors: host, fungus and oral microenvironment-modifying factors. The morphologies of *Candida albicans* transform from yeast, to pseudo-hyphal, hyphal and mycelial forms (Coronado-Castellot *et al.*, 2013). *Candida albicans* is present in a yeast form as a commensal (Vazquez *et al.*, 2014), but can transform into hyphae when suitable environmental conditions prevail. The hyphal form penetrates host tissue and is responsible for the disease pathogenesis (Mukherjee *et al.*, 2015).

Candida albicans, can be rounded and oval-shaped yeast, measuring 3-30 μm in diameter, reproduces asexually through budding, where protoplasmic buds (blastoconidia) emerge from the

mother cell and grow until they finally detach and form daughter cells. The daughter cells sometimes do not detach and form chains of cells called pseudo hyphae or germ tubes (Coronado-Castellote *et al.*, 2013). These extensions can be mistaken for hyphae. Under certain conditions yet to be clearly described, these hyphal forms may further multiply and form dense mycelia.

It appears as though *candida albicans* must go through a saprophytic phase in order to produce clinical lesions. Then over time, nutritional and environmental variations modulate its conversion to the mycelial or invasive form with its virulence. Thus, it can evade macrophages and cause infection (Coronado-Castellote *et al.*, 2013).

The formation of germ tubes and the presence of certain glycoproteins in the fungal wall facilitates adherence to cell membranes and therefore biofilm formation (Chandra *et al.*, 2001). The presence of germ tubes and the production of phospholipase C further enhances invasion of adjacent tissues (Pereira *et al.*, 2015). While the infection progresses, *Candida* alters the host defense mechanisms (e.g., inhibiting phagocytosis) and further induces suppressor T lymphocytes and mannan found in its cell wall, which interferes with antigen presentation, inhibiting the immune defense response (Coronado-Castello *et al.*, 2013).

Thus, hydrolytic enzyme produced by *candida albicans* which causes tissue damage results from direct action of the micro-organisms and from host defenses during tissue invasion. The host's defenses that are compromised include IgE-mediated immune allergic reactions against the fungal antigens and delayed hypersensitivity reactions. This is demonstrated in chronic mucocutaneous candidiasis.

The diagnosis of oral candidiasis is essentially clinical and is based on the recognition of the lesions by the clinician and confirmed by the microscopic identification of *candida albicans*. The biopsy techniques available for the isolation of *candida albicans* in the oral cavity include cytological smears and incisional biopsy, which is indicated for cases of hyperplastic candidiasis because this type could present dysplasia (Garcia-Cuesta *et al.*, 2014). Direct demonstration of *Candida albicans* may be done by wet mount preparation with potassium hydroxide or Gram staining techniques. It may be cultured on Sabouraud dextrose agar (SDA) and species identification may be presumptively done by observing colonial morphology or more specifically

using CHROMagar. Furthermore, distinction among *Candida* species may be afforded by the germ tube test and API technique (Deorukhkar *et al.*, 2014) .

Various diffusion methods can be used to determine the antifungal activities of the extracts. These include the agar disk-diffusion method; antimicrobial gradient method; agar well diffusion method; agar plug diffusion method and the cross-streak method. The agar disk-diffusion technique, developed in the 1940's (Heatley *et al.*, 1944) is the most widely used method in clinical microbiology laboratories for routine antimicrobial susceptibility testing. Although this method cannot be used to test all fastidious bacteria accurately, it has been standardized to test certain fastidious bacterial pathogens like *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *streptococci*, *Neisseria meningitis* and *Haemophilus parainfluenzae*, using specific culture media, different incubation conditions and interpretive criteria for inhibition zones. Several bioassays such as disk-diffusion, well diffusion and broth or agar dilution are well known and commonly used to compare fungi and antimicrobial testing (Balouiri *et al.*, 2016). In this study, the agar disk diffusion method was used to compare the antifungal activity of Gymnemic acid to Chlorhexidine Gluconate 0.02% and Nystatin. This is a standardized method and is recommended by Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2008).

2.6 Management of oral candidiasis

Even though considerable advances have been achieved since the initiation of systemic antifungal therapy in the 1950s, the present antifungal agents are far from perfect. Some antifungals have to be endured at high doses for prolonged periods, while others are not readily bioavailable to target organs. In addition, no single antifungal agent is appropriate for all patients because of patient-specific medical conditions, hypersensitivities, risk of drug interactions, the location of the infection and the presence of specific confounders such as antifungal-resistant species (Garcia-Cuesta *et al.*, 2014). The latter is commonly caused by poor patient compliance when taking medication for protracted time periods. These problems, together with the fact that candidiasis is mostly an autoinfection, supports the need to inhibit *candida albicans'* morphological transformation and thereby prevent dissemination of the infection.

The pharmacological treatment of oral candidiasis can be categorized into two categories. Topical drugs, which are applied to the affected mucosal site to treat superficial infections and systemic drugs that are prescribed for widespread infections and when topical therapy is insufficient (Garcia-Cuesta *et al.*, 2014).

Topical antifungal agents, such as nystatin, miconazole, amphotericin B and clotrimazole are used as the first-line treatment for mild to moderate cases of oral candidiasis (Lyu *et al.*, 2016; Akpan *et al.* 2002).

Nystatin is a membrane-active polyene macrolide produced by *Streptomyces noursei* strains and is present in different forms, including oral suspension, oral pastille and topical cream (Kaur *et al.*, 2010; Wong *et al.*, 2014). Nystatin is not absorbed from the wall of gastrointestinal tract when used orally (Samaranayake *et al.*, 2009). This makes the topical use of nystatin the most common route of administration in dentistry, as systemic exposure is not significant. Nystatin is also used in the prophylaxis of oral and systemic candidiasis in newborns, infants and immunocompromised patients, as it is known to have low incidence of drug interactions and is cost effective, especially in developing countries (Campos *et al.*, 2012).

The management of oral candidiasis also involves the use of anti-microbial mouth rinses such as Chlorhexidine gluconate (CHX). Chlorhexidine gluconate is a polybiguanide (bisbiguanide) and by virtue of its cationic properties has a wide range of topical antimicrobial properties. The anti-*candida albicans* efficacy of CHX is due to its fungicidal activity (Ellepola *et al.*, 1999) and inhibition of fungal adhesion to mucosal epithelial cells (Barasch *et al.*, 2004). Chlorhexidine is well tolerated and has few side effects. A 0.12-0.2% concentration of CHX rinse has been used in the treatment and prevention of dental caries and periodontal disease for more than two decades (Barasch *et al.*, 2004).

The generation of systemic antifungal drugs effectively started with the introduction of amphotericin B-deoxycholate in 1958 (Lewis *et al.*, 2011). The search for new antifungal drugs has mainly focused on how to reduce toxicity, enhance bioavailability, improve the antifungal spectrum and counteract resistance. This search has led to the introduction of the first generation triazoles, fluconazole and itraconazole. Both triazoles showed a broader spectrum of antifungal activity than the imidazoles that was earlier introduced and had better-improved safety profiles

compared with amphotericin B and ketoconazole (Rex *et al.*, 1994). These drugs improved the management of invasive fungal infections immensely.

The last major milestone in antifungal discovery in the 20th century was the introduction of echinocandins. Echinocandins are semisynthetic lipopeptides that inhibit the synthesis of β -1, 3-d-glucan in susceptible fungi. This inhibition leads to the destruction of cell walls (Rueda, et al., 2014). These antifungal agents target mainly glucan-rich cell walls, which are not present in mammalian cells. (Mora-Duarte *et al.*, 2002; Maertens *et al.*, 2004; Pappas *et al.*, 2007). However, echinocandins do not act against some common opportunistic yeast (*Cryptococcus species*) and less common molds (i.e., *Fusarium*, *Scedosporium*, and *Mucorales*) that often develop as breakthrough infections in severely immunocompromised patients.

Table 1 below summarizes the commonly used topical and systemic antifungal agents (Garcia-Cuesta., *et al.*, 2014).

Table 1: Antifungal agents commonly used for *Candida* spp

Antifungal agents	Formulation	Dose	Mechanism of action
Nystatin	Suspension 60ml	4-6ml every 6h	Binds ergosterol
Chlorhexidine (gluconate)	Mouth wash	Rinse 5 ml for one minute	Binds onto the cell wall
Fluconazole	Tablets Suspension	50 - 100mg 10mg/ml 10mg/ml	Inhibits ergosterol biosynthesis, by acting 10mg/ml

Itraconazole	Capsule	100-200mg/day	on the P-450-dependent enzyme.
Ketoconazole	Gel 2% Tablets Suspension 30 or 10cc	3 times/ day 200mg 1-2 times day	
Miconazole	Gel	100mg every 6h	
Amphotericin B	50mg for infusion	100-200mg every 6h	Binds to fungal cell membranes thus altering membrane permeability
Clotrimazole	Gel 1% Tablets 10mg	3 times / day 5 times / day	

2.7 Gymnemic acid

The derivatives of Gymnemic acids are several acylated tigloyl, methylbutyryl group substituted members derived from deacylgymnemic acid (DAGA) which is a 3-O- β -glucuronide of gymnemagenin ($3\beta, 16\beta, 21\beta, 22a, 23, 28$ - hexahydroxy -olean-12-enc). Gymnemic acid a comprises of gymnemic acids A₁, A₂, A₃ and A₄ and named gymnemagenin.

Gymnemic acid is a purified extract from *Gymnema sylvestre*, a slow growing, perennial, medicinal plant found in Central and Western India, Tropical Africa and Australia is regarded as one of the plants with potent antimicrobial and antifungal activity (Komalavalli *et al.*, 2000; Vedyappan *et al.*, 2013).

Pharmacologic efficacy of many plants for the treatment of several medical conditions has been described by traditional medicine practitioners (Ramasamy *et al.*, 2009). There is an increase in reported cases of antimicrobial properties of medicinal plants from different parts of the world. The World Health Organization estimates that the extracts and active components of plants are used as folk medicine in traditional management of 80% of population of the world (Shaik *et al.*, 1994).

Gymnema sylvestre is referred to as "gurmar" in Hindi, which means "sugar destroying". Beside its anti-sweetening ability, *Gymnema* has also been tested as a hypoglycemic agent in combination with insulin, with encouraging results. Preliminary research showed that taking 200 mg/d of gymnema extract reduces the required insulin dose by half and lowered Haemoglobin A1c (HbA1c) in both type 1 and type 2 diabetes (Pothuraju *et al.*, 2014). Extracts of the plant are also reported to increase the number of beta cells in the pancreas and hence modulate the internal production of insulin (Prabhakar *et al.*, 2011).

A few patients were able to reduce the dose of conventional hypoglycemic drugs, such as glyburide or tolbutamide when taking them with 400mg of this plant extract (Prabhakar *et al.*, 2011). The mechanism of action of the plant seems to occur by increasing the endogenous insulin production or by increasing the serum C-peptide levels. *Gymnema Sylvester* has hypolipideamic action where it cause decreases the total cholesterol levels, triglycerides and LDL-C, while enhancing the levels of HDL-C (Prabhakar *et al.*, 2011; Singh *et al.*, 2017).

In this study, Gymnemic acid (a plant extract of *Gymnema Sylvester*) was investigated, based on its antimicrobial and antifungal properties studies reporting a wide spectrum of bacteria and fungi, including *Candida albicans*. Vedyappan *et al.*, (2013) has shown that the antifungal activity of Gymnemic acid is attributed to inhibition yeast-to-hypha conversion in *Candida species* and also non-toxic to *candida albicans* (Vedyappan *et al.*, 2013).

2.8 Method of analysis

The extraction of Gymnemic acid from the *Gymnema sylvestre* plant involves preparation of plant extracts and isolation of Gymnemic acid from aqueous extract of *Gymnema sylvestre*. The

powdered plant materials can be successively extracted by using different solvents' non-polar to polar methods. These solvents include ethanol, de-ionised water and petroleum benzene (Krishna *et al*, 2012).

Isolation of Gymnemic acid from the aqueous extract is usually done with the thin layer chromatographic technique. The latter has been shown to be effective in generating metabolite constituents of various plant compounds and their purification. However, a commercially prepared Gymnemic acid will be used for this study.

Periodic acid–Schiff (PAS) is the stain of preference for identifying *candida albicans*.

The principle of staining is based on the fact that periodic acid oxidizes certain tissue elements to produce dialdehydes. The aldehydes produced will then react with the fuchsin-sulfurous acid which combines with the basic rosaniline to form a magenta-colored compound. Periodic acid is a good oxidant for this reaction because it does not excessively oxidize the aldehydes to carboxylic acid, which would then produce a weak Schiff reaction. The intensity of the reaction depends to some extent on the length of treatment with the periodic acid and Schiff's solutions (da Silva *et al*, 2017).

This method of PAS staining has been used for a long time with good result and this will be used for this study.

2.9 Clinical benefit

C. albicans is known to cause both local oral and systemic infections. *C. albicans* cells occur in different morphological states (hypha, pseudohypha and yeast) and under certain conditions can undergo white-opaque phenotype switching (Vediyappan *et al.*, 2013). The ability to change from yeast or pseudohyphal states to the hyphal growth state is important for the development of systemic infections and is evident by the limited virulence of various *C. albicans* mutants that are defective in the formation of hypha (Lo *et al.*, 1997; Noble *et al.*, 2010). Gymnemic acid has been shown to inhibit yeast-to-hypha conversion of *Candida* (Vediyappan *et al.*, 2013). The purpose of this study is to compare its antifungal properties to chlorhexidine, a commonly used as a topical agent in the management of oral candidiasis.

CHAPTER THREE: METHODOLOGY

3.1 Aim

The aim of the study was to determine the effectiveness of Gymnemic acid to inhibit *candida albicans* growth and hyphae development.

3.2 Hypothesis

Two conjectures were made as the premise for the study.

1. H₀: GA produces inhibition zones for *candida albicans* equal to those caused by conventional anti-fungals.
2. H_A: Hyphal growth in *candida albicans* after treatment with GA is of equal length to treatment with conventional anti-fungals.

3.3 Objectives

- To determine the inhibition zone around the impregnated disc.
- To determine and compare the bud growth across the three treatments: Gymnemic acid, Nystatin and Chlorhexidine.
- To determine and compare the hyphal growth across the three treatments (Gymnemic acid, Nystatin and Chlorohexidine).

3.4 Methodology

3.4.1 Overview

This *in vitro* laboratory study was based on the established principles of disk diffusion testing to measure the antifungal activity of Gymnemic acid, using Kirby-Bauer method (Bauer, 1966). Gymnemic acid was compared with two commonly used antifungal agents, namely Nystatin and Chlorhexidine digluconate, while deionized water served as the control. These treatments were tested on *candida albicans* (ATCC 36810). The study was conducted in the Department of Oral Medicine and Periodontology and the Dental Research Laboratory (DRL) at the Faculty of Dentistry, University of the Western Cape, Tygerberg campus.

Disc diffusion tests were carried out on the agar plates containing the prepared *Candida* (3 plates each week). Samples of *candida albicans* were transferred using a sterile loop from underneath and at the edge of each disc, for each medicament, and transferred to bovine serum vials (n=24). This was repeated for week 2 and week 3 (n=72). These vials were incubated for 24 hours at 37°C. At 2, 4, 6 and 24 hours, 10 µl from each vial was inoculated onto a glass slide (n=16/week). Each half of the glass slide contained the smear from “under” and “edge”, from which bud surface area and hyphal length measurements were done after staining. Only 32 slides were sent for PAS staining (representing hour 6 and hour 24); 8 for each medicament.

3.6 Material and methods

3.6.1 Study design

Experimental comparative study.

3.6.2 Sample size

Inhibition zone diffusion tests were completed in triplicate for three consecutive weeks. Each agar plate (n=9) contained the test materials: Gymnemic acid, Nystatin, Chlorhexidene and De-ionised water (control).

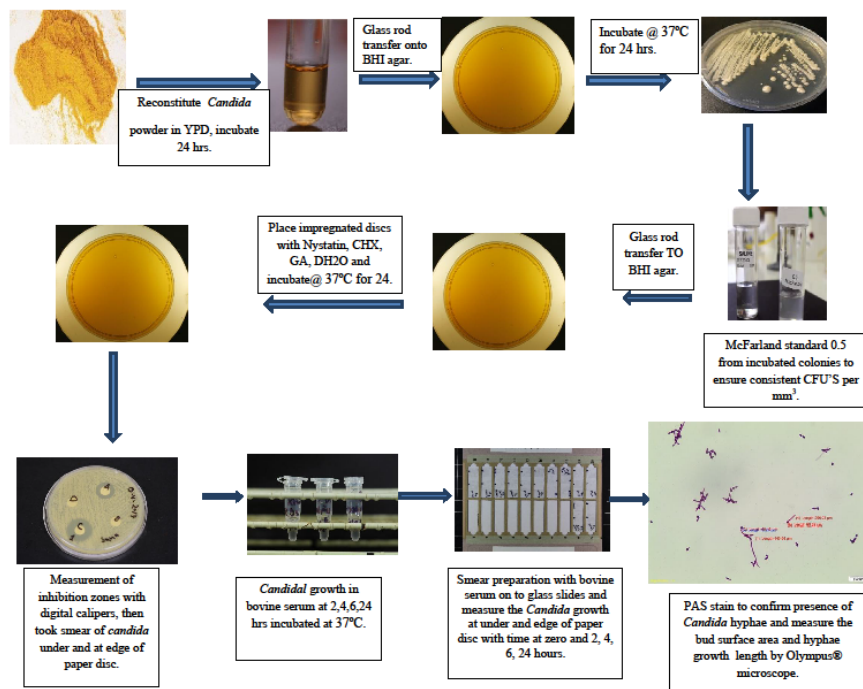


Figure 1: Flow diagram illustrating all the steps involved in this study.

3.7 Media preparation

3.7.1 Phosphate buffered saline

Phosphate buffered saline (PBS) (Oxoid, BR0014G) was prepared by dissolving 1 tablet in 100mL sterile de-ionised water and sterilized at 121°C for 15 minutes.

3.7.2 Brain heart infusion broth

Brain heart infusion (BHI) broth was prepared by dissolving 37g BHI powder (Sigma-Aldrich, 53286-500G) in 1L of sterile de-ionised water, dissolved thoroughly and autoclaved at 121°C for 15 minutes.

3.7.3 BHI agar plates

The mixture is cooled to 45°C and plates are poured. Brain heart infusion Agar plates were prepared by adding 5% agar (Sigma-Aldrich, A6686-500G) in BHI broth and thereafter autoclaving the agar at 121°C for 15 minutes.

3.7.4 Yeast peptone dextrose (YPD)

Suspend 50 g in 1 liter of de-ionised water then autoclave for 15 minutes at 121 °C (Sigma-Aldrich, Y1375-250G).

3.7.5 Seaboard dextrose broth (SD)

Seaboard dextrose broth was prepared by dissolving 37g SD powder (Sigma-Aldrich, S3306-100G) in 1L of sterile de-ionised water, dissolved thoroughly and autoclaved at 121°C for 15 minutes.

3.8 Preparation of discs for susceptibility test

Six-millimeter blank diameter antibiotic susceptibility discs (Lasec SA Pty Ltd, Cape Town, South Africa) were selected, placed onto 4 glass Petri dishes and sterilized in an autoclave at 121°C for 4 minutes. Gymnemagenin (Sigma-Alrich, product code: 52181, CAS no: 22467-07-8) was mixed with de-ionised water at a weight ratio of 1:1 (1gram:1ml) and known as Gymnemic acid. The sterile discs were aseptically infused with 40µl of the 1:1 Gymnemic acid aqueous extracts (Harun et al, 2013), 100µL Chlorhexidine gluconate and 100µL of Nystatin at concentrations of 40µg/ml, (0.12% w/v) (Driscoll et al., 2012). The fourth disc was infused with 100µL sterile de-ionised water, as negative control. The infused discs were air-dried and stored at 4°C under sterile conditions.

3.9 Preparation of cultures

Candida albicans was chosen as a test organism because it is the most common organism implicated in oral infections (Wade, 2013). *Candida albicans* strain (ATCC 36810) strain was resuscitated in Brain heart infusion broth and cultured on a fresh Sabouraud Dextrose (SDagar) plate. These plates were incubated at 37°C for 24 hours.

After incubation, a sterile loop was used to transfer a well isolated colony onto a glass slide, which was Gram stained to confirm its morphology. The pure culture agar plates were stored at 4°C to inhibit the growth of *candida albicans*. At week 2 and week 3, a new *candida albicans* inoculum was harvested from the stored agar plates and transferred onto Yeast Peptone Dextrose (YPD) agar media (*BD Difco, USA*) and incubated in a *Memmert 854* incubator for 24 hours at 37°C. A sterile loop was used to transfer the *Candida* inoculum onto a new SD agar plate, which

was incubated for 24 hours at 37°C (Figure 1). This was repeated each week for the duration of the study.

3.10 Establishing the McFarland standard

The McFarland Standards are turbidity standards used to approximate the amount of microorganisms present within a liquid suspension. The standards are used to visually compare the turbidity of a suspension with the turbidity of the appropriate commercially available standards (Sutton *et al.*, 2011).

A 24-hour *Candida* isolate was standardized in phosphate buffered saline to 0.5 McFarland on a DensiChek (*BioMerieux, North Carolina, USA*) for measuring bacterial densities in suspensions. This process involved the transfer of a newly prepared culture into Phosphate buffered saline in order to form a suspension of the selected culture. The suspension was calibrated according to the 0.5 McFarland standards (approximately 1.5×10^8 CFU/ml) (Andrews, 2001).

3.11 Antifungal susceptibility assays

The BHI agar plates were inoculated with 100µl of standardized culture by spreading the sample over the agar surface using a sterile glass rod. At this stage the plates were ready to undergo antifungal disk diffusion testing within 15 minutes.

The infused disks were aseptically firmly placed on the agar plates with sterile needles. The whole procedure from Preparation of Pure *Candida* cultures to Preparation of Agar disks for sensitivity diffusion tests was repeated in triplicate each time over three weeks (9 plates/tests).

3.12 Antifungal activity screening and measurements

The three plates per group were incubated for 24 hours at 37°C. The plates were inoculated then discs were placed and then incubated . The diameters of growth inhibition zones around each disk were measured across three points with a digital caliper and recorded in millimeters. The inhibition zones were compared to the NCCLS recommendation. Discs without inhibition zones were considered as not having antifungal activity.

3.13 Hyphal growth assay and measurement

3.13.1. Hyphal growth and bud surface area

Rationale for “under” and “edge” of disc *candida albicans* collection: Although visible inhibition was not present for GA, it is the objective of the study to determine the influence of GA on the *C. albicans*. It is therefore important to assess the *candida albicans* at the “edge” of the paper disc for GA, NYS and CHX. NYS and CHX could also have affected the *candida albicans* at the edge of inhibition.

A sterile loop was used to collect *candida albicans* underneath and at the edge of the inhibition zone of each treatment disc and it was subsequently incubated in Bovine Serum Albumin (BSA) at 37°C for 24 hours (n=24 vials /week). Bovine serum albumin, a globular protein (~66,000 Da) is used in numerous biochemical applications due to its stability and lack of interference within biological reactions (Nita *et al.*, 2016).

At 2, 4, 6 and 24 hours, the vials were placed in the Vortex at a speed of 600 rpm for 5 seconds, where after the vials were incubated again for two hours to stimulate hyphal growth.

A 10 µl inoculum was smeared onto the glass slide. Each half of the glass slide contained the smear from “under” and “edge” for each medicament (deionized water, Nystatin, Chlorhexidine and Gymnemic acid). Bud surface area from the images of the microscopic measurements it was noted that the buds were more round than oval and hyphal length was measured from these glass slides, using the Olympus® microscope. One slide for each week at hour 6 and hour 24 were sent for PAS staining.

3.13.2 Periodic acid–Schiff (PAS) Stain

For each medication, 1 slide per week was sent for PAS staining (n=8/week). The inoculated slides were air-dried, dipped into de-ionised water and stained with Periodic acid–Schiff (PAS) solution for 5 minutes, to determine the presence or absence of *candida albicans* hyphae. The exposed slides were rinsed well with de-ionised water, after which they were exposed to Schiff’s reagent for 20 minutes. Each of the slides were washed in running tap water for about 10 minutes, after which they were counterstained with a haemtoxylin stain for about 2 minutes. The counterstained slides turned blue under Scott’s Tap Water and dehydrate.

3.13.3 *Candida* hyphae and bud surface area measurement

The prepared slides were observed until at least 15 *Candida albicans* cells were seen in the field of view. Three *Candida albicans* cells were randomly chosen and measurements for the *Candida albicans* hyphae length and bud surface area under an Olympus® microscope. Hyphal presence, length and bud shape and surface area were measured using Olympus Stream image analysis software 40/0.65 (Bx41) with magnification 100x. The bud size and shape was assessed from left-to-right and from top-to-bottom and bud surface area was calculated using the formula:

$$\text{Area} = \pi ab$$

$$\text{Area} = 3.14 \times (\text{left-to-right}/2) \times (\text{top-to-bottom}/2)$$

Olympus® Stream image analysis software is an innovative software system for image analysis that helps convert the Olympus microscope into a high performance analysis tool, for display, capture and analysis of biological images.

CHAPTER FOUR

RESULTS

4.1 Antifungal inhibition zone measurement after 24 hours incubation

Inhibition zones were detected in the agar plates for Nystatin and Chlorhexidine (Figure 2, Table 2).



Figure 2: Inhibition zones of *candida albicans*; A: CHX B: GA C: NYS D: DH₂O

The inhibition zones for each of the materials over the three weeks are tabulated in Table 2 with standard deviations (SD) for each.

Table 2: Inhibition zone in mm with the SD

	Week 1 mean	SD	Week2 mean	SD	Week3 mean	SD	Average of the 3 weeks' mean	SD of the 3 weeks' mean
CHX	16.66	1.15	16	0	15.66	0	16.11	0.8635
NYS	20.33	0.577	19.33	0.577	20	0.577	19.88	0.577
GA	0	0	0	0	0	0	0	0
DH₂O	0	0	0	0	0	0	0	0

The results in Table 2 indicate that NYS had the largest average inhibition zone 19.88 (± 0.577), followed by CHX 16.11 (± 0.8635). GA and DH₂O had no any inhibition zone. There was no significant difference with regard to inhibition zones for all the treatments between week 1, 2, and 3.

4.2. Bud surface area measurement of *Candida Spp*

Despite the fact that NYS and CHX had inhibition zones, *candida albicans* was still present at the edge of the disc, but none were present underneath the disc. In order to assess the effect of the various testing medicaments on *candida albicans*, bud surface area was evaluated at the margin of the inhibition zone and underneath the paper disc.

The buds were oval in shape and the surface area was calculated (Table 3).

Table 3: Bud surface area (in mm²) for *candida albicans* isolated from the edge of the discs impregnated with test compounds.

Name	Hours0.w1	Hours2.w1	Hours4.w1	Hours6.w1	Hours 24.w1
CHX	4655.35	7088.57	5601.76	2848.83	3265.00
GA	5054.40	5502.22	4707.01	3195.85	2713.45
NYS	4680.66	6039.39	6640.18	2884.94	3670.13
Name	Hours0.w2	Hours2.w2	Hours4.w2	Hours6.w2	Hours24.w2
CHX	4852.65	5332.62	4159.89	3545.95	3966.41
GA	5002.45	4672.90	5104.55	2767.32	853.90
NYS	3435.32	4971.88	7181.72	3070.27	3619.67
Name	Hours0.w3	Hours2.w3	Hours4.w3	Hours6.w3	Hours24.w3
CHX	6863.30	5197.91	5534.47	3496.37	2928.87
GA	4907.32	4878.03	7034.96	3005.53	2677.73
NYS	9537.83	7632.12	6287.88	3037.37	4614.27

At 0, 2, 4, 6 and 24 hours, no significant difference in bud surface area was seen between the various medicaments for the 3 weeks. The experiment was done three times; once per week in order to ensure reproducibility of the results. Only GA showed a significantly smaller bud surface area (853.90) after 24 hours of incubation in week 2 (Table 3).

In general, a sharp decrease in mean values of bud surface area was noted for all the medicaments in each week from hours 4 to hours 6.

Table 4 illustrates the mean bud surface area values of week 1, 2 and 3 combined. Non-overlap in Figure 3 indicates a significant difference ($p=0.05$).

Table 4: Mean values of bud surface area (in mm²) for *C. albicans* isolated from the edge of the discs impregnated with test compounds CHX, NYS, GA

	Hour 0 mean	Hour 2 mean	Hour 4 mean	Hour 6 mean	Hour 24 mean	Hours 0-24 mean
CHX	5457.1	5873.03	5098.70	3297.05	3386.76	4622.53
NYS	5884.60	6214.46	6703.26	2989.56	3968.02	5113.49
GA	4988.05	5017.17	5615.50	2997.52	2081.69	4138.51

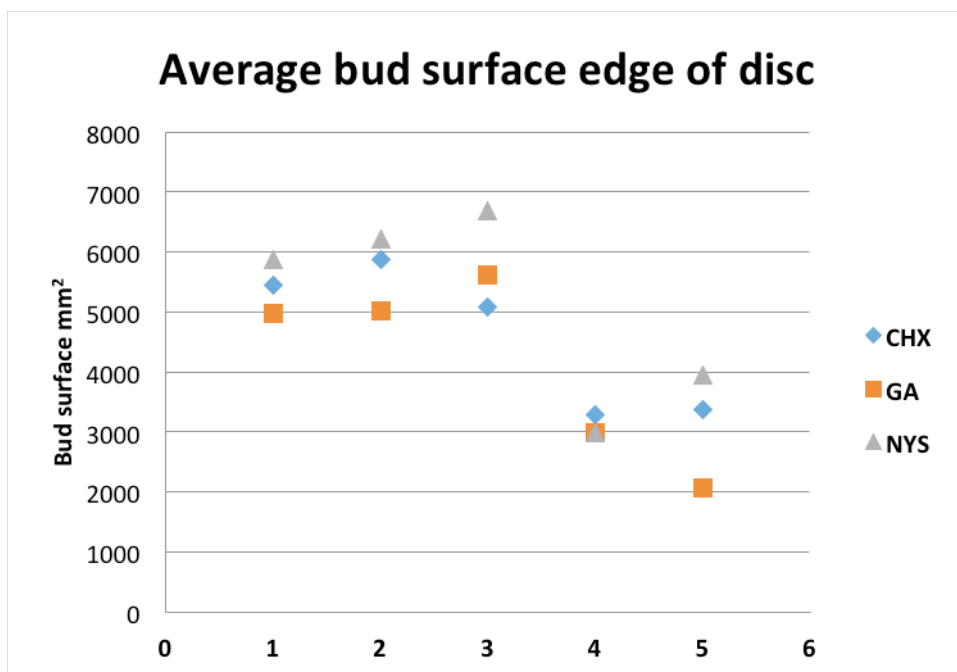


Figure 3: Mean values of the bud surface area (in mm²) at the edge of the inhibitory zone

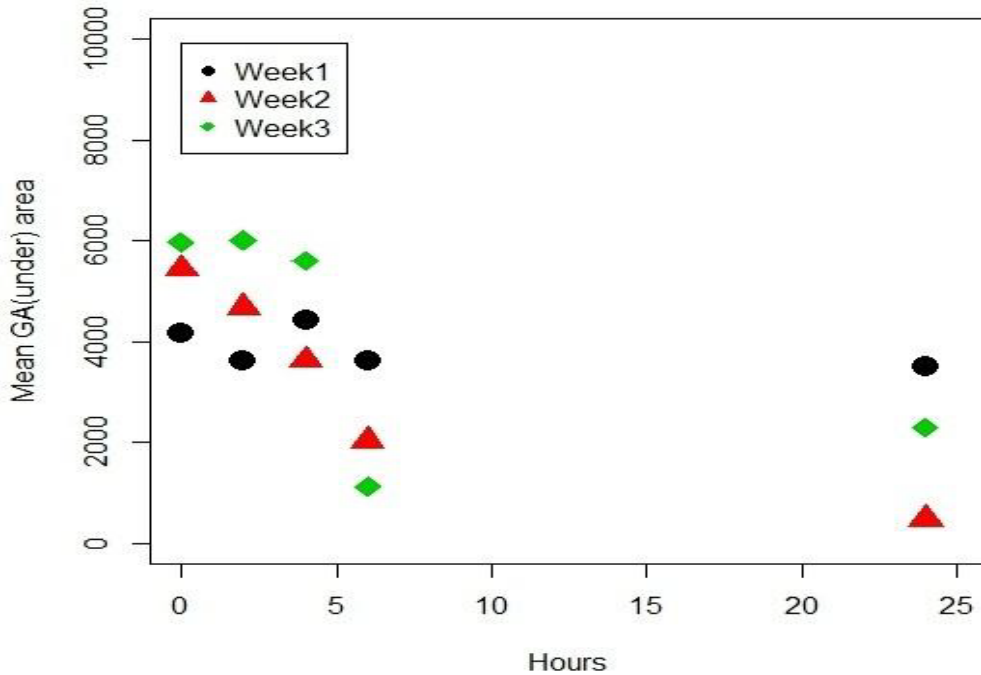
Table 4: shows that mean the bud surface values of GA at the edge for the time intervals 0, 2, 4, 6, 24 and 0-24 hours. The smallest mean value over the 0-24 hour period was (4138.51) this mean was significantly smaller than CHX (4622.53) and NYS (5133.49) with a ($p < 0.0001$) for this 0-24 hour period.

4.3. GA under bud surface

Table 5: Mean values of bud surface area in *candida albicans* isolated from the under of the discs impregnated with test compound GA .

	Hour 0	Hour 2	Hour 4	Hour 6	Hour 24
Week 1	4171.92	3607.37	4426.28	3606.52	3499.96
Week 2	5436.74	4670.85	3620.99	2016.29	471.48
Week 3	5961.99	6015.95	5601.37	1123.51	2291.40

Figure 4: Bud size of GA under for week 1-3 per hours. Figure 5 and Table 5 indicates that no



significant difference existed between weeks 1, 2 and 3 ($p=0.34$) but there was a sharp drop in mean values from 4-6 hours. A similar trend was observed in Table 4 but between the 4-6 hours a significant decrease in bud size ($p=0.0003$) was noted. The significance level between DH_2O and GA under for 0, 2, 4, 6 and 24 hours is presented in Table 6 .

Table 6: Mean and SD of bud surface area in *candida albicans* isolated from the (edge/under) of the discs impregnated with test compound GA relative to DH₂O

	0hrs	SD 0hrs	2 hrs	SD 2 hrs	4 hrs	SD 4 hrs	6hrs	SD 6hrs	24 hrs	SD 24 hrs	0- hrs 24 hrs	SD0-24 hrs
Control DH₂O	5144.87	1778.60	6109.61	1854.07	6806.17	2351.53	2684.69	883.73	3020.93	889.01	4753.25	1551.38
GA edge	4988.05	864.29	5017.72	2131.59	5615.51	1666.12	2989.56	745.12	2081.69	1110.56	4138.51	1303.53
GAunder	5190.22	2418.86	4764.72	2915.61	4549.55	2830.32	2248.77	1499.14	2087.62	1569.39	3768.18	2246.66

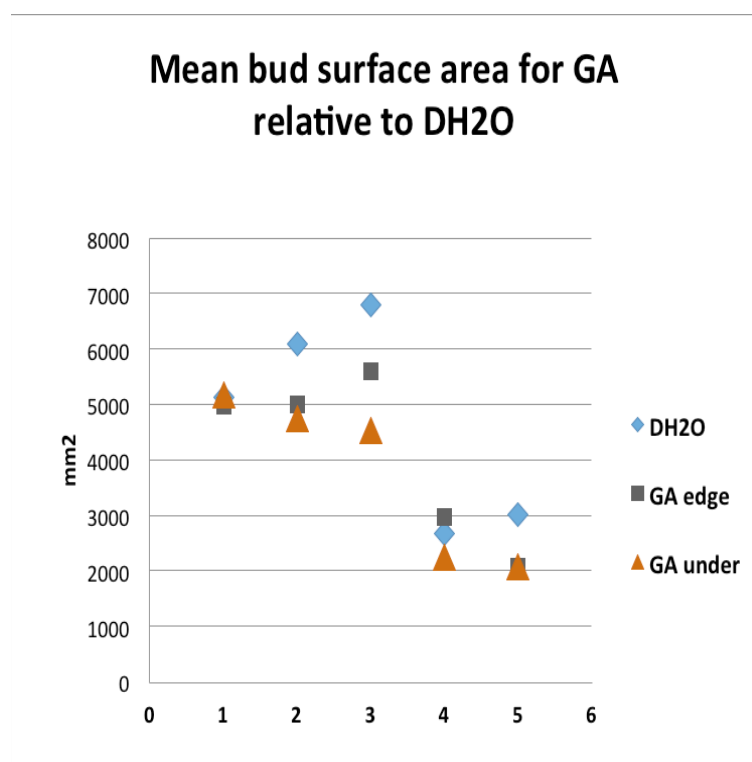


Figure 5: Mean bud surface area for GA relative to DH₂O

Two way ANOVA of Variance was applied to Table 6 bud surfaces for GA vs DH₂O controlled group areas, stratified by time (hours), site GA (under /edge).

GA (under /edge) was statistically significant compared to the control DH₂O ($p= 0.0047$). The bud surface area for GA and DH₂O between 0-24 hours was significant ($p<0.0001$).

Table 6 shows the standard deviation (SD) of the mean values for the bud surface specific time interval (0-24 hours). The mean values from Table 6 were ± 1.4 x (standard error) intervals in order to prevent overlap and allow a visual interpretation. Non-overlap from left to right (Figure 5) indicates a significant difference between the two means ($p=0.05$).

Figure 5 show that hours 0, 2 and 4 do not differ significantly. A significant drop in bud surface exist between hour 4 to 6 for GA (under/edge) as well as the DH₂O. There was a significantly smaller bud surface for GA (under/edge) at 24 hours compared to the DH₂O bud size. The overall mean value of the control (DH₂O) was significantly greater than GA (under/edge) for the 24 hour period. The mean bud surface area for GA (under/edge) over the 0-24 hour period did not differ significantly, although the bud surface area of GA (under) was consistently smaller than GA (edge) at 2, 4 and 6 hours, indicating growth inhibition of *candida albicans*.

Bud size	4hrs	6hrs	% reduction 4 to 6 hrs
GA edge	5615.50	2997.52	53.37%
GA under	4549.54	2248.77	49.42%
Control DH₂O	6806.17	2684.69	39.44%

Table 7: Reduction in bud size in *candida albicans* isolated from the (edge /under) of the discs impregnated with test compound GA relative to DH₂O at hours 4 to 6

4.4 Hyphal growth for GA, NYS, CHX

Table 8 illustrates that at 0 hours, there was no hyphae formation associated with any of the medicaments.

Table 8: Hyphae length in *candida albicans* isolated from the edge of the discs impregnated with test compounds .

Names	Hours0.w1	Hours2.w1	Hours4.w1	Hours6.w1	Hours24.w1
CHX	NA	391.672	562.158	455.232	922.934
GA	NA	224.706	526.424	326.156	529.584
NYS	NA	411.11	522.832	421.35	315.134
Names	Hours0.w2	Hours2.w2	Hours4.w2	Hours6.w2	Hours24.w2
CHX	NA	360.92	435.794	1276.776	803.406
GA	NA	346.106	403.126	755.508	551.684
NYS	NA	411.928	497.644	565.18	721.76
Names	Hours0.w3	Hours2.w3	Hours4.w3	Hours6.w3	Hours24.w3
CHX	NA	449.782	889.128	925.982	551.986
GA	NA	229.154	614.206	243.614	294.928
NYS	NA	455.42	544.076	630.3	1533.856

Table 8 shows the variance between the mean values for week 1, 2 and 3 at the 0, 2, 4, 6 and 24-hour time intervals. There was a significant difference between the mean values for GA edge ($p=0.05$) compared with NYS and CHX (Figure 7).

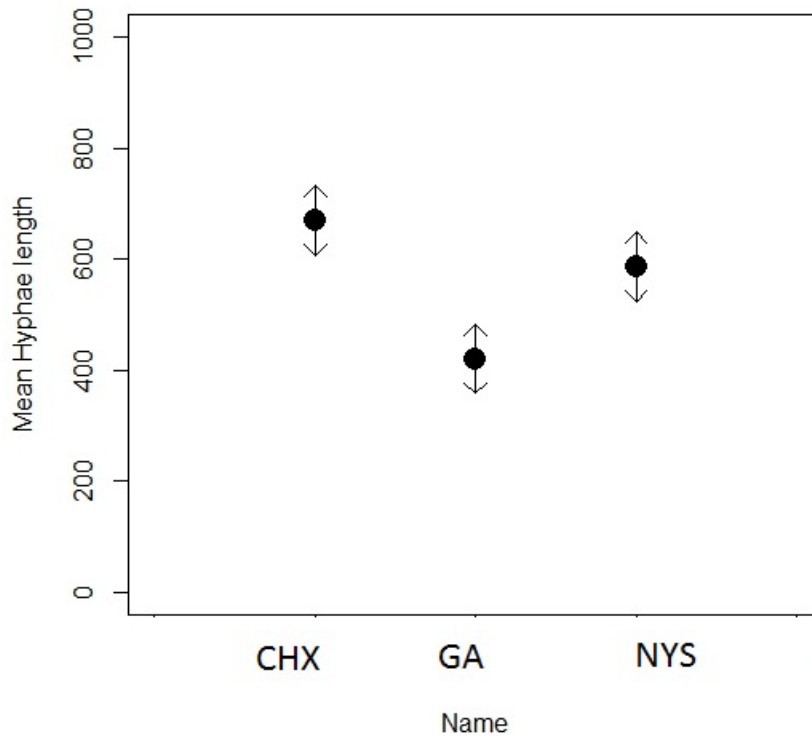


Figure 7: Mean values for GA, CHX, and NYS 0-24 hrs for weeks 1, 2, 3

Statistical analysis for GA (under) was done for week 1, 2, 3 at the times 0, 2, 4, 6 and 24

Table 9: Hyphae length in *candida albicans* isolated from GA (under) over the 24-hour period for weeks 1, 2, 3

	0hrs	2hrs	4hrs	6hrs	24hrs
Week1	NA	333.192	582.160	370.282	336.534
Week2	NA	549.612	452.676	404.546	419.584
Week3	NA	544.742	802.782	307.600	431.912

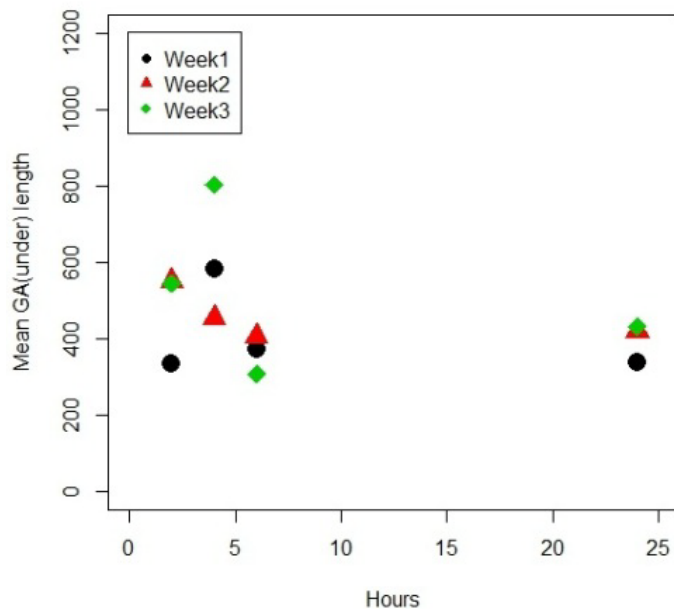


Figure 7: GA under hyphae length

For GA (under) there was no significant difference in the hyphal length between weeks 1, 2, 3 ($p=0.16$). Comparing the hyphal growth to the control (DH₂O) will provide insight into the true effect of GA on the *candida albicans*.

Table 10 provides the mean and SD of hyphae length for GA relative to the control (DH₂O).

	2hrs	2hrs SD	4hrs	4hrs SD	6hrs	6hrs SD	24hrs	24hrs SD	0-24 overall	0-24SD
Control DH₂O	432.3	101.68	2635.78	3324.85	670.38	251.01	1518.26	978.36	1314.18	1163.97
GA edge	266.66	93.38	2081.73	2227.10	441.76	319.73	458.73	279.55	812.22	729.94
GA under	575.85	166.85	1668.65	2027.41	360.81	164.70	396.01	130.55	725.33	622.37

Table 10: Mean and SD for GA (under /edge) vs DH₂O hyphae length

Two-way ANOVA analysis of variance for hyphal length was completed considering two variables namely the (medicament) GA (under/edge) and time (Hours 0, 2, 4, 6 and 24). There were significant differences in the mean value ($p=0.035$) between GA (under/edge) compared to

DH₂O over the 24 hour periods. Significant differences were found between the various time intervals 0, 2, 4, 6 and 24 ($p=0.0001$). The trend of the mean values for each hour interval indicated that the mean values for GA (under/edge) is smaller than DH₂O, indicating that GA (under/edge) sufficiently inhibited the hyphae growth. There was no significant difference between GA (under/edge) over the 24 hour time period ($p=0.66$).

Table 9 shows the overall DH₂O mean value (1163.97), which is much greater than GA under (622.37) and GA edge (729.94) -Table 10.

Interestingly, the hyphae length also a shared a similar trend with bud size at 4 hours. After 4 hours the means of DH₂O and GA (under/edge) is considerably greater than the means for these three groups at hour 2, 6 and 24.

Table 10 (Figure 8) shows the SD values plotted at ± 1.4 (standard error) limits and confirms the significantly high SD values at the means of hours 4 compared to hour 2 and 6.

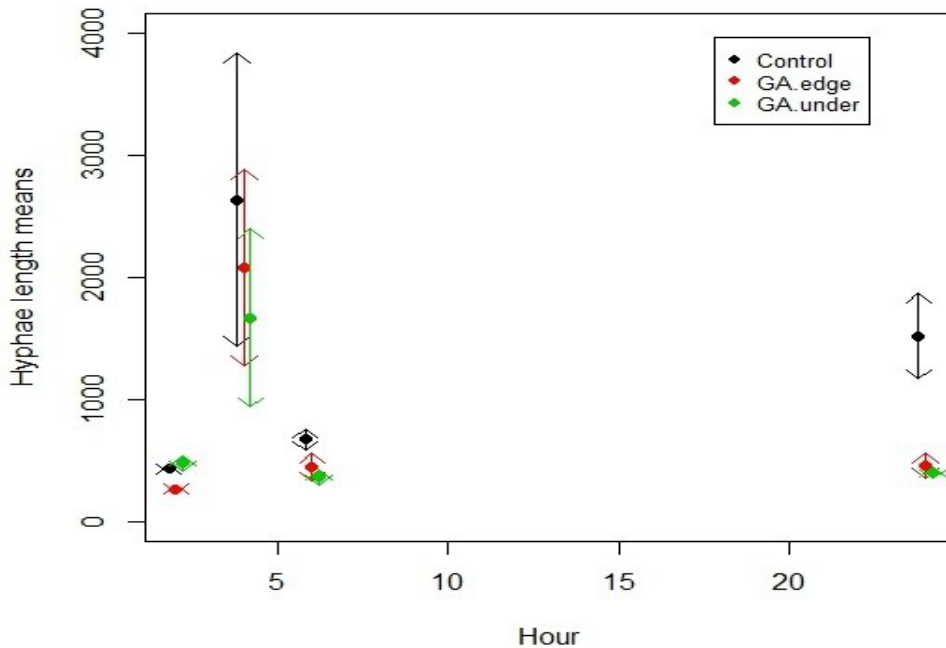


Figure 8: SD of Hours for Hyphae growth

When these high SD values of (Table 10) are considered, a clear picture of the results can be obtained if the statistical analysis is complemented with $Y = \log(\text{Hyphae length})$. The log values are used in order to normalize the large SD values in order to obtain a statistical analysis with the

Y-value that will be more representable of the hyphae growth.

Two-way ANOVA analysis can be completed on Table 11.

Table 11: Y value of the log (Hyphae) length

	2hrs	2hrs SD	4hrs	4hrs SD	6hrs	6hrs SD	24hrs	24hrs SD	0-24 overall	0-24 SD
Control DH₂O	6.041	0.253	7.193	1.161	6.439	0.396	7.128	0.659	6.700	0.617
GA edge	5.521	0.388	7.042	1.128	5.409	0.583	5.968	0.580	6.1100	0.669
GA under	6.104	0.375	6.944	0.923	5.801	0.426	5.923	0.372	6.193	0.524

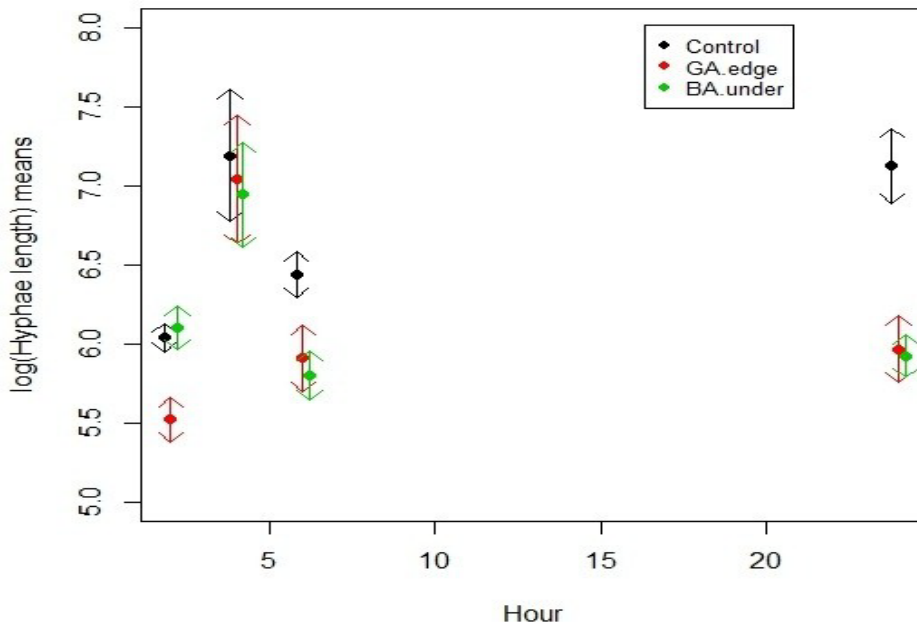


Figure 9: Y-values log (Hyphae) length at Hours 0, 2, 4, 6 and 24

At 6 and 24 hour time intervals, GA (under/edge) had significantly shorter hyphal growth ($p < 0.0001$) than DH_2O , while at 4 hours, no significant difference in hyphae length was seen. GA (under/edge) had shorter hyphae with a smaller SD.

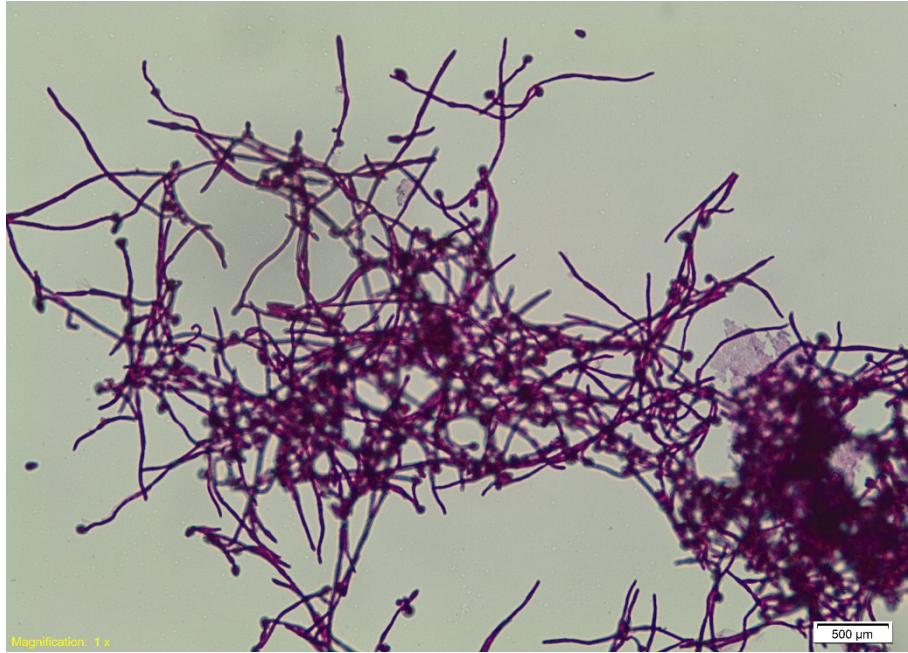


Figure 10: Hyphal formation under DH_2O treatment at hour 6

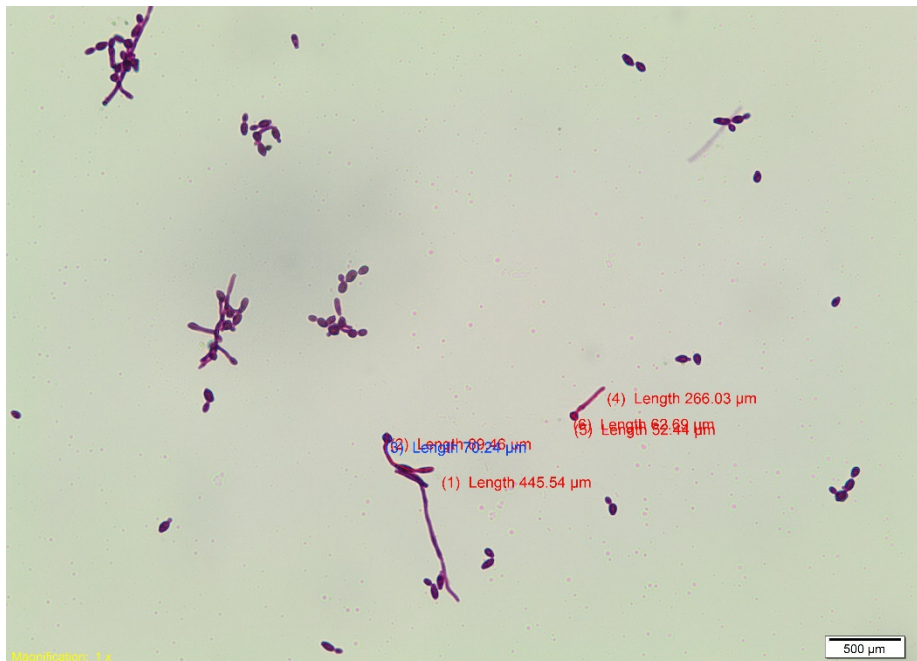


Figure 12: Hyphal formation at the edge of GA disc at hour 6

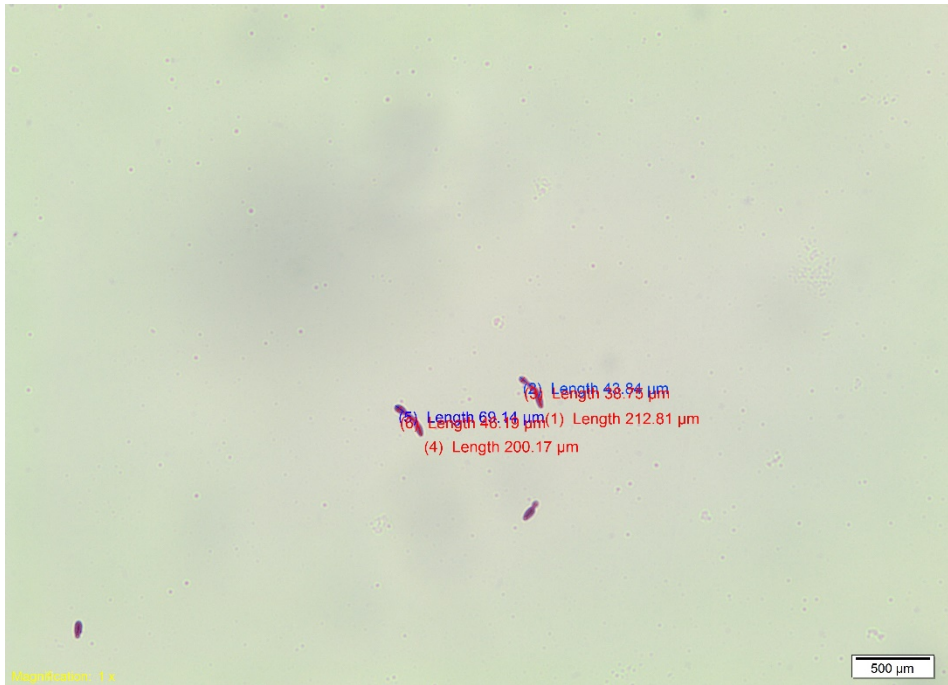


Figure 13: Hyphal formation under the GA disc at hour 6

CHAPTER FIVE

DISCUSSION

5.1 Inhibitory zone

There was no zone of inhibition around GA and DH₂O discs, while CHX and NYS discs exhibited significantly large inhibition zones (Figure 2). This indicates that the two conventional medications have inhibited the yeast around them. This is in agreement with previous studies that used different purified aqueous fractions of GA (Vediyappan *et al.*, 2013).

For both GA and DH₂O, no significant inhibition of *candida albicans* growth was found underneath and at the edge of the disc. This is because GA alters the growth of Candida (Vediyappan *et al.*, 2013) but does not kill it as does NYS and CHX.

It would be presumed that the *Candida* under the paper disc from the disc diffusion test would be affected by the Gymnemic acid. Hence the bud surface area and the hyphae growth was assessed and compared to the CHX, NYS, and DH₂O.

5.2 Bud size

An increase in the cell surface of the bud indicates that the cell is due to undergo cell division. During this phase, the cell volume may double in size (Chaffin, 1984). Three *candida albicans* cells were measurements of bud surface area per slide. Although GA did not show any visible inhibition at the edge of the paper disc, upon incubation of a smear taken from the edge of the paper discs, the initial bud surface area at hour 0 for GA was already smaller than the cell surface of CHX and NYS respectively (Table 4, Figure 3). Although there was no visible inhibition, the *candida albicans* was affected by the GA, it was noted that from 0-2 hours CHX and NYS bud surfaces progressively increased, but GA showed little change. From 2-4 hours, CHX showed a progressive decrease in bud size; NYS increased at a faster rate than GA. The greatest decrease in bud size occurred from 4-6 hours for all medications. Only GA progressively decreased in bud size from 6 to 24 hours. NYS increased in bud size from 6-24 hours and CHX showed little change in bud surface area (Figure 8). In general, at hour 2 the bud surface increased until 4 hour, where the largest cell surface area was noted for all medicaments; the cell surface recorded

at 6 hours was smaller than at 4 hour. The reason for this large discrepancy in size is that the two daughter cell separate after 4 hour and are separated before hour 6. The same phenomenon was demonstrated for all treatments (Figures 6, 7 and Table 4) (Chaffin, 1984).

The trends of the mean values in Table 6 are representative of the treatment (GA under /edge) and hours (0-24) since the ANOVA on the interaction effect indicated a ($p=0.3650$). Therefore because there were no significant differences between the bud size of the *Candida* found under the disc versus at the edge of the disc for week 1, 2 and 3 the conclusion could be reached that the GA was equally effective in the inhibition of bud size growth under the disc compared to the edge of the disc. This conclusion corroborates the findings of other authors that GA is effective in modifying the pathogenicity of *Candida* (Vediyappan *et al.*, 2013).

The bud sizes from the GA treatment were significantly ($p=0.0001$) smaller than CHX and NYS throughout the three weeks (Table 4, Figure 3, 7).

The bud surface from week 2 hour 24 for GA deviated significantly compared to the other time intervals of the weeks (Table 3). From the images of the microscopic measurements it was noted that the buds were more round than oval. This resulted in the buds having a smaller size at 24 hours at week 2. In the other weeks (1, 3) the buds at 24 hours had clear daughter cells attached to them, but in week 2 at 24 hours very few attached daughter cells were visible. The working theory was that the some of the buds measured were daughter cells and this resulted in the decrease of the mean value for week 2 at the 24 hour time interval (Chaffin, 1984).

Figure 5 and 7 best demonstrates the relationship between cell surface area of GA under and GA edge, in relation to DH₂O and the incubation time. At Hour 0, the surface areas of the buds were similar in size. During the progression from 0-4 hours, GA under had a decrease in bud surface area as compared to GA edge and DH₂O. This illustrates that the buds from under the paper disc were inhibited to a greater extent than GA edge and DH₂O respectively. DH₂O buds increased in size while, GA (under) continuously caused a decrease in bud size compared to GA (edge).

From Figure 7 and Table 7, it is a clear that the mean bud size of GA (under) is smaller than GA (edge), but the difference was not statistically significant. The percentage of size reduction for GA (under) was 49%, GA (edge) was 53% and the control (DH₂O) was 39%. The bud size reduction between 4 and 6 hours was significantly different between GA and DH₂O.

For hour 4, the bud size of GA (under) reduced 33.2% more than that of DH₂O. GA (under) reduced 16% more than DH₂O at hour 6. This indicates that the daughter cell that formed between the end of hour 4 but before hour 6, were also smaller for GA (under) versus DH₂O.

Table 7, shows the reduction of surface area size from a larger value at 4 hours to a lower value at 6 hours. Three *candida albicans* cells were observed and measurements of buds per slide. The reduction in size of the bud to less than 50% of the original 4 hour size indicates that the cell could have either returned back to single cell status or have a smaller non divided double cell. It has been noted that apical cells grow and divide while sub-apical cells become arrested in the cell cycle because they have insufficient cytoplasm. Once the cytoplasm is sufficient the sub-apical cells will re-enter the cell cycle.

The absence of any significant difference between GA under and GA edge indicates that GA had an inhibitory effect on *candida albicans*. Even though growth was not completely inhibited, GA had an effect on the growth pattern of *candida albicans* by retarding development in interphase; thereby retarding the doubling time (Figure 5, 6 and Table 7). The overall mean bud surface values from 0-24 hours indicates that GA (under) and GA (edge) were 20% and 12.9% smaller, respectively than the control (DH₂O). The greatest reduction in the bud size would occur where GA contacted the buds, but even those at the periphery of the paper disc would benefit from the diffusion effect of GA. Clinically this could be translated as: areas in close proximity to GA would benefit from antifungal effects. This will assist the body's defense mechanism to clear the *candida albicans* infection.

5.3 Hyphae length

Hyphal development is also an important pathogenic factor (Sudbery *et al.*, 2004). Three *candida albicans* cells were measurements of hyphal per slide. At hour 0, hyphae length base-line readings showed no hyphal growth across all treatments. (This lack of hyphal growth was represented with "NA" in table 4.3.1). The progressive growth inhibition for the study medicaments and other medicaments would be clearly observed under the microscope.

Over the 24 hour period for the three weeks, GA treatment significantly reduced the hyphal growth as compared to CHX and NYS treatments ($p=0.45$) (Figure 7 and Table 8). There were no significant differences found in hyphal length of GA under for week 1, 2 and 3 ($p=0.16$).

The reduced hyphal growth at 6 hours in Table 8 compared to the length of the hyphae for the periods of observation (0, 2, 4 and 24 hours) could indicate retardation of the hyphae growth over the full period of growth for 6 hours.

GA (under/edge) showed no significant differences over the 24 hours, with the exception at 2 hours (Figure 9, 10 and Table 9, 10), where a significant difference in hyphal growth between GA (under /edge) was seen, with large SD of the lengths between the measured hyphae. An increase in bud size correlated with an increase in hyphal length, from 0 to 2 hours. This is likely to be the period where *candida albicans* showed the greatest growth (Sevilla, 1986). Thereafter, hyphal length in isolates from GA (under/edge) consistently decreased significantly ($p=0.0001$) for both GA (edge) and DH₂O (hours; 2, 4, 6, and 24). This could imply that relative hyphal growth started responding to GA after 2 hours. The images below in figures 11, 12 and 13 show that in the DH₂O group, there was predominant hyphae growth from the buds. In the GA (under/edge), many buds did not have hyphal growth (Vediyappan *et al.*, 2013).

CHAPTER SIX

CONCLUSION

The complete eradication of *Candida* may not be desirable as it may disrupt the balance of normal flora, thus treatment with natural substances such as GA may be beneficial. Since *Candida*'s ability to switch between yeast, hyphal and pseudo hyphal morphologies is one of its virulence factors (Miller and Johnson, 2002), targeting prevention of this transition will impact its pathogenesis and clinical presentation. GA reduced the hyphae length and bud size of *Candida*, but unlike NYS and CHX, did not inhibit the Candidal growth. The reduction in bud size and hyphae length indicated that GA reduced the pathogenic potential of *Candida*, rendering it less virulent.

Clinical relevance of this study the GA and its extracts it the study indicated no toxicity (Vediyappan et al., 2013), but interrupted the yeast to hyphae conversion. This in vitro study would then provide the viability for future tests as an ingredient in *Candida* treatment for various clinical scenario, for example, in denture cream, xerostomia treatment, after radiotherapy, HIV, children in orphanages where *Candida* is considered a communicable disease.

RECOMMENDATIONS

Further studies need to be done on real time with video in order to view the daughter cell formation and hyphal growth. The dose responses to GA versus the growth rate could be used to establish the ideal dose of GA.

LIMITATIONS

The rationale for the study was to assess the efficacy of a commercially available Gymnemic acid in the concentration as per the established literature. Therefore the serial dilution of the commercially available Gymnemic acid for the establishment of the lowest concentration of Gymnemic acid for *candida spp* bud and hyphae alterations was not in the scope of this study.

The longest hyphae growth was selected, but when the microscopic images are critically evaluated, the buds that did not develop hyphae became apparent. Despite this limitation in the study, when one considers the % of buds in the field of view that did not develop hyphae significant results in favor of GA was still established.

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