The effect of subinhibitory concentrations of gentian violet on germ tube formation by *Candida albicans* and its adherence to oral epithelial cells

Tumane Daniel Mafojane

Degree of Masters of Dentistry in Oral medicine and Periodontology

Research report submitted to the faculty of Health sciences, University of the Witwatersrand, Johannesburg, in the partial fulfilment of the requirements for the

Degree of Master of Dentistry, Johannesburg

22 May 2017

DECLARATION

I, Tumane Daniel Mafojane, declare that this research report is my own work. It is being submitted for the degree of Master of Dentistry to the University of The Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……

…….day of……2017.

DEDICATION

To my family that supported me…..

PUBLICATIONS AND PRESENTATIONS

Oral presentation:

Mafojane T.

The effect of gentian violet on the adherence ability and germ tube formation of *C. albicans.* Research day, 2015, School of Oral Health Sciences, University of The Witwatersrand, Johannesburg.

Mafojane T., Shangase S., Patel M.

The effect of gentian violet on the adherence ability and germ tube formation of *C. albicans.* 46th Scientific meeting of the South Africa division – IADR, 3RD September 2015.

Mafojane T., Shangase S., Patel M.

The effect of subinhibitory concentrations of gentian violet on the germ tube formation by *C. albicans* and its adherence to oral epithelial cells. Submitted to Archives of Oral Biology for publication.

ABSTRACT

Background: Oral candidiasis is mostly caused by *Candida albicans* and occurs in immunocompromised individuals. The virulence factors of *C. albicans* include adherence to the oral epithelial cells or prosthesis, germ tube formation and hydrolytic enzyme production. It can be treated with antifungal agents and antimicrobial mouthrinses. Affordable and effective mouth rinses including gentian violet offer viable alternatives in developing countries. Studies have shown that gentian violet has antifungal properties both in *vivo* and in *vitro*. However, it is difficult to maintain the antifungal or inhibitory concentration in the oral cavity due to the diluting effect of the saliva. The effect of these sub-inhibitory concentrations has not been established. Therefore, the aim of this study was to investigate the effect of sub-inhibitory concentrations of gentian violet on the germ tube formation by *C. albicans* and its adherence ability to the oral epithelial cells.

Methods: Ten strains of *C. albicans* isolated from each of the denture wearers, healthy individuals and HIV positive patients were used in the study. Gentian violet (0.5%) was purchased from the local pharmacy. Antifungal property was determined using a microdilution technique. Minimum fungicidal concentrations were recorded. Sub-inhibitory concentration (0.000244%) of gentian violet was selected and its effect on the adherence ability was investigated using well described laboratory techniques. Briefly, gentian violet exposed *C. albicans* cells were incubated with oral epithelial cells. Epithelial cells were harvested, glass slides were prepared, stained with the Gram stain and viewed under the microscope. The number of adherent yeast cells per 100 epithelial cells was measured. The effect of five sub-inhibitory concentrations, including the MFC, on germ tube formation was determined using a well described technique. Briefly, *C. albicans* cells were exposed to the various concentrations of gentian violet in the presence of horse serum. Cells were harvested,

slides were prepared, stained with crystal violet and viewed under the microscope. Germ tube formation in 100 yeast cells was recorded. In both the experiments, water was used as a control. The results were compared to the control using the Kruskal-wallis test.

Results: The minimum fungicidal concentrations ranged from 0.000122% to 0.000977% of gentian violet solution. Sub-inhibitory concentrations of gentian violet reduced the adherence ability of *C. albicans* significantly ($p<0.01$) compared to the control. For the strains isolated from denture wearers, normal healthy individuals and HIV positive patients, the reduction in adherence was 55%, 56% and 56% respectively. The sub-inhibitory concentrations of GV significantly ($p<0.01$) inhibited germ tube formation compared to the controls. The inhibition was concentration dependent, with a decreased effect on germ tube formation as the gentian violet became diluted. Up to 98% reduction was noted at the concentration of 0.000244% gentian violet.

Conclusion: At high concentrations, gentian violet completely killed all *C. albicans* and at low concentrations it can inhibit the adherence of *C. albicans* cells to oral epithelial cells and inhibit the hyphae formation. These results suggest that the beneficial effects of gentian violet would last beyond the fungicidal concentrations in the treatment of candidiasis. Therefore, gentian violet can be used as a topical antifungal agent in patients with candidiasis.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Professor Mrudula Patel (Department of Oral Biological Sciences, University of the Witwatersrand) and Professor Sindisiwe Shangase (Department of Oral medicine and Periodontology) for their supervision and guidance in this project.

Ms Leticia Letsatsi the staff member and intern (Department of Oral Biological Sciences, University of the Witwatersrand) for her assistance with the laboratory experiments.

University of The Witwatersrand, Faculty Research Office for the funding of this project.

TABLE OF CONTENTS

PAGES

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMNS

AIDS:	Acquired Immuno- Deficiency Syndrome
ALS:	Agglutinin like sequence
ANOVA:	Analysis of variance
C. albicans:	*Candida albicans*
C3d:	Complement receptor type
CFU/ml:	Colony forming units per millilitre
CHX:	Chlorhexidine gluconate
CMJAH:	Charlotte-Maxeke Johannesburg Academic Hospital
GV:	Gentian violet
HIV:	Human immunodeficiency virus
hrs:	hours
MFC:	Minimum Fungicidal concentration
MFC:	minimum fungicidal concentration
rpm:	revolutions per minute
SD:	standard deviation
WHO:	World Health Organizations
˚C:	degrees centigrade
nm:	nanometre
ml:	millilitre
µl:	microliters

CHAPTER 1

INTRODUCTION

Oral candidiasis is mostly caused by *Candida albicans* and it occurs in immunocompromised individuals, denture wearers and sometimes in normal individuals. The virulence factors of *C. albicans* include adherence to the oral epithelial cells or prosthesis, germ tube formation and hydrolytic enzymes production. Systemic dissemination of infection can be debilitating with serious consequences. *C. albicans* organisms isolated from the oral cavities of HIV positive patients are known to be more virulent than the isolates from the HIV negative patients (Sweet *et al.*, 1995).

Oral candidiasis can be treated with antifungal agents and adjunctive antimicrobial mouthrinses. Affordable and effective mouth rinses including gentian violet and medicinal plant products offer viable alternatives in developing countries (Maley and Arbiser, 2013). Studies have shown that gentian violet has antifungal properties both *in vivo* and *in vitro* (Nyst *et al.*, 1992; Traboulsi *et al.*, 2008; Ying *et al.*, 2010). However, it is difficult to maintain the therapeutic concentration in the oral cavity due to the diluting effect of the saliva. This reduces the efficacy of the antifungal drug against the microbes with regards to its antimicrobial effect. Advances in research have suggested a novel approach to therapy in which the antifungal agent aims to reduce the virulence of *C. albicans* as opposed to focusing on the antimicrobial effect. This would be ideal where the causative organism is part of the normal flora, as is the case with *C. albicans* in the oral cavity (Gauwerky *et al.*, 2009). Pathogenic characteristics such as biofilm and germ tube formation, adherence mechanism and production of tissue damaging enzymes are possible targets of new therapeutic agents.

The effect of gentian violet on the germ tube formation and adherence to the oral epithelial cells by *C. albicans* isolated from HIV positive patients including denture wearers has not been established.

1 Literature review

Infections caused by fungi such as *Candida* are most frequently superficial occurring on moist mucosal surfaces of individuals suffering with a mild debilitation (Williams and Lewis, 2011). Oral candidiasis is one of them. However, systemic dissemination of fungal infection in immune-compromised patients may cause deep mycosis with life threatening debilitating consequences. Mortality rate of individuals with systemic candidiasis has been reported to be 30-50% (Vazquez, 2010) and even as high as 71% - 79% (Akpan and Morgan, 2002).

1.1 Oral Candidiasis

Oral candidiasis is the most common fungal infection in humans (Sharon and Fazel, 2010). It is an infection that is caused by yeast like fungus of the genus *Candida*, a normal commensal of the mouth and gastrointestinal flora. Oral candidiasis affects the very young or the very old with the carriage rate in the general population ranging from 20-70% without any symptoms (Ghannoum and Radwan, 1990). Several predisposing factors such as xerostomia which can be brought about as a consequence of impaired salivary function, the use of broad-spectrum antibiotics, and other immunosuppressive conditions can place the individual at risk of developing candidiasis. Most often this happens due to the weakening of host defense (Williams and Lewis, 2011). Recently there has been a significant increase in the incidence of all forms of candidiasis. This reflects changes in medical practise such as greater use of

invasive surgical procedure, wide spread use of immunosuppressive drugs as well as broad-spectrum antibiotics and by the escalation of HIV- infection and acquired immuno-deficiency syndrome AIDS (Akpan and Morgan, 2002).

The incidence of oral candidiasis isolated from the oral cavity of various population groups has been widely reported. A 45% incidence has been found in neonates, 45% - 65% in healthy children and 30% - 45% of healthy adults and a slightly higher incidence of 50% - 65% in people who wear removable dentures. It has also been found in 65% – 88% of patients living in long term frail care facilities, in 90% of patients with leukemia on chemotherapy and 95% of patients who are HIV positive (Akpan and Morgan, 2002).

However, in patients where the host's immune response is depressed or when changes occur in oral flora balance, overgrowth can be anticipated (Rivera-Hidalgo and Stanford, 1999). Clinically oral candidiasis can cause significant amount of discomfort, pain and dysguesia. In HIV positive patients it can dissaminate and cause oesophageal candidiasis which can cause difficulty in swollowing and chest pain (Williams and Lewis, 2011).

1.2 Types of oral candidiasis

Human infections caused by *Candida* can be broadly categorized into systemic and superficial. Systemic infections generally develop in individuals who are severely immune-compromised, has indwelling devices and intravenous catheters. Whilst these infections are relatively rare, they are often associated with high mortality. The superficial infections found on moist mucosal surfaces such as those in the mouth are more prevalent and less damaging to the host (Williams *et al.,* 2013).

The clinical characteristics of oral candidiasis vary and different classifications have been used. Samaranayake *et al.,* 2009 proposed a classification of oral candidiasis based on primary and secondary forms. In his classification, the primary forms are divided into acute form (pseudo membranous and erythematous), chronic form (hyperplasic, nodular, plaque-like and erythematous) and *Candida* – associated lesions namely denture stomatitis such as angular stomatitis, median rhomboid stomatitis and linear marginal erythema (Samaranayake *et al.,* 2009). Candida associated forms disease such as linear marginal erythema has been described in HIV positive patients (Grbic *et al.,* 1995). Denture induced stomatitis affects many individuals with removable complete dentures; the key risk factors with such patients are ill fitting dentures, poor denture hygiene and colonization of the denture fitting surface and mucosa by *C. albicans* (Gendreau and Loewy, 2011). Studies have shown that *Candida* associated denture stomatitis is a common infection that affects approximately 50% to 65% of denture wearers (Akpan and Morgan, 2002; Darwazeh *et al.*, 2001).

Severe denture stomatitis is generally associated with poor denture hygiene and *Candida* infection (Budtz-Jörgensen and Bertram, 1970). In immunosuppressed patients, systemic mycosis infection can involve one organ or be disseminated causing endocarditis and meningitis (de Almeida and Scully, 2015).

Oral candidiasis is commonly caused by *C. albicans*. However, yeast other than *C. albicans* with pathogenic potential has been isolated from the lesions (Samaranayake et al., 2009).

1.3 *Candida albicans*

Candida albicans is a round, budding yeast that produces a pseudo-mycelium both in laboratory cultures and in tissues. When incubated at room temperature or at 37^{o}C on a Sabouraud agar, it has smooth, soft, cream coloured colonies with a yeasty odour. The surface growth of the colonies consists of oval yeast while the submerged growth consists of pseudo mycelium (Jawetz et al, 1980).

Some species of fungi can grow in either yeast or mould phase and are termed dimorphic fungi. Dimorphic fungi of *Candida spp* appear in different forms: Blastopores, true hyphae (germ tube) and pseudo hyphae. All species of *Candida* can produce yeast cells but only *C. albicans* can form true hyphae (germ tubes). These are non-constricted extensions which form when the yeast cell is incubated in serum-containing medium at 37^{o}C degrees celsius (Bagg *et al.*, 2006). The formation of a germ tube is a critical virulence factor in the organism's ability to cause infection.

Candida albicans is an oppertunistic fungal pathogen. It is carried in the oral cavities of 70-80% healthy individuals (Odds, 1988). It can cause superficial infections such as thrush and denture stomatitis; however, in more severe cases it can cause life threatening systemic mycoses. Although *C. albicans* is the commonest species involved in human infection; other spp found in the oral cavity, for example; *C. tropicalis, C. pseudotropicalis, C. guillierimondii, C. krusei, C. lusitaniae, C. parapsilosis, C. stellatoidea.* and *C. glabrata* are also pathogenic to humans (Samaranayake *et al.*, 2009).

Candida dubliniensis, an opportunistic fungus is one of the *Candida* species that shares many phenotypic features such as hyphae formation with *C. albicans* and therefore may be misidentified in laboratories. However, it differs from *C. albicans* with respect to epidemiology, some of the virulence characteristics and the ability to develop fluconazole resistance in vitro. It was first described in 1995 from an HIV positive patient and it is mostly isolated from oral candidiasis lesions. Nevertheless, it is capable to causing extra-oral infections. In a nine year study in Kuwait showed that of 368 isolates identified as *C. dubliniensis*; 67.1% came from respiratory specimens, 11.7% from oral swabs, 9.2% from urine, 3.8% from blood, 2.7% from vaginal swabs and 5.4% from other sources (Gutiérrez et al., 2002, Khan et al., 2012, Loreto et al., 2010).

1.4 Pathogenicity of *C. albicans*

C. albicans has the ability to produce virulence factors such as adhesion, hyphae formation and secretion of extracellular enzymes that enhance colonisation on biotic and abiotic surfaces and invade host tissue (Cannon and Chaffin, 1999). These virulence factors can be affected by endogenous and exogenous factors including pH values, immunocompromised conditions and even the stage of infection. Wearing a dental prosthesis produces a micro-environment favourable to the growth of *Candida* with low oxygen and low ph. This may be caused by reduced salivary flow under the surfaces of the denture or poor oral hygiene. Growth of *Candida* and adherence to oral epithelial cells is often enhanced by a high carbohydrate intake particularly glucose (Akpan and Morgan, 2002; Darwazeh *et al.*, 2001; Gasparoto *et al.*, 2009).

Candida albicans organisms isolated from the oral cavities of HIV positive patients are known to have increased virulence and altered genotype than the isolates from the HIV negative patients (Sweet *et al.*, 1995, Ollert *et al.*, 1995). The severity and chronicity of oral candidiasis in AIDS patients has been attributed to the loss of T-helper cell function and not so much the opportunistic infections.

A study has shown that adhesion to buccal epithelial cells, phospholipase production and germ tube formation in *Candida* are reduced after the exposure to different subinhibitory concentrations of Chlorhexidine (Ellepola and Samaranayake, 2000). Medicinal plant extracts such as *Dodonaea viscosa var. angustifolia* and *Scutellaria baicalensis* have also shown to cause changes in the virulence properties of *C. albicans* (Ellepola and Samaranayake, 2000; Naicker and Patel, 2013).

1.4.1 Adherence and production of hydrolytic enzymes

Adherence of *C. albicans* to host cells is seen as an essential step in the establishment of disease (Yang, 2003). Adherence occurs between the *C. albicans* cell wall and host cell surface, therefore cell wall components are associated with the pathogenicity (Cannon and Chaffin, 1999). Therefore, fungal cell walls components such as mannose, C3d receptors, mannoprotein, and saccharins involved in the adherence process of the organism (Kanbe *et al.*, 1991) forms a crucial step in the pathogenesis of oral candidiasis. Adhesins and lectins which are proteins found on the external surface of the cell wall are important in the adhesion to host cells, inert polymers and proteins. Host cell receptors are also important in the adhesion. For the epithelial cell adhesion, several adhesins have been identified. Most of them are mannoproteins (Calderone, 1993). In addition, extracellular polymeric material

found on the cell surface and floccular fibrillary surface layer also contributes towards cellular adhesion (McCourtie and Douglas, 1984, Critchley and Douglas, 1987). The organism can adhere to oral epithelial cells and evade host defence systems by penetrating the epithelium.

The ability of *C. albicans* to produce secretory enzymes such as proteinases and phospholipase play an important role in its pathogenicity (Ying *et al*., 2010). They facilitate host tissue destruction and penetration. These enzymes also facilitate the adherence of *C. albicans* to the host cells (Martin and Lamb, 1982). Adherence ability is also linked to the hyphae formation. It has also been suggested that the hyphal form of *C. albicans* can adhere, penetrate fissures on the denture fitting surface and invade oral mucosa (Ramage *et al.*, 2004).

1.4.2 Germ tube or hyphae formation

Germ tubes are the initial projections observed when *Candida* switches from yeast form to hyphal growth. The ability to switch between the yeast form and the filamentous form is thought to be an important virulence property of C. *albicans* (Yang, 2003). The change into different morphologies contributes to colonisation and dissemination within the host tissues (Figure 1.1), and thereby promotes infection (Braun and Johnson, 1997).

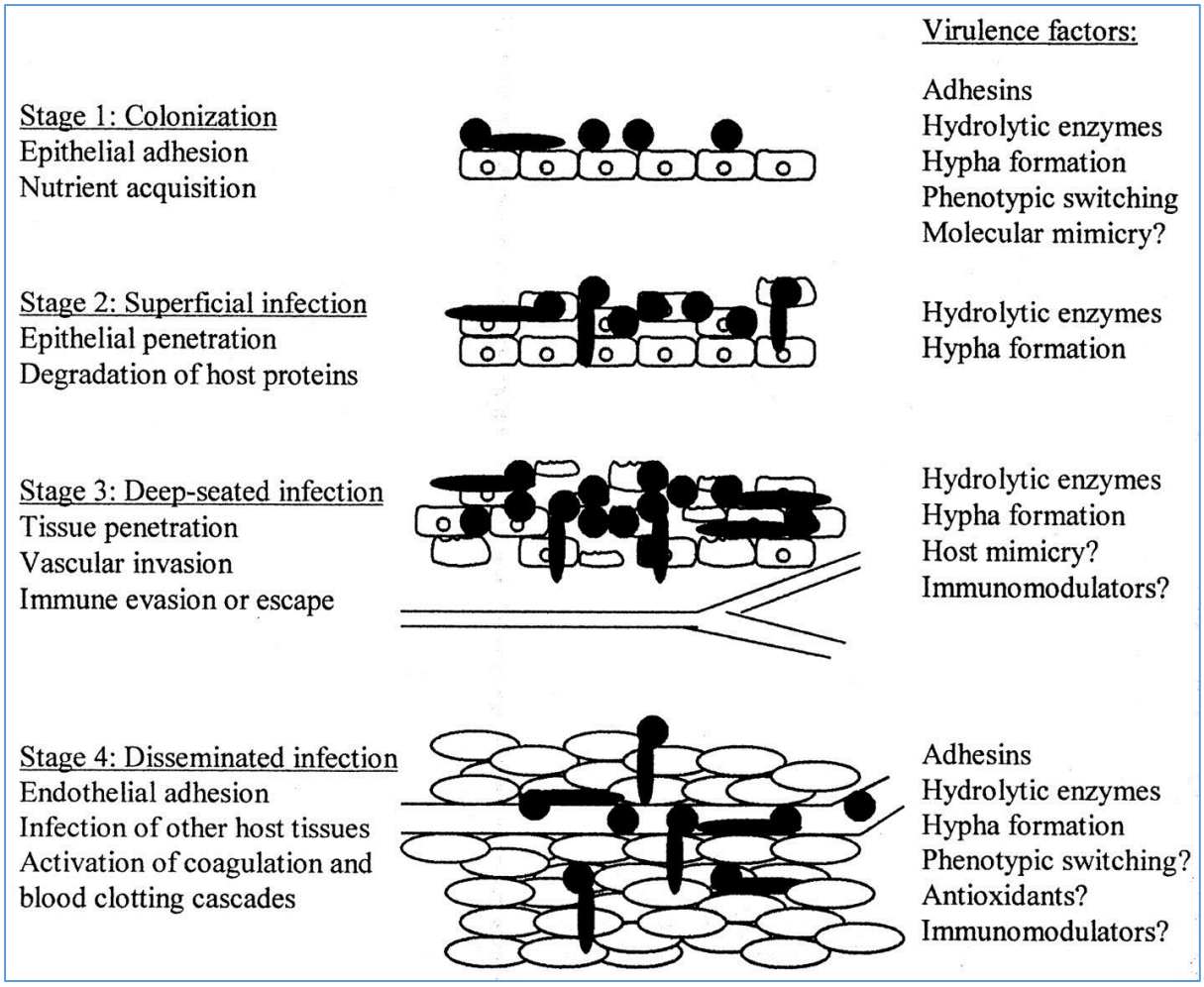

Figure 1.1: Adhesion, penetration and dissemination of *C. albicans* into host tissue. (Naglik *et al*. 2003)

Because hyphae and pseudo hyphae are invasive (i.e. they invade the agar substratum when they grow in the laboratory), it is postulated that this property could promote tissue penetration during the early stages of infection (Sudbery *et al.*, 2004). Formation of hyphae helps the fungi to overcome the host defence system e.g. Macrophages cannot phagocytose the tube form of organism. Germ tube bearing cells can adhere more to the epithelial cells compared to the oval cells (Kimura and Pearsall, 1980).

1.4.3 Biofilm formation

The use of medical implants such as catheters, pacemakers, prosthetic heart valves, and joint replacements has increased dramatically over the years (Hawser and Douglas, 1994). These devices can become colonized by microorganisms which form a biofilm consisting of cells and organized matrix of extracellular polymeric material which can cause implant associated infection. The release of microorganisms from the biofilm may initiate an acute disseminated infection frequently leading to the subsequent removal of the implant device (Hawser and Douglas, 1994). The infections can be caused by Gram-positive bacteria, notably staphylococci. However, the infection caused by Gram-negative bacteria and fungi can be more serious and can result in candidaemia (Dougherty, 1988; Hawser and Douglas, 1994). Fungal infections are caused by the pathogenic *Candida* species, particularly *C. albicans*, *C. tropicalis*, and *C. parapsilosis.*

These organisms have been regarded as increasingly important nosocomial pathogens (Wey *et al.*, 1988). Medical devices such as urinary catheters, prosthetic heart valves, silicone voice prostheses and cerebrospinal fluid shunts have been shown to be strongly associated with *Candida* infections (Odds, 1988). Levine *et al.*, (1988) reported a case of *C. albicans* infection following a total knee arthroplasty. The fluid culture of the aspirate was positive for *C. albicans* as a result of septic arthritis secondary to fungal infection (Levine *et al.*, 1988).

1.5 Treatment of oral candidiasis

Treatment of oral candidiasis should start with the practise of good oral hygiene. This should include brushing of the tongue and teeth twice daily, interproximal flossing and professional scaling and polishing at least every six months (Sharon and Fazel, 2010).

A number of topically or systemically antifungal agents are available for the treatment of oral candidiasis. Treatment options vary from topical delivery of polyenes agents such as nystatin and amphotericin B given four times a day to a systemic azole weekly dose (fluconazole) or single dose per day for a week of itraconazole (Samaranayake *et al.*, 2009).

Nystatin and amphotericin B are commonly used for the treatment of oral candidiasis. However, apart from the high cost that makes these drugs financially inaccessible to patients; poor solubility, stability, absorption, toxicity, and the development of resistance are problematic with existing drugs (Perlin, 2010). Although fungal resistance to topical agent is rare (Perlin, 2010), a significant resistance in *C. albicans* isolated from cancer patients with prolonged neutropenia has been reported (Lambert and O'Grady, 1992).

Additionally, Chlorhexidine a Bis-bisguanide antimicrobial antiseptic compound can be used in a mouth rinse form. It acts on the cell membrane causing leakage of intracellular material, respiratory inhibition and cytoplasmic coagulation (Camacho *et al.*, 2007). Chlorhexidine gluconate (0.2% aqueous solution) mouth rinse may be used as a supplement to topical or oral antifungal therapy (Sharon and Fazel, 2010). Although other topical antifungal agents work well, the need for frequent dosing, unpleasant taste, as well as the short contact time between the antifungal agent and the affected oral tissues can contribute to treatment failures (Sharon and Fazel, 2010).

1.6 Gentian violet and oral candidiasis

Gentian violet (hexamethyl pararosaniline) or crystal violet or methyl violet is a water soluble triphenymethane dye. It is derived from coal tar and has been used in Gram staining of bacteria. It has been used for more than a century as an antiseptic, and it used to be the only

treatment available for mucosal candidiasis. It has been used as an antibacterial, antifungal, anti-trypanosomal, antiviral, anti-angiogenic and antitumour agent (Maley and Arbiser, 2013, Samantha Hsieh *et al*., 2016). More recently, gentian violet has been used as mono therapy and an adjunct to treat oral candidiasis in HIV infected individuals, especially in developing countries (Maley and Arbiser, 2013).

Despite more than a century of use, no cases of cancer or toxicity have been reported and definitively linked to this compound (Maley and Arbiser, 2013). The low cost and ease of application of gentian violet makes a suitable alternative to other topical agents, e.g. Nystatin, for the treatment oral candidiasis especially in resource-limited settings (Jurevic *et al.,* 2011). However, it is not very popular because it stains oral mucosa which compromises appearance and patients are stigmatised.

An effective concentration that would not show any oral staining and yet be effective has been established (Jurevic *et al.,* 2011). In addition, a short term exposure of sub-therapeutic concentration of gentian violet has shown to reduce (6-8%) the hydrolytic enzyme production by *C. albicans* (Ying *et al*., 2010). When a study compared the efficiency of 0.5% gentian violet, ketoconazole and nystatin mouthrinses in the treatment of candidiasis in HIV positive patients, no significant difference between the cure rates was found. The conclusion from the study was that gentian violet was effective in the treatment of oral candidiasis (Nyst *et al.,* 1992).

These studies have shown that gentian violet has antifungal properties (Gomes-de-Elvas *et al*., 2012, Riley and Flower, 1950). However, it is difficult to maintain the therapeutic concentration in the oral cavity due to the constant salivary flow. In addition, a different approach to therapy has been suggested by some investigators, where the virulence of *C.*

albicans can be targeted instead of an antimicrobial property. This would be ideal where the causative organism is a part of the normal flora, such as *C. albicans* in the oral cavity. Pathogenic characteristics such as germ tube and adherence mechanism, biofilm formation, production of tissue damaging enzymes can be a possible target of new drugs (Cannon and Chaffin, 1999; Gauwerky *et al.*, 2009). The effect of gentian violet on the *C. albicans* isolated from the oral cavities of HIV positive patients and denture wearers has not been studied. In addition, the effect of gentian violet on the adherence properties of *C. albicans* has also not been established.

1.7 Aim

The study investigated the effect of sub-inhibitory concentrations of gentian violet on the germ tube formation by *C. albicans* and its adherence to oral epithelial cells.

1.8 Objectives

- To determine the Minimum Fungicidal Concentrations (MFC) of gentian violet against *C. albicans* isolated from the oral cavities denture wearers, normal healthy individuals and HIV positive patients.
- To study the effect of sub-inhibitory concentrations of gentian violet on the germ tube formation by *C. albicans* and its adherence to oral epithelial cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cultures and the test product

Ten cultures of *C. albicans* isolated from the oral cavities of denture wearers, ten from normal healthy individuals and ten from HIV positive patients were obtained from the Oral microbiology laboratory (Table 2.1).

Table 2.1: *C. albicans* strains used in the study

***Candida albicans* strains (n=30)**		
Denture wearers	**Normal healthy individuals**	**HIV positive patients**
D1	N3	H2.1
D3	N8	H3.1
D4	N9	H16.1
D5	N22	H24.1
D7	N33	H25.1
D9	N35	H26.1
D26	N40	H64.1
D28	N43	H77.1
D29	N44	H187.1
D30	N47	H196.1

These cultures were obtained under ethical clearance previously and therefore an ethical wavier was obtained from the Human Research Ethics Committee of University of the Witwatersrand (W-CJ-140425-2). For this study, cultures were grown on Sabouraud Dextrose Agar at 37^{o}C for 48 hours. Gentian violet (0.5%) was purchased from the local pharmacy. Fresh inoculums with optical density of 0.2 (405 nm) containing approximately 10^5 - 10^6 organisms per milliliter was prepared and used in all the experiments.

2.2 Minimum fungicidal concentration (MFC) study

MFC study was performed in order to determine sub-inhibitory concentrations for the subsequent experiments. It was performed using a microtitre double dilution technique. A two-fold dilution of gentian violet was prepared using sabouraud broth. One hundred microliter of each of the diluted gentian violet concentrations was added to each of the 96 well round bottom microtitre plate. Fresh inoculums with an optical density of 0.2 (405 nm) containing approximately 10^5 - 10^6 organisms per milliliter was prepared and each well inoculated with 100 µl of inoculum. Plates were incubated aerobically at 37^{o}C for 48 hrs (Figure 2.1).

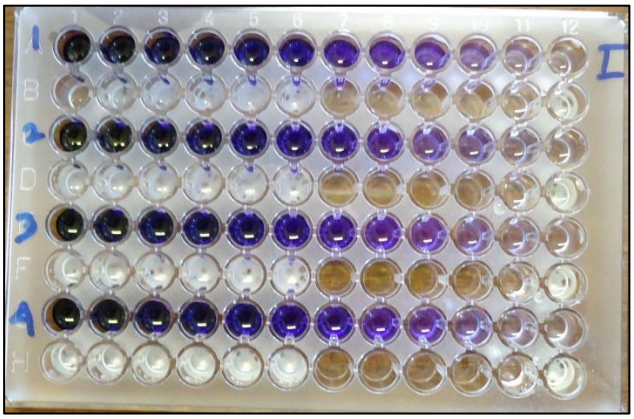

Figure 2.1: Microtitre plate wells containing various dilutions of gentian violet and inoculum. The numbers 1 to 4 represents different strains.

Chlorhexidine gluconate (0.2% aqueous solution) was used as a positive control and distilled water as a negative control. After incubation, each well was sub-cultured on a Sabouraud Dextrose agar. The lowest concentration with no growth was recorded as the MFC for that test organism (Figure 2.2).

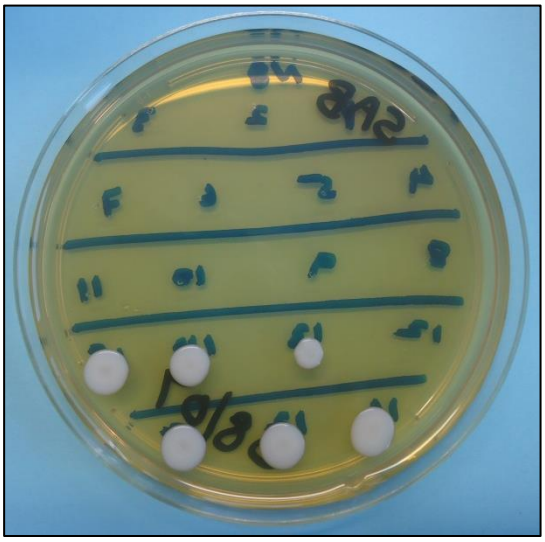

Figure 2.2: Culturing of microtitre plate wells on Sabouraud agar plate and the *C. albicans* growth.

Four sub-inhibitory concentrations for germ tube assay were selected whilst only one concentration for adherence assay was selected. The sub-inhibitory concentrations selected for germ tube formation were 0.000488%, 0.000244%, 0.000122%, 0.000061%, 0.000031%, and for adherence assay was 0.000244%.

2.3 Adherence assay

Adherence assays was performed using a technique previously described by (Ghannoum and Radwan, 1990) with modifications. Buccal epithelial cells were harvested from the oral cavity of healthy laboratory (*Candida* non-carriers) human volunteers from the Oral and Dental hospital at Charlotte-Maxeke Johannesburg Academic Hospital (CMJAH). These volunteers were screened for the *Candida* carriage and once found to be non-carriers they

were selected for the study. None were taking antibiotics at any time during the study. A single dental practitioner from the Oral medicine and Periodontology department from the university of the Witwatersrand collected buccal epithelial cells. This was done by rubbing gently on the oral buccal mucosa using a sterile cotton wool swab. Buccal epithelial cells were dislodged from the swab and suspended in 2 ml distilled water and centrifuged at 5000 rpm for 5 minutes, the supernatant was removed and the cells washed three times. *C. albicans* strains were grown into a Sabouraud Dextrose broth containing sub-inhibitory concentration (0.000244%) of gentian violet for 2 hours at 37^{o}C with constant shaking at 60 rpm. A control containing water was also used.

After incubation, yeast cells were harvested and centrifuged at 5000 rpm for 5 minutes, washed three times and re-suspended into 2 ml sterile distilled water. *C. albicans* cells were standardized to 10^{7} cells/ml using haemocytometer. Two millilitre of treated yeast cells and 2 ml of epithelial cells were mixed and incubated at 37^{o}C for three hours. Thereafter, the non-adherent *C. albicans* yeast cells were separated from epithelial cells using a 20 µm pore nylon filter paper, washed twice and the epithelial cells were re-suspended in 2 ml distilled water. Smears on glass slides were prepared and the epithelial cells and adherent yeast cells were stained with crystal violet for one minute (Figure 2.3).

The number of yeast cells adhering to 100 buccal epithelial cells was counted using light microscopy with the magnification of x 1000. The number of adherent yeast cells in the presence of gentian violet treatment was compared to the control.

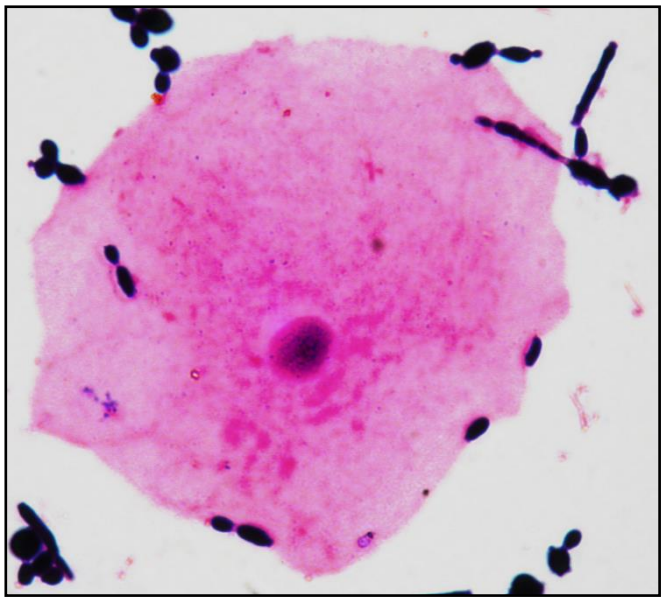

Figure 2.3: Light microscopy picture of oral epithelial cell. Pink: epithelial cell, black: *C. albicans* cells.

2.4 Germ tube formation

The effect on germ tube formation was examined using a technique described by (Naicker and Patel, 2013) with modifications. Horse serum (2ml) containing sub-inhibitory concentrations of gentian violet (0.000488%, 0.000244%, 0.000122%, 0.000061%, and 0.000031%) were inoculated with 10 µl of culture inoculum. Horse serum without gentian violet was included as a control. Test tubes were incubated for 3 hours at 37^{o}C. Smears of each culture were prepared, heat fixed and stained with potassium permanganate for one minute. Fifty cells per smear were randomly selected and viewed under the light microscope and the number of germ tubes per 100 cells was recorded. Cells were considered to have germinated if the germ tube formation was at least double the length of the cell. Number of germ tube formation in the presence of sub-inhibitory concentrations of gentian violet was compared to the control without gentian violet.

2.5 Statistical Analysis of data

The effect of gentian violet on the germ tube formation by *C. albicans* and its adherence property was compared to the control using the Kruskal-wallis test and Wilcoxon Rank-Sum Test. A p value <0.05 was considered statistically significant in all the analysis.

CHAPTER 3

RESULTS

3.1 Minimum fungicidal concentration (MFC) study

Results of the MFC assays are shown in Table 3.1 and 3.2. The MFC ranged from 0.000122 to 0.000977% of gentian violet solution. On observation there was no difference in the MFC values for the strains of *C. albicans* isolated from the different study groups was noted. Based on these results 1 sub-inhibitory concentration (0.000244%) and 5 sub-inhibitory concentrations (0.000488%, 0.000244%, 0.000122%, 0.000061% and 0.000031%) were selected for the adherence assays and germ tube assays respectively.

3.2 Adherence assay

The results of the adherence assay are shown in Table 3.3 and Figure 3.1. Sub-inhibitory concentrations of gentian violet reduced the adherence ability of *C. albicans* significantly ($p<0.01$) compared to the control. The effect was similar for all the study groups. For the strains isolated from denture wearers, normal healthy individuals and HIV positive patients, the reduction in adherence due to gentian violet exposure compared to the control was 55%, 56% and 56% respectively.

Table 3.1: Minimum Fungicidal Concentration of gentian violet against *C. albicans* strains

Group	*C. albicans*	Repeats	MFC (% gentian violet)			Median
			1	2	3	
Denture wearer	D1	3	0.000488	0.000488	0.000244	0.000488
	D3	3	0.000244	0.000488	0.000488	0.000488
	D4	3	0.000244	0.000244	0.000244	0.000244
	D5	3	0.000244	0.000122	0.000488	0.000244
	D7	3	0.000244	0.000244	0.000244	0.000244
	D9	3	0.000488	0.000977	0.000488	0.000488
	D26	3	0.000244	0.000122	0.000244	0.000244
	D28	3	0.000488	0.000977	0.000488	0.000488
	D29	3	0.000977	0.000488	0.000977	0.000977
	D30	3	0.000977	0.000195	0.000977	0.000977
Normal healthy individuals	N3	3	0.000488	0.000488	0.000488	0.000488
	N8	3	0.000122	0.000488	0.000488	0.000488
	N9	3	0.000244	0.000244	0.000244	0.000244
	N22	3	0.000977	0.000488	0.000488	0.000488
	N33	3	0.000244	0.000488	0.000244	0.000244
	N35	3	0.000122	0.000244	0.000488	0.000244
	N40	3	0.000244	0.000488	0.000488	0.000488
	N43	3	0.000244	0.000977	0.000977	0.000977
	N44	3	0.000977	0.000488	0.000488	0.000488
	N47	3	0.000122	0.000244	0.000122	0.000122
HIV positive patients	H2.1	3	0.000488	0.000488	0.000244	0.000488
	H3.1	3	0.000244	0.000244	0.000488	0.000244
	H16.1	3	0.000244	0.000244	0.000244	0.000244
	H24.1	3	0.000488	0.000488	0.000488	0.000488
	H25.1	3	0.000244	0.000488	0.000244	0.000244
	H26.1	3	0.000488	0.000488	0.000488	0.000488
	H64.1	3	0.000244	0.000244	0.000977	0.000244
	H77.1	3	0.000977	0.000488	0.000488	0.000488
	H187.1	3	0.000244	0.000244	0.000244	0.000244
	H196.1	3	0.000244	0.000488	0.000244	0.000244

Table 3.2: Summary results of minimum fungicidal concentration of gentian violet against *C. albicans* strains

Group	***C. albicans* strain**	**Range of MFC (% gentian violet)**
Denture wearer	10 (3 repeats)	0.000122 – 0.000977 (1.2 µg/ml – 9.7 µg/ml)
Normal healthy individuals	10 (3 repeats)	0.000122 – 0.000977 (1.2 µg/ml – 9.7 µg/ml)
HIV positive patients	10 (3 repeats)	0.000244 – 0.000977 (2.4 µg/ml – 9.7 µg/ml)

3.3 Germ tube formation

The results of the germ tube formation assay are shown in Tables 3.4, 3.5, 3.6, 3.7 and Figure 3.2. The effect of five concentrations of gentian violet on the germ tube formation was studied. The results showed that the sub-inhibitory concentration of gentian violet significantly ($p<0.01$) inhibited germ tube formation compared to the controls. The inhibition was concentration- dependent with the effect on germ tube formation decreasing as the gentian violet became diluted. Percentage reductions in the germ tube formation at various concentration of gentian violet are shown in Table 3.8. Up to 100% and 98% reduction was noted at the concentration of 0.000488% and 0.000244% gentian violet respectively.

Table 3.3: Effects of gentian violet on the adherence ability of *C. albicans*

Denture wearers			**Normal healthy individuals**			**HIV positive patients**		
Strain	**No. of *C. albicans* adherent to 100 epithelial cells**		**Strain**	**No. of *C. albicans* adherent to 100 epithelial cells**		**Strain**	**No. of *C. albicans* adherent to 100 epithelial cells**	
	Control	GV		Control	GV		Control	GV
D1	374	230	N3	300	79	2.1	256	107
D3	510	180	N8	276	159	3.1	412	204
D4	300	100	N9	304	254	16.1	518	198
D5	444	159	N22	464	245	24.1	386	220
D7	492	184	N33	480	111	25.1	248	80
D9	484	249	N35	260	138	26.1	276	127
D26	280	98	N40	468	220	64.1	308	165
D28	394	84	N43	272	127	77.1	429	230
D29	275	138	N44	344	96	187.1	298	134
D30	400	228	N47	328	115	196.1	520	150
Mean	**395.3**	**165**		**349.6**	**154.4**		**365.1**	**161.5**
SD	**88.23712**	**59.51284**		**87.3145**	**63.24942**		**102.9611**	**50.50468**
	55% reduction			**56% reduction**			**56% reduction**	

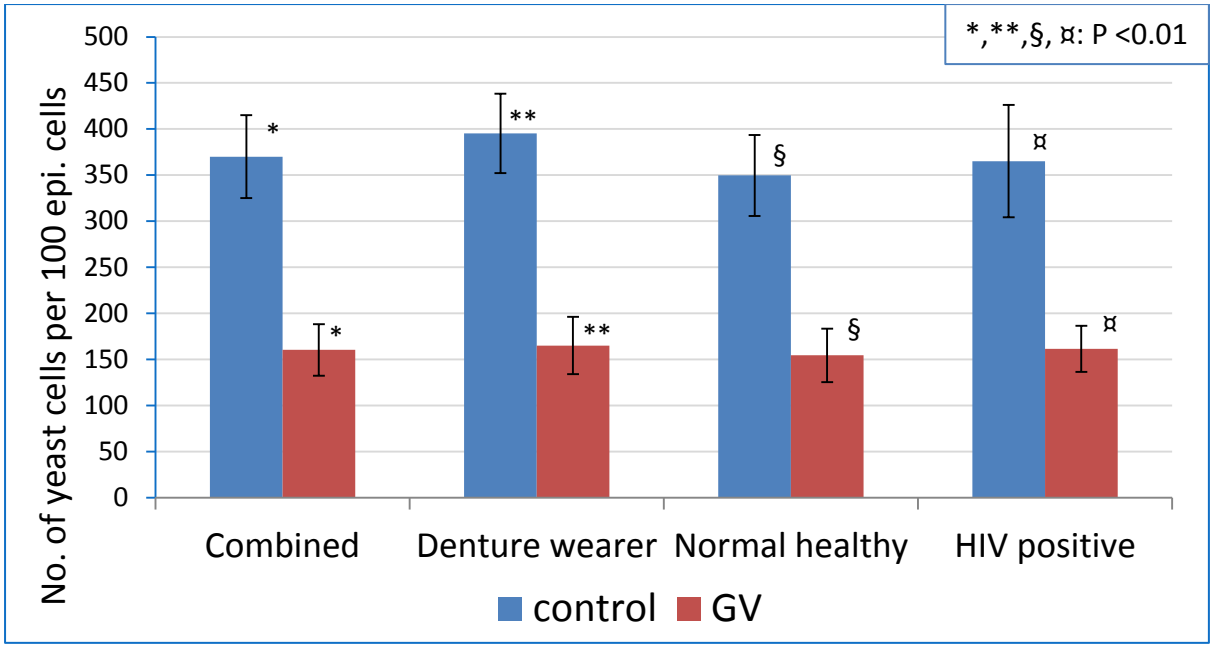

Figure 3.1: The effect of gentian violet (GV) on adherence of *C. albicans* to oral epithelial cells. Combined: Results of all three study groups to gather

Table 3.4: The effect of gentian violet on the germ tube formation ability of *C. albicans* isolated from denture wearers.

***C. albicans* strain**	***C. albicans* cells with germ tube formation (%) at various concentrations of gentian violet**					
	0% (control)	**0.000488%**	**0.000244%**	**0.000122%**	**0.000061%**	**0.000031%**
D1	55	0	0	2	28	48
D3	54	0	0	0	42	35
D4	49	0	0	0	6	25
D5	29	0	0	3	21	34
D7	42	0	16	34	34	62
D9	70	0	0	24	59	65
D26	75	0	10	37	19	69
D28	24	0	0	7	42	24
D29	42	0	0	24	33	57
D30	80	0	0	12	57	73
Mean±SD	**52±18.7**	**0±0**	**2.6±5.6**	**14.3±14.3**	**34.1±16.6**	**49.2±18.5**

Table 3.5: The effect of gentian violet on the germ tube formation ability of *C. albicans* isolated from normal healthy individuals.

***C. albicans* strain**	***C. albicans* cells with germ tube formation (%) at various concentrations of gentian violet**					
	0% (control)	**0.000488%**	**0.000244%**	**0.000122%**	**0.000061%**	**0.000031%**
N3	70	0	0	0	31	41
N8	74	0	0	0	22	51
N9	49	0	0	9	23	45
N22	42	0	0	0	7	45
N33	77	0	0	0	10	62
N35	62	0	0	0	46	46
N40	55	0	0	1	19	32
N43	79	0	0	12	43	60
N44	50	0	0	0	23	42
N47	48	0	0	0	44	46
Mean±SD	**60.6±13.5**	**0±0**	**0±0**	**2.2±4.4**	**26.8±13.8**	**47±8.8**

Table 3.6: The effect of gentian violet on the germ tube formation ability of *C. albicans* isolated from HIV positive patients.

***C. albicans* strain**	***C. albicans* cells with germ tube formation (%) at various concentrations of gentian violet**					
	0% (control)	**0.000488**	**0.000244**	**0.000122**	**0.000061**	**0.000031**
H2.1	68	0	0	5	5	43
H3.1	52	0	0	0	0	45
H16.1	60	0	0	2	2	28
H24.1	54	0	0	6	6	53
H25.1	60	0	0	3	3	44
H26.1	38	0	0	0	0	18
H64.1	44	0	0	3	1	18
H77.1	41	0	0	0	0	11
H187.1	53	0	0	5	3	12
H196.1	30	0	0	0	0	24
Mean±SD	**50±11.6**	**0±0**	**0±0**	**2.4±2.4**	**2±2.2**	**29.6±15.4**

Table 3.7: Summary of results of the effect of gentian violet on the germ tube formation ability of *C. albicans*. Where *, **, §, ¤: p<0.01. Results of concentration 0.000488% are not given because of the zero numbers of cells with germ tube.

	***C. albicans* cells with germ tube formation (%) at various concentrations of gentian violet**				
	0 (Control)	0.000244	0.000122	0.000061	0.000031
Combined	54.2 *,**,§,¤	0.86*	6.3**	20.9§	41.9¤
Normal healthy	60.6	0	2.2	26.8	47
Denture wearer	52	2.6	14.3	34.1	49.2
HIV positive	50	0	2.4	2	29.6

Table 3.8: Summary of results of the effect of gentian violet (GV) on the germ tube formation ability of *C. albicans* (Percentage reduction). Results of concentration 0.000488% are not given because of 100% reduction across the study groups.

	% reduction in germ tube (GT) formation at various concentrations of gentian violet compared to the controls [(GT in control - GT in test)/GT in control] x 100			
	0.000244	0.000122	0.000061	0.000031
Combined	98.41	88.38	61.44	22.69
Normal healthy	100	96.37	55.78	22.44
Denture wearer	95	72.50	34.42	5.38
HIV positive	100	95.20	96.00	40.80

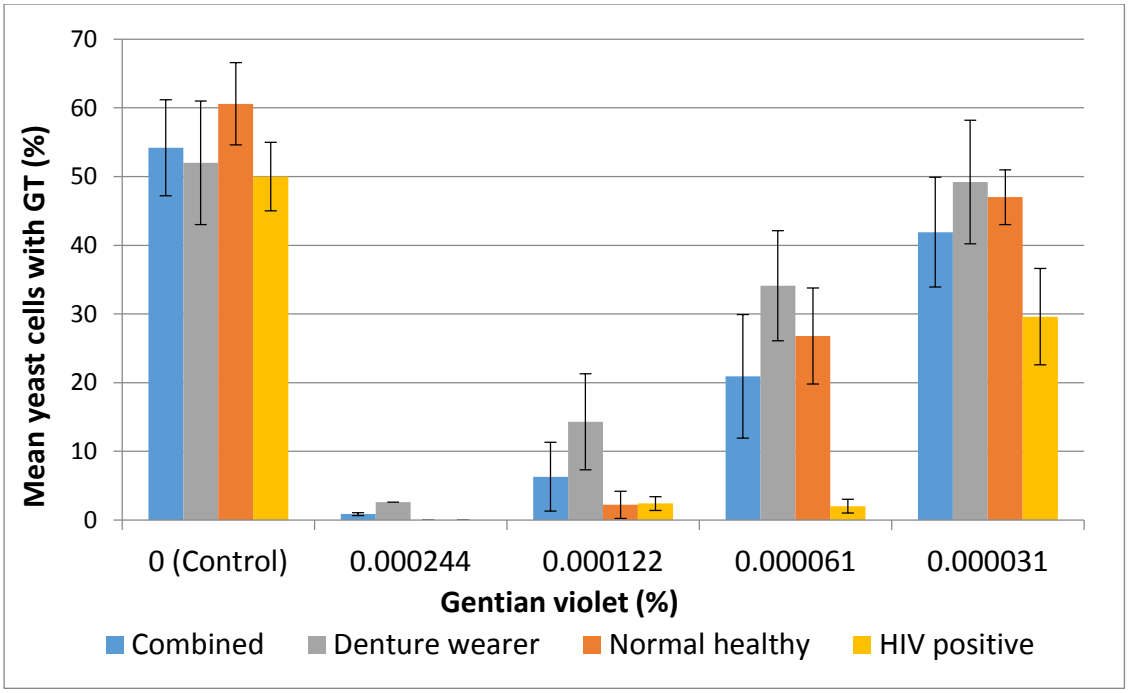

Figure 3.2: The effect of gentian violet on germ tube formation in *C. albicans.* Results of concentration 0.000488% are not given because of the zero numbers of cells with germ tube.

CHAPTER 4

DISCUSSION

Candida albicans is normal commensal yeast that usually exists within the oral cavity, vagina and gastrointestinal tract. It is an opportunistic pathogen and therefore causes infections such as candidiasis in the oral cavity, vagina and deep tissues. It can also cause serious infection such as candidemia. Oral candidiasis is more prevalent in immunocompromised patients and it is normally threated with antifungal mouth rinses and antifungal agents (Akpan and Morgan, 2002). Gentian violet has been used for many years to treat oral and vaginal candidiasis particularly in the resource poorer countries. Its antifungal activity has been established (Maley and Arbiser, 2013). However, due to the constant flow of saliva the therapeutic concentrations cannot be maintained. Therefore, this study was undertaken to investigate the effect of sub-inhibitory concentrations on the virulence properties such as adherence ability and germ tube formation. The results showed that at high concentrations, gentian violet kills *C. albicans* and at low concentrations it inhibits the ability to adhere to the epithelial cells and form germ tube. Both these properties are important in the development of this infection. This suggests that at the sub-inhibitory concentrations, gentian violet would still exhibit a beneficial property by rendering *C. albicans* avirulent.

4.1 MFC of gentian violet

In this study MFC values of gentian violet against *C. albicans* were 1.2 µg to 9.7 µg/ml (0.000122 to 0.00097%). These results are comparable to the MFC readings reported by Ying *et al.,* in 2010 (8 µg/ml) and Traboulsi *et al.,* in 2008 (1 µg/ml). However, low MFC values (0.5 µg/ml) were reported by Kondo *et al.* (2012). They also reported that *Candida* species

other than *C. albicans* requires higher concentrations of gentian violet. In their study, 1 µg/ml and 50 µg/ml of gentian violet was required to kill *C. parapsilosis* and *C. glabrata* respectively. Although these concentrations can kill *Candida,* usually the therapeutic doses used in the oral cavity or vagina are much higher (0.5%). In the oral cavity this high dose is justified because it soon becomes diluted due to the salivary flow. MFC values suggest that while becoming diluted it will still be effective until it is diluted more than 1000 times (from 0.5% to 0.000122%).

In the MFC studies the effect of gentian violet is measured after 24 hours of incubation period therefore the concentrations required are usually low. If a time-kill study is performed as reported by Kondo *et al.* (2012), in first 30 minutes of treatment the effective concentration would be 50 µg/ml of gentian violet (0.005%). This is much lower than the recommended concentration (0.5%). These results suggest that the initial therapeutic concentration can be reduced. Gentian violet at 0.5% concentration stains the oral cavity and the patient compliance is poor. For this reason, studies were conducted to determine the concentration which stained the oral cavity a minimum to none (Jurevic *et al.,* 2011) and the antifungal property of that concentration was determined. The effective concentration which did not stain the oral cavity was found to be 0.00165% solution of gentian violet (Jurevic *et al.,* 2011). In the clinical trial this concentration proved to be as effective as nystatin (Mukherjee *et al.,* 2017). In this study the results showed that the use of 0.00165% gentian violet solution twice a day for 14 days in HIV positive patients with oral candidiasis, either cured or improved the patients (68.5%) and it was comparable to the use of conventional nystatin (67.8%). This concentration was effective probably because 0.00165% would become diluted in the day and still be effective until it becomes 0.0001% (also found in our

study) and by end of the day the second application would take care of the night time treatment.

There is no doubt that in the clinical trials 0.5% solution is effective (Wright *et al.,* 2009, Nyst *et al.,* 1992), however lower concentrations should be used which will still be effective and increase the patient compliance. Present research was undertaken to study the effect (other than fungicidal) of gentian violet lower than the MFC values. The results showed that at the concentrations lower than the MFC values, gentian violet inhibits the virulence properties of *C. albicans*.

4.2 Effect of gentian violet on the adherence

Adhesion of fungus to the host cell is the most important initial step in the disease process. Once in contact with the host cells, *Candida* spp. produce adhesins which are responsible for the adhesion process. The sub-inhibitory concentrations of gentian violet ($p<0.01$) significantly inhibit the adherence of *C. albicans* to epithelial cells. It is possible that gentian violet may have inhibited the production of adhesins by the *C. albicans* cells or caused a damage to the adhesins already present in the outer envelopes which may have prevented adherence. In *C. albicans,* genes such as the agglutinin like sequence (ALS) family encodes cell-wall glycoproteins, some of which are implicated in adherence to host surfaces (Gaur and Klotz, 1997; Hoyer, 2001). Gentian violet may have affected ALS genes thus causing interference with the synthesis of adhesins, and reducing the adherence of *C. albicans* to oral epithelial cells. Fibrillar-floccular layer present on the cell wall mannoprotein of *C. albicans* also facilitate adhesion of *C. albicans* to host cells (Olsen, 1990; Tronchin *et al*., 1984). Gentian violet may affecte this fibrillar layer.

These results cannot be compared with any other studies because there are no studies that have investigated the effect on the adherence. However, at sub-inhibitory concentrations many antimicrobial compounds have shown this property. Chlorhexidine gluconate (CHX) which is a broad-spectrum antimicrobial agent interferes with adherence of *C. albicans* to buccal epithelial cells by disturbing the outer layer (Tobgi *et al.,* 1987; Bobichon and Bouchet, 1987) whereas octenidine and pirtenidine which are structurally similar to CHX disrupt the ultrastructure of its cell wall (Ghannoum *et al.,* 1989). Phytochemicals present in some medicinal plants have also shown the ability to prevent adherence of *C. albicans* to the epithelial cells. *Dodonaea angustifolia* has shown to inhibit *C. albicans* adherence (Patel *et al.,* 2009). At sub-inhibitory concentration Eugenyl Acetate, a component of the essential oil compound derived from clove oil can also inhibit the adherence of *C. albicans* to the host cells (De Toledo *et al,* 2001, Musthafa *et al.,* 2016).

In addition to the adherence to epithelial cells, *C. albicans* is known to adhere to artificial surfaces (Odds, 1988, Gendreau and Loewy, 2011). *C. albicans* is capable of forming biofilms which are self-sufficient communities and they are resistant to antimicrobial agents. Medical devices such as urinary catheters, prosthetic heart valves, silicone voice prostheses, endotracheal tubes, and cerebrospinal fluid shunts have been shown to be strongly associated with *Candida* infections (Odds, 1988). Levine *et al.,* (1988) reported a case of *C. albicans* infection following a total knee arthroplasty. Denture wearers also contract candidiasis due to the adherence of *C. albicans* to denture acrylic (Gendreau and Loewy, 2011). Although present investigations did not study the effect of gentian violet on the adherence ability to the artificial surfaces, a study has shown that gentian violet can significantly reduce the adherence of *C. albicans* to the urinary catheters and the results were comparable to chlorhexidine gluconate (Camacho *et al.,* 2007). In addition, Traboulsi *et al.,* (2011) has also

shown that at concentration of 0.18 to 0.3 µg/ ml, gentian violet can reduce the biofilm formation and at 4 µg/ml it can disrupt the biofilm. Perhaps the use of gentian violet in the coating of medical devices and tubes can be explored.

Following the attachment to the host cells, *C. albicans* produces germ tube or hyphae to facilitate the tissue penetration. The effect of gentian violet on the germ tube formation was also studied.

4.3 Effect of gentian violet on the germ tube formation

Following the attachment of host tissue cells, *C. albicans* invades host cells by producing hyphae. Induced endocytosis and active penetration are the two routes of invasions. The active penetration can occur through the intracellular junctions or through host cells due to the physical force of the elongation of hyphae. Hydrolytic enzymes also assist in this penetration and tissue destructions (Martin *et al.,* 2011). A therapeutic chemical can inhibit this active penetration by inhibiting the hyphae formation. At sub-inhibitory concentrations, a commonly used antimicrobial compound chlorhexidine gluconate is known to inhibit the hyphae formation in *C. albicans* and *C. dubliniensis* (Ellepola and Samaranayake, 2000, Ellepola, 2011).

The sub-inhibitory concentrations of gentian violet also inhibited the production of germ tube which means that it would intercept the second step of pathogenesis. Up to 98% reduction in the germ tube formation was recorded at the concentration of 0.000244% (2.4 µg/ ml) gentian violet. In contrast, Ying *et al*. (2010), reported only 11% reduction at 2 µg/ml. The discrepancy in the results cannot be explained. Sub-inhibitory concentrations of plant extracts

such as *Coriandrum sativum* (coriander) and *Ocimum gratissimum* (Alfavaca) have been shown to inhibit germ tube formation of *C. albicans* (Nakamura *et al.* 2004, Silva *et al.* 2011). Biology of hyphal formation is a complex process and the growth of the hyphal tube requires cell wall generation. This effect may have been due to cell wall damage as the studies by Naicker and Patel, (2013) and Sangetha *et al.* (2009) who investigated the antimicrobial activity of the medicinal plant, *Dodonaea viscosa* var. *angustifolia* and *Cassia spectabilis* respectively against *C. albicans* at an ultrastructural level, found notable alterations in the cell wall and membrane.

4.4 Side effects and toxicity of gentian violet

Although the use of gentian violet is recommended (WHO, 2009) due to its efficacy, cost-effectiveness and safety at 0.5-1.0%, the toxicity of this compound should be discussed. In clinical trials during topical applications of gentian violet, a small number of patients experienced cracked lips, dry mouth and local irritation (Nyst *et al.* 1992, Wright *et al.* 2009). Usually the major concern is discolouration of the mucosa. Some case reports have described toxicity of gentian violet causing mucous membrane lesions and irritation in the treatment of oral candidiasis (Slotkowski and Redondo, 1966, Verbov, 1976). There are random reports of toxicity in babies such as necrosis, difficulty in breast feeding and obstructive laryngo-tracheitis due to the treatment of gentian violet (John 1968, Utter 1990, Baca *et al.* 2001).

Previous studies have shown that gentian violet also interacts with the DNA of cells and therefore the topic of oncogenic potential of this chemical has become important (Rosenkranz, 1971, Au *et al.,* 1978, 1979). Mice fed with extremely high doses of gentian violet for two years have shown increase in hepatocellular carcinoma (Culp *et al.,* 2006) and

thyroid cancer (Littlefield *et al.,* 1989). However, gentian violet has been used for a century and no reported cancer cases have been linked to the use this chemical. Due to lack of evidence of toxicity, cost effectiveness and efficacy, it has been recommended by the WHO and approved by FDA allowing the sale over the counter (Maley and Arbiser, 2013).

4.5 *C. albicans* strains

C. albicans is carried by normal healthy as well as immunocompromised individuals and if the opportunity is given this fungus can cause infection. Strains of *C. albicans* isolated from the normal healthy individuals, denture wearers and HIV positive patients were included just to have variety of strains rather than the comparison of population groups. From the observation it can be deduced that, in spite of where *C. albicans* strains were isolated from, the beneficial effect of gentian violet on the killing, adherence and hyphae formation was seen. Comparison of the results between the groups was not the aim of this study and therefore comparison statistics was not performed. However, the results showed that gentian violet would be effective as a topical therapeutic agent in any type of patients suffering from candidiasis.

4.6 Clinical implications

Candida albicans causes two common opportunistic infections, oropharyngeal and vaginal candidiasis. As this study has also shown, during treatment high concentration of gentian violet would kill *C. albicans.* However, these body cavities are exposed to constant secretions of fluids which would dilute the therapeutic dose of gentian violet. This study has also shown that at these diluted concentrations, gentian violet would still be effective by inhibiting the

adherence of *C. albicans* to the host cells and by inhibiting the hyphae formation which are crucial in the pathogenesis. This suggests that it has therapeutic property even at very low sub-therapeutic concentrations.

CHAPTER 5

LIMITATIONS, FUTURE RESEARCH AND CONCLUSIONS

5.1 Limitations

Thirty strains of *C. albicans* were included in this study and the experiments were performed only once due to the time and financial constraint.

Only the *C. albicans Candida* were included in the study due to the time and financial constraint.

5.2 Future research

- A time-kill study can be conducted where it can show concentrations required to kill *C. albicans* at different time periods. Results of the MFC studies are generally read at 24 hours.
- *Candida* other than *C. albicans* occurs in the oral cavities of individuals which are also known to cause infections. Therefore, the efficacy of gentian violet against non-*C. albicans Candida* can be studied.
- Hydrolytic enzymes such as proteinase, phospholipase and lipases produced by *C. albicans* causes tissue damage. It would be interesting to study the effect of gentian violet on the production of these hydrolytic enzymes.
- Denture wearers commonly suffer from denture stomatitis which is also a type of candidiasis. The use of gentian violet in the denture wearers has not been studied.
- There is little research available with regards to the use of gentian violet in the coating of the medical devices, catheters and endoscope tubes which can be investigated.

5.3 Conclusions

In vitro and *in vivo* studies have shown the antifungal activity and efficacy of gentian violet against *C. albicans*. Therefore, mucosal candidiasis such as oral and vaginal caused by *C. albicans* can be treated by topical application of gentian violet. However, in these body cavities, the therapeutic compounds become diluted due to the constant secretions of fluids. This study showed that at high concentrations, gentian violet can completely kill *C. albicans* and at low concentrations it can inhibit the adherence of *C. albicans* cells to oral epithelial cells. In addition, at low concentrations gentian violet can inhibit the hyphae formation. During pathogenesis, these properties allow establishment, colonization and penetration of *C. albicans* and facilitate tissue destruction in the host. These results suggest that the beneficial effects of gentian violet would last beyond the fungicidal concentrations in the treatment of candidiasis. Gentian violet can be used as a topical antifungal agent in patients with candidiasis.

REFERENCES

Akpan, A., & Morgan, R. (2002). Oral candidiasis. *Postgraduate Medical Journal, 78(922), 455-459.*

Au, W., Butler, M.A., Bloom, S.E., Matney, T.S. (1979). Further study of the genetic toxicity of gentian violet. *Mutation Research. Feb;66(2);103-112.*

Au, W., Pathak, S., Collie, C.J., Hsu, T.C. (1978). Cytogenetic toxicity of gentian violet and crystal violet on mammalian cells in vitro. *Mutation Research. Nov;58(2-3);269-276.*

Baca, D., Drexler, C., Cullen E.(2001). Obstructive laryngotracheitis secondary to gentian violet exposure. *Clinical Pediatrics. Apr;40(4);233-235.*

Bagg, J., MacFarlane, T. W., Poxton, I. R., Smith, A. J. (2006). *Essentials of microbiology for dental students*: Oxford University Press. 2nd ed.

Braun, B. R., Johnson, A. D. (1997). Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1. *Science, 277(5322), 105-109.*

Bobichon, H., Bouchet, P.(1987). Action of chlorhexidine on budding *Candida albicans*: scanning and transmission electron microscopic study. *Mycopathologia. Oct;100(1);27-35.*

Budtz-Jörgensen, E., & Bertram, U. (1970). Denture stomatitis I. The etiology in relation to trauma and infection. *Acta Odontologica Scandinavica, 28(1), 71-92.*

Camacho, D., Gasparetto, A., & Svidzinski, T. (2007). The effect of chlorhexidine and gentian violet on the adherence of *Candida spp*. to urinary catheters. *Mycopathologia, 163(5); 261-266.*

Cannon, R., & Chaffin, W. (1999). Oral colonization by *Candida albicans. Critical Reviews in Oral Biology and Medicine, 10(3); 359-383.*

Calderone, R.A. (1993). Recognition between *Candida albicans* and host cells. *Trends in Microbiology May; 56(1); 55-58.*

Critchley, I.A., Douglas, L.J. (1987). Role of glycosides as epithelial cell receptors for Candida albicans. *Journal of General Microbiology. Mar;133(3);637-643.*

Culp S.J., Mellick, PW., Trotter, R.W., Greenlees, K.J., Kodell, R.L., Beland, F.A. (2006). Carcinogenicity of malachite green chloride and leucomalachite green in B6C3F1 mice and F344 rats. *Food and Chemical Toxicology. Aug;44(8):1204-1212.*

Darwazeh, A. M.-G., Al-Refai, S., & Al-Mojaiwel, S. (2001). Isolation of *Candida species* from the oral cavity and fingertips of complete denture wearers. *The Journal of Prosthetic Dentistry, 86(4); 420-423.*

de Almeida, O. P., & Scully, C. (2015). Fungal infections of the mouth. *Brazilian Journal of Oral Sciences, 1(1); 19-26*.

De Toledo,L.G., Ramos, M.A., Spósito,L., Castilho, E.M., Pavan, F.R., De Oliveira Lopes. E., Éde. O., Zocolo, G.J., Silva, F.A., Soares, T.H., dos Santos, A.G., Taís Maria Bauab, T.M., & De Almeida, M.T., (2016). Essential Oil of *Cymbopogon nardus* (L.) Rendle: A Strategy to combat fungal infections caused by *Candida Species*. *International Journal of Molecular Science.17(8); 1252-1267.*

Dougherty, S. H. (1988). Pathobiology of infection in prosthetic devices. *Review of Infectious Diseases, 10(6); 1102-1117.*

Ellepola, A., & Samaranayake, L. (2000). The effect of brief exposure to sub-therapeutic concentrations of chlorhexidine gluconate on the germ tube formation of oral *Candida albicans* and its relationship to post-antifungal effect. *Oral Diseases, 6(3), 166-171*.

Ellepola, A. N. B. (2011). The effect of brief exposure to sub-therapeutic concentrations of chlorhexidine gluconate on germ tube formation of oral *Candida dubliniensis*. *Mycoses, 54: e330–e335.*

Gasparoto, T. H., Dionisio, T. J., de Oliveira, C. E., Porto, V. C., Gelani, V., Santos, C. F., Lara, V. S. (2009). Isolation of *Candida dubliniensis* from denture wearers. *Journal of Medical Microbiology, 58(7), 959-962*.

Gaur, N., Klotz, S.A (1997). Expression, cloning and characterization of a *Candida albicans* gene, ALA1 that conferes adherence properties upon Saccharomyces cerevisiae fro extracellular matrix proteins. *Journal of infection and immunity. Dec; 65 (12): 5289-5294*

Gauwerky, K., Borelli, C., Korting, H. C. (2009). Targeting virulence: a new paradigm for antifungals. *Drug Discovery Today, 14(3), 214-222*.

Gendreau, L., & Loewy, Z. G. (2011). Epidemiology and etiology of denture stomatitis. *Journal of Prosthodontics, 20(4); 251-260.*

Ghannoum, M.A., Abu Elteen, K., Stretton, R.J., Whittaker, P.A.(1989). Effects of octenidine and pirtenidine on adhesion of *Candida species* to human buccal epithelial cells in vitro. *Archives of Oral Biology. 35(4):249-253.*

Ghannoum, M. A., & Radwan, S. S. (1990). *Candida* adherence to epithelial cells: CRC Press.1st ed:270.

Gomes-de-Elvas, A. R, Palmeira-de-Oliveira, A., Gaspar, C., Gouveia, P., Palmeira-de-

Oliveira, R., Pina-Vaz, C., Rodrigues, A. G., Martinez-de-Oliveira, J. (2012). In vitro assessment of gentian violet anti- candida activity. *Gynecology and Obstetrics Investigations ,74(2):120-4.*

Grbic, J. T., Mitchell-Lewis, D. A., Fine, J. B., Phelan, J. A., Bucklan, R. S., Zambon, J. J., & Lamster, I. B. (1995). The relationship of candidiasis to linear gingival erythema in HIV-infected homosexual men and parenteral drug users. *Journal of Periodontology,* 66(*1), 30-37.*

Gutiérrez, J., Morales, P., González, M. A., Quindós, G. (2002). *Candida dubliniensis*, a new fungal pathogen. *Journal of Basic Microbiology, 42(3):207-27.*

Hawser, S. P., & Douglas, L. J. (1994). Biofilm formation by *Candida species* on the surface of catheter materials in vitro. *Infection and Immunity, 62(3), 915-921*.

Hoyer, L.L., Fundyga R., Hecht, J.E., Kapteyn, J.C., Klis, F.M., Arnold, J. (2001). Characterization of Agglutinin-like Sequence Genes from Non-*albicans Candida* and Phylogenetic Analysis of the ALS family. *Genetics.* Apr; *157 (4); 1555-1567.*

Jawetz, E. M., Mellsick, J.L., Adelberg, I. (1980). *Review of medical microbiology*. 19th ed

John, R.W. (1968). Necrosis of Oral Mucosa after Local Application of Crystal Violet. *British Medical Journal* .Jan 20; *1(5585); 157–158.*

Jurevic, R., Traboulsi, R., Mukherjee, P., Salata, R., Ghannoum, M. (2011). Identification of gentian violet concentration that does not stain oral mucosa, possesses anti-candidal activity and is well tolerated. *European Journal of Clinical Microbiology and Infectious Diseases, 30(5), 629-633*.

Kanbe, T., Li, R., Wadsworth, E., Calderone, R., Cutler, J. (1991). Evidence for expression of the C3d receptor of *Candida albicans* in vitro and in vivo obtained by immunofluorescence and immunoelectron microscopy. *Infection and Immunity, 59*(5), *1832-1838.*

Khan, Z., Ahmad, S., Joseph, L., Chandy, R. (2012). *Candida dubliniensis*: an appraisal of its clinical significance as a bloodstream pathogen. *PLoS One,7(3):e32952.* doi: 10.1371/journal.pone.0032952. Epub 2012 Mar 2.

Kimura, L.H., Pearsall, N.N. (1980). Relationship between germination of *Candidia albicans* and increased adherence to human buccal epithelial cell. *Journal of Infection and Immunity. May (2), 464-468.*

Kondo, S., Tabe, Y., Yamada, T., Misawa, S., Oguri, T., Ohsaka, A., Miida, T. (2012) Comparison of Antifungal Activities of Gentian Violet and Povidone-Iodine Against Clinical Isolates of Candida Species and Other Yeasts: A Framework to Establish Topical Disinfectant Activities. *Mycopathologia 173 (1): 21-25.*

Lambert, H., & O'Grady, F. (1992). Antifungal agents. *Antibiotics and chemotherapy. London: Churchill Livingstone, 27-37.*

Levine, M., Rehm, S. J., & Wilde, A. H. (1988). Infection with *Candida albicans* of a Total Knee Arthroplasty: Case Report and Review of the Literature. *Clinical Orthopaedics and Related Research, 226, 235-239.*

Littlefield, N.A., Gaylor, D.W., Blackwell, B.N., Allen, R.R. (1989). Chronic toxicity/carcinogenicity studies of gentian violet in Fischer 344 rats: two-generation exposure. *Food and Chemical Toxicology. Apr;27(4);239-247.*

Loreto, E. S., Scheid, L. A., Nogueira, C. W., Zeni, G., Santurio, J. M., Alves, S. H. (2010). *Candida dubliniensis*: epidemiology and phenotypic methods for identification. *Mycopathologia,169(6):431-43.* doi: 10.1007/s11046-010-9286-5. Epub 2010 Feb 21.

Maley, A. M., & Arbiser, J. L. (2013). Gentian Violet: A 19th Century Drug Re-Emerges in the 21st Century. *Experimental Dermatology*. *Dec; 22(12): 775–780.*

McCourtie, J., Douglas, L.J. (1985). Extracellular polymer of Candida albicans: isolation, analysis and role in adhesion. *Journal of General Microbiology*. Mar;131(3):495-503.

Mukherjee, P.K., Chen H., Patton, L.L., Evans, S., Lee, A., Kumwenda, J., Hakim J., Masheto, G., Sawe, F., Pho, M.T., Freedberg K.A., Shiboski, C.H., Ghannoum, M.A., Salata, R.A., Oral HIVAIDS Research Alliance (OHARA) AIDS Clinical Trials Group (ACTG) 5265 Team. (2017). *AIDS. Jan 2;31(1):81-88*

Martin, M.V., Lamb, D.J. (1982). Frequency of *Candida albicans* serotypes in patients with denture-induced stomatitis and in normal denture wearers. *Journal of Clinical Pathology. Aug;35(8):888-891.*

Martin, R., Wachtler, B., Schaller, B.M., Wilson, D., Hube, B. (2011). Host-pathogen interactions and virulence-associated genes during *Candida albicans* oral infections. *International journal of Medical Microbiology. June 301, (5),417-422*

Musthafa, K.S., Hmoteha, J., Thamjarungwongc, B., Voravuthikuncha, S.P., (2016). Antifungal potential of eugenyl acetate against clinical isolates of *Candida species Microbial Pathogenesis. Oct, 99, 19–20.*

Naglik, J.R., Challacombe, S.J., Hube, B. (2003). *Candida albicans* secreted Aspartyl Proteinases in virulence and pathogenesis. Microbiology and molecular biology reviews, Sept, 400–428.

Naicker, S. D., & Patel, M. (2013). *Dodonaea viscosa* var. *angustifolia* Inhibits Germ Tube and Biofilm Formation by *C. albicans*. *Evidence-Based Complementary and Alternative Medicine, volume 2013, Article ID 261978, 1-6.*

Nakamura C.V., Ishida, K., Faccin, L.C., Dias Filho B.P.,Garcia Cortez, D.A., Rozental, S., de Souza, W., Ueda-Nakamura, T. (2004).In vitro activity of essential oil from *Ocimum gratissimum* L. against four *Candida species*. *Research in Microbiology,155, 579–586.*

Nyst, M., Perriens, J., Kimputu, L., Lumbila, M., Nelson, A., & Piot, P. (1992). Gentian violet, ketoconazole and nystatin in oropharyngeal and esophageal candidiasis in Zairian AIDS patients. *Annales de la Société belge de médecine tropicale, 72(1), 45-52.*

Odds, F. C. (1988). *Candida and candidosis: a review and bibliography*: Bailliere Tindall.

Ollert, M. W., Wende, C., Görlich, M., McMullan-Vogel, C. G., Borg-von Zepelin, M., Vogel, C.-W., & Korting, H. C. (1995). Increased expression of *Candida albicans* secretory proteinase, a putative virulence factor, in isolates from human immunodeficiency virus-positive patients. *Journal of Clinical Microbiology, 33(10), 2543-2549.*

Olsen, I.(1990). Oral adhesion of yeasts. *Acta Odontol Scand. Feb;48(1):45-53.*

Patel M., Gulube, Z., Dutton, M.(2009). The effect of *Dodonaea viscosa var. angustifolia* on *Candida albicans* proteinase and phospholipase production and adherence to oral epithelial cells. *Journal of Ethnopharmacology 124, 562–565.*

Perlin, D. S. (2010). Antifungal drug resistance in developing countries *Antimicrobial Resistance in Developing Countries* (pp. 137-156): Springer.

Ramage, G., Tomsett, K., Wickes, B. L., López-Ribot, J. L., & Redding, S. W. (2004). Denture stomatitis: a role for *Candida* biofilms. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology, 98(1), 53-59.*

Riley, K., Flower, A. H. (1950). A comparison of the inhibitory effect of Castellani's paint

and of gentian violet solution on the in vitro growth of *Candida albicans*. *Journal of Investgaive Dermatology, Nov;15(5):355-61.*

Rivera-Hidalgo, F., & Stanford, T. W. (1999). Oral mucosal lesions caused by infective microorganisms I. Viruses and bacteria. *Periodontology 2000, 21(1), 106-124*.

Rosenkranz, H.S., Carr, H.S. (1971). Posible hazard in usse of gentian violet. *British medical journal. 183 (5776), 702-703*

Samantha Hsieh, B. S., Eric, L., Maranda, B. S., Tarek Salih, B. S., et alMaria de la Paz Flores, B. S., Joaquin Jimenez, M. D. (2016). The Purple Dye That Heals. *JAMA Dermatology,*152(4):495. doi:10.1001/jamadermatol.2015.4239

Samaranayake, L. P., Keung Leung, W., & Jin, L. (2009). Oral mucosal fungal infections. *Periodontology 2000, 49(1), 39-59.*

Sangetha, S., Zuraini, Z., Suryani, S., Sasidharan, S.(2009). In situ TEM and SEM studies on the antimicrobial activity and prevention of *Candida albicans* biofilm by *Cassia spectabilis* extract. *Micron. 40, 439–443.*

Sharon, V., & Fazel, N. (2010). Oral candidiasis and angular cheilitis. *Dermatologic Therapy. 23(3), 230-242*.

Silva, F., Ferreira, S., Duarte, A., Mendonça, D.I., Domingues, F.C. (2011). Antifungal activity of *Coriandrum sativum* essential oil, its mode of action against *Candida* species and potential synergism with amphotericin B. *Phytomedicine. 2011 Dec 15;19(1):42-47.*

Slotkowski, E.L, Redondo, D.(1966). Mucosal irritation following use of gentian violet. *American Journal of Disease of Children. Jul;112(1):40-42.*

Sudbery, P., Gow, N., & Berman, J. (2004). The distinct morphogenic states of *Candida albicans*. *Trends in Microbiology, 12(7), 317-324.*

Sweet, S., Cookson, S., & Challacombe, S. (1995). *Candida albicans* isolates from HIV-infected and AIDS patients exhibit enhanced adherence to epithelial cells. *Journal of Medical Microbiology, 43*(6), *452-457.*

Tobgi, R. S., Samaranayake, L.P., Macfarlane, T.W. (1987) Adhesion of *Candida albicans* to buccal epithelial cells exposed to chlorhexidine gluconate. *Journal of Medical and Veterinary Mycology 25, 335-338.*

Traboulsi, R. S., Mukherjee, P. K., & Ghannoum, M. A. (2008). In vitro activity of inexpensive topical alternatives against *Candida spp*. isolated from the oral cavity of HIV-infected patients. *International Journal of Antimicrobial Agents, 31*(3), *272-276*.

Traboulsi, R.S., Mukherjee, P.K., Chandra, J., Salata, R.A., Jurevic, R., Ghannoum, M.A. (2011). Gentian violet exhibits activity against biofilms formed by oral *Candida* isolates obtained from HIV-infected patients. Gentian violet exhibits activity against biofilms formed by oral *Candida* isolates obtained from HIV-infected patients. *Antimicrobial Agents and Chemothererapy. Jun;55(6);3043-3045.*

Tronchin,G., Poulain,D., Vernes,A.(1984) Cytochemical and ultrastructural studies of *Candida albicans*. *Archives of Microbiology, Octob, 139,(2), 221–224.*

Utter, A.R.(1990). Gentian violet treatment for thrush: can its use cause breastfeeding problems? *Journal of human lactation.Dec;6(4);178-80.*

Verbov, J. Oral irritation with gentian violet. (1976). *British Medical Journal. Sep 11;2(6036):639.*

Wey, S. B., Mori, M., Pfaller, M. A., Woolson, R. F., & Wenzel, R. P. (1988). Hospital-acquired candidemia: the attributable mortality and excess length of stay. *Archives of internal medicine, 148(12); 2642-2645*.

Williams, D., & Lewis, M. (2011). Pathogenesis and treatment of oral candidosis. *Journal of Oral Microbiology. Jan ,(3);5771-5781*.

Williams, D. W., Jordan, R. P., Wei, X.-Q., Alves, C. T., Wise, M. P., Wilson, M. J., & Lewis, M. A. (2013). Interactions of *Candida albicans* with host epithelial surfaces. *Journal of Oral Microbiol, Oct;5,(1); 1-8.*

Wright, S.C., Maree, J.E., Sibanyoni, M. (2009). Treatment of oral thrush in HIV/AIDS patients with lemon juice and lemon grass (*Cymbopogon citratus*) and gentian violet. *Phytomedicine. Mar;16(2-3);118-24.*

Yang, Y.-L. (2003). Virulence factors of *Candida* species. *Journal of Microbiology Immunology and Infection. 36(4;: 223-228.*

Ying, S., Qing, S., & Chunyang, L. (2010). The effect of gentian violet on virulent properties of *Candida albicans*. *Mycopathologia. 169(4); 279-285*.

CHAPTER 7

APPENDIX A

7.1 Media and reagents

Sabouraud Dextrose Agar

Sabouraud Agar powder (Merck, South Africa) 60g

Distilled water 1L

Ingredients were boiled until completely dissolved. The solution was then autoclaved at 121 °C for 10 minutes. The mixture was then cooled to 45 °C and poured into Petri dishes.

Sabouraud Dextrose Broth

Sabouraud Broth powder (Merck, South Africa) 20g

Distilled water 1L

Ingredients were added together, mixed well and autoclaved at 121 °C.

Potassium permanganate solution

Potassium permanganate 5g

Distilled water 100ml

Ingredients were added together, mixed well and stored at room temperature.

APPENDIX B

Statistical analysis

7.2 Adherence assay

Tumane Mafojane

Adherence stats.

```
-------------------------------------------------------------------------------
-
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Prof Adherence 2015 June.log
  log type:  text
 opened on:  30 Jun 2015, 18:22:48

. use "Prof Adherence 2015 June.dta"

.
. *Questions: 1 Does plant extract have any effect on the adherence?
. *Lump all the results togather (compare controls to tests)
.
. sktest adherence

                    Skewness/Kurtosis tests for Normality
                                                              ------- joint ------
    Variable |    Obs    Pr(Skewness)    Pr(Kurtosis)  adj chi2(2)     Prob>chi2
-------------+-----------------------------------------------------------------
   adherence |     60       0.1466          0.0452          5.79          0.0552

.
. ttest adherence, by(control)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      30       160.3    10.25089    56.14645    139.3346    181.2654
       1 |      30         370    16.77772    91.89537    335.6857    404.3143
---------+--------------------------------------------------------------------
combined |      60      265.15    16.77308    129.9237    231.5872    298.7128
---------+--------------------------------------------------------------------
    diff |            -209.7    19.66145                -249.0567   -170.3433
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t = -10.6655
Ho: diff = 0                     degrees of freedom =       58

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 1.0000

.
. ranksum adherence, by(control)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

     control |      obs    rank sum    expected
-------------+---------------------------------
           0 |       30         467         915
           1 |       30        1363         915
-------------+---------------------------------
    combined |       60        1830        1830

unadjusted variance     4575.00
adjustment for ties       -0.89
                        ---------
adjusted variance       4574.11

Ho: adherence(control==0) = adherence(control==1)
           z =  -6.624
    Prob > |z| =   0.0000

. *MEAN ADHERENCE IS MUCH HIGHER IN CONTROLS AND THIS IS SIGNIFICANT (T TEST VALID)
.
```

```
. *2 Is there a difference between the results of different groups?
. *(compare controls and tests separately and compare the reduction as well)
.
. bysort type2:ttest adherence, by(control)

-------------------------------------------------------------------------------
-
-> type2 = Denture

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      10         165    18.81961    59.51284    122.4271    207.5729
       1 |      10       395.3    27.90303    88.23712     332.179     458.421
---------+--------------------------------------------------------------------
combined |      20      280.15    31.08302    139.0075    215.0925    345.2075
---------+--------------------------------------------------------------------
    diff |            -230.3    33.65645               -301.0096   -159.5904
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -6.8427
Ho: diff = 0                                     degrees of freedom =       18

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 1.0000

-------------------------------------------------------------------------------
-
-> type2 = Normal

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      10       154.4    20.00122    63.24942    109.1541    199.6459
       1 |      10       349.6    27.61127     87.3145     287.139     412.061
---------+--------------------------------------------------------------------
combined |      20         252    27.86877    124.6329      193.67      310.33
---------+--------------------------------------------------------------------
    diff |            -195.2    34.09444               -266.8298   -123.5702
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -5.7253
Ho: diff = 0                                     degrees of freedom =       18

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 1.0000

-------------------------------------------------------------------------------
-
-> type2 = HIV

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      10       161.5    15.97098    50.50468    125.3711    197.6289
       1 |      10       365.1    32.55916    102.9611    291.4461    438.7539
---------+--------------------------------------------------------------------
combined |      20       263.3    29.27322    130.9138    202.0304    324.5696
---------+--------------------------------------------------------------------
    diff |            -203.6    36.26529               -279.7905   -127.4095
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -5.6142
```

```
Ho: diff = 0                                     degrees of freedom =       18

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 1.0000

. 
. bysort type2:ranksum adherence, by(control)

-------------------------------------------------------------------------------
-
-> type2 = Denture

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

     control |      obs     rank sum    expected
-------------+---------------------------------
           0 |       10          55         105
           1 |       10         155         105
-------------+---------------------------------
    combined |       20         210         210

unadjusted variance      175.00
adjustment for ties        0.00
                         ----------
adjusted variance        175.00

Ho: adhere~e(control==0) = adhere~e(control==1)
                z =  -3.780
    Prob > |z| =   0.0002

-------------------------------------------------------------------------------
-
-> type2 = Normal

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

     control |      obs     rank sum    expected
-------------+---------------------------------
           0 |       10          55         105
           1 |       10         155         105
-------------+---------------------------------
    combined |       20         210         210

unadjusted variance      175.00
adjustment for ties        0.00
                         ----------
adjusted variance        175.00

Ho: adhere~e(control==0) = adhere~e(control==1)
                z =  -3.780
    Prob > |z| =   0.0002

-------------------------------------------------------------------------------
-
-> type2 = HIV

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

     control |      obs     rank sum    expected
-------------+---------------------------------
           0 |       10          55         105
           1 |       10         155         105
-------------+---------------------------------
    combined |       20         210         210
```

```
unadjusted variance      175.00
adjustment for ties        0.00
                        ---------
adjusted variance        175.00

Ho: adhere~e(control==0) = adhere~e(control==1)
            z =  -3.780
    Prob > |z| =   0.0002

.
. *CONCLUSIONS ARE THE SAME
.
. log close
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Prof Adherence 2015 June.log
  log type:  text
 closed on:  30 Jun 2015, 18:22:48
-------------------------------------------------------------------------------
```

7.3 Germ tube assay

-> Concentration = Control

Variable	Obs	Mean	Std. Dev.	Min	Max
Number Cells	30	54.2	15.17802	24	80

. Regress Number Cells

Source	SS	df	MS		
Model	0	0	.	Prob > F	= .
Residual	106276.978	179	593.726133	R-squared	= 0.0000
Total	106276.978	179	593.726133	Adj R-squared	= 0.0000
				Root MSE	= 24.366

Number Cells	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
_cons	20.71111	1.816171	11.40	0.000	17.12725	24.29497

. regress Number Cells i. Concentration

Source	SS	df	MS		
Model	78071.5778	5	15614.3156	Prob > F	= 0.0000
Residual	28205.4	174	162.1	R-squared	= 0.7346
Total	106276.978	179	593.726133	Adj R-squared	= 0.7270
				Root MSE	= 12.732

```
 Number Cells |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
--------------+----------------------------------------------------------------
Concentration |
            2 |   .8666667   3.287349     0.26   0.792    -5.621547     7.35488
            3 |         6.3   3.287349     1.92   0.057    -.1882135    12.78821
            4 |   20.96667   3.287349     6.38   0.000     14.47845    27.45488
            5 |   41.93333   3.287349    12.76   0.000     35.44512    48.42155
      Control |        54.2   3.287349    16.49   0.000     47.71179    60.68821
              |
        _cons | -7.11e-15   2.324507    -0.00   1.000    -4.58786     4.58786
------------------------------------------------------------------------------

. one way Number Cells concentration, tabulate bonf

Concentration |       Summary of Number Cells
           on |       Mean   Std. Dev.       Freq.
--------------+------------------------------------
            1 |          0          0          30
            2 |   .86666667   3.3909955          30
            3 |        6.3   10.215438          30
            4 |   20.966667   18.509053          30
            5 |   41.933333   16.846023          30
      Control |        54.2   15.178024          30
--------------+------------------------------------
        Total |   20.711111   24.366496         180

                    Analysis of Variance
    Source              SS         df      MS         F     Prob > F
------------------------------------------------------------------------
Between groups      78071.5778      5   15614.3156     96.33     0.0000
 Within groups       28205.4     174       162.1
------------------------------------------------------------------------
    Total          106276.978     179   593.726133

Bartlett's test for equal variances:  chi2 (4) = 66.8847 Prob>chi2 = 0.000

note: Bartlett's test performed on cells with positive variance:
      1 multiple-observation cells not used

                Comparison of Number Cells by Concentration
                            (Bonferroni)
Row Mean-|
Col Mean |         1         2         3         4         5
---------+------------------------------------------------------------
       2 |    .866667
         |      1.000
         |
       3 |       6.3   5.43333
         |      0.854     1.000
         |
       4 |   20.9667      20.1   14.6667
```

```
       |     0.000     0.000     0.000
       |
     5 |   41.9333   41.0667   35.6333   20.9667
       |     0.000     0.000     0.000     0.000
       |
 Control |      54.2   53.3333      47.9   33.2333   12.2667
       |     0.000     0.000     0.000     0.000     0.004

. log off
      name:  <unnamed>
       log:  E:\REsearch\Dr Dan.log
  log type:  text
 paused on:  10 Jun 2015, 14:06:53
```

APPENDIX C

7.4 Ethics clearance certificate

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Medical School Room 10M07, 10th Floor. Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-140425-2 25/04/2014

TO WHOM IT MAY CONCERN:

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

Investigator: Prof M Patel, Dr T Mafojane.

Project title: The effect of sub-therapeutic concentrations of gentian violet on germ tube formation by C. albicans and its adherence to oral epithelia cells

Reason: This study uses existing stock *C. albicans* cultures. There are no human participants.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON - JONES
HREC (MEDICAL)
2014 -04- 25
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat : Anisa Keshav, Zanele Ndlovu.