

**THE EFFECT OF *DODONAEA VISCOSA* VAR. *ANGUSTIFOLIA*
(L.F) ON THE ULTRASTRUCTURE OF *CANDIDA ALBICANS*
CELL WALL AND BIOFILM FORMATION**

Serisha Devi Naicker



Degree of Master of Science in Medicine by research only

Dissertation submitted to the Faculty of Health Sciences, University of the
Witwatersrand, Johannesburg, in fulfillment of the requirements for the
degree of Master of Science in Medicine.

Johannesburg, 2012

DECLARATION

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

..... (Signature of candidate)

..... day of 2012.

DEDICATION

To the underdog,
“When you know better, you do better” – Maya Angelou

PUBLICATIONS AND PRESENTATIONS

Poster Presentation:

Naicker, S and Patel, M.

The effect of *Dodonaea viscosa* var. *angustifolia* (L.F) on the ultrastructure of *Candida albicans* cell wall and biofilm formation.

4WARD 2011, 4th Federation of Infectious Diseases Societies of Southern Africa (FIDSSA) Congress, Elangeni Hotel, Durban.

ABSTRACT

Oral candidiasis is an infection prevalent in immunocompromised individuals. The main causative agent is *Candida albicans*. Many antifungal agents are available and are effectively used. However, due to the development of drug resistance, toxicity and poor solubility resulting in poor absorption; medicinal plants have been investigated. *Dodonaea viscosa* var. *angustifolia*, an indigenous South African plant has shown to have an antifungal effect including inhibition of adherence of *C. albicans* to oral epithelial cells; which is the crucial first step of infection. This study investigated the effect of the crude extract on the ultrastructure of *C. albicans* cell wall, which might be responsible for the reduced adherence to oral epithelial cells. The effect of the plant extract on *C. albicans* germ tube and biofilm formation was also studied since biofilm structure allows for high resistance to antifungal agents and host defense mechanisms.

Crude plant extracts were prepared using dried leaves and acetone. Three *C. albicans* strains were used throughout the study. Minimal fungicidal concentrations of plant extract were determined using a microdilution technique. Three subinhibitory concentrations 3.125, 1.562 and 0.781 mg/ml were selected for further studies. The effect of these subinhibitory concentrations of plant extract on the *C. albicans* cell wall structure, cell membrane, germ tube formation, biofilm formation and cell wall proteins were studied using transmission electron microscopy, light microscopy, scanning electron microscopy and SDS-PAGE respectively.

The subinhibitory concentrations of crude plant extract rendered *C. albicans* cell wall thinner and at some places caused cell wall breakage and disruption. This effect increased with a decrease in plant extract concentration. The cell membrane was also damaged by the plant extract showing increased undulation. This effect was not concentration dependent. The subinhibitory concentrations decreased *C. albicans* germ tube formation and the effect increased with an increase in concentration. Biofilm formation was reduced by the plant extract and in addition, hyphal formation by cells within the biofilm was also reduced. However, SDS-PAGE showed that on a molecular level, the plant extract did not remove any specific adhesin proteins from the cell wall.

The crude plant extract of *D. viscosa* var. *angustifolia* at high concentrations, kills *C. albicans* and at low concentrations, renders the surviving cells avirulent. Therefore it has the potential to be developed into an effective therapeutic agent to treat and prevent oral candidiasis. However, further research is required to identify the mode of action of the extract, the specific chemicals responsible for the effect, and the cytotoxicity.

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my incredible supervisor **Dr Mrudula Patel** (Department of Clinical Microbiology and Infectious Diseases, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand and National Health Laboratory Service), for always supporting me and guiding me throughout these two years. Her assistance and trust was invaluable and I am grateful for that always. Her hardworking attitude inspires me to become a better person in everything I do and strive to achieve.

I would like to thank **Prof Adriano Duse**, (Head of Department of Clinical Microbiology and Infectious Diseases, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand and National Health Laboratory Service) for the support and allowing me to use the laboratory facility.

I would like to thank the **National Research Fund** and the **Postgraduate Merit Award** (University of the Witwatersrand) for funding my studies and the **Medical Research Council** (South Africa) for funding this project.

I acknowledge my **fellow laboratory colleagues** (Department of Clinical Microbiology and Infectious Diseases, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand) for their advice, help and support; **Prof Foluso Owotade** (Department of Clinical Microbiology and Infectious Diseases, School of Pathology, Faculty of Health Sciences,

University of the Witwatersrand) for statistical assistance; **the staff of the Infection Control Laboratory** (Clinical Microbiology and Infectious Diseases, National Health Laboratory Service, Johannesburg) for use of equipment and consumables; **Prof Margot Hosie** and **Deran Reddy** (School of Anatomy, Faculty of Health Sciences, University of the Witwatersrand) for guiding me through the treacherous journey of yeast TEM.

I am forever grateful to my **parents Dean and Devi Naicker** for sacrificing so much for me to pursue my dreams; **family and friends** for their guidance, support, love and understanding; especially my brother **Eloshan**, for never giving up and always supporting my decisions.

TABLE OF CONTENTS

	Page
DECLARATION.....	i
DEDICATION.....	ii
PUBLICATIONS AND PRESENTATIONS.....	iii
ABSTRACT.....	iv
ACKNOWLEDGEMENTS.....	vi
TABLE OF CONTENTS.....	viii
LIST OF FIGURES.....	xiv
LIST OF TABLES.....	xix
LIST OF ABBREVIATIONS AND ACRONYMS.....	xxi
 CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW.....	 1
1.1 Oral Candidiasis.....	1
1.2 <i>Candida albicans</i>	4
1.2.1 Morphology.....	4

1.2.2 Pathogenicity.....	6
1.2.3 Cell wall.....	8
1.2.3.1 Structure.....	8
1.2.3.2 Proteins and Mannoproteins.....	10
1.2.3.3 Adherence.....	12
1.2.4 Factors related to <i>Candida albicans</i> pathogenicity.....	14
1.2.4.1 Phenotypes.....	14
1.2.4.2 Cell Surface Hydrophobicity.....	14
1.2.4.3 Adhesin genes.....	16
1.2.4.4 Germ tube and adherence.....	18
1.2.4.5 Enzymes and adherence.....	19
1.2.4.6 Lipids and adherence.....	20
1.2.5 Factors relating to host cells.....	20
1.2.6 Environmental Factors that affect <i>Candida albicans</i> adhesion.....	21
1.2.7 Hydrolytic Enzymes.....	22
1.2.8 <i>Candida albicans</i> Biofilm.....	23
1.3 Treatment of oral candidiasis	25
1.3.1 Antifungal agents.....	25
1.3.2 Mouthrinses.....	27
1.3.3 Drug combinations.....	28
1.3.4 Natural products.....	29
1.3.4.1 Medicinal Plants.....	29
1.3.4.1.1 <i>Dodonaea viscosa</i> var. <i>angustifolia</i>	30

1.4 Aim.....	33
--------------	----

1.5 Specific Objectives.....	33
------------------------------	----

CHAPTER 2: MATERIALS AND METHODS

2.1 Cultures.....	34
-------------------	----

2.2 Plant Material.....	34
-------------------------	----

2.3 Plant Extraction.....	35
---------------------------	----

2.4 Minimum Fungicidal Concentration study.....	35
---	----

2.5 Transmission Electron Microscopy of planktonic <i>Candida albicans</i>	36
--	----

2.6 Effect of crude plant extract on germ tube formation by <i>Candida albicans</i>	38
---	----

2.7 Scanning Electron Microscopy and Light Microscopy of the <i>Candida albicans</i> biofilm.....	41
--	----

2.8 Protein analysis of the <i>Candida albicans</i> cell wall.....	42
--	----

2.9 Statistical Analysis.....	45
-------------------------------	----

2.9.1 Sample Size Estimation.....	45
-----------------------------------	----

2.9.2 Minimum Fungicidal Concentration Study.....	47
---	----

2.9.3 Effect of plant extract on <i>Candida albicans</i> cell wall.....	47
---	----

2.9.4 Effect of plant extract on <i>Candida albicans</i> cell membrane.....	47
2.9.5 Effect of plant extract on <i>Candida albicans</i> germ tube formation	48
2.9.6 Effect of plant extract on <i>Candida albicans</i> biofilm formation and protein analysis of the <i>Candida albicans</i> cell wall.....	48
 CHAPTER 3: RESULTS	
3.1 Identification of <i>Candida albicans</i> strains.....	49
3.2 Minimum Fungicidal Concentration study.....	49
3.3 Transmission Electron Microscopy of planktonic <i>Candida albicans</i> cells.....	49
3.4 Effect of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on germ tube formation.....	64
3.5 Effect of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on <i>Candida albicans</i> biofilm formation.....	68
3.5.1 Light Microscopy.....	68
3.5.2 Scanning Electron Microscopy.....	73
3.6 Effect of the crude plant extract on cell wall proteins of <i>Candida albicans</i>	81
 CHAPTER 4: DISCUSSION.....	
4.1 Antifungal activity of Medicinal Plants.....	87

4.2 Effect of the crude plant extract of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on <i>Candida albicans</i> cell wall and cell membrane.....	89
4.3 Effect of the crude plant extract on <i>Candida albicans</i> germ tube formation.....	93
4.4 Effect of the crude plant extract on <i>Candida albicans</i> biofilm formation.....	95
4.5 Effect of the crude plant extract on cell wall proteins of <i>Candida albicans</i>.....	99
4.6 Plant chemicals with antimicrobial effect.....	102
4.7 <i>Dodonaea viscosa</i> var. <i>angustifolia</i> and the oral cavity.....	103
4.8 Future Research.....	104
4.9 Technical difficulties and possible shortcomings.....	105
 CHAPTER 5: CONCLUSION.....	 108
 CHAPTER 6: APPENDICES	
 Appendix A: Composition and preparation of Media, Buffers and Stains	 109
Appendix B: Statistical Results.....	113
Appendix C: Document Scans.....	117

CHAPTER 7: REFERENCES.....	120
-----------------------------------	------------

LIST OF FIGURES

Figure 1.1	Erythematous candidiasis in a patient's mouth	2
Figure 1.2	Morphology of <i>Candida albicans</i> colonies cultured on a Sabouraud dextrose agar plate	5
Figure 1.3	Germ tubes (arrows) formed by <i>Candida albicans</i> cells after a three hour incubation in horse serum	7
Figure 1.4	The six layers of the <i>Candida albicans</i> cell wall (taken from Calderone and Braun, 1991)	9
Figure 1.5	Light microscope image showing the adherence of yeast cells (black) to an epithelial cell (stained pink)	13
Figure 1.6	Leaves and twigs of <i>Dodonaea viscosa</i> var. <i>angustifolia</i>	31
Figure 1.7	Map of South Africa with highlighted areas showing the location of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> (taken from van Wyk <i>et al.</i> , 2002).....	31
Figure 2.1	Transmission electron micrographs showing the grading of cell wall thickness	39
Figure 2.2	Transmission electron micrographs showing the grading of cell membrane undulation	40

Figure 2.3	Schematic diagram showing the method of extraction of proteins from the <i>Candida albicans</i> cell wall.....	44
Figure 3.1	The effect of different concentrations of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on the cell wall of <i>Candida albicans</i> cells	54
Figure 3.2	<i>Candida albicans</i> cells with 3+ cell wall thickness that were predominately observed in the control samples.....	55
Figure 3.3	<i>Candida albicans</i> cells with 1+ cell wall thickness that were predominately seen in the test samples	56
Figure 3.4	The effect of different concentrations of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on the cell membrane of <i>Candida albicans</i> cells	60
Figure 3.5	<i>Candida albicans</i> cells with 1+ cell membrane undulation were predominately observed in the control samples	61
Figure 3.6	<i>Candida albicans</i> cells with 3+ cell membrane undulation were predominately observed in the test samples.....	62
Figure 3.7	Transmission electron micrographs of cytoplasmic changes when cells were exposed to subinhibitory concentrations of plant extract.....	63
Figure 3.8	The percentage of cells for three <i>Candida albicans</i> strains that formed germ tubes in horse serum with and without exposure to the three subinhibitory concentrations of crude plant extract	66

Figure 3.9	The effect of crude plant extract on the germ tube formation by <i>Candida albicans</i> cells	67
Figure 3.10	Light micrograph at 400X magnification depicting the <i>Candida albicans</i> biofilm grown in the absence of plant extract (control)	69
Figure 3.11	Light micrograph at 400X magnification depicting the <i>Candida albicans</i> biofilm grown in the absence of plant extract (control)	70
Figure 3.12	Light micrograph at 400X magnification illustrating the effect of 3.125 mg/ml crude plant extract of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on <i>Candida albicans</i> biofilm formation	71
Figure 3.13	Light micrograph at 400X magnification illustrating the effect of 1.562 mg/ml crude plant extract of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on <i>Candida albicans</i> biofilm formation.....	72
Figure 3.14	Scanning Electron Microscope Micrograph illustrating the extracellular polymeric material of a <i>Candida albicans</i> biofilm in the control samples.....	78
Figure 3.15	Scanning Electron Microscope Micrograph showing the effect of the crude plant extract of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on <i>Candida albicans</i> biofilm formation.....	79

Figure 3.16	Scanning Electron Microscope Micrograph showing the effect of the crude plant extract of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on <i>Candida albicans</i> biofilm formation.....	80
Figure 3.17	Sodium Dodecyl Sulphate – Polyacrylamide Agarose Gel Electrophoresis analysis of the cell wall protein extracts from the three <i>Candida albicans</i> strains after exposure to the crude plant extract and water (control)	82
Figure 3.18	Sodium Dodecyl Sulphate – Polyacrylamide Agarose Gel Electrophoresis analysis of the cell wall protein extracts from the three <i>Candida albicans</i> strains after exposure to the crude plant extract and water (control)	83
Figure 3.19	Sodium Dodecyl Sulphate – Polyacrylamide Agarose Gel Electrophoresis analysis of the cell wall protein extracts from the three <i>Candida albicans</i> strains after exposure to the crude plant extract and water (control)	84
Figure 4.1	Diagram illustrating the summary of results of the effect of the crude plant extract of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on <i>Candida albicans</i>	107
Figure B1	Frequency of cell wall thickness (frequency weights) by the different treatments of plant extract and water	113
Figure B2	One way Analysis of Variances test results for the effect of different concentrations of plant extract on the <i>Candida albicans</i> cell wall	113
Figure B3	Pairwise comparison with Scheffe test results for changes caused by the different concentrations of plant extract on the <i>Candida albicans</i> cell wall	114

Figure B4	Median test results for changes caused by the different concentrations of plant extract on the <i>Candida albicans</i> cell wall	114
Figure B5	Frequency of cell membrane changes (frequency weights) by the different treatments of plant extract and water	114
Figure B6	One way Analysis of Variances test results for the effect of different concentrations of plant extract on the <i>Candida albicans</i> cell membrane.....	115
Figure B7	Pairwise comparison with Scheffe test results for changes caused by the different concentrations of plant extract on the <i>Candida albicans</i> cell membrane.....	115
Figure B8	Median test results for changes caused by the different concentrations of plant extract on the <i>Candida albicans</i> cell membrane	115
Figure B9	One way Analysis of Variances results for the effect of different concentrations of plant extract on germ tube formation	116
Figure B10	Pairwise comparison with Scheffe test for the effect of different concentrations of plant extract on germ tube formation	116
Figure C1	Ethical clearance	117
Figure C2	Random Sampling Grid	118
Figure C3	Random Number Table	119

LIST OF TABLES

Table 2.1	The volumes (in millilitres) of the various components needed to make up the stacking gel and the resolving gels (BioRad)	46
Table 3.1	Identification of <i>Candida albicans</i> strains	51
Table 3.2	Minimum Fungicidal Concentration of the plant extract against <i>Candida albicans</i>	52
Table 3.3	The effect of different concentrations of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on the <i>Candida albicans</i> cell wall	53
Table 3.4	The effect of different concentrations of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on the <i>Candida albicans</i> cell membrane.....	59
Table 3.5	The effect of crude plant extract on the germ tube formation of <i>Candida albicans</i>	65
Table 3.6	Scanning Electron Microscope micrograph results for <i>Candida albicans</i> strain ATCC 90028 showing the effect of the crude plant extract at three subinhibitory concentrations on biofilm formation	75
Table 3.7	Scanning Electron Microscope micrograph results for <i>Candida albicans</i> strain A71 showing the effect of the crude plant extract at three subinhibitory concentrations on biofilm formation.....	76

Table 3.8	Scanning Electron Microscope micrograph results for <i>Candida albicans</i> strain A79 showing the effect of the crude plant extract at three subinhibitory concentrations on biofilm formation.....	77
Table 3.9	Molecular weights of the proteins extracted from <i>Candida albicans</i> cells after exposure to crude plant extract and water (control) as seen on the various percentage gels.....	85

LIST OF ABBREVIATIONS AND ACRONYMNS

AIDS:	Acquired Immuno-Deficiency Syndrome
ALS:	Agglutinin-like Sequence
ANOVA:	Analysis of Variances
ATCC:	American Type Culture Collection
βME:	Beta-Mercaptoethanol
BEC:	Buccal Epithelial Cells
CFU/ml:	Colony Forming Units per millilitre
CSH:	Cell Surface Hydrophobicity
ECM:	Extracellular Material or Matrix
g:	Gram
HIV:	Human Immunodeficiency Virus
kDa:	kilo-Dalton
L.F:	Linneaus Fillius
MFC:	Minimum Fungicidal Concentration

mg/ml:	Milligram per millilitre
ml:	Millilitre
mm:	Millimetre
PBS:	Phosphate Buffered Saline
rpm:	Revolutions per minute
SD:	Standard Deviation
SDS:	Sodium Dodecyl Sulphate
SDS-PAGE:	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SEM:	Scanning Electron Microscopy
TEM:	Transmission Electron Microscopy
μl	Microlitre
μm	Micrometre

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Oral candidiasis is an infection that commonly occurs in immunocompromised individuals such as cancer, transplant and HIV positive patients. The main causative agent of candidiasis is *Candida albicans*. Many antifungal agents such as amphotericin B, nystatin, and fluconazole are available and are effectively used (Mims *et al.*, 2004). However, due to the toxicity of these drugs and development of drug resistance, medicinal plants have been investigated as an alternative treatment. *Dodonaea viscosa* var. *angustifolia*, an indigenous South African plant has proved to have an antifungal effect. The crude plant extract also inhibits the adherence of *C. albicans* to oral epithelial cells, which is the crucial first step of infection (Patel *et al.*, 2009).

This study investigated the effect of the crude extract on the ultrastructure of *C. albicans* cell wall, which might be responsible for the reduced adherence to oral epithelial cells. The effect of the plant extract on *C. albicans* germ tube and biofilm formation was also studied since biofilm structure allows for high resistance to antifungal agents and host defense mechanisms.

1.1 Oral Candidiasis

Oral candidiasis is an opportunistic infection of the oral cavity (Figure 1.1) and there is a high incidence of infection amongst the elderly and immunocompromised individuals such as HIV positive, cancer, transplant and surgery patients (Akpan and Morgan, 2002). Oral candidiasis has been grouped into the following categories acute



Figure 1.1: Erythematous candidiasis in a patient's mouth.

pseudomembranous candidiasis (thrush), acute atrophic candidiasis, chronic atrophic candidiasis (denture stomatitis) and chronic hyperplastic candidiasis (Epstein *et al.*, 1984). A high percentage of elderly denture-wearers can acquire denture stomatitis, which is an infection of the oral mucosa that is promoted by a close-fitting upper denture (Douglas, 2002).

The etiological agent of oral candidiasis is *Candida albicans* although other *Candida* species such as *C. tropicalis*, *C. dubliniensis*, and *C. glabrata* have been implicated in this disease (Cannon and Chaffin, 1999). *Candida albicans* is the most common species of *Candida* isolated from patients with oral candidiasis (Patel *et al.*, 2006).

The presence of *C. albicans* in the oral cavity does not indicate infection since *C. albicans* is a minor component of the oral flora in many individuals and they have no clinical symptoms (Cannon and Chaffin, 1999). There have been several studies done on the *Candida* carrier rate in the general population and these have shown that asymptomatic carriage varies geographically in healthy and compromised individuals. In Germany, the carrier rate in HIV-positive and HIV-negative subjects were 73.8% and 13.8% respectively (Schmidt-Westhausen *et al.*, 1991). In Thailand, the carrier rate in HIV-positive and HIV-negative subjects were 66.6% and 10.8% respectively (Teapaisan and Nittayananta, 1998). In South Africa, the carrier rate in HIV-positive and HIV-negative subjects were 75% and 68% respectively (Hauman *et al.*, 1993). Reports have shown that the carrier rate also varies in different regions of South Africa. In one province, the Western Cape, the rate was higher, with 68% of HIV-negative subjects and 75% of HIV-positive patients carrying yeasts (Hauman *et al.*,

1993). In unknown HIV status subjects, the rate was 30.4% in a rural area of South Africa and 58% in a semi urban part of the Gauteng province (Patel *et al.*, 2006).

Most of these commensal *C. albicans* cells proliferate as budding yeasts and hyphae are rare. However, when the epithelium barrier is breached due to trauma or hormonal alterations, invasion of submucosal tissue may result and in many instances, there is conversion to hyphal growth (Nisengard and Newman, 1994). Therefore since *C. albicans* is an opportunistic pathogen, in certain parts of the population; it is able to cause superficial infections such as thrush or denture stomatitis and in more severe cases, it causes life-threatening systemic mycoses (Jayatilake *et al.*, 2005). Predisposing factors for *C. albicans* infections include immunosuppressive therapy, antibiotic therapy, use of indwelling devices and intravenous catheters, HIV-infection, diabetes and old age (Ramage *et al.*, 2001). *Candida albicans* accounts for the majority of systemic infections with mortality rates ranging between 50-100% (Tayel *et al.*, 2010). Superficial *Candida* infections such as denture stomatitis are much less serious but can be troublesome and are encountered very frequently (Douglas, 2002).

1.2 *Candida albicans*

1.2.1 Morphology

Candida albicans grows as spherical to oval budding yeast cells 3-5 µm by 5-10 µm in size. These are called blastospores and *C. albicans* also forms elongated, filamentous cells joined end to end called pseudohyphae. Cultures grow on Sabouraud medium as creamy-white, flat or hemispherical colonies (Figure 1.2) with a beer-like aroma (Samaranayake, 2002). *Candida albicans* is a dimorphic fungus therefore it can exist as both a yeast and hyphal form at different stages of its life cycle, whereas other

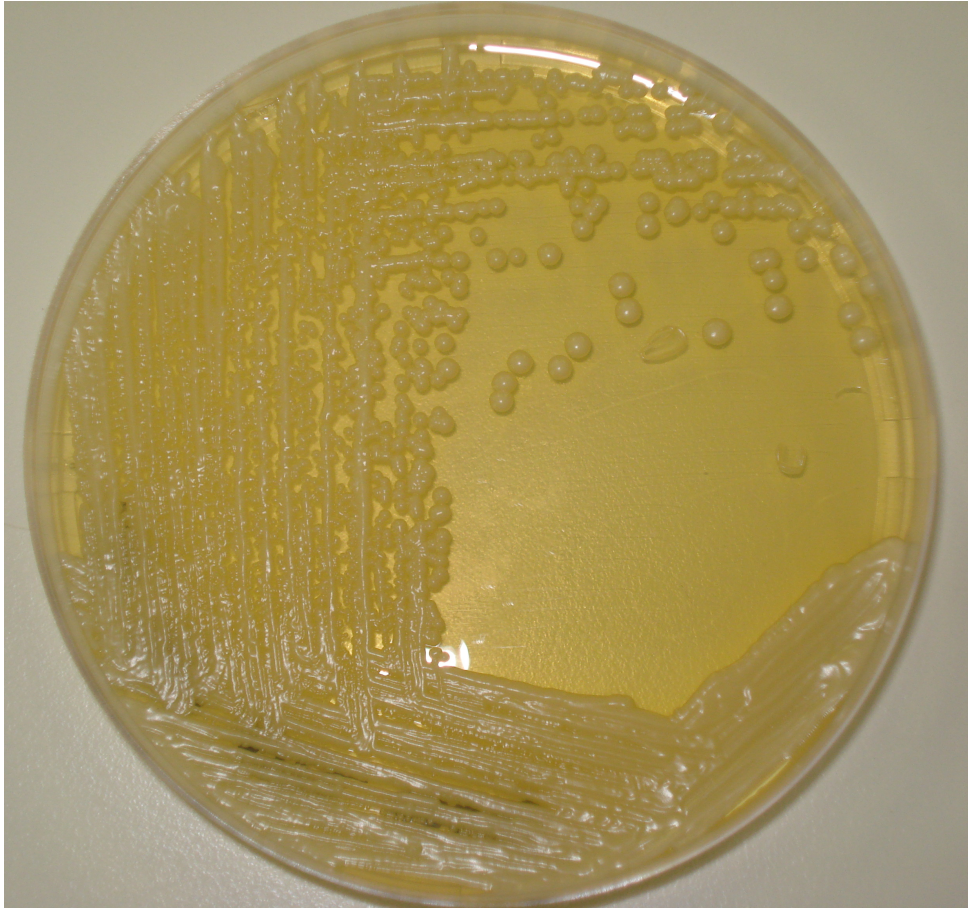


Figure 1.2: Morphology of *Candida albicans* colonies cultured on a Sabouraud dextrose agar plate.

fungi exist as one form only. It has been observed that the ability to switch between yeast and hyphal morphologies can play a role in the virulence of *C. albicans* (Sudbery *et al.*, 2004). Environmental factors can induce the switch from a yeast form to a hyphal form for example serum inoculated with *C. albicans* yeast cells can induce hyphal formation (Figure 1.3) at a growth temperature of 37°C within 3 hours (Brawner and Cutler, 1986).

1.2.2 Pathogenicity

Candida albicans possesses many virulent properties, such as the ability to adhere to host tissues and prostheses (e.g. dentures), the potential to switch (e.g. rough to smooth colony formation), modify surface antigens, the ability to form hyphae that helps in tissue invasion, and the ability to produce a range of hydrolytic enzymes such as extracellular phospholipases and proteinases which appear to break down physical defense barriers of the host (Samaranayake, 2002).

The invasive mechanisms of *C. albicans* are not understood in detail; however, few factors considered to be important are protein-protein interactions between *Candida* cell walls and host cells. The morphological forms of *C. albicans* and the physiology of the host are also important. It has been shown that hyphae, pseudohyphae and blastoconidia can be important pathogenic characteristics of *C. albicans* (Lemar *et al.*, 2002). It has also been observed that yeast-related factors such as adhesion to host cells, cell surface hydrophobicity (CSH) and secretion of several degradative enzymes are important in promoting virulence (Samaranayake *et al.*, 2003). Adherence occurs between moieties of the *C. albicans* cell wall and host surfaces therefore pathogenicity also relates to cell wall components (Cannon and Chaffin, 1999).

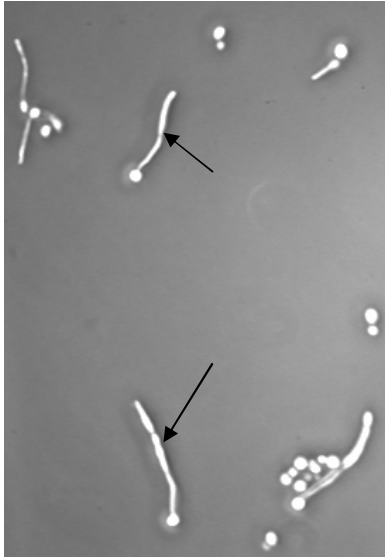


Figure 1.3: Germ tubes (arrows) formed by *Candida albicans* cells after a three hour incubation in horse serum.

1.2.3 Cell wall

1.2.3.1 Structure

The cell wall of *C. albicans* (Figure 1.4) is important in pathogenicity since it is required for growth, provides rigidity and protection against osmotic insult and is also the site of contact between the organism and its environment (Calderone and Braun, 1991). It is mainly composed of polysaccharides such as glucan, chitin and mannan. Polysaccharides make up about 80-90% of the cell wall, while the rest of it is mostly made up of protein. Among the polysaccharides, chitin is a minor constituent and makes up about 1-10% of the cell wall. Glucan is a major constituent since it makes up about 47-60% of the cell wall. Chitin and glucan are microfibrillar polysaccharides and are found throughout the cell wall but are mainly concentrated near the plasma membrane hence provide rigidity to the cell wall. Mannan is a major constituent of the cell wall and makes up about 40% of the cell wall. Mannan is a dominant component found throughout the surface of the cell wall (Cannon and Chaffin, 1999).

Proteins make up about 6-25% and lipids make up about 1-7% of the fungal cell wall (Calderone and Braun, 1991). The outer layer of the *C. albicans* cell wall also has fimbriae, which are composed of mostly mannoproteins and appear as a dense network of radially projecting fibrils (Chaffin *et al.*, 1998; Cannon and Chaffin, 1999). These fibrillar structures on the cell wall surface have been shown to play a role in the adhesion of *C. albicans* to a range of surfaces (Douglas, 1987).

Proteins found at the external surface of the cell wall like adhesins and lectins also play an important role in adhesion to host surfaces (Castillo *et al.*, 2008). Adhesins

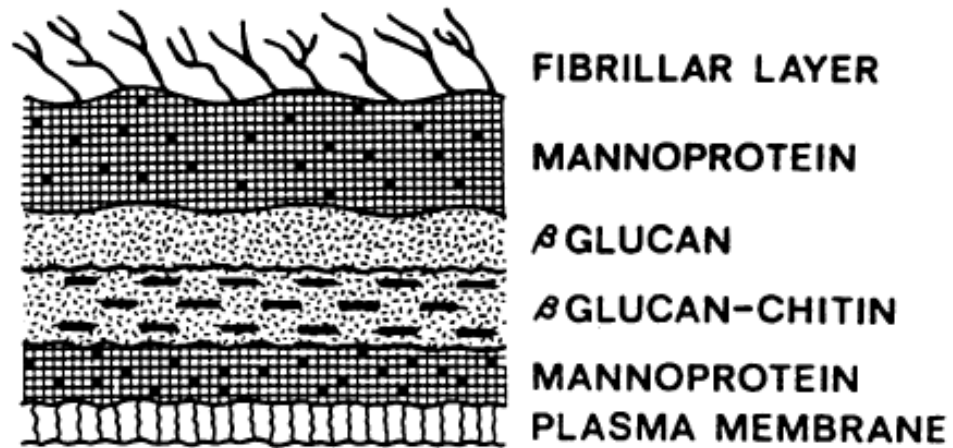


Figure 1.4: The six layers of the *Candida albicans* cell wall (Calderone and Braun, 1991).

are moieties found on the fungal surface that allow for the binding of *C. albicans* to other cells (host or microbial), inert polymers, or proteins. Many different experimental approaches and reagents have been used to identify *C. albicans* adhesins (also referred to as binding proteins or receptors) and host ligands or receptors. These experiments have shown that *C. albicans* has many adhesins and that there may be more than one adhesin that recognizes a host ligand or cell. Several cell wall proteins have been identified as possible adhesins for epithelial cells. Most of the adhesins identified are mannoproteins; they consist of glycoproteins composed of a mannan oligosaccharide linked to proteins by glycosidic bonds (Calderone, 1993). Both the protein and/or carbohydrate portions have been found to be involved in adherence (Cannon and Chaffin, 1999).

1.2.3.2 Proteins and Mannoproteins

Experiments have identified cell wall surface mannoproteins and glycoproteins that are involved in adherence. Most studies focused either on extraction of cells with sodium dodecyl sulphate (SDS) or dithiothreitol (DTT) or on digestion of the cell wall by zymolyase. Thereafter, these extracts were analysed by crossed immuno-electrophoresis or SDS-polyacrylamide gel electrophoresis (PAGE) with Western blotting. Dithiothreitol treatment releases components associated with the outermost layers of the cell wall, whereas zymolyase digestion releases residual wall mannoproteins that are covalently linked to structural cell wall glucans (Calderone and Braun, 1991).

These techniques have been used to study the cell surface macromolecules of both yeast and hyphal (or germ tube) forms of *C. albicans*. Studies show that the *C.*

albicans cell wall has complex protein-containing components and that there are qualitative and quantitative differences in protein composition between yeast and hyphal cell walls. Hyphal formation is thought to play an important role in yeast adherence therefore differences in protein composition between the yeast and hyphal forms indicate that the proteins specific to hyphal cell walls may play a role in adherence. A complex array of genuine cell wall-bound protein components were extracted from both yeast and hyphal forms and their molecular weights ranged between 13 kDa and 650 kDa (Casanova *et al.*, 1992). However, only a few of these proteins have been identified that are thought to play a role in adherence. A low molecular weight protein with a size of 19 kDa was found on the hyphal cell wall only and was identified as an adhesin (Ponton and Jones, 1986). Glycoproteins that may act as adhesins with molecular weights ranging from 55 to 60 kDa (Fukayama and Calderone, 1991), 60 to 68 kDa (Casanova *et al.*, 1992), 80kDa (Casanova and Chaffin, 1991) and 165kDa (Fukayama and Calderone, 1991) have been identified. High molecular weight mannoproteins of sizes: 180 kDa (Casanova *et al.*, 1989), 235-250 kDa (Ponton and Jones, 1986), and 260 kDa (Casanova *et al.*, 1989) have also been identified. All these cell wall components play an important role in adherence to host cells, which is the initial stage of infection.

Other components of the cell wall have also shown to affect yeast adhesion. Extracellular polymeric material (EPM) found on the cell surface contains surface components, mainly mannoprotein, responsible for adhesion. The protein portion of the mannoprotein adhesin is probably more important in adhesion to buccal epithelial cells (BECs) than the carbohydrate portion hence may provide the predominant interaction between yeast cell and epithelial cell (Critchley and Douglas, 1987).

Candida albicans produces floccular and fibrillar surface layers. The floccular surface layer mediates adhesion to oral mucosal cells. The fibrillar surface layer is synthesised by *C. albicans* in the presence of high sugar concentrations during the exponential phase of growth (McCourtie and Douglas, 1984). It is possible that mannan-containing moieties found on the yeast cell wall also mediate adhesion (Sandin, 1987). Chitin could also be an adhesin involved in yeast adherence but this needs to be evaluated further since chitin is mainly located in the inner layer of the yeast cell wall (Douglas, 1987).

1.2.3.3 Adherence

Adherence of *C. albicans* cells to oral mucosal cells (Figure 1.5) is very important because of the constant secretion of saliva in the oral cavity. Yeast cells in the oral cavity will be washed away by saliva since the oral cavity is a continuous-flow environment therefore the yeast cells need to adhere to the surface of mucous membranes. Additionally, the growth conditions in the oral cavity are not suitable unless the yeast cells adhere (Tronchin *et al.*, 1991). Saliva flow and major organic constituents of saliva allow for the physical removal of microorganisms. These organic constituents are proteins and glycoproteins, such as mucin, and can aggregate microorganisms together, hence allow for their clearance from the mouth by swallowing. There are also many other antimicrobial factors present in saliva and these include antibodies such as immunoglobulin A (IgA), lysozyme, lactoferrin, and the sialoperoxidase system (Marsh and Martin, 1999). Adherence of yeast cells allow for colonization of the oral cavity and therefore adherence is the first step of infection (Tronchin *et al.*, 1991). Epithelial cells are constantly being lost by desquamation therefore the progeny of the adherent yeast cells need to attach to newly exposed cells

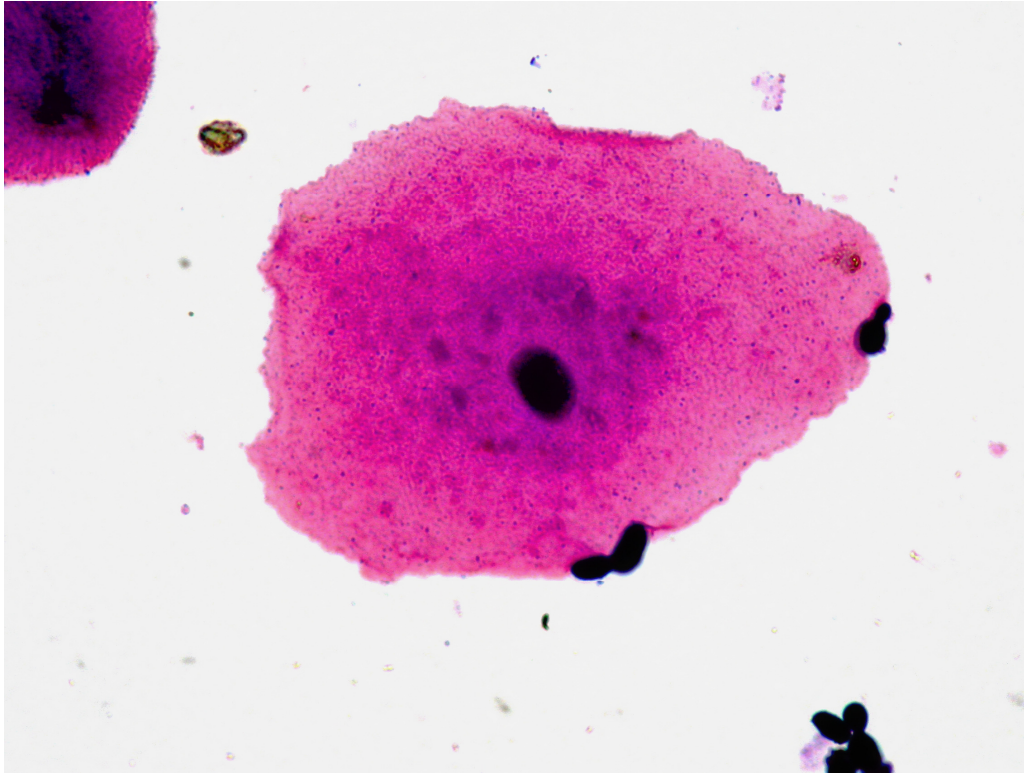


Figure 1.5: Light microscope image showing the adherence of yeast cells (black) to an epithelial cell (stained pink).

in order to maintain colonization. The ability of *C. albicans* to adhere is an important virulence determinant and it is influenced by the organism's ability to change to the hyphal form (Douglas, 1987). It has been observed that adherent strains of *C. albicans* are more pathogenic than strains that are less adherent therefore there is a positive correlation between adherence and pathogenicity (Hoyer, 2001).

Adherence is mediated by interactions between microbial adhesins, which are adhesive structures found on the cell surface, and complementary host receptors. There are many factors involved that affect yeast adhesion (Douglas, 1987). These factors could relate to yeast or host cells; or there could be environmental factors affecting adhesion (Olsen, 1990).

1.2.4 Factors related to *Candida albicans* pathogenicity

1.2.4.1 Phenotypes

There are differences in adhesive ability for various *Candida* species and strains of *C. albicans* (Cannon and Chaffin, 1999). It was shown that *C. albicans* adhesion varied significantly under the same growth conditions depending on the phenotypic state of the organism. Between the two phenotypes ('white' and 'opaque') it was observed that pathogenic isolates ('white') were more adhesive to BECs, whereas there was no significant difference between the phenotypes with respect to adherence to plastic (Kennedy *et al.*, 1988).

1.2.4.2 Cell Surface Hydrophobicity

Hydrophobic interactions may also play a role and it was shown that pathogenic *Candida* species exhibit different degrees of cell surface hydrophobicity (CSH). This

relates to yeast adhesion (Douglas, 1987). Hydrophobic forces are long range reversible forces which are thought to contribute to adherence by maintaining the fidelity of adhesin-receptor bonds (Samaranayake *et al.*, 2003).

Cell surface hydrophobicity contributes to the pathogenicity of *C. albicans* since hydrophobic cells show a higher adherence to epithelial cells, endothelial cells and extracellular matrix proteins than hydrophilic cells. The CSH status of *C. albicans* can vary in response to different environmental conditions and growth phases (Singleton *et al.*, 2001). In a study, it was seen that CSH increased at a lower growth temperature of 23°C and the results suggested that it contributed to adherence to BECs although it was not the predominant mechanism (Hazen, 1989). Cell surface hydrophobicity is also thought to be involved in the adhesion of *C. albicans* to plastic medical devices and acrylic dentures. Microbial structures that contribute to CSH include outer membrane protein, lipoprotein, phospholipid, lipopolysaccharide and fimbriae (Anil *et al.*, 2001). Cell surface hydrophobicity of *C. albicans* correlates with the concentration of surface fibrils found on the cell wall exterior (Hazen and Hazen, 1992).

Experiments have been done to identify several specific surface proteins that appear to contribute to CSH and affect cell attachment to host targets. This has been achieved by partial cell wall digestion to release proteins in the cell wall matrix. One *C. albicans* gene called *CSH1* encoding a candidate protein responsible for CSH was cloned and analysed. The knockout of this gene resulted in a decrease in CSH and reduced adherence of *C. albicans* to fibronectin. Therefore the results suggest that this

specific gene encodes a hydrophobic surface protein that is implicated in adherence to host cells (Singleton *et al.*, 2001).

Candida albicans isolates exposed to sub-therapeutic concentrations of the antiseptic mouthwash, chlorhexidine gluconate significantly reduced their CSH. Adhesion to denture acrylic, BECs, and germ tube formation was dramatically affected by chlorhexidine (Anil *et al.*, 2001). It has also been found that when *C. albicans* cells were exposed to non-antibiotic, antimicrobial agents such as chlorhexidine acetate or cetylpyridinium chloride, there was a decrease in hydrophobicity and this in turn decreased binding of yeast cells (Jones *et al.*, 1991; 1995).

1.2.4.3 Adhesin genes

Many cell-surface adhesins have been identified and characterized biochemically. These surface proteins may play a role in the adherence of *C. albicans* to extracellular matrix proteins and host cell surfaces. Genetic and molecular biology approaches have allowed for the investigation of adhesin function by isolating and expressing these genes in heterologous hosts (Cannon and Chaffin, 1999). *Saccharomyces cerevisiae* has been used in many of these experiments since *S. cerevisiae* cells have a lesser ability to adhere to a variety of surfaces than *C. albicans* cells hence gene sequences thought to be involved in adherence have been isolated, cloned and expressed in *S. cerevisiae* to possibly confer adherence on this non-adherent yeast (Fu *et al.*, 1998). One gene family of *C. albicans* called the agglutinin-like sequence (ALS) has been studied extensively since it encodes for adhesins that were observed to play a role in adherence. Members of the ALS family are related to *S. cerevisiae* agglutinin genes that enable cell-cell interactions during mating of haploid cells

(Cannon and Chaffin, 1999). The gene family includes at least nine genes and has also been found in other *Candida* strains such as *C. dubliniensis* and *C. tropicalis*. The proteins encoded by these genes have been found on the surface of *C. albicans* cells in the host environment and therefore these proteins have a widespread presence in the host and play an important role in host-pathogen interactions (Hoyer, 2001).

One gene, *ALSI*, which has been studied, has been found to play a role in adherence. This gene was isolated and expressed in *S. cerevisiae* cells causing these transformants to adhere to human endothelial and epithelial cells. Therefore these results suggest that *ALSI* may encode a candidal adhesin involved in adherence (Fu *et al.*, 1998).

Another *C. albicans* adherence gene, *ALAI*, which is homologous to *ALSI*, has been the focus of many gene expression experiments. Gaur and Klotz (1997) cloned the *ALAI* gene and expressed it in *S. cerevisiae* cells. These transformed yeast cells were able to bind to magnetic beads coated with various extracellular membrane proteins, i.e. fibronectin, laminin and collagen IV, and to human BECs. Adherence to BECs was also increased therefore implying that the adhesin may be multifunctional, recognizing multiple ligands and mediating adherence to different tissues. However, the only difference between wild-type *C. albicans* and the *ALAI* transformant of *S. cerevisiae* was their adherence ability. *Candida albicans* cells aggregated after attachment to ECM protein-coated magnetic beads and BECs. Autoaggregation occurred when transformants were induced by calcium ions, however, the calcium ions did not induce adherence therefore concluding that adherence and aggregation

are two separate events. Some researchers later showed that overexpression of the *ALAI* gene resulted in both adherence and aggregation (Gaur *et al.*, 1999).

Other *C. albicans* adherence genes, *AAF1/CAD1* and *α INT1* have also been cloned and partially characterized (Barki *et al.*, 1993; 1994). Expression of *α INT1* in *S. cerevisiae* resulted in adherence to epithelial cells, formation of germ tube-like structures similar to *C. albicans* germ tubes, and autoaggregation (Gaur *et al.*, 1999). The germ tube-like structures in *S. cerevisiae* showed an enhanced autoaggregation similar to *C. albicans* germ tubes (Gaur and Klotz, 1997).

1.2.4.4 Germ tube and adherence

Candida albicans cells bearing germ tubes are more adherent to BECs than yeast forms of *C. albicans* (Kimura and Pearsall, 1980). These authors discovered that there was a strong correlation between germination and increased adherence of *C. albicans* to BECs. Partial inhibition of germination by cysteine resulted in reduced adherence therefore implying that germination plays an important role in adherence. Germ tube/BEC complexes are resistant to dissociation with heat and SDS, but they are susceptible to inhibitors of transglutaminase (TGase) cross-linking enzymes. Transglutaminase enzymes facilitate the formation of an innate host barrier defense by cross-linking substrate proteins in stratified squamous epithelium. This suggests that germ tubes have unique microbial adhesive properties by exploiting mammalian TGases in order to adhere to the oral mucosa of the host (Bradway and Levine, 1993).

Early investigators suggested that the difference in adherence capacity between yeasts and germ tubes might be due to antigens found on germ tubes (Sundstrom and Kenny,

1984). A developmentally regulated gene, *HWPI* (hyphal wall protein 1) was identified by immunofluorescence. The protein encoded by this gene, Hwp1, bound to exogenous ligands and was only expressed on hyphal surfaces (Staab *et al.*, 1996). It was observed that the *HWPI* gene had similar properties to the *ALS1* and *ALA1* genes. Sundstrom (1999) used *hwp1* null mutants and complemented strains to determine the mechanism of binding by the Hwp1 protein. The recombinant Hwp1 protein and germ tube surfaces of *C. albicans* showed TGase activity. The adherence of germ tubes and recombinant Hwp1 to BECs was resistant to thermal dissociation in the presence of SDS but was susceptible to transglutiminase inhibitors. These results support an adherence mechanism involving cross-linking of Hwp1 to unidentified proteins associated with BECs through BEC - TGase activity. Other adhesins operate through lectin-like or hydrophobic interactions, whereas, Hwp1 has the ability to cross-link to host proteins therefore making it a unique adhesin.

1.2.4.5 Enzymes and adherence

Enzymes such as extracellular proteinases and phospholipases have also shown to facilitate adherence and invasion of tissue. Investigators have also observed that *C. albicans* adherence to BECs was inhibited by the proteinase inhibitor, pepstatin; therefore indicating that proteinase may play a role in adherence (Martin and Lamb, 1982). However, the exact mechanism of how proteinases are involved in adherence is not known. There are probable mechanisms, for example the proteinase cleaves some component on the host that is masking a host cell receptor and this cleavage allows for the binding of a specific yeast adhesin to the host receptor (Calderone and Braun, 1991). This is similar to a mechanism described for a bacterial system (Beachey, 1981). It is also possible that proteinases may modify adhesins on the yeast

surface and thereby allowing for adhesion (Cannon and Chaffin, 1999). Recently, other enzymes such as surface-located phosphatases have also been found to play a role in fungal adherence (Portela *et al.*, 2010).

1.2.4.6 Lipids and adherence

Cellular lipids may also be involved in adherence (Olsen, 1990). Certain surface lipids isolated from yeast and mycelial forms of *C. albicans* inhibited the adherence of yeast cells to BECs. Surface lipids isolated from a membrane-free preparation of yeast cell walls and from whole epithelial cells also blocked adherence. The lipid extracts were analysed and contained various classes that inhibited adherence therefore both the yeast cells and epithelial cells may have surface lipids involved in their mutual adherence (Ghannoum *et al.*, 1987).

1.2.5 Factors relating to host cells

There are host cell receptors that can recognize *C. albicans* adhesins and this affects adherence. One of the first discovered host receptors or ligands that recognized an adhesin was fibronectin (Skerl *et al.*, 1984). It has also been observed that yeast cells bind to lactosylceramide, which is a major glycosphingolipid that is widely distributed in epithelial tissues. Therefore this BEC glycosphingolipid is also a receptor for *C. albicans* and this interaction mediates yeast colonization (Jimenez-Lucho *et al.*, 1990). Another host component is fibrin clots since in some experiments, *C. albicans* readily adhered to fibrin (Samaranayake *et al.*, 1988). It is possible that the fibrinogen binding factor of *C. albicans* is a mannoprotein found on the cell wall surface (Guinet and Bruneau, 1988).

Human donors also affect the binding to BECs for example *C. albicans* adhered in greater numbers to BECs from AIDS patients than to BECs from healthy individuals or transplant patients (Schwab *et al.*, 1997). *Candida albicans* isolates from candidiasis patients exhibited, on average, higher adhesiveness to BECs than isolates from asymptomatic yeast carriers (Kornev and Velichko, 1987).

1.2.6 Environmental Factors that affect *Candida albicans* adhesion

Divalent cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) have been shown to increase yeast adhesion to BECs and acrylic surfaces. At high concentrations, these cations also caused aggregation of *C. albicans* therefore implying that ion-binding mechanisms and electrostatic forces may be important in adhesion to BECs and acrylic. pH could also play a role in yeast adhesion since experiments have shown that adherence of *C. albicans* varied with the pH of the test medium. It was observed that maximal adherence occurred at pH 3, while less adhesion occurred under neutral pH conditions (Samaranayake and MacFarlane, 1982a).

Sugars, both endogenous and exogenous, may affect the oral carriage of *C. albicans* cells by modifying their adhesive properties. Adherence of *C. albicans* to BECs increased after cultivation in media containing galactose and maltose (McCourtie and Douglas, 1981; Samaranayake and MacFarlane, 1982b).

Saliva may also play a role in yeast adhesion. It was observed that salivary secretory immunoglobulin A anti-*Candida* antibodies inhibited *C. albicans* binding to BECs therefore suggesting that IgA antibodies can inhibit the adherence of *C. albicans* to

the oral mucosa. It was also shown that the removal of antibodies increased adherence in some cases (Epstein *et al.*, 1982).

Broad spectrum antibacterial drugs have been shown to promote candidal infection (Velichko and Karaev, 1987). The antibiotic, tunicamycin, inhibited adherence by decreasing mannoprotein synthesis, although it did not affect chitin or glucan synthesis (Douglas and McCourtie, 1983). Indigenous bacterial flora may interfere with yeast adhesion. It was observed that *C. albicans* adherence to denture acrylic surfaces *in vitro* was significantly reduced by *Streptococcus salivarius* (Samaranayake *et al.*, 1980). Lectins have also been implicated in affecting yeast adhesion. Addition of concanavalin A, a lectin that binds to α -D- mannosyl residues, to BECs decreased *C. albicans* adherence (Sandin, 1987).

1.2.7 Hydrolytic Enzymes

Candida albicans produces a range of hydrolytic enzymes that have various roles in the infection process. The two enzymes that have received the most attention are proteinases and phospholipases and are important virulence determinants (Haynes, 2001). Experiments have shown that in mice, the proteinase-producing strain was more virulent than the proteinase-deficient strain (MacDonald and Odds, 1983; Kwon-Chung *et al.*, 1985). These researchers postulated that proteinases may act on the epithelial tissues whereby it may uncover sites on the tissue in order for the fungal cells to adhere and hence penetrate (Kwon-Chung *et al.*, 1985). Proteinases, in addition to having a direct effect on adhesion, may also act on the yeast surface to modify adhesins or on host surfaces to expose ligands (Cannon and Chaffin, 1999). Proteinases may also be involved in counteracting the host immune response since it

has been observed that proteinase is able to resist phagocytosis and intracellular killing (MacDonald and Odds, 1983).

Phospholipases may be involved in host cell penetration, adhesion to epithelial cells, invasion of epithelium and interaction with host signal transduction pathways (Schaller *et al.*, 1999). These enzymes are also considered important virulence determinants. Experiments involving the disruption of a gene that encodes for a phospholipase showed a decrease in virulence and a reduced ability of *C. albicans* to penetrate host cells. Therefore suggesting that phospholipases play a role in causing damage to host cell membranes since these enzymes degrade the phospholipid constituents present in membranes (Leidich *et al.*, 1998).

Surface-located acid phosphatases of *C. albicans* may also play an important role in the pathogenesis of the organism. A study was done to determine whether ectophosphatase activity influences fungal adhesion in HIV positive patients. The results showed that *C. albicans* isolates from HIV positive subjects had higher ectophosphatase activity and this resulted in higher adhesion to epithelial cells; therefore suggesting that the activity of acidic surface phosphatases may play a part in the early stages of infection allowing for successful establishment and colonization (Portela *et al.*, 2010).

1.2.8 *Candida albicans* Biofilm

Commensal *C. albicans* yeast cells usually exist within biofilms in the oral cavity; biofilms are spatially organized, heterogenous communities of fungal cells encased in a matrix consisting of extracellular polymeric substances (Jin *et al.*, 2004). Scanning

electron microscopy studies have shown that *C. albicans* biofilms consist of a dense network of yeasts, hyphae, and pseudohyphae, as well as a matrix material (Hawser *et al.*, 1998). Cells within biofilms have unique phenotypic characteristics as compared to planktonic cells. *Candida albicans* colonizes oral epithelia developing a biofilm that can disseminate into the bloodstream and cause fatal systemic infections in immunocompromised patients. *Candida albicans* biofilms can form on prostheses, for example denture acrylic, and medical implants such as catheters and joint replacements. These devices are a risk factor and are often associated with *C. albicans* infections (Baillie and Douglas, 1998).

Candida albicans biofilm formation is initiated by the adherence of planktonic cells to a surface possibly through cell wall-located adhesion molecules and the cells aggregate into a microcolony. Once the first layer of cells is formed, an extracellular matrix made up of proteins and polysaccharides is produced to consolidate the early biofilm (Garcia-Sanchez *et al.*, 2004). Often, individual yeast cells detach from the mature biofilm and cause infection which is responsive to drug therapy. However, the biofilm itself is highly resistant to antifungal agents and host defense mechanisms as compared to planktonic yeast cells. It has been reported that *C. albicans* biofilms are resistant to many clinical antifungal agents, including amphotericin B and fluconazole due to the fact that these antifungals can not actively penetrate the biofilm matrix (Baillie and Douglas, 1999; Al-Fattani and Douglas, 2006). Therefore there is persistence of *C. albicans* infection on inert, inserted surfaces or superficial mucosae and infection is difficult to treat (Cao *et al.*, 2008). Hence there is a need to develop an antimicrobial agent that can actively penetrate the biofilm and therefore eradicate the biofilm completely or reduce biofilm formation.

1.3 Treatment of oral candidiasis

1.3.1 Antifungal agents

Treatment of oral candidiasis has been a major problem since the eukaryotic yeast cells are very similar to host cells therefore differentiation between host and yeast by the specific antifungal agent has to be taken into consideration. There are a few antifungal agents that are used clinically and this is limited by drug-safety considerations, their narrow spectrum of activity, efficacy and development of resistance (Endo *et al.*, 2010).

Expensive antifungal agents such as amphotericin B and the azoles (e.g. fluconazole and miconazole) have been used extensively in the treatment of candidiasis due to the limited treatment options available for opportunistic fungal infections (Perlin, 2010). The antifungal activities of these antifungal agents are due to the inhibition of synthesis of ergosterol or direct interaction with ergosterol. Ergosterol is the major component of the fungal cell membrane hence functions as a bioregulator of membrane fluidity, asymmetry and integrity. Fluconazole affects the synthesis of ergosterol by inhibiting enzymes involved in the biosynthesis pathway hence resulting in a depletion of ergosterol thereby leading to the formation of a yeast plasma membrane with altered structure and function. Amphotericin B, a polyene antifungal, can directly bind to the ergosterol membrane. The binding results in the formation of pores in the membrane hence leads to altered permeability, leakage of vital cytoplasmic components and eventually the cell dies from oxidative damage (Ghannoum and Rice, 1999).

Antifungal agents have been extensively used to treat candidiasis in immunocompromised individuals. However, the widespread and incorrect use of these antifungal agents has led to the emergence of drug resistance in several common pathogenic fungi. In developing countries, the emergence of resistance is low due to the limited availability of drugs. There is a trend in the emergence of resistance seen in developed countries whereby the frequent use of antifungals led to the development of drug resistance (Perlin, 2010). There is evidence suggesting that extended or repeated courses of fluconazole led to the development of resistance (Ruhnke *et al.*, 1994; Marr *et al.*, 2001).

Fluconazole is extensively used in the treatment of oral candidiasis and therefore there have been many reports on the decreased *in vitro* and *in vivo* susceptibility to fluconazole of *C. albicans* isolates from HIV patients (Fekete-Forgacs *et al.*, 2000). Certain virulence traits of *C. albicans* isolates have been found to be responsible for the development of resistance. This therefore implies that there is a correlation between antifungal susceptibility and virulence factors. It has been reported that fluconazole resistant *C. albicans* strains show an increased adherence to BECs and plastic, increased germ tube formation, increased proteinase enzyme production and increased extracellular phospholipase activity (Fekete-Forgacs *et al.*, 2000).

Furthermore, resistance to oral miconazole therapy was observed in three out of 72 South African HIV/AIDS patients that had previously received antifungal treatment (Blignaut *et al.*, 1999) therefore as antifungal drugs become readily available in developing countries; antifungal resistance is inevitable. This drives the need to develop new antifungal agents.

1.3.2 Mouthrinses

Other strategies include using mouthrinses containing antimicrobial agents such as chlorhexidine gluconate and triclosan as effective treatment against oral candidiasis. They are usually used in combination with antifungal drugs. Chlorhexidine is a broad-spectrum antimicrobial that has been effective in the treatment and prevention of candidiasis. Its mode of action involves the non specific binding of the drug's positively charged groups to negatively charged sites on the epithelial cell surface. It has also been shown to readily bind to both mucosal and denture materials then slowly released therefore resulting in prolonged antimicrobial activity in the oral cavity (Audus *et al.*, 1992).

Many studies have evaluated chlorhexidine gluconate for its *in vitro* antifungal properties against *C. albicans* (Giuliana *et al.*, 1997; MacNeill *et al.*, 1997; Giuliana *et al.*, 1999; Patel and Coogan, 2008). It has also been reported that pretreatment of denture acrylic surfaces with chlorhexidine resulted in reduced adherence of yeasts to the dentures (McCourtie *et al.*, 1986). In addition, sub-therapeutic concentrations of chlorhexidine reduced germ tube formation and hydrophobicity of *C. albicans* (Ellepola and Samaranayake, 1998; 2000). Triclosan has also shown to be effective against *C. albicans in vitro* (Hernandez-Richter *et al.*, 2000). The antifungal activity of chlorhexidine gluconate and triclosan on *C. albicans* strains isolated from HIV-positive patients was investigated *in vitro*. Both mouthrinses were found to be effective against the *C. albicans* isolates (Patel and Coogan, 2008).

There have been conflicting results involving the use of chlorhexidine gluconate in the treatment of candidiasis in leukemia and bone marrow transplantation patients.

These clinical trials showed that chlorhexidine was effective in decreasing *C. albicans* infection since there was a significant reduction in disease severity in patients that used the mouthrinse compared to those that used a placebo or no mouthrinse at all (Ferretti *et al.*, 1988; Thurmond *et al.*, 1991; Epstein *et al.*, 1992), however, it does not eradicate the yeast completely as seen in other clinical reports (Spijkervet *et al.*, 1989; Wahlin, 1989; Weisdorf *et al.*, 1989). A study conducted by Patel and Coogan (2008) showed that a mouthrinse containing fluoride and triclosan significantly reduced the salivary *Candida* counts in HIV positive patients compared to a mouthrinse containing chlorhexidine. Therefore more *in vivo* studies need to be performed to determine the efficacy of chlorhexidine gluconate in the treatment of oral candidiasis.

1.3.3 Drug combinations

Drug combinations have also been considered as a therapy strategy and these include combining antifungal drugs such as amphotericin B with antibiotics like tigecycline or doxycycline. These combinations have proven to be effective against planktonic cells and biofilms of *C. albicans* (Miceli *et al.*, 2009; Ku *et al.*, 2010). Another approach has been the combination of fluconazole and a pure compound isolated from pomegranate peel and this was shown to be effective against candidiasis. These approaches are advantageous since the synergistic effect increases antifungal activity as compared to individual agent activity. The benefits include broad spectrum of activity, improved safety and a reduction in antifungal resistance. However, this novel approach requires more investigation by *in vivo* studies (Endo *et al.*, 2010).

1.3.4 Natural products

1.3.4.1 Medicinal Plants

In developing countries, more than 80% of the population relies on plants for their medicinal needs (Masoko *et al.*, 2005). Natural products derived from medicinal plants are an abundant source of biologically active compounds and these have resulted in the development of new chemicals for pharmaceutical products. Many pathogens have developed resistance to many current therapeutic agents, such as antifungal drugs and antibiotics, therefore medicinal plants can be used as alternative therapeutic agents. Medicinal plants have been used traditionally for the treatment of oral diseases. Several plant-derived chemicals have been investigated for their efficacy against oral microbial pathogens (Palombo, 2009).

Numerous plant extracts have shown antibacterial properties against oral bacteria. The crude extract of *Piper cubeba* (cubeb or tailed pepper) has shown good antimicrobial activities against a number of oral pathogens (Silva *et al.*, 2007). Mastic gum is resin that is exuded by the *Pistacia lentiscus* tree and used in the preparation of foods and as a remedy for oral malodour was found to exhibit antimicrobial activity (Sterer, 2006). The antimicrobial activity of traditional South African medicinal plants used as chewing sticks against oral pathogens and *C. albicans* has also been established (More *et al.*, 2008).

It has been shown that plant extracts and organic solvent extracts of South African medicinal plants such as *Allium sativum* (garlic), *Glycyrrhiza glabra* (liquorice root), *Tulbaghia violacea* (wild garlic), *Warburgia salutaris* (pepper-bark tree) and *Dodonaea angustifolia* (sand olive) are effective against *C. albicans* infection. Several

active compounds in plants with antifungal properties have been identified. Allicin is an active compound in garlic with antimicrobial and antifungal properties. Saponin is present in many medicinal plants and has shown to have antifungal activity (Motsei *et al.*, 2003).

There has been an increase in the incidence of human systemic mycoses caused by *C. albicans* and other *Candida* species in immunocompromised individuals and therefore the number of deaths due to mycoses has increased. Antifungal agents such as amphotericin B and the azoles are expensive and many clinical strains have acquired resistance to these drugs (Masoko *et al.*, 2007). Other problems using antifungal drugs as a strategy against *C. albicans* infection include shortage of medication and patients returning with recurring symptoms therefore plants can be used as a source for new biologically active compounds (Motsei *et al.*, 2003).

1.3.4.1.1 *Dodonaea viscosa* var. *angustifolia*

Dodonaea viscosa var. *angustifolia* (Figure 1.6) belongs to the *Sapindaceae* family and is widely distributed throughout South Africa (Figure 1.7). It has many medicinal properties and has been used traditionally to treat various ailments. A mixture of the leaves and tips of the twigs are boiled in water and traditionally used to treat colds, fever, flu, measles, arthritis, sore throats, oral thrush, pneumonia, tuberculosis and externally as a soothing mixture for skin rashes (van Wyk *et al.*, 2002).

Generally, this plant species contains di- and tri- terpenes, saponins, flavonoids and a complex mixture of other phenolic compounds. Chemical studies have isolated and characterized several flavonoids, diterpenoid acids, some biologically active saponins,



Figure 1.6: Leaves and twigs of *Dodonaea viscosa* var. *angustifolia*. A: Leaves, B: Whole plant.

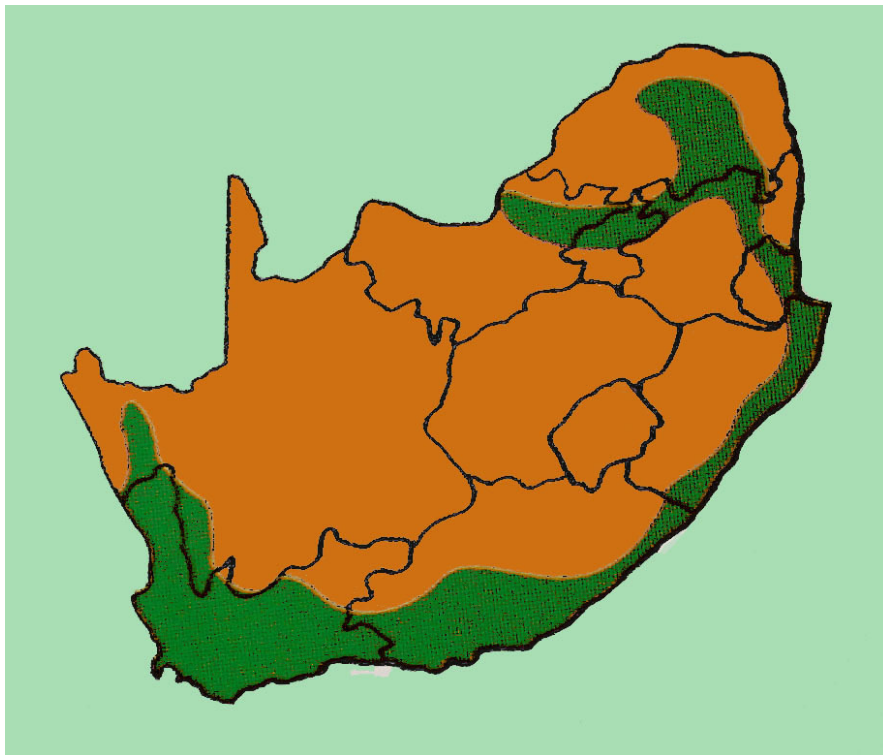


Figure 1.7: Map of South Africa with highlighted areas showing the location of *Dodonaea viscosa* var. *angustifolia* (van Wyk *et al.*, 2002).

plant acids, a novel acid ester, essential oils, sterols, and tannins from the aerial parts and seeds of this plant (Rani *et al.*, 2009).

Essential oils, aqueous and alcoholic extracts from the leaves have shown antibacterial, hypotensive and antihelmintic activities, as well as cardiac depressant and coronary-constricting properties. A saponin mixture from the seeds has shown to have phagocytosis enhancing, analgesic and molluscicidal properties. Trimethoxy flavones from leaves, seeds and bark have shown to have antiviral activity against polio-, rhino- and picorna- viruses (Rani *et al.*, 2009). Crude extracts of the leaves are effective against both HIV Type 1 and HIV Type 2 (Asres *et al.*, 2001). In addition to its antimicrobial and antibacterial activities against a wide range of microorganisms, aqueous and alcoholic extracts of this plant has shown antiulcer activity, wound healing, antioxidant, anti-inflammatory, analgesic and antipyretic properties (Amabeoku *et al.*, 2001). A leaf mixture has been used to treat tooth aches and this plant also has antifungal activity against *C. albicans* isolated from HIV-infected patients (Patel and Coogan, 2008).

Patel *et al.*, (2009) examined the effect of this plant extract on the virulence of *C. albicans*. In this study, *C. albicans* was exposed to a subinhibitory concentration of crude plant extract and mixed with a medium containing oral epithelial cells. The results showed that the extract inhibited the adherence of *C. albicans* to oral epithelial cells. The researchers postulated that the plant extract could have interfered with adhesin synthesis or mechanically disrupted adhesins of *C. albicans*. They also suggested that some cell wall damage could have occurred due to exposure to the

plant extract. Therefore this current study examined the effect of *D. viscosa* var. *angustifolia* on the ultrastructure of the *C. albicans* cell wall and biofilm formation.

1.4 Aim

The aim of this study was to determine the effect of the *Dodonaea viscosa* var. *angustifolia* extract on the *C. albicans* cell wall and biofilm formation.

1.5 Specific Objectives

- To obtain a crude acetone extract of *Dodonaea viscosa* var. *angustifolia*, determine minimum fungicidal concentration and select three subinhibitory concentrations of plant extract for further studies.
- To study the effect of the subinhibitory concentrations of plant extract on planktonic *C. albicans* cells under the transmission electron microscope.
- To determine the effect of the plant extract on germ tube formation by *C. albicans* under the light microscope.
- To examine the effect of the plant extract on *C. albicans* biofilm formation under the light microscope and scanning electron microscope.
- To analyze the molecular effect of the plant extract on the proteins of *C. albicans* involved in adherence using SDS-PAGE.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cultures

Candida albicans ATCC 90028 and two clinical isolates of *C. albicans* were obtained from the Oral Microbiology laboratory. Clinical strains were isolated from the oral cavity of HIV positive patients. Permission from The Committee for Research on Human Subjects (Medical), University of the Witwatersrand, Johannesburg (Appendix C, Figure C1, Page 116) was obtained to use these strains. *Candida albicans* strains were cultured on Sabouraud Dextrose agar (Merck, South Africa) and incubated at 37°C for 48 hours. They were identified using a Gram stain (Gram's staining kit, Diagnostic Media Products NHLS), a germ tube technique and API 32C sugar assimilation tests (Biomerieux). *Candida albicans* colonies were emulsified in phosphate buffered saline and the optical density was adjusted to 0.3 at 405 nm wavelength on a spectrophotometer which gave approximately 10^6 - 10^7 cells/ml. This suspension was used as an inoculum for all the experiments throughout the study.

2.2 Plant material

Dodonaea viscosa var. *angustifolia* plant material was collected from the Pypeklipberg, Mkhunyane Eco Reserve, Mpumalanga province of South Africa. The plant was verified as family *Sapindaceae*, *Dodonaea viscosa* var. *angustifolia* Benth by Rane Reddy, a taxonomist, from the Herbarium at the University of the Witwatersrand, Johannesburg and thereafter was used in subsequent experiments. Voucher specimen number J 94882 was deposited in the Herbarium (Patel and Coogan, 2008). For the study, twigs with leaves were collected.

2.3 Plant extraction

Leaves were dried under shade and milled to a fine powder. One gram of finely ground plant material was mixed with 10 ml acetone in McCartney bottles (United Scientific) and vortexed for five minutes. The mixture was transferred into eppendorf tubes (United Scientific) and centrifuged at 10 000 rpm for 10 minutes. The supernatant was collected and transferred into a preweighed 100 ml beaker. This process was repeated three times to allow for a thorough plant extraction. The supernatants were dispensed into the same beaker (Eloff, 1999). The solvent was then removed from the crude plant extract under a cold air stream at 7°C. The beaker was weighed again. The quantity of dry crude plant extract was calculated by subtracting the weight of empty beaker from the weight with the dry plant extract. On average, one gram of dried leaves extraction yielded 0.156 g dried crude extract. As required, the plant extract was reconstituted in acetone to obtain a final concentration of 50 mg/ml. Plant extracts were freshly prepared for each experiment.

2.4 Minimum Fungicidal Concentration study

The MFC was determined using a modified microtitre double dilution technique (Patel and Coogan, 2008). The plant extract samples were diluted 1:2 in Sabouraud Dextrose Broth (Merck, South Africa) and diluted further in seven doubling dilutions to 1:256. For each well, the quantity of crude plant extract was calculated. One hundred microlitres of each dilution was transferred into wells of a 96-well microtitre plate (Corning Incorporated, Adcock Ingram). Water instead of plant extract was included in one well as a negative control. Chlorhexidine was used in one well as a positive control. Acetone (vehicle fluid) was also used as a control. The wells were then inoculated with 100 µl of the *C. albicans* inoculum and incubated at 37°C for 48

hours. After incubation, each well was cultured onto Sabouraud Dextrose Agar plates and examined for *C. albicans* growth. The lowest dilution with no growth was considered the MFC and it was recorded as mg/ml. These experiments were repeated three times for each strain. Three concentrations below the MFC that showed some growth were selected as subinhibitory concentrations and used throughout the study. They were 3.125, 1.562, and 0.781 mg/ml and these concentrations were used in subsequent experiments.

2.5 Transmission Electron Microscopy of planktonic *Candida albicans* cells

The effect of the crude plant extract on the cell wall and cell membrane of planktonic cells was observed using a technique described by Ishida *et al.*, (2009) with minor modifications. Two millilitres of Sabouraud Dextrose Broth containing the three subinhibitory concentrations of crude plant extract was inoculated with 100 µl of 10^6 - 10^7 cfu/ml of test culture and incubated at 37°C while shaking at 60 rpm for six hours. Water instead of plant extract was used as a control. Cultures were centrifuged at 5000 rpm for 10 minutes and *C. albicans* cells were suspended in sterile distilled water. Cells were washed three times, resuspended into sterile distilled water and prepared for transmission electron microscopy. Cells were further washed in phosphate buffered saline (PBS) with pH 7.3 and fixed in a solution of 2.5% (v/v) gluteraldehyde overnight at 4°C. After fixation, yeasts were washed three times in PBS (pH 7.3) and thereafter post-fixed for two hours in 1% (v/v) osmium tetroxide at 4°C. Thereafter cells were washed for three times again in PBS (pH 7.3), dehydrated in successive ethanol washes (50%, 70%, 80%, 95% and 100%) and embedded in Spurr's Epoxy resin (Agar Scientific). Ultrathin sections (60 - 90 nm) were cut with glass knives by an ultramicrotome (ULTRACUT, Reichert Jung, Austria) thereafter

stained with uranyl acetate and lead citrate (Ishida *et al.*, 2009) and viewed under the Transmission Electron Microscope (JEOL JEM 100S, JEOL LTD, TOKYO) at 15 000X magnification. These experiments were repeated three times for all three test strains.

For each sample, 50 cells were thoroughly examined and observations were recorded. On initial observation, it was found that the samples including the control had a mixture of cells having different thicknesses of cell wall. Therefore thickness of cell wall was graded as 1+ to 3+ from thinnest to the thickest respectively (Figure 2.1, Page 39). In each sample, the number of cells with varied cell wall thickness was recorded. Cell wall measurements were also calculated by using a random number table (Appendix C, Figure C3, Page 118). This table was superimposed onto each TEM micrograph by Adobe PhotoShop (Creative Suite 1). A random sampling grid (Appendix C, Figure C2, Page 117) was then used to obtain three measurements of the cell wall. This was physically measured in millimetres using a 15 cm ruler, an average was taken from the three measurements and this measurement value was then converted to micrometres. Thereafter, an average was calculated for all the cells in each cell wall thickness category and a final measurement was obtained. The final calculated measurements were as follows; 1+ cell wall: 0.33 μ m, 2+ cell wall: 0.46 μ m and 3+ cell wall: 0.83 μ m. The number of cells in each category from the test samples was calculated and compared to the controls using statistical analysis by ANOVA, the Scheffe test and the median test. Refer to Section 2.9.3, Page 47 for full explanation of statistical analysis performed.

Damage of the cell membrane seen as an undulation was graded as 1+ to 3+, from lowest to highest respectively (Figure 2.2, Page 40). In each sample, 50 cells were randomly selected and the number of cells in each category was calculated. The number of cells in each category from the test samples was compared to the controls using statistical analysis by ANOVA, the Scheffe test and the median test. Refer to Section 2.9.4, Page 47 for full explanation of statistical analysis performed.

2.6 Effect of crude plant extract on germ tube formation by *Candida albicans*

The effect of the plant extract on germ tube formation by *C. albicans* was studied using a technique described by Mackenzie (1962) with some modifications. One millilitre of horse serum containing the three subinhibitory concentrations of crude plant extract was inoculated with 50 µl of 10^7 cfu/ml of test culture inoculum. Horse serum without plant extract was also inoculated and used as a control. Cultures were then incubated at 37°C. After three hours, culture smears were prepared on glass slides, heat-fixed and stained with Gram's crystal violet for one minute. The slides were then viewed under the light microscope at 1000X magnification (Nikon YS100). Fifty cells were examined and the number of cells with germ tube was recorded. Cells were considered germinated if they had a germ tube at least twice the length of the cell. These experiments were repeated three times for each strain and three subinhibitory concentrations. The number of yeast cells with germ tube exposed to the various test concentrations were compared to the unexposed controls using an ANOVA test and the Scheffe test. Refer to Section 2.9.5, Page 48 for full explanation of statistical analysis performed.

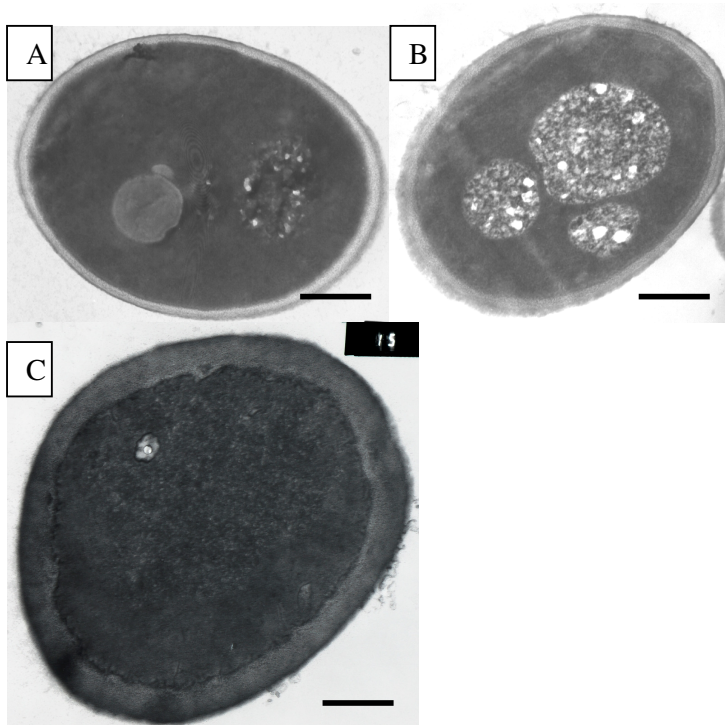


Figure 2.1: Transmission electron micrographs showing the grading of cell wall thickness. A: 1+ thickness, B: 2+ thickness, C: 3+ thickness.
Bar = 0.7 μm .

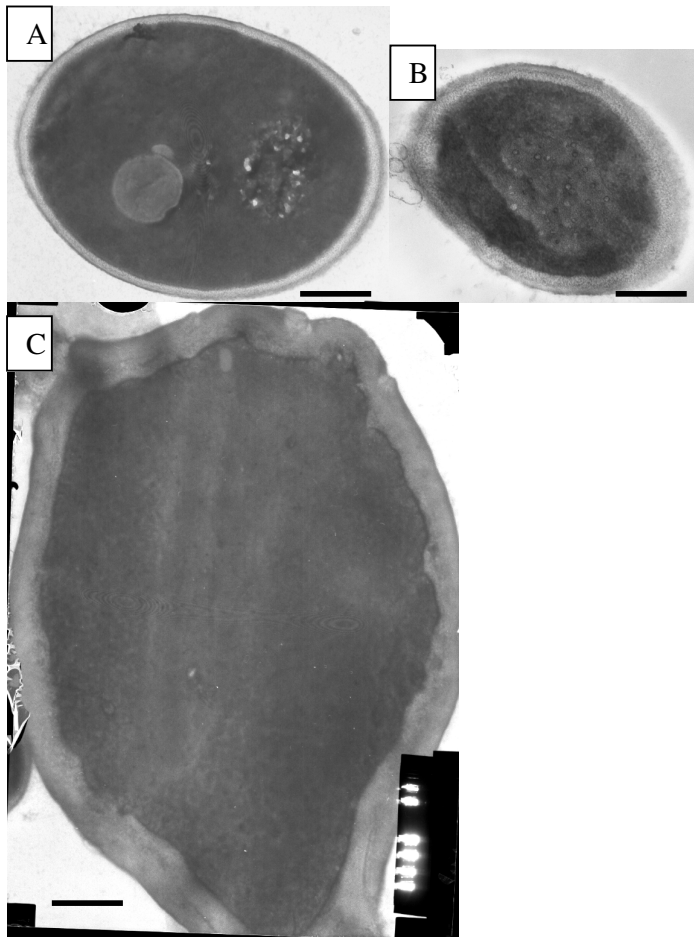


Figure 2.2: Transmission electron micrographs showing the grading of cell membrane undulation. A: 1+ undulation, B: 2+ undulation, C: 3+ undulation. Bar = 0.7 μm .

2.7 Scanning Electron Microscopy and Light Microscopy of the *Candida albicans* biofilm

The effect of the crude plant extract on biofilm formation was studied using a technique described by Bandara *et al.*, (2010) with some modifications. Four Thermanox plastic cover slips with 25 mm diameter (Nulge Nunc International) were coated with 1 ml of filter sterilized human saliva in six well tissue culture plates (costar, Corning Incorporated, Adcock Ingram) at 37°C for one hour. The saliva was then removed and a 1 ml *C. albicans* suspension containing 10^6 cfu/ml was placed into each well covering the coverslips and incubated at 37°C. Yeast cells were allowed to adhere to the plastic cover slips for three hours. The non adherent cells were removed by washing cover slips in PBS. Three coverslips with adherent yeast cells were exposed to Sabouraud Dextrose Broth containing the three subinhibitory concentrations of plant extract for a week. The fourth coverslip was covered with only Sabouraud Dextrose Broth and used as an unexposed control. Every alternate day, the medium with and without plant extract was changed. The preparation for viewing under the Scanning Electron Microscope (JEOL 840S, JEOL LTD, Tokyo) involved washing the cover slips in PBS, and thereafter fixing in 1 ml 2.5% (v/v) gluteraldehyde overnight at 4°C. The cover slips were then washed in PBS and dehydrated in a series of ethanol washes (70% for 10 minutes, 95% for 10 minutes, and 100% for 20 minutes). The cover slips underwent critical point drying then mounted onto aluminium stubs and viewed at various magnifications (Bachmann *et al.*, 2002). These experiments were repeated three times for each strain. The observations of the tests were recorded and compared to the control by visual analysis.

For the light microscopy, biofilms were grown onto cover slips as described in this section and stained with crystal violet for one minute, dried and examined at 400X magnification under a light microscope (Nikon YS100). The observations of the tests were recorded and compared to the control by visual analysis.

2.8 Protein analysis of the *Candida albicans* cell wall

The protein content of the *C. albicans* cell wall after exposure to the crude plant extract was studied by cell wall extraction and Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) described by Casanova and Chaffin (1991) with some minor modifications. Yeast cells were first exposed to two subinhibitory concentrations of plant extract i.e. 1.562 mg/ml and 0.781 mg/ml. Fifteen millilitres of Sabouraud Dextrose Broth containing the subinhibitory concentrations of plant extract was inoculated with 0.5 ml of 10^6 - 10^7 cfu/ml of test culture inoculum and incubated at 37°C while shaking at 60 rpm. Water, instead of plant extract, in Sabouraud Dextrose Broth was used as a control. Yeast cells were then harvested at 16 hours after incubation, washed three times and resuspended into 1 ml sterile distilled water. A cell count by haemocytometer was done to make the number of cells in the plant extract samples approximately equal to the number of cells in the control. The reason for this was to standardize the number of cells in each test sample hence keeping the protein extraction for each sample consistent. In all experiments, the control had approximately 5×10^5 cells/ml and the two plant extract samples were adjusted accordingly by incubating repeats therefore for concentration 1.562 mg/ml, there were three tubes added together to make up the amount of cells needed and for 0.781 mg/ml, there were two tubes added.

Cells were resuspended in 0.5 ml of solution containing ammonium carbonate (Merck, South Africa) at pH 8.64 and 1% (v/v) β -mercaptoethanol (β ME) for an incubation period of 30 minutes at 37°C. The suspension was centrifuged for 10 minutes at 6017 rpm and the supernatant (containing the β ME-extracted material) removed and stored at -20°C. β -mercaptoethanol acts as a reducing agent and reduces any disulphide bridges present that contribute to the protein structure of the cell wall and thereby makes the cell wall proteins more accessible. For this experiment; the pellet was reconstituted, washed once with 0.5 ml cold water and once with 0.5 ml of 0.6M potassium chloride (Merck, South Africa). Thereafter, the pellet was resuspended in 0.5 ml of 0.6M potassium chloride containing 0.5 mg/ml zymolyase (Inqaba Biotech) and incubated at 28°C for two hours with gentle agitation. Zymolyase is a β -glucanase that hydrolyses linkages in glucose polymers hence releasing the entire population of proteins and glycoproteins found on the surface of the yeast cell wall. Spheroplast (cells without cell walls) formation was examined microscopically thereafter. The mixture was then centrifuged for 10 minutes at 6017 rpm, the supernatant carefully removed and further centrifuged for 30 minutes at 14 680 rpm. The supernatant was removed and this extract was then used in the SDS-PAGE experiments (Casanova and Chaffin, 1991). See Figure 2.3 for illustration of method.

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis was performed on the extracted material as described by Laemmli (1970). Permission was granted by Dr Colleen Flanagan from the School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg for the use of the SDS-PAGE equipment. A 5% stacking gel was used throughout and three different percentage

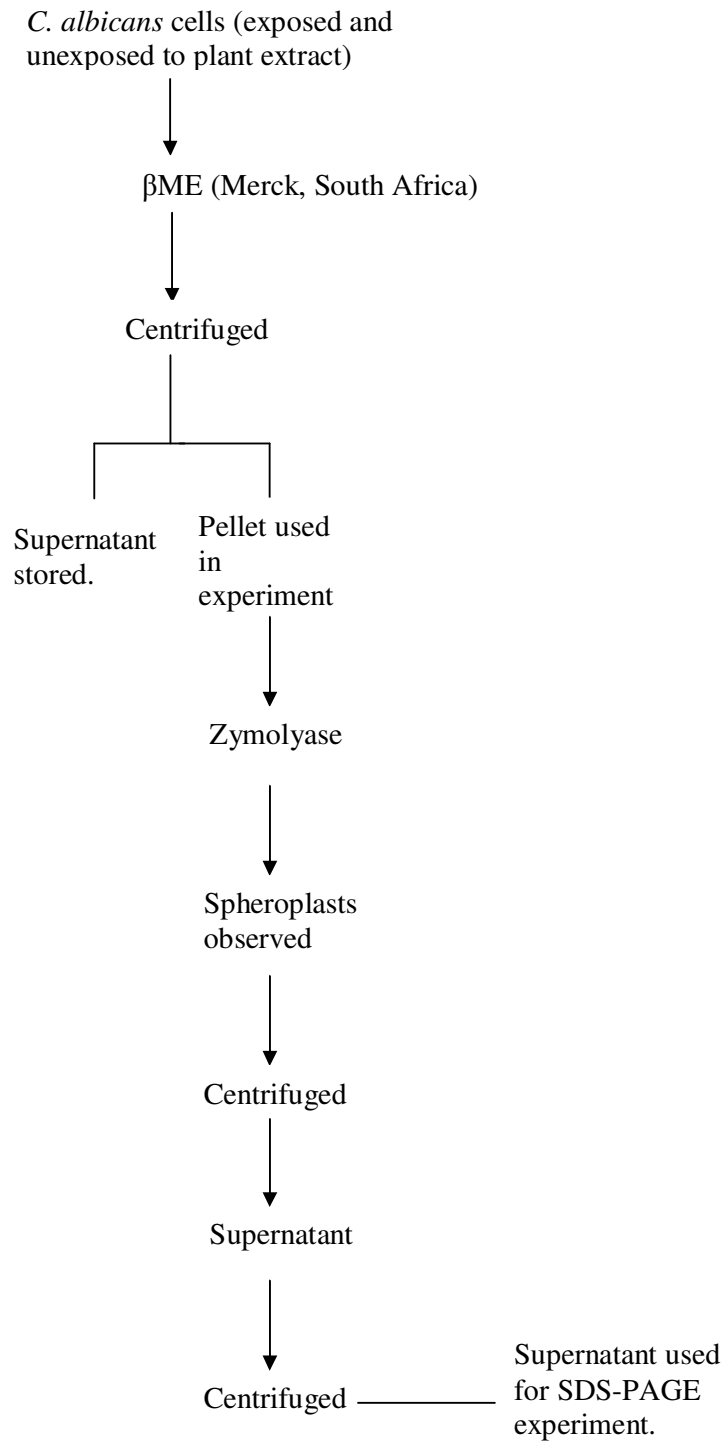


Figure 2.3: Schematic Diagram showing the method of extraction of proteins from the *Candida albicans* cell wall. See Section 2.8 for further explanation.

resolving gels (6%, 8% and 12%) were prepared to analyze the protein extracts. Each gel was made up to a final volume of 10 ml (Table 2.1).

Twenty microlitres of each sample was mixed with 20 µl of 2X SDS sample buffer (BioRad) and heated for 10 minutes on a 65 °C heating block. Thirty microlitres of each sample was loaded into separate wells of each gel. Ten microlitres of a molecular marker was also loaded into one well for each gel. The gel was run at 100 V until the dye front passed from the stacking gel into the resolving then the voltage was increased to 150 V until the dye front reached the bottom of the gel. The gels were then stained with Coomassie Brilliant Blue G (Sigma) staining solution overnight and thereafter the gels were destained with Coomassie destaining solution every hour until the protein bands were blue whereas the rest of the gel was entirely destained. For this study, it was determined that complete destaining of each gel took approximately 5 hours. The gels were viewed under a gel docking system (BioRad) and analyses of the bands on the gel were done using the Quantity One software (BioRad).

2.9 Statistical Analysis

2.9.1 Sample Size Estimation

A statistician was consulted at the beginning of the project and it was decided that five strains of *C. albicans* will be used. However, as the project progressed it was seen that counting 150 cells in total for three *C. albicans* strains for the TEM aspect of this project alone was already time-consuming and tedious. Therefore the statistician was consulted again and a sample size calculation was done on the data that had been acquired. From the frequency weights calculated for cells with 1+, 2+ and 3+ cell wall

Table 2.1: The volumes (in millilitres) of the various components needed to make up the stacking gel and the resolving gels (BioRad).

% Gel	Component Volume
5% stacking gel	Water - 6.8
	30% (v/v) acryl-bisacrylamide mix (BioRad) -1.7
	1.5M Tris buffer (pH 6.8) (Sigma) - 1.25
	10% (w/v) SDS (Sigma) - 1.0×10^{-1}
	10% (w/v) Ammonium persulphate (Sigma) - 1.0×10^{-1}
	TEMED (Sigma) - 1.0×10^{-2}
6% resolving gel	Water - 5.3
	30% (v/v) acryl-bisacrylamide mix - 2.0
	1.5M Tris buffer (pH 8.8) - 2.5
	10% (w/v) SDS - 1.0×10^{-1}
	10% (w/v) Ammonium persulphate - 1.0×10^{-1}
	TEMED - 8.0×10^{-3}
8% Resolving gel	Water - 4.6
	30% (v/v) acryl-bisacrylamide mix - 2.7
	1.5M Tris buffer (pH 8.8) - 2.5
	10% (w/v) SDS - 1.0×10^{-1}
	10% (w/v) Ammonium persulphate - 1.0×10^{-1}
	TEMED - 6.0×10^{-3}
12% Resolving gel	Water - 3.3
	30% (v/v) acryl-bisacrylamide mix - 4.0
	1.5M Tris buffer (pH 8.8) - 2.5
	10% (w/v) SDS - 1.0×10^{-1}
	10% (w/v) Ammonium persulphate - 1.0×10^{-1}
	TEMED - 4.0×10^{-3}

thickness (Appendix B, Figure B1, Page 112), 57% of cells had 3+ cell wall thickness in the control sample and 45% of cells had 3+ cell wall thickness exposed to the highest plant extract concentration (3.125 mg/ml). Therefore the effect size was 15% with alpha equal to 0.05 and power being 0.90. The eventual sample size was 380 cells in each group. It was seen that three *C. albicans* strains with three repeats for each experiment with three concentrations of plant extract would be adequate since 450 cells in each group were counted which is much more than the eventual sample size.

2.9.2 Minimum Fungicidal Concentration Study

Descriptive statistics were used for the MFC study since the median MFC was the most important value and hence calculated.

2.9.3 Effect of plant extract on *Candida albicans* cell wall

The effect of the three concentrations of plant extract and the control was compared using one - way ANOVA and then the Scheffe test was performed for pairwise comparisons after ANOVA. However, the data was not normally distributed therefore a non parametric test was performed. The non – parametric test selected was the median test. A P-value of < 0.05 was considered statistically significant.

2.9.4 Effect of plant extract on *Candida albicans* cell membrane

The effect of the three concentrations of plant extract and the control on the cell membrane was compared using one - way ANOVA and then the Scheffe test was performed for pairwise comparison after ANOVA. However, the data was not normally distributed therefore a non parametric test was performed. The non –

parametric test selected was the median test. A P-value of < 0.05 was considered statistically significant.

2.9.5 Effect of plant extract on *Candida albicans* germ tube formation

The effect of the three concentrations of plant extract and the control on germ tube formation was compared using one - way ANOVA and then the Scheffe test was performed for pairwise comparison after ANOVA. A P-value of < 0.05 was considered statistically significant.

2.9.6 Effect of plant extract on *Candida albicans* biofilm formation and protein analysis of the *Candida albicans* cell wall

There were no statistical analyses performed on the results from the effect of the plant extract on biofilm formation and the protein analysis of the *C. albicans* cell wall since these results were purely observational whereby the differences between the subinhibitory concentrations and control samples were visually recorded and compared.

CHAPTER 3: RESULTS

3.1 Identification of *Candida albicans* strains

All three test strains were successfully identified using cultural characteristics, Gram reaction, cell morphology, germ tube production and API 32C sugar assimilation tests. The results are shown in Table 3.1, Page 51. These cultures were used in subsequent experiments.

3.2 Minimum Fungicidal Concentration study

The results of the MFC of crude plant extract for each of the three strains used in the experiment are shown in Table 3.2, Page 52. There were three repeats done for each strain and the median MFC was recorded. The lowest concentration that showed no viable growth was recorded as the MFC. The aim of this project was to determine the effect of the subinhibitory concentration of plant extract on *C. albicans*, meaning the concentration that will not completely eliminate the organism but may have some effect on the cell wall and biofilm formation. Therefore three concentrations lower than the MFC were selected. The three subinhibitory concentrations used in the subsequent experiments were 3.125 mg/ml, 1.562 mg/ml and 0.781 mg/ml. Water and acetone had no effect on the *C. albicans* strains. There was no growth in the chlorhexidine well.

3.3 Transmission Electron Microscopy of planktonic *Candida albicans* cells

The effect of the plant extract on the cell wall of planktonic *C. albicans* cells was studied. On first observation, it was noted that the cell wall of *C. albicans* cells in the control (unexposed to the plant extract) samples did not have a uniform cell wall

thickness therefore grading of the thickness of cell wall was necessary. Fifty cells per sample were randomly selected and the cell wall thickness was graded as 1+ being the thinnest, 2+ being intermediate thickness and 3+ the thickest. The number of cells in each category was recorded (Table 3.3, Page 53). Three repeats were performed for each strain therefore these results are reproducible and accurate. The results for the three strains were added together. The sum, mean and standard deviation (SD) values were calculated. The mean number of cells within each category of grading for cell wall thickness of both control and test samples are represented as a percentage in Figure 3.1, Page 54.

The results showed that many plant exposed cells had a thin cell wall compared to the control samples. This can be deduced further by Figure 3.2, Page 55 and Figure 3.3, Page 56. Similarly, the number of cells with a thick cell wall decreased after exposure to the plant extract. Cells with intermediate thickness of cell wall also decreased in plant exposed cells. It can also be seen that as the concentration of plant extract decreased, the number of cells with a thin cell wall increased and the cells with a thick cell wall decreased. This suggests that the plant extract had reduced the thickness of cell wall of *C. albicans* and the effect increased with a decrease in concentration. These changes were statistically significant ($P < 0.0001$).

Table 3.1: Identification of *Candida albicans* strains.

Strain	Cultural characteristics on Sabouraud dextrose agar	Gram reaction	Germ tube production	API 32C Profile	Identification
ATCC 90028	Spherical, creamy-white colonies with a beer-like aroma.	Purple, round oval cells with budding.	Positive	6566130	88% <i>Candida albicans</i>
A71 clinical isolate	Spherical, creamy-white colonies with a beer-like aroma.	Purple, round oval cells with budding.	Positive	6576130	75.9% <i>Candida albicans</i>
A79 clinical isolate	Spherical, creamy-white colonies with a beer-like aroma.	Purple, round oval cells with budding.	Positive	2542130	96.8% <i>Candida albicans</i>

Table 3.2: Minimum Fungicidal Concentration of the crude plant extract against *Candida albicans*.

Strain	Acetone extract	Minimum Fungicidal Concentration (mg/ml)	Median Minimum Fungicidal Concentration (mg/ml)
ATCC90028	1	6.25	
	2	6.25	
	3	12.5	6.25
A71	1	6.25	
	2	6.25	
	3	6.25	6.25
A79	1	6.25	
	2	6.25	
	3	6.25	6.25

Negative control: Water – Growth of *Candida albicans*

Positive control: Chlorhexidine – No growth of *Candida albicans*

Vehicle fluid: Acetone – Growth of *Candida albicans*

Table 3.3: The effect of different concentrations of *Dodonaea viscosa* var. *angustifolia* on the *Candida albicans* cell wall.

	Number of <i>Candida albicans</i> cells/50 cells											
Strains	Control (water)			3.125 mg/ml			1.562 mg/ml			0.781 mg/ml		
	1+	2+	3+	1+	2+	3+	1+	2+	3+	1+	2+	3+
ATCC												
90028	3	5	42	12	13	25	18	11	21	26	16	8
	7	11	32	11	10	29	22	8	20	26	10	14
	7	16	27	12	10	28	21	12	17	24	10	16
A71	14	22	14	17	16	17	31	9	10	31	9	10
	8	22	20	13	14	23	23	14	13	16	17	17
	3	19	28	20	12	18	18	17	15	30	14	6
A79	2	16	32	6	13	31	12	17	21	23	8	19
	4	12	34	6	10	34	20	14	16	22	11	17
	3	18	29	2	13	35	19	10	21	18	7	25
Sum	51	141	258	99	111	240	184	112	154	216	102	132
Mean	5.7	15.7	28.7	11	12.3	26.7	20.4	12.4	17.1	24	11.3	14.7
±SD	±14.8	±5.5	±8.1	±5.6	±2.1	±6.5	±5.1	±3.3	±4	±5	±3.5	±5.9

Where 1+: Thinnest cell wall, 2+: Intermediate thickness, 3+: Thickest cell wall

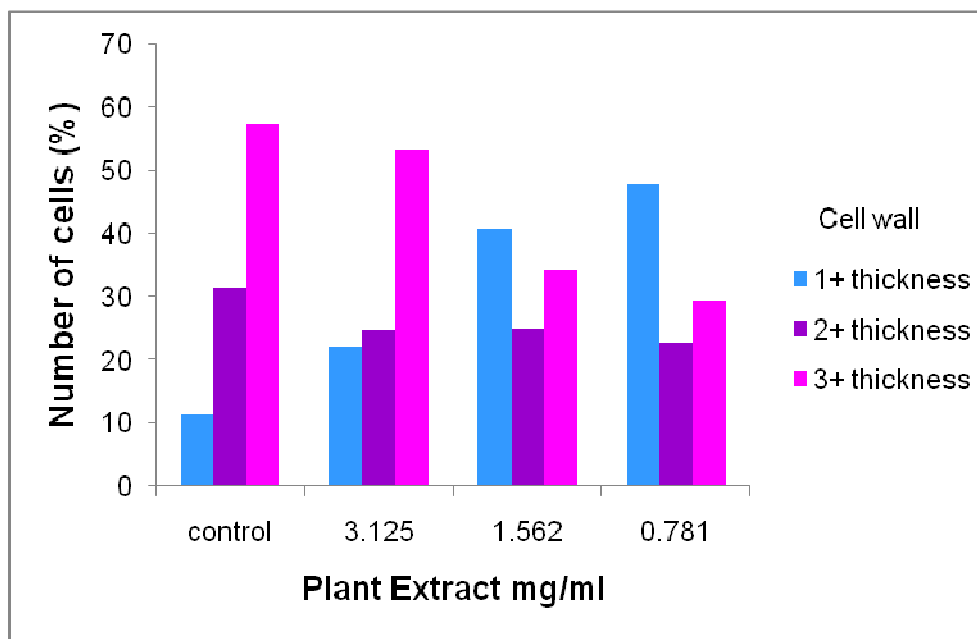


Figure 3.1: The effect of different concentrations of *Dodonaea viscosa* var. *angustifolia* on the cell wall of *Candida albicans* cells.

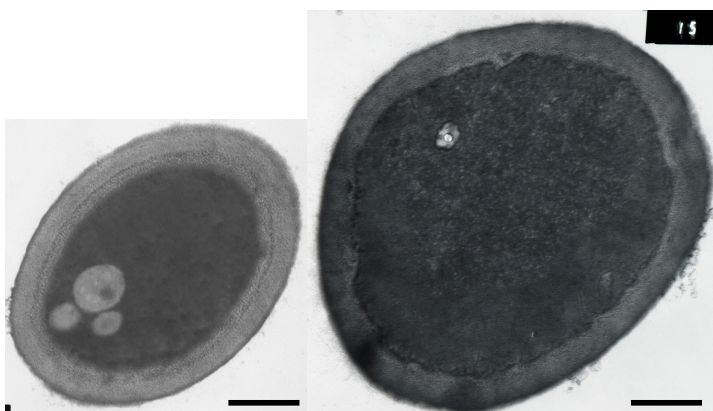


Figure 3.2: *Candida albicans* cells with 3+ cell wall thickness that were predominately observed in the control samples. Bar = 0.7 μm .

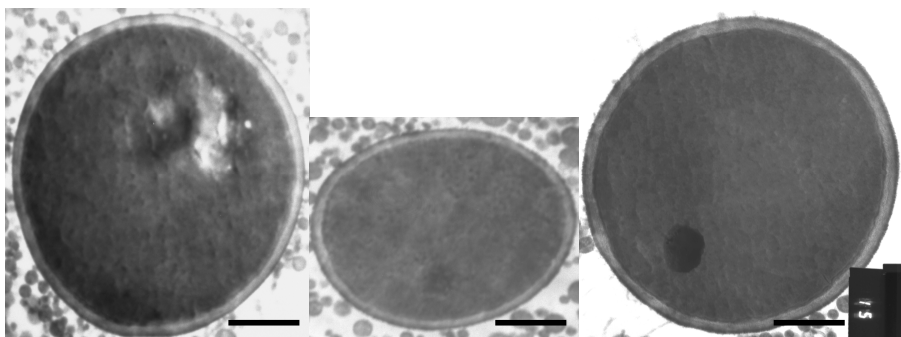


Figure 3.3: *Candida albicans* cells with 1+ cell wall thickness that were predominately seen in the test samples. Bar = 0.7 μm .

The effect of the plant extract on the cell membrane of planktonic *C. albicans* cells was studied. Cell membrane damage was measured by determining membrane undulation. The undulation of cell membrane varied even in the control samples therefore grading became necessary. Fifty cells per sample were randomly selected and the cells were graded as 1+ being minimal, 2+ being intermediate and 3+ being severe undulation. The number of cells within each category of grading in each sample including the control was recorded (Table 3.4, Page 59). Three repeats were performed for each strain therefore these results are reproducible and accurate. The sum, mean and standard deviation (SD) for each sample was calculated. The number of cells within each category of grading for cell membrane undulation of both control and test samples are represented as a percentage in Figure 3.4, Page 60. Data from each concentration was compared with the control and the results showed that the changes caused by the plant extract were statistically significant ($P < 0.0001$).

The results showed that the number of cells with minimal undulation decreased in plant extract exposed *C. albicans* cells compared to the cells in the control samples. This can be deduced further by the TEM micrographs in Figure 3.5, Page 61 and Figure 3.6, Page 62. Cells with severe undulation increased in cells exposed to the plant extract. There were minimal changes in cells with intermediate undulation exposed to the plant extract. The results also showed that the effect is not concentration dependent because the number of cells with severe undulation in each test concentration is similar. These results suggest that the subinhibitory concentrations of crude plant extract causes damage to the cell membrane of *C. albicans*.

Candida albicans cells in each test sample were further examined for any cellular changes compared to the cells in the control samples. The results showed that there was a reduction in the density of the cytoplasm in the cells treated with the plant extract compared to the untreated cells. Copper grids were viewed at various magnifications and the observations are shown in Figure 3.7, Page 63. Visual observations show that the plant extract also has an effect on the cytoplasm of *C. albicans* cells and some of the cells had disrupted cell wall (Figure 3.7 b and Figure 3.7 d).

Table 3.4: The effect of different concentrations of *Dodonaea viscosa* var. *angustifolia* on the *Candida albicans* cell membrane.

	Number of <i>Candida albicans</i> cells/50 cells											
Strains	Control (water)			3.125 mg/ml			1.562 mg/ml			0.781 mg/ml		
	1+	2+	3+	1+	2+	3+	1+	2+	3+	1+	2+	3+
ATCC												
90028	44	5	1	22	14	14	12	14	24	23	11	16
	44	6	0	24	14	12	28	8	14	18	10	22
	47	3	0	9	15	26	14	15	21	13	13	24
A71	44	5	1	18	17	15	3	18	29	7	3	40
	43	7	0	3	9	38	6	15	29	12	7	31
	46	4	0	3	4	43	5	12	33	5	6	39
A79	43	4	3	1	6	43	2	4	44	1	7	42
	46	4	0	3	5	42	3	8	36	11	6	33
	46	3	1	4	7	39	6	10	37	8	6	36
Sum	403	41	6	87	91	272	79	104	267	98	69	283
Mean	44.8	4.5	0.7	9.7	10.1	30.2	8.8	11.5	29.7	10.9	7.7	31.4
±SD	±1.5	±1.3	±1	±9.1	±4.9	±13.4	±8.3	±4.4	±9.1	±6.7	±3.1	±9

Where 1+: Minimal undulation, 2+: Intermediate undulation, 3+: Severe undulation

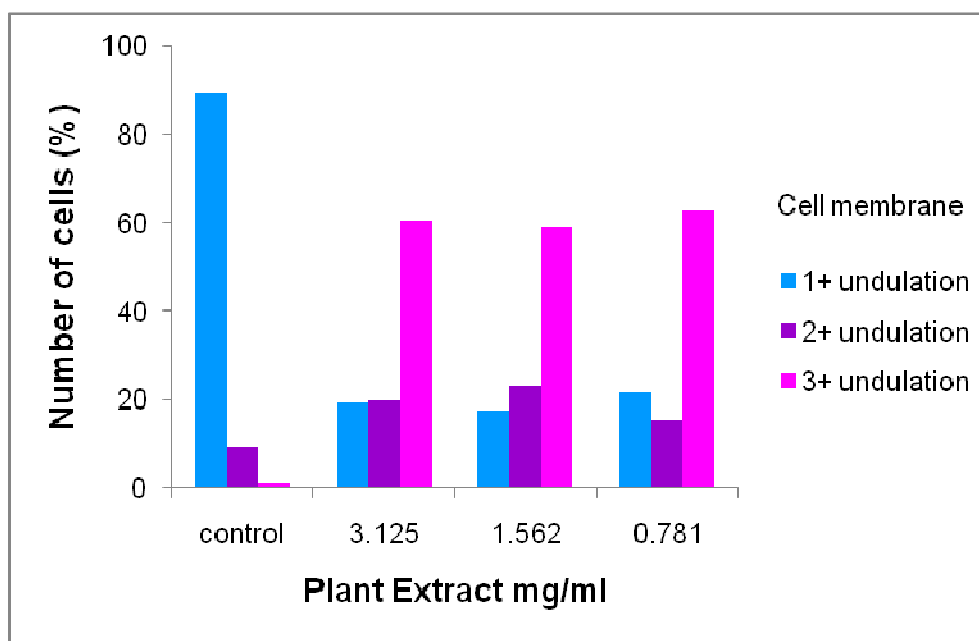


Figure 3.4: The effect of different concentrations of *Dodonaea viscosa* var. *angustifolia* on the cell membrane of *Candida albicans* cells.

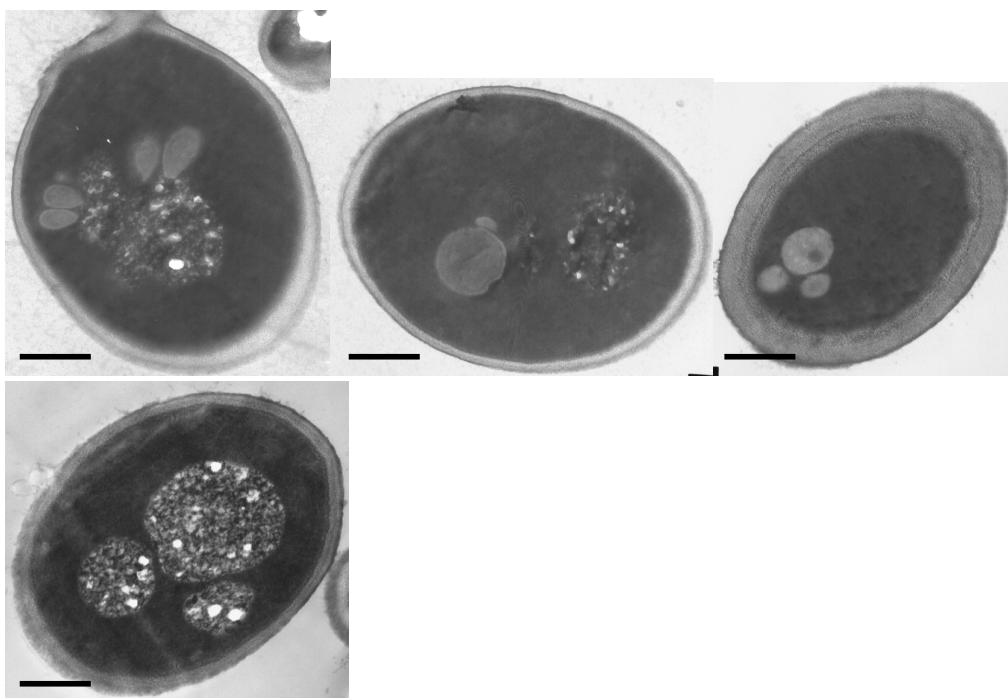


Figure 3.5: *Candida albicans* cells with 1+ cell membrane were predominately observed in the control samples. Bar = 0.7 μm.

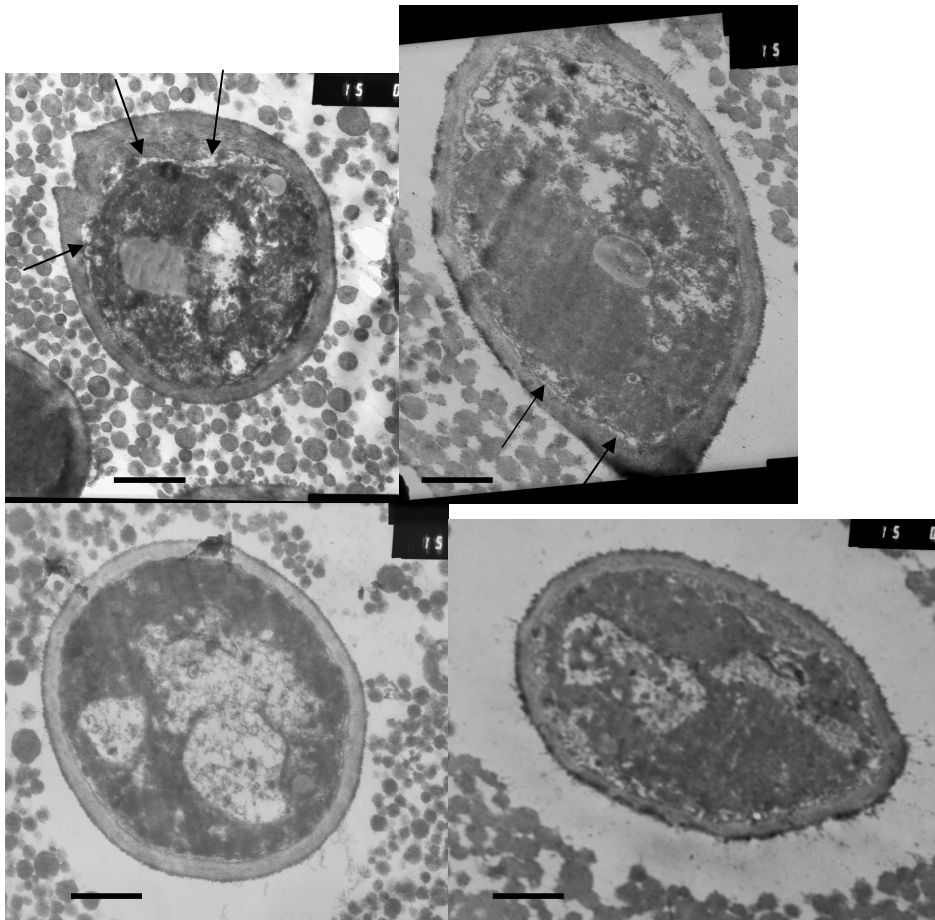


Figure 3.6: *Candida albicans* cells with 3+ cell membrane undulation were predominately observed in the test samples. Bar = 0.7 μm . Arrows indicate severe undulation and detachment of cell membrane from cell wall.

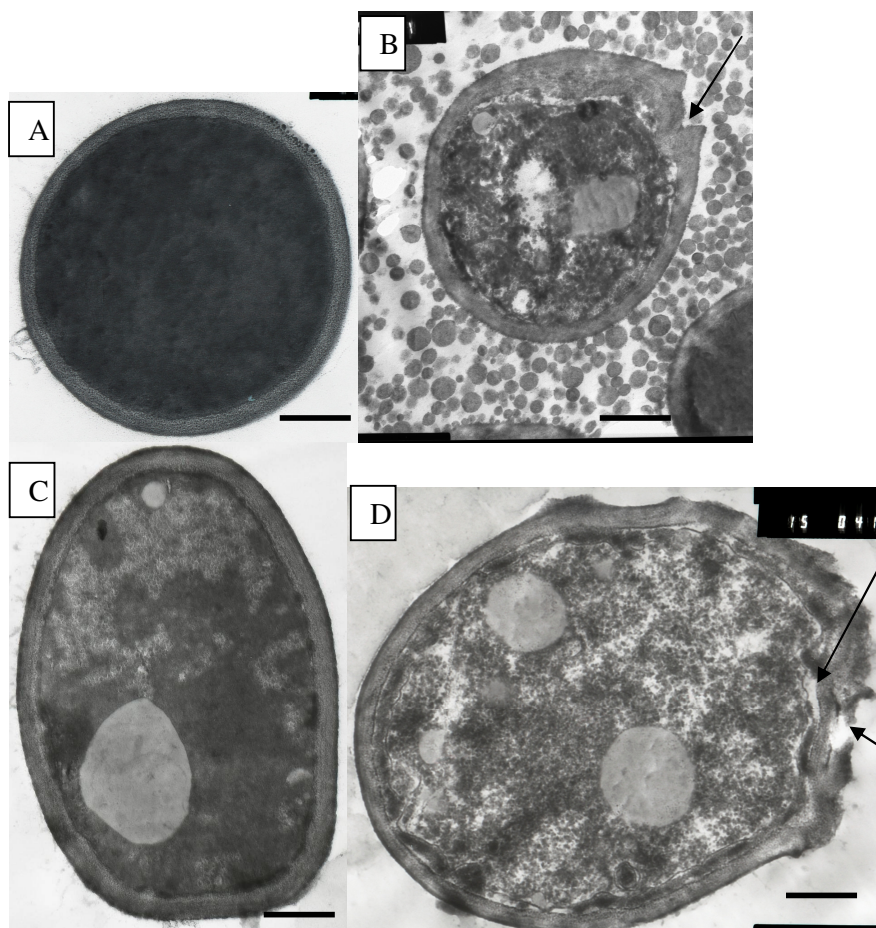


Figure 3.7: Transmission electron micrographs of cytoplasmic changes when cells were exposed to subinhibitory concentrations of plant extract. A: control, B and C: plant extract. Arrows in B and D indicate cell wall breakage, disruption and detachment of cell membrane from cell wall. Bars in A = 0.5 μm , and B, C and D = 0.7 μm .

3.4 Effect of *Dodonaea viscosa* var. *angustifolia* on germ tube formation

The effect of the plant extract on germ tube formation of planktonic cells was studied. Three repeats were performed for each strain therefore these results are reproducible and accurate. In each experiment, the number of cells with germ tube from 50 cells was recorded and converted into a percentage. The results of the three test strains are listed in Table 3.5, Page 65 and graphically represented in Figure 3.8, Page 66. The combined results of all three strains are represented in Figure 3.9, Page 67. The number of cells with germ tube in the plant exposed samples was compared with the cells in the control samples.

The results showed that a reduced number of *C. albicans* cells produced germ tubes in the presence of plant extract compared to the control. All three strains behaved similarly. The reduction in germ tube formation by plant extract was statistically significant with a P value of < 0.0001 and an Fstat value of 121.73 (df = 35). The effect of the inhibition of germ tube formation by the plant extract was concentration dependent. An increase in concentration of plant extract increased the inhibition of germ tube formation.

Table 3.5: The effect of crude plant extract on the germ tube formation of *Candida albicans*.

	No. of <i>Candida albicans</i> cells with germ tube/50 cells (%)			
Strain	Control	3.125 mg/ml	1.562 mg/ml	0.781 mg/ml
ATCC 90028	23 (46)	5 (10)	7 (14)	14 (28)
	20 (40)	3 (6)	5 (10)	19 (38)
	21 (42)	2 (4)	9 (18)	16 (32)
A71	25 (50)	2 (4)	10 (20)	18 (36)
	20 (40)	6 (12)	9 (18)	17 (34)
	27 (54)	3 (6)	14 (28)	19 (38)
A79	18 (36)	4 (8)	9 (18)	14 (28)
	25 (50)	2 (4)	7 (14)	16 (32)
	26 (52)	3 (6)	8 (16)	18 (36)
Mean	23 (46)	3 (7)	7 (17)	17 (21)
±SD	±3.1 (6.3)	±1.4 (2.8)	±2.5 (5)	±1.9 (8.3)

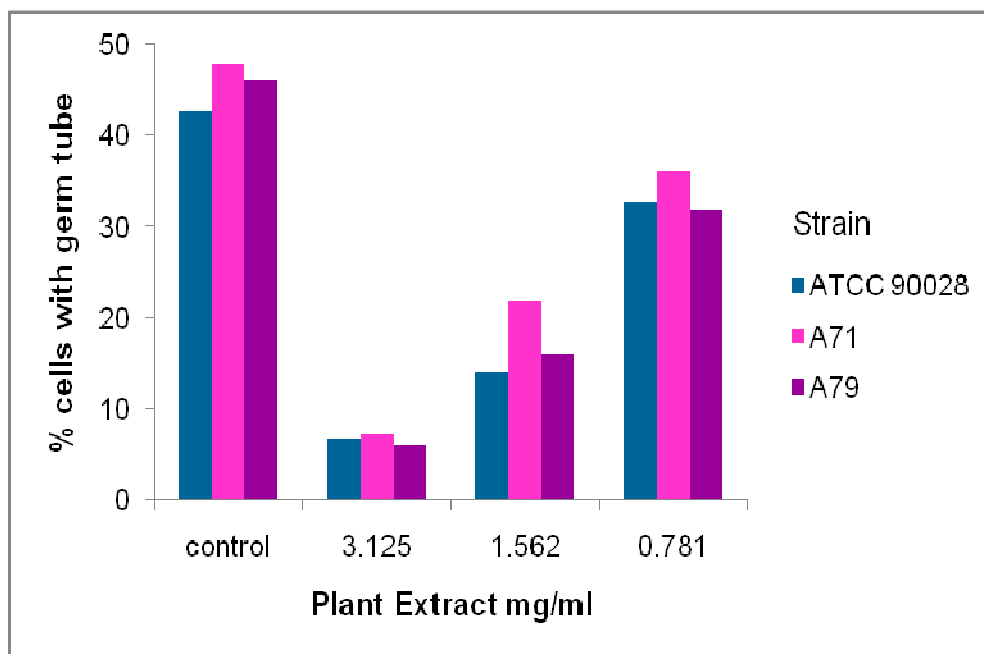


Figure 3.8: The percentage of cells for three *Candida albicans* strains that formed germ tubes with and without exposure to the three subinhibitory concentrations of crude plant extract.

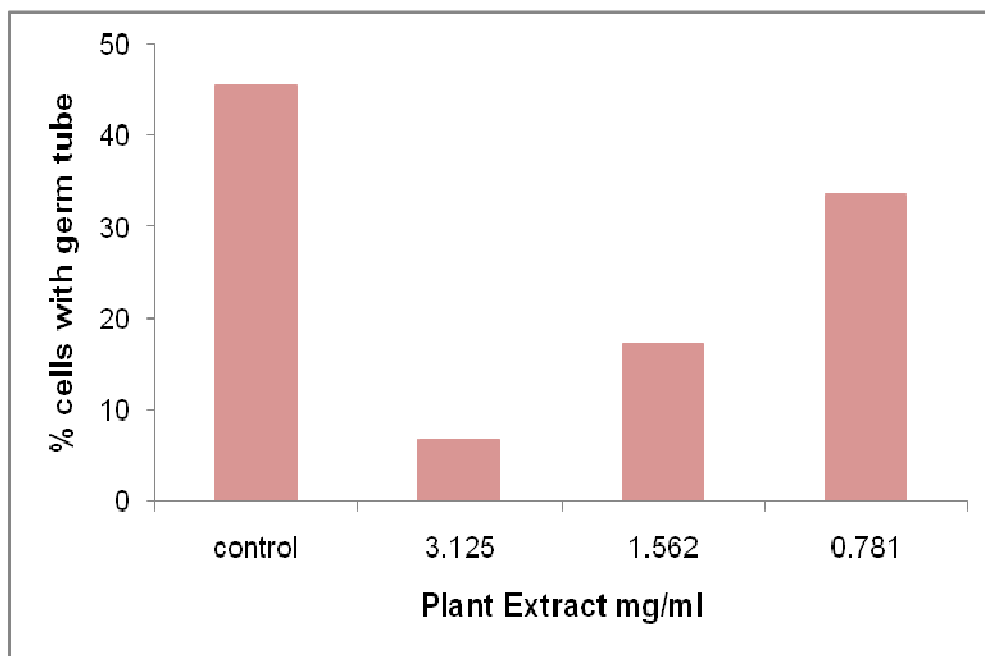


Figure 3.9: The effect of crude plant extract on the germ tube formation by *Candida albicans* cells.

3.5 Effect of *Dodonaea viscosa* var. *angustifolia* on *Candida albicans* biofilm formation

3.5.1 Light Microscopy

The effect of the plant extract on *C. albicans* biofilm formation was studied under the light microscope and pictures were taken capturing various areas on a plastic cover slip. The observations are shown in Figures 3.10 to 3.13, Pages 69 to 72. The control biofilm had confluent growth with extensive hyphae formation whereas the biofilm grown in the presence of plant extract had reduced adherent cells with short germ tubes. The three subinhibitory concentrations showed similar results. These results suggest that the plant extract reduced biofilm and hyphae formation. All three strains behaved similarly.

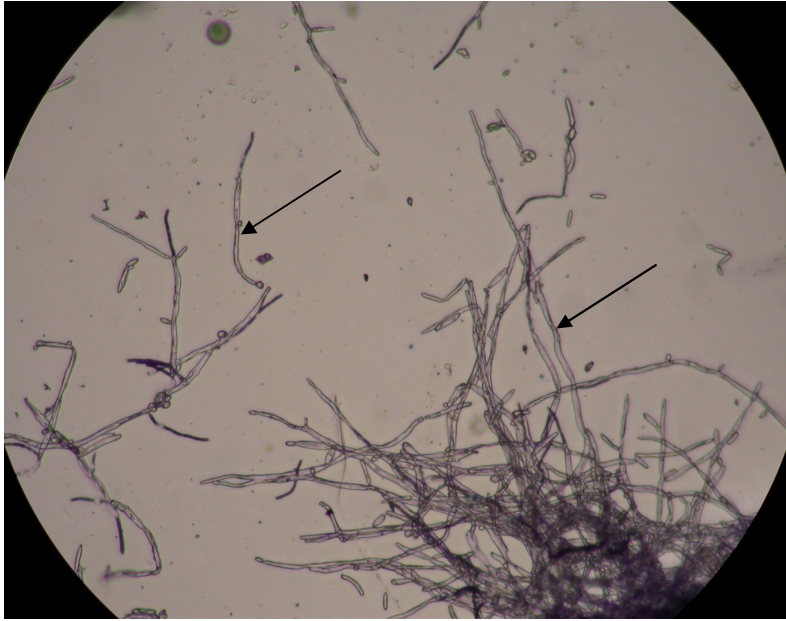


Figure 3.10: Light micrograph at 400X magnification depicting the *Candida albicans* biofilm grown in the absence of plant extract (control). Arrows show extensive hyphae formation by *Candida albicans*.

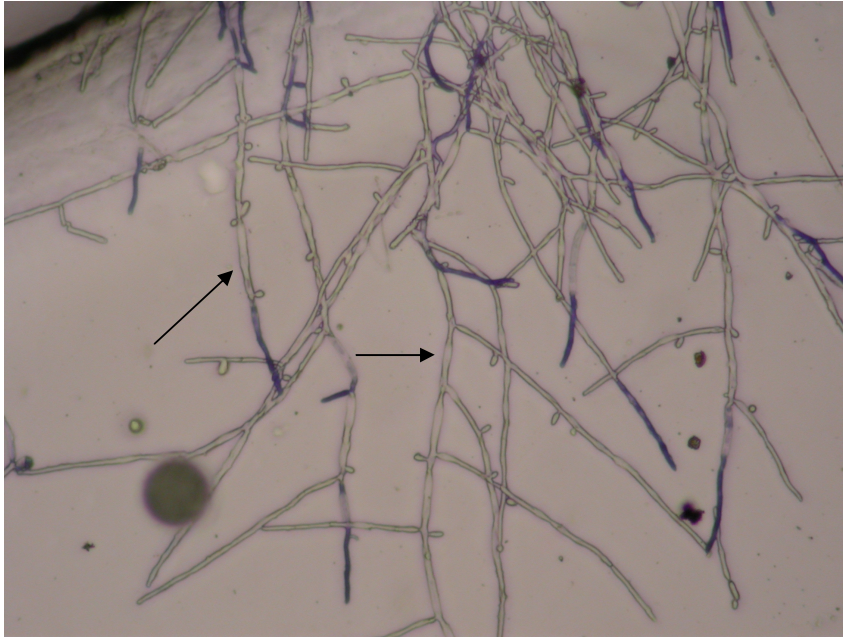


Figure 3.11: Light micrograph at 400X magnification depicting the *Candida albicans* biofilm grown in the absence of plant extract (control). Arrows show extensive hyphae formation by *Candida albicans*.

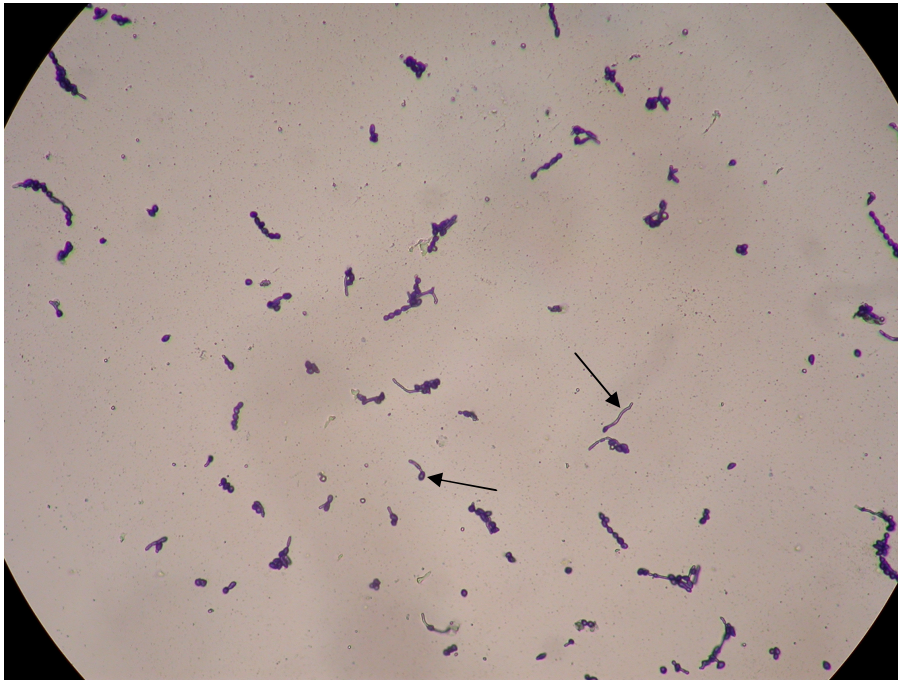


Figure 3.12: Light micrograph at 400X magnification illustrating the effect of 3.125 mg/ml crude plant extract of *Dodonaea viscosa* var. *angustifolia* on *Candida albicans* biofilm formation. Arrows show *Candida albicans* cells with short hyphae.

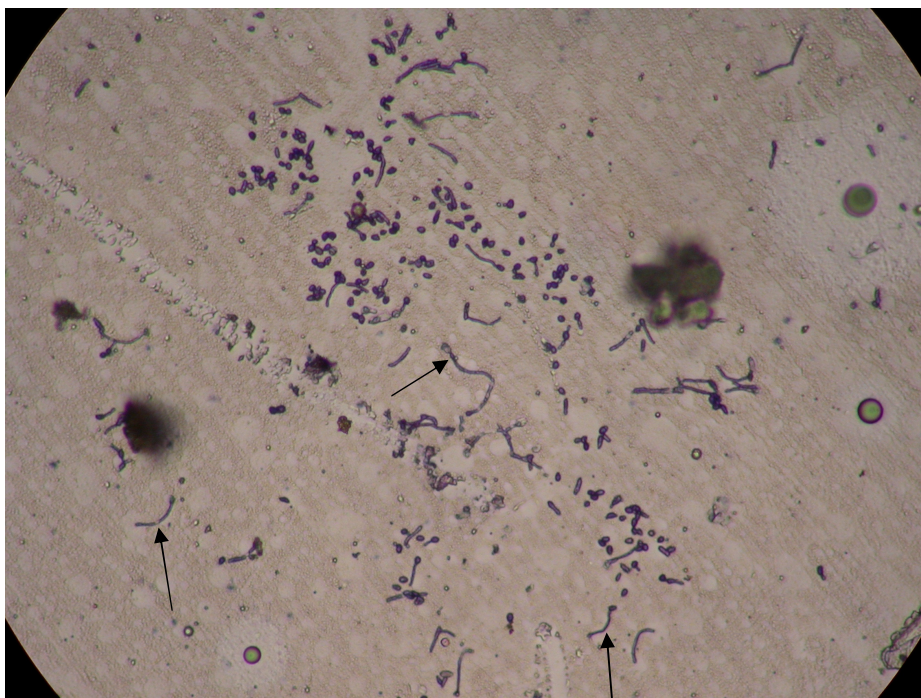


Figure 3.13: Light micrograph at 400X magnification illustrating the effect of 1.562 mg/ml crude plant extract of *Dodonaea viscosa* var. *angustifolia* on *Candida albicans* biofilm formation. Arrows show *Candida albicans* cells with short hyphae.

3.5.2 Scanning Electron Microscopy

The effect of the plant extract on biofilm formation was also studied using scanning electron microscopy. The results are shown in Tables 3.6 to 3.8, Pages 75 to 77. Biofilm formation was reduced by the plant extract and it was concentration dependent. As the concentrations increased, biofilm formation and hyphae formation decreased. The adherent cells in the test samples had two or three times reduced hyphal formation compared to the unexposed cells in the control sample. The plant extract exposed cells were morphologically different and abnormal compared to the control cells. Control cells had extracellular polymers present surrounding cells which were maintaining the biofilm structure and adhering it to the surface. These polymers were absent in the test samples. All three strains showed similar results.

The results for *C. albicans* strain ATCC 90028 are shown in Table 3.6, Page 75, the control biofilm on plastic coverslips and the cover slips exposed to the crude plant extract of *D. viscosa* var. *angustifolia* at concentrations 3.125 mg/ml, 1.562 mg/ml and 0.781 mg/ml are shown. It can be seen visually that the plant extract has an effect on biofilm formation of *C. albicans* at all concentrations compared to the control. A decrease in plant extract concentrations increased the adherent cells and hyphae formation.

The results for *C. albicans* strain A71 are shown in Table 3.7, Page 76, the control biofilm on plastic coverslips and the cover slips exposed to the crude plant extract of *D. viscosa* var. *angustifolia* at concentrations 3.125 mg/ml, 1.562 mg/ml and 0.781 mg/ml are shown. It can be seen visually that the plant extract has an effect on biofilm

formation of *C. albicans* at all concentrations compared to the control. A decrease in plant extract concentration increased the adherent cells and hyphae formation.

The results for *C. albicans* strain A79 are shown in Table 3.8, Page 77, the control biofilm on plastic coverslips and the cover slips exposed to the crude plant extract of *D. viscosa* var. *angustifolia* at concentrations 3.125 mg/ml, 1.562 mg/ml and 0.781 mg/ml are shown. It can be seen visually that the plant extract has an effect on biofilm formation of *C. albicans* at all concentrations compared to the control. A decrease in plant extract concentrations increased the adherent cells and hyphae formation.

Additional images were captured illustrating the extracellular polymeric material (EPM) that was only seen in the control and this is shown in Figure 3.14, Page 78. Cells that showed an abnormal cell shape were found only on cover slips exposed to plant extract at all test concentrations (Figure 3.15, Page 79 and Figure 3.16, Page 80). Extracellular debris was also seen on the cover slips exposed to plant extract but this was assumed to be plant material that precipitated with the growth medium (Figure 3.15, Page 79 and Figure 3.16, Page 80).

Table 3.6: Scanning Electron Microscope micrograph results for *Candida albicans* strain ATCC 90028 showing the effect of the crude plant extract at three subinhibitory concentrations on biofilm formation.

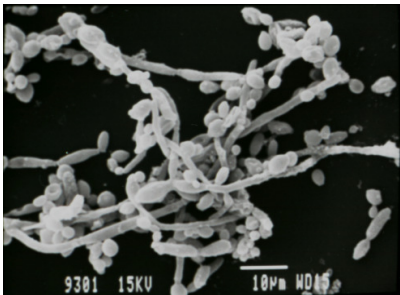
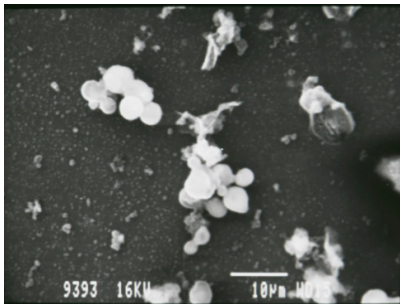
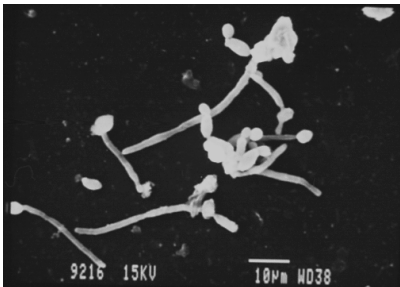
Control	3.125 mg/ml	1.562 mg/ml	0.781 mg/ml
 Note extensive hyphae formation and the quantity of cells in the biofilm are also high. Magnification: 1700X.	 Note no hyphae formation and some abnormal cells. Magnification: 2000X.	 Note cells with short hyphae. Magnification: 2200X.	 Note the long hyphae and increased quantity of biofilm. Magnification: 1500X.

Table 3.7: Scanning Electron Microscope micrograph results for *Candida albicans* strain A71 showing the effect of the crude plant extract at three subinhibitory concentrations on biofilm formation.

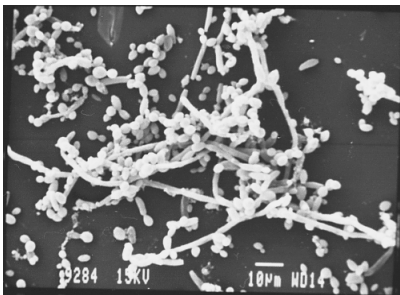
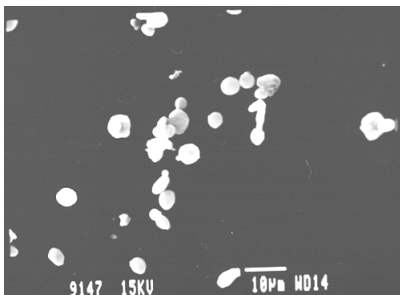
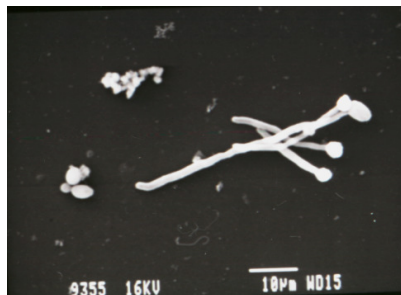
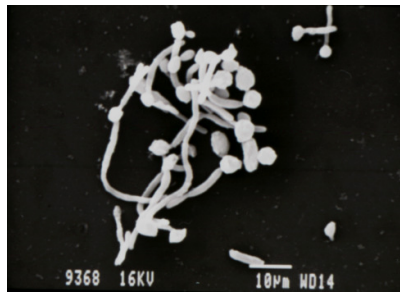
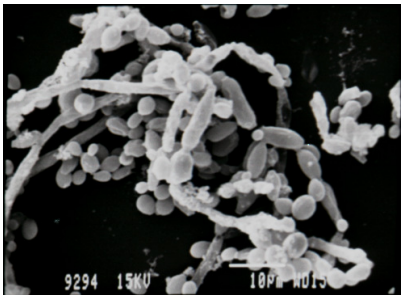
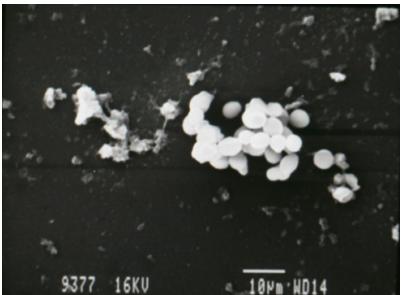
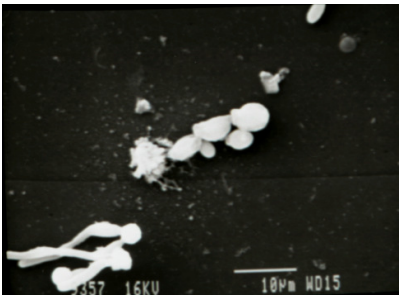
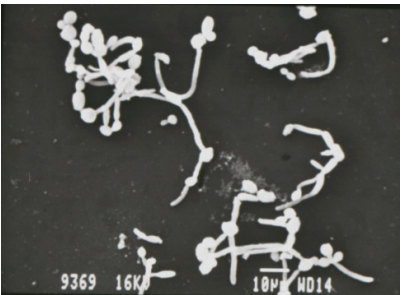
Control	3.125 mg/ml	1.562 mg/ml	0.781 mg/ml
 Note the extensive hyphae formation. Quantity of cells in the biofilm is also high. Magnification: 1000X.	 Note short hyphae formation and cells with abnormal shape. Magnification: 1500X.	 Note hyphae formation. Magnification: 1700X.	 Note long hyphae formation. Magnification: 1500X.

Table 3.8: Scanning Electron Microscope micrograph results for *Candida albicans* strain A79 showing the effect of the crude plant extract at three subinhibitory concentrations on biofilm formation.

Control	3.125 mg/ml	1.562 mg/ml	0.781 mg/ml
 Note the extensive hyphae formation. Quantity of cells in the biofilm is also high. Magnification: 2000X.	 Note no hyphae formation and some abnormal cells. Magnification: 1500X.	 Note short hyphae and some abnormal cells. Magnification: 2200X.	 Note long hyphae and increased quantity of biofilm. Magnification: 1000X.

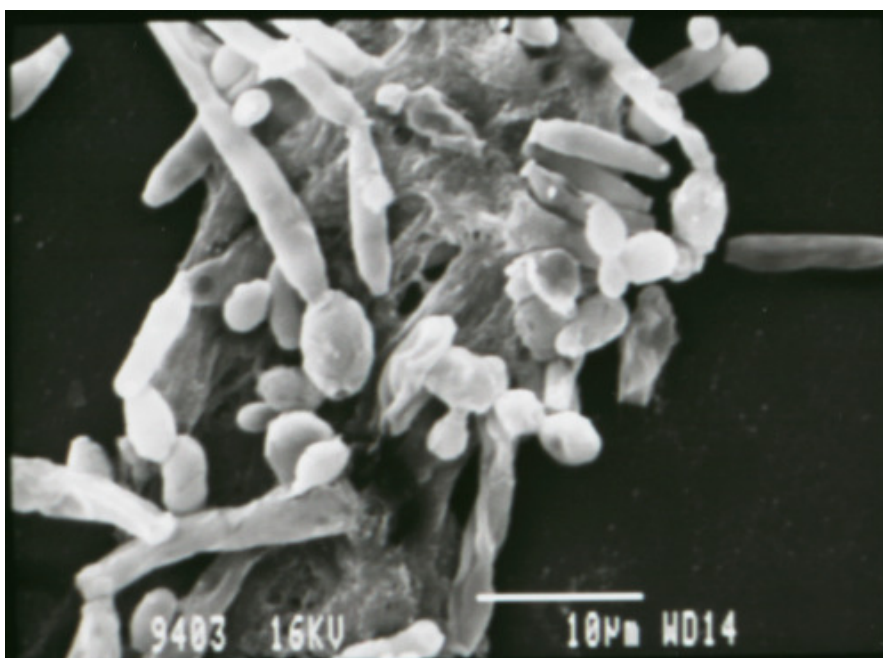


Figure 3.14: Scanning Electron Microscope Micrograph illustrating the extracellular polymeric material of a *Candida albicans* biofilm in the control samples.

Magnification: 2700X.

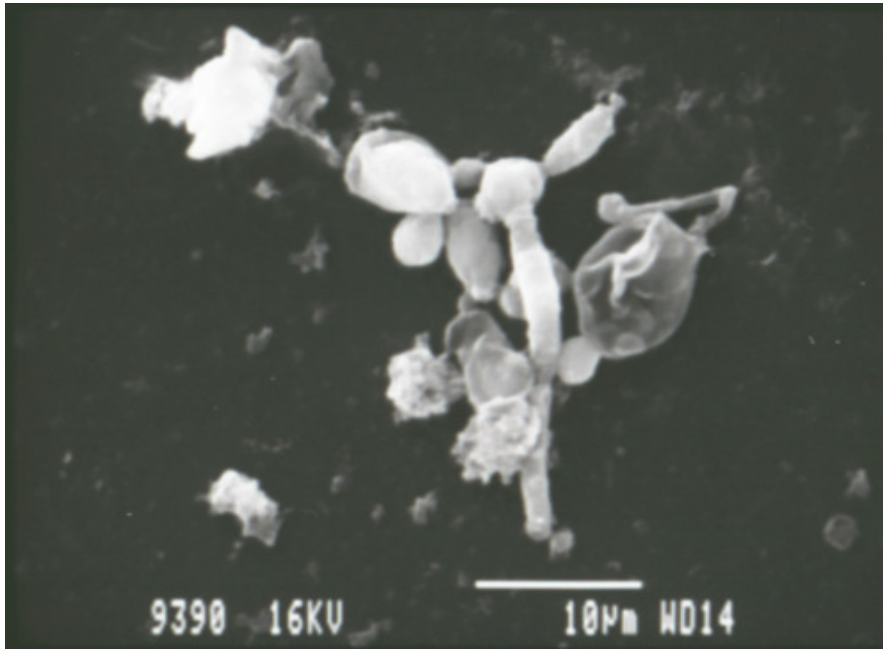


Figure 3.15: Scanning Electron Microscope Micrograph showing the effect of the crude plant extract of *Dodonaea viscosa* var. *angustifolia* on *Candida albicans* biofilm formation. Note the abnormal cell shape and debris. Magnification: 2700X.

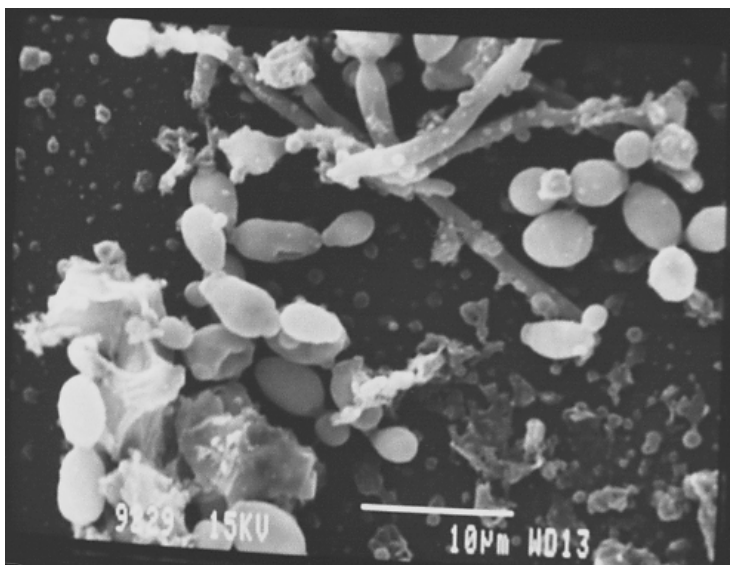


Figure 3.16: Scanning Electron Microscope Micrograph showing the effect of the crude plant extract of *Dodonaea viscosa* var. *angustifolia* on *Candida albicans* biofilm formation. Note the abnormal cell shape and debris. Magnification: 3000X.

3.6 Effect of the crude plant extract on cell wall proteins of *Candida albicans*

The cell wall proteins from the three *C. albicans* strains were extracted from both the control and yeast cells exposed to the crude plant extract. Three gels were run and the acrylamide concentration of the resolving gel only differed i.e. 6%, 8% and 12%. The acrylamide concentration of the stacking gel remained constant at 5%. The effect of the plant extract on the cell wall proteins (adhesins) was visually observed by comparing the cell wall proteins present in the control sample and the cell wall proteins present in the test samples on the different percentage gels and this is represented in Figures 3.17 to 3.19, Pages 82 to 84.

The results from the three gels (Figures 3.17 to 3.19, Pages 82 to 84) illustrated that the proteins extracted from the control sample and the test samples were the same for all strains. It was seen that there were approximately 20 bands that ranged between 9kDa and 56kDa (Table 3.9, Page 85) in all the test samples as well as the control sample for the three *C. albicans* strains. It was deduced that there was no difference between the proteins present in extracted cell walls of exposed and unexposed *C. albicans* cells, which suggest that no adhesins were removed from the cell wall by the plant extract.

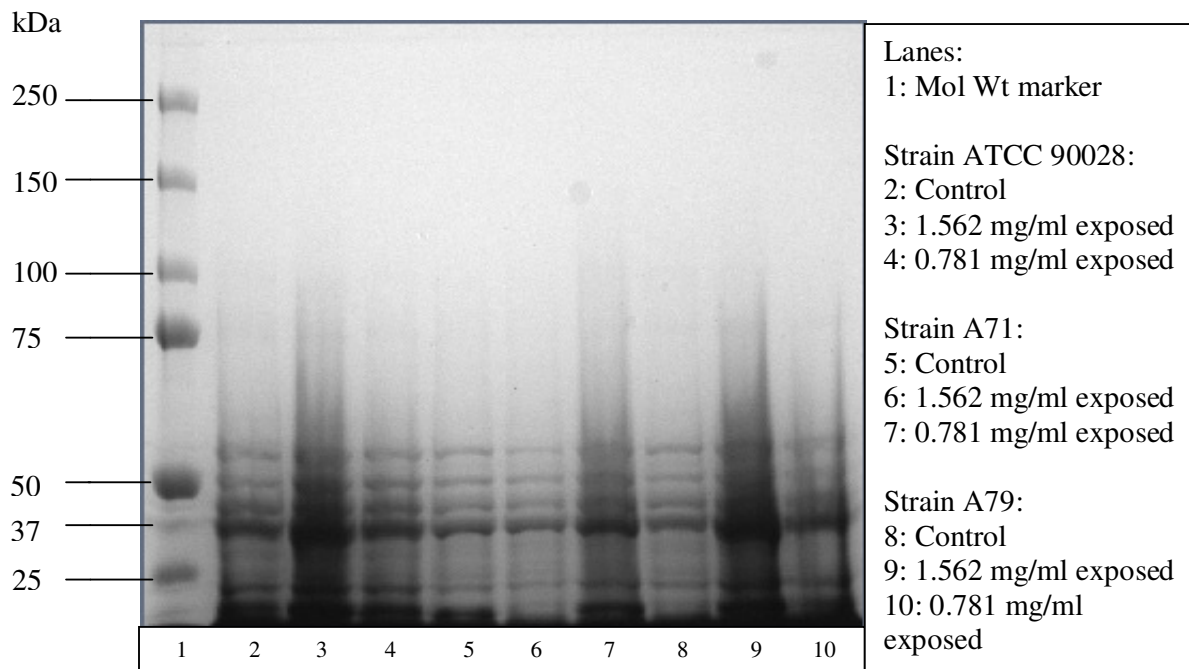


Figure 3.17: Sodium Dodecyl Sulphate – Polyacrylamide Agarose Gel

Electrophoresis analysis of the cell wall protein extracts from the three *Candida albicans* strains after exposure to the crude plant extract and water (control). The gel (acrylamide percentage: 5% stacking gel/6% resolving gel) was stained with Coomassie Brilliant Blue.

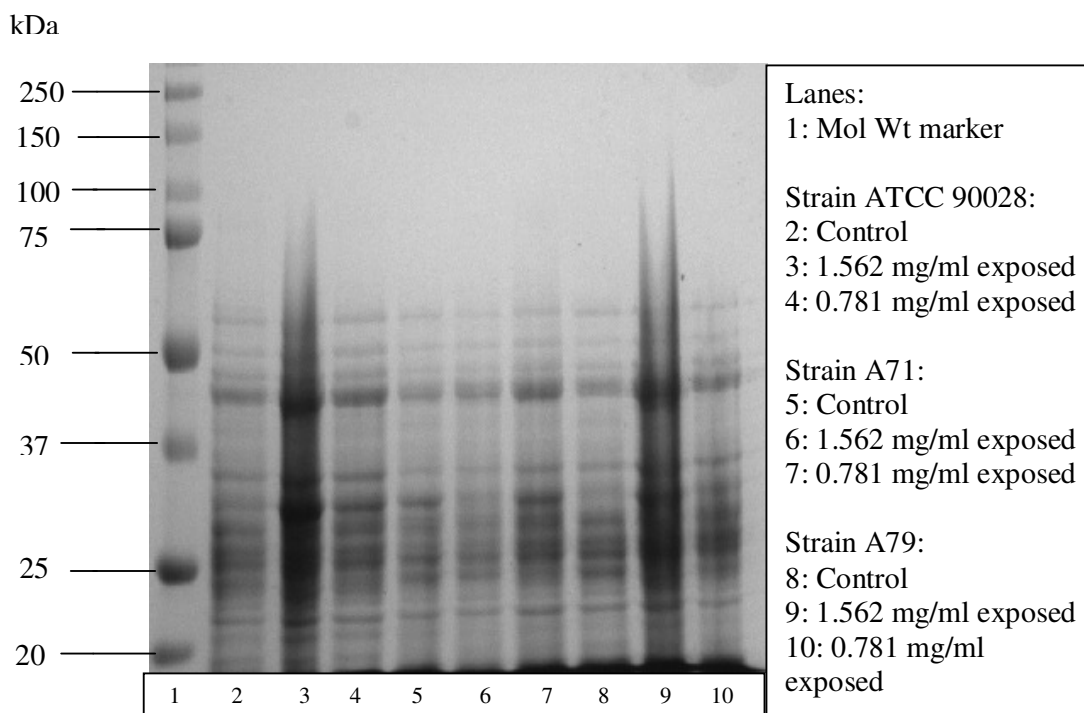


Figure 3.18: Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis

analysis of the cell wall protein extracts from the three *Candida albicans* strains after exposure to the crude plant extract and water (control). The gel (acrylamide percentage: 5% stacking gel/8% resolving gel) was stained with Coomassie Brilliant Blue.

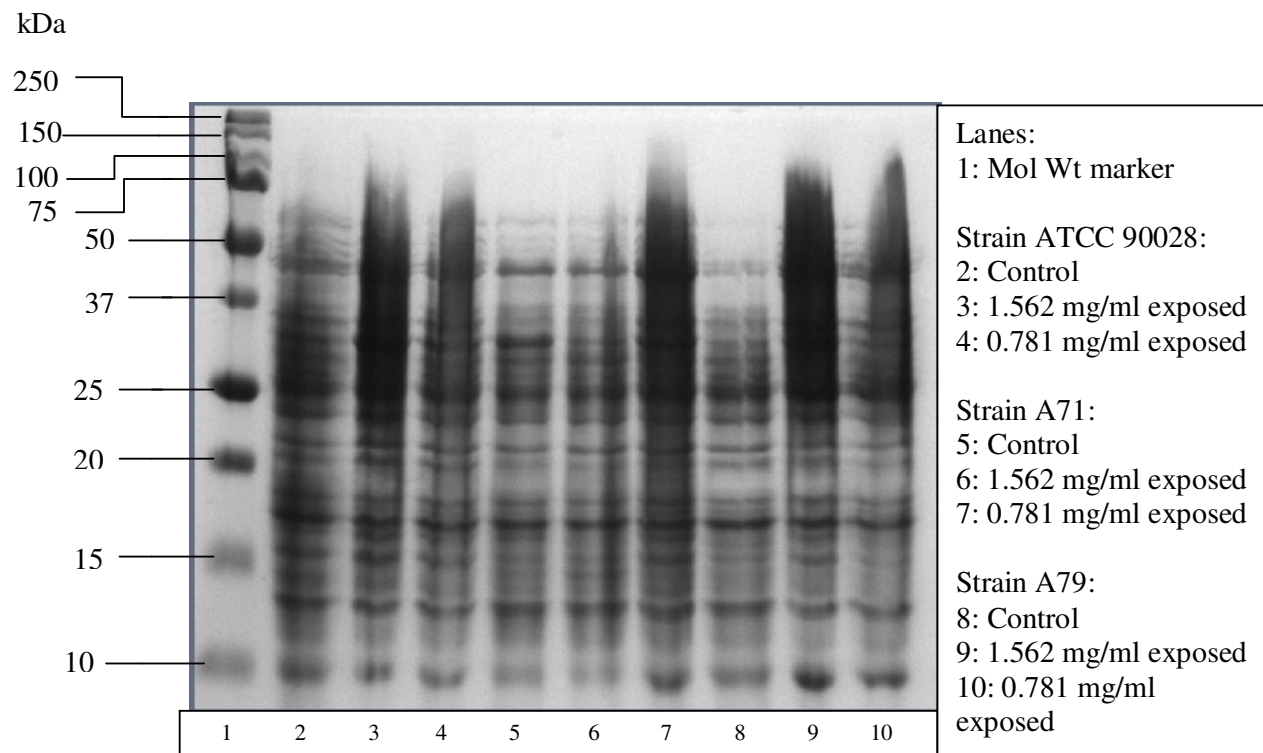


Figure 3.19: Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis

analysis of the cell wall protein extracts from the three *Candida albicans* strains after exposure to the crude plant extract and water (control). The gel (acrylamide percentage: 5% stacking gel/12% resolving gel) was stained with Coomassie Brilliant Blue.

Table 3.9: Molecular weights of the proteins extracted from *Candida albicans* cells after exposure to crude plant extract and water (control) as seen on the various percentage gels.

Band Number	Molecular Weight (kDa)
1	9
2	12
3	15
4	16
5	17
6	19
7	20
8	22
9	24
10	26
11	28
12	30
13	33
14	36
15	42
16	43
17	46
18	50
19	55
20	56

CHAPTER 4: DISCUSSION

Oral candidiasis is a major problem in immunocompromised patients such as patients with HIV, cancer and organ transplants. Elderly denture wearers also suffer from oral candidiasis. Untreated oral candidiasis can cause disseminated candidiasis and systemic infections (Samaranayake, 2002). Compared to the antibacterials available, the number of suitable antifungals is limited. Some of the currently used antifungals are polyenes, azoles and flucytosine. Polyenes and azoles act on the cell membrane whereas flucytosine inhibits fungal DNA synthesis. These drugs are toxic because fungal cells are eukaryotic thus they are similar to host cells which causes unpleasant drug side effects. In addition, there are problems with drug solubility and stability hence leading to poor absorption of these existing drugs. Drug resistance is also increasing therefore new agents to control fungal infections are needed (Mims *et al.*, 2004).

A novel approach of therapy has been suggested by some researchers, where the virulence of an organism can be targeted instead of an antimicrobial approach (Gauwerky *et al.*, 2009). In this approach, the causative organism may not be killed but it could be rendered avirulent. This would be ideal in instances 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 formation, biofilm formation, production of hydrolytic enzymes and the *C. albicans* cell wall (which is responsible for germ tube formation and adherence to oral epithelial cells) could be possible targets in the development of new antifungal drugs (Cannon and Chaffin, 1999; Gauwerky *et al.*, 2009).

In this study, the antifungal property of the plant *D. viscosa* var. *angustifolia* was investigated. The results showed that the crude extract of this plant inhibits germ tube formation in *C. albicans* cells in the planktonic form. It also reduces biofilm formation and hyphae production in cells within a biofilm form. Studies have shown that this plant has an antifungal property at high concentrations by killing *C. albicans* cells and at low concentrations by inhibiting the adherence to oral epithelial cells (Patel and Coogan, 2008; Patel *et al.*, 2009). This study showed that *C. albicans* adherence was inhibited due to damage to the cell wall and cell membrane caused by exposure to the crude plant extract of *D. viscosa* var. *angustifolia*. However, none of the adhesin proteins responsible for adherence were missing due to exposure to the plant extract. This suggests that there might be other mechanisms responsible for the inhibition of *C. albicans* adherence to oral epithelial cells. These results are discussed in detail. Refer to Figure 4.1, Page 106 for a summary of the results.

4.1 Antifungal activity of Medicinal Plants

Many plants have been explored for their antifungal, particularly anticandidal, activity. When an ethnomedical survey in Tanzania was undertaken, 36 plant species were identified as those traditionally used for the treatment of *Candida* infection, 36% of which had scientifically proven efficacy (Runyoro *et al.*, 2006a). Laboratory studies have shown that many plants have anti-*Candida* activity, including the commonly used *Allium sativum* (garlic), *Glycyrrhiza glabra* (liquorice root), huangqin (a Traditional Chinese Medicinal herb), *Carpobrotum edulis* (sour fig) and Cuban oregano named *Plectranthus barbatus* Andrews (Franzblau and Cross, 1986; Motsei *et al.*, 2003; Runyoro *et al.*, 2006b). *Dodonaea viscosa* var. *angustifolia* is a medicinal plant that has an anticandidal effect (Patel and Coogan, 2008).

In this experiment, the median MFC of the crude plant extract of *D. viscosa* var. *angustifolia* was 6.25 mg/ml for all three *C. albicans* strains. These concentrations are generally considered high for a crude plant extract. If these crude extracts are researched further for their phytochemical analysis and an active ingredient identified, the required concentration for the active ingredient would be higher than the MFC of the crude extract. Therefore low concentrations are considered advantageous. However, some studies suggest the use of crude plant extract as a therapeutic agent. In that case, high MFC values are also beneficial. For example, the methanol extract of *Cassia spectabilis*, a plant commonly used in traditional medicine showed a MFC of 6.25 mg/ml, which was used on mice infected with *C. albicans* at 2.5 g/kg once a day for three days and it demonstrated beneficial effects (Sangetha *et al.*, 2009). Therefore the high concentrations of crude plant extract of *D. viscosa* var. *angustifolia* can be used to treat *C. albicans* infections particularly if it is used in the mouth as a topical application in the form of a mouthrinse to treat oral candidiasis.

Mouthrinses containing antimicrobial agents such as chlorhexidine gluconate and triclosan have been used to treat oral candidiasis. Chlorhexidine is a broad-spectrum antimicrobial that has been effective in the treatment and prevention of candidiasis. The main reason that chlorhexidine gluconate-containing mouthrinses have been effective against candidiasis is that it binds to mucosal surfaces and denture materials hence is slowly released after the initial rinse therefore the correct concentration is maintained and its effect remains active for a long period of time (Audus *et al.*, 1992). This phenomenon is called substantivity of the formula. In this study, the substantivity of *D. viscosa* var. *angustifolia* was not evaluated however, the crude extract of this plant has a potential to be used as an anti-*Candida* agent.

Furthermore, at concentrations lower than the median MFC, the crude extract of *D. viscosa* var. *angustifolia* at a concentration of 0.781 mg/ml is known to inhibit the adherence of *C. albicans* to oral epithelial cells which is the crucial step required in the pathogenicity of this organism. The authors suggested that *D. viscosa* var. *angustifolia* had some effect on the cell wall of *C. albicans* which may have caused the inhibition of adherence (Patel *et al.*, 2009). In the present study, three subinhibitory concentrations of 3.125, 1.562 and 0.781 mg/ml were selected to study the effect on the ultrastructure of cell wall and cell wall related virulence factors such as germ tube and biofilm formation by *C. albicans*.

4.2 Effect of the crude plant extract of *Dodonaea viscosa* var. *angustifolia* on *Candida albicans* cell wall and cell membrane

Many factors affect the adherence of *C. albicans* to oral epithelial cells. These are *C. albicans* cells, host cells and the environment in which the interaction is occurring (Olsen, 1990). Experiments done by Patel *et al.*, (2009) where the effect of *D. viscosa* var. *angustifolia* on adherence was established, the yeast cells were first exposed to the plant extract and then challenged with oral epithelial cells. This suggests that the plant extract may have caused some changes in or on the surface of the *C. albicans* cells and this phenomenon was responsible for the beneficial effect. Therefore in the present study, an attempt was made to examine the morphological changes in the *C. albicans* cells caused by this plant extract using Transmission Electron Microscopy (TEM).

The results showed that the crude plant extract of *D. viscosa* var. *angustifolia* reduced the thickness of the cell wall compared to the control. Although in the control

samples, uniform thickness of the cell wall was expected, it was not observed. Instead, cells with a mixture of thicknesses were seen. Therefore grading was done in all the test as well as control samples. There were an increased number of cells with thin cell wall in the samples treated with the plant extract. It can be seen from Figure 3.1, Page 54 that as the plant extract concentration decreases, the cell wall became thinner. It was an unusual result because one would expect an increased effect with an increase in concentration. Instead, the results showed that at increased concentrations of plant extract, some of the cells were killed and the surviving cells had a thinner cell wall compared to the control. At a concentration of 3.125 mg/ml, ultrathin sections under the electron microscope showed a very low cell number compared to the other concentrations. Therefore it can be seen that even at a concentration of plant extract lower than the MFC, *C. albicans* cells are still being killed.

At much lower concentrations, that is 1.562 mg/ml and 0.781 mg/ml, the effect of the plant extract on the *C. albicans* cell wall can be studied since fewer cells were killed at these concentrations. The number of cells with 1+ cell wall thickness increased as concentration decreased therefore it can be deduced that these changes were concentration dependent, which was further verified by statistical analyses. There was a significant difference between the control and test samples ($P < 0.0001$).

Some plant treated cells had a very thin cell wall with damage and disruption. Similar results were shown by Tyagi and Malik (2010) when they studied the antifungal effect of the herb, *Cymbopogon citrotus* (lemon grass) on *C. albicans*. Lemon grass essential oil (LGO) vapour caused extensive internal damage and it was also seen that cell wall thickness varied and it appeared disrupted at some places. LGO - treated cells showed

distorted morphology and shape but the cell wall thickness was the same as the control. Sangetha *et al.*, (2009) investigated the antimicrobial activity of the medicinal plant, *Cassia spectabilis* against *C. albicans* at an ultrastructural level. Their TEM observation also showed untreated cells having a typical cell wall structure and exposed cells with notable alterations in the cell wall and membrane.

It can be deduced further that high concentrations of crude plant extract will reduce the number of *C. albicans* cells and the surviving cells will have a damaged cell wall. Severe damage to the cell wall may allow leakage of cell organelles and therefore the cell may become dysfunctional. Mild damage to the cell wall can cause some dysfunction in *C. albicans*. It can affect cell division, budding and hyphae formation. It could also affect the adherence of this organism to surfaces such as epithelial cells, acrylic dentures and prostheses. In this study, the effect of *D. viscosa* var. *angustifolia* on hyphae formation and the ability of adherence to plastic surfaces were studied and the results are discussed in Sections 4.3 and 4.4.

The cell membrane was also significantly damaged by the crude plant extract since the number of cells with 3+ cell membrane undulation (severe undulation) increased when yeast cells were exposed to the subinhibitory concentrations. However, the effect of the plant extract was not dependent on the plant extract concentration since it can be seen from Figure 3.4, Page 60 that there was a general increase in the number of cells with 3+ cell membrane undulation at all concentrations of plant extract as compared to the control. The effect of the plant extract on the *C. albicans* cell membrane was further verified by statistical analyses and it was shown that there was a significant difference between the control and the subinhibitory concentrations ($P <$

0.0001). It can be deduced that the crude plant extract of *D. viscosa* var. *angustifolia* also has an effect on the *C. albicans* cell membrane hence this could also affect the ability of this organism to cause infection.

The effect of the crude plant extract on the internal organization of the yeast cells can be seen in Figure 3.7, Page 63. The cytoplasm of the control cell is denser compared to the cells treated with plant extract. It can be seen that the plant extract has made the cytoplasm granular in appearance and large vacuoles are also present suggesting that cell permeability might be affected. Similar results were found by Lemar *et al.*, (2002) when they studied the effect of garlic extract on *C. albicans*. Garlic increased formation of vacuoles in the cytoplasm and the cytoplasm became granular suggesting damage to the cell membrane which monitors the flow of chemicals and fluids. Lemon grass essential oil vapour has also shown similar effect causing extensive internal damage such as irregular plasmalemma and wide vacuoles with large lipid globules (Tyagi and Malik, 2010). The mode of action of antifungal drugs, such as polyenes and azoles, is their effect on the cell membrane of fungal cells. The cytoplasmic membrane of cells treated with azasterols showed several changes as compared to the control. These changes included the presence of evaginations, discontinuity and detachment from the cell wall (Ishida *et al.*, 2009). The crude plant extract of *D. viscosa* var. *angustifolia* showed similar results in this study.

The *C. albicans* cell wall and membrane is not only important in adherence to host tissue and inert surfaces but also involved in germ tube formation since the development of a germ tube involves the remodeling of the cell wall. Germ tube formation allows for tissue invasion and penetration thus is also an important

virulence factor involved in the *C. albicans* infection cycle. Therefore the effect of the plant extract of *D. viscosa* var. *angustifolia* on germ tube formation was studied.

4.3 Effect of the crude plant extract on *Candida albicans* germ tube formation

Candida albicans is a dimorphic fungus therefore it exists as both a yeast and hyphal form at different stages of its life cycle. Both the yeast and hyphal forms of *C. albicans* are found in infected tissue therefore both morphological forms are involved in the pathogenesis of *C. albicans*. However, it has been observed that the hyphal form is more important for pathogenesis since hyphae adhere more strongly to mammalian cells (for example BECs), promote tissue penetration, and provide a mechanism to escape the attack of macrophages (Pitarch *et al.*, 2002). Hence the ability to switch between yeast and hyphal morphologies plays a role in the virulence of *C. albicans* (Sudbery *et al.*, 2004). *Candida albicans* yeast-to-hyphae transition is required for virulence and is known to occur in response to a wide variety of inducing conditions present in host tissues including serum, body temperature, N-acetylglucosamine, neutral pH, amino acids, and certain human hormones (Banerjee *et al.*, 2008).

In this study, yeast-to-hyphae transition was induced using horse serum where the test is called germ tube formation. A germ tube is described as an initial elongating structure that will become a hypha. The results showed that the crude plant extract of *D. viscosa* var. *angustifolia* had an effect on hyphal formation since in Figure 3.8, Page 66 and Figure 3.9, Page 67, the percentage of cells with hyphae decreased when the yeast cells were exposed to the extract. Even at a low concentration of 0.781 mg/ml of plant extract, there was a significant effect. The statistical analyses further verified the significance between the control and the test samples ($P < 0.0001$). This

correlated with studies done with essential oil from *Coriandrum sativum* (coriander) and *Ocimum gratissimum* (Alfavaca) where the concentrations lower than the MFC inhibited germ tube formation of *C. albicans* (Nakamura *et al.*, 2004; Silva *et al.*, 2011). The results obtained in the present experiment are also similar to experiments involving conventional mouthrinses such as chlorhexidine, where sub-therapeutic concentrations of chlorhexidine were found to suppress germ tube formation (Ellepola and Samaranayake, 2000) therefore providing further evidence that the crude plant extract of *D. viscosa* var. *angustifolia* has the potential to become an effective antifungal agent. The plant extract of *D. viscosa* var. *angustifolia* also showed inhibition at values lower than its MFC therefore this could be advantageous in reducing the pathogenicity of *C. albicans* since germ tube formation is an important virulence factor.

The biology of hyphal formation involves a complex process made up of various components. In the transition of yeast to hyphal morphology, hyphal growth is defined by a polarisome and a Spitzenkorper at the tip of the growing hyphae. This complex together with the GTPase hydrolyzing enzyme plays an important role in hyphal formation. Once these structures are at the tip of the growing hyphal tube, the Golgi structure of hyphal *C. albicans* cells is then relocalized to the growing tip of the cell and the nucleus is repositioned and divided. One nucleus migrates into the mother cell and the other moves out toward the tip of the elongating germ tube. This complex movement of the nucleus during initial stages of hyphal development is mediated by microtubules (Whiteway and Bachewich, 2007). This allows for the elongation of hyphae from the yeast form to which it is attached and thereby eventually leading to tissue adhesion and invasion.

It can then be deduced that the plant extract has an indirect effect on the cell wall by affecting hyphal formation since the *C. albicans* cell wall is the outermost cellular structure responsible for the shape of the cell. The transition from yeast to hyphal morphologies requires cell wall remodeling, whereby there are changes in its composition and organization. The cell wall is a flexible and dynamic structure that is constantly changing in response to environmental signals as well as to different stages of the cell cycle. The cell wall is also the initial point of contact between the cell and its environment thus it contributes to interactions between the cell and the host. For example host cell recognition and adhesion to host receptors (Pitarch *et al.*, 2002). New antifungal agents targeting the yeast cell wall specifically will be of great value in the treatment of oral candidiasis especially since drug resistance has emerged and the existing drugs target the cell membrane which increases toxicity.

4.4 Effect of the crude plant extract on *Candida albicans* biofilm formation

Commensal *C. albicans* yeast cells usually exist within biofilms in the oral cavity; biofilms are spatially organized heterogeneous communities of fungal cells encased in a matrix consisting of extracellular polymeric substances (Jin *et al.*, 2004). *Candida albicans* biofilms can form on prostheses, for example denture acrylic, and medical implants such as catheters and joint replacements. These devices are a risk factor and are often associated with *C. albicans* infections. Cells within biofilms have unique phenotypic characteristics as compared to planktonic cells (Baillie and Douglas, 1998). They are highly resistant to antifungal agents since antifungals can not actively penetrate the extracellular polymeric matrix that encases the cells within a biofilm. *Candida albicans* biofilm formation is initiated by the adherence of planktonic cells to a surface possibly through cell wall-located adhesion molecules and the yeast cells

aggregate into a microcolony. Once the first layer of cells is formed, an extracellular matrix made up of proteins and polysaccharides is produced to consolidate the early biofilm (Garcia-Sanchez *et al.*, 2004). Often, individual yeast cells detach from the mature biofilm and cause infection which is responsive to drug therapy. However, the biofilm itself is highly resistant to antifungal agents and host defense mechanisms as compared to planktonic yeast cells. It has been reported that *C. albicans* biofilms are resistant to many clinical antifungal agents, including amphotericin B and fluconazole (Baillie and Douglas, 1999). Therefore the ability to form a biofilm is an important virulence factor and a novel antifungal agent is needed to prevent biofilm formation.

In this experiment, the effect of the crude plant extract of *D. viscosa* var. *angustifolia* on biofilm formation was evaluated. From the light and SEM micrographs, it can be seen that the crude plant extract reduced *C. albicans* biofilm formation compared to the controls and the results were similar for all three strains. When the *C. albicans* biofilm was grown in the presence of plant extract, the adherent cells were less efficient in hyphae formation thereby reducing virulence. The essential oil of *Coriandrum sativum* (coriander) also showed similar results (Furletti *et al.*, 2011). The crude methanol extract of *Cassia spectabilis*, also reduced biofilm constituents in *C. albicans* cells but the required concentration (6.25 mg/ml) was very high (Sangetha *et al.*, 2009). Whereas in this current study, the crude plant extract of *D. viscosa* var. *angustifolia* significantly reduced biofilm formation at a concentration as low as 0.781 mg/ml. *Candida albicans* biofilm development involves three steps: adhesion, biofilm growth, and maturation. In this study, treated cells showed reduced biofilm formation and the biofilms mainly consisted of yeasts, pseudohyphae and some cells with abnormal shapes, which suggest the plant extract may affect some metabolic steps

responsible for biofilm formation. It may have altered the cell surface hydrophobicity (CSH) which is an important factor in *C. albicans* adherence and germ tube formation (Hazen and Hazen, 1992). It contributes to the interaction between the cells and surfaces playing a critical role in biofilm formation. The plant extract may have even affected adhesins such as the Hwp1 protein which is only expressed by hyphae and is responsible for biofilm formation (Nobile *et al.*, 2006).

In this study, the results also showed that the extracellular polymeric matrix was present in the biofilm grown without the plant extract but it was absent in the biofilms which were grown in the presence of plant extract. The extracellular polymeric matrix generally restricts the penetration of antifungal drugs rendering treatment ineffective (Baillie and Douglas, 1998). Use of this plant extract in conjunction with an antifungal drug would be ideal where the extracellular polymeric material will be absent and the few *C. albicans* cells that are still present will have an unrestricted exposure to the antifungal drug. Use of traditional medicine with conventional medicine as an additional therapeutic agent has been found to have a synergistic effect (Mukherjee *et al.*, 2005; Endo *et al.*, 2010).

The plant extract of *D. viscosa* var. *angustifolia* reduces the adherence of *C. albicans* to epithelial cells (Patel *et al.*, 2009) therefore it could also reduce adherence to inert, inserted surfaces like dentures and thereby reduce biofilm formation. Biofilm formation is critical in the development of denture stomatitis and even though antifungal drugs are used to treat it, infection reestablishes soon after treatment (Chandra *et al.*, 2001) therefore an antifungal agent that can reduce biofilm formation would be extremely useful.

The effect of baicalein (BE); a major component of the Chinese herb, *Scutellaria baicalensis*, was evaluated against *C. albicans* biofilm formation. Baicalein inhibited biofilm formation since growth mainly consisted of yeasts and pseudohyphae. The researchers also found the effect was dose- and time- dependent since higher BE concentrations were more effective and BE inhibited biofilms at different stages of biofilm development when it was added at various intervals over a 24 hour incubation period (Cao *et al.*, 2008). This suggests that if extracts of *D. viscosa* var. *angustifolia* are developed into a mouthrinse and used every day, it will not allow *C. albicans* biofilm formation in the oral cavity which will be an additional advantage.

The *C. albicans* cell wall is important in biofilm formation and development due to its adhesive and structural properties. Any antifungal agent targeting the cell wall could be advantageous in eliminating *C. albicans* infection especially on inert or biological surfaces in the oral cavity. It can be deduced from the various aspects of this study that the plant extract of *D. viscosa* var. *angustifolia* has an effect on the *C. albicans* cell wall, hyphal formation and biofilm formation which are all interrelated and they are important virulence factors.

Proteins like adhesins and glycoproteins expressed on the *C. albicans* cell wall have been shown to play an important role in adherence (Castillo *et al.*, 2008). Patel *et al.*, (2009) postulated that the *D. viscosa* var. *angustifolia* plant extract could have interfered with adhesins found on the *C. albicans* cell wall surface that are involved in adherence. Therefore an attempt was made to compare the cell wall proteins present in the *C. albicans* cells exposed to the plant extract and those in cells without exposure to the plant extract.

4.5 Effect of the crude plant extract on cell wall proteins of *Candida albicans*

The cell wall of *C. albicans* is made up of the polysaccharides: mannan, chitin, glucan, as well as proteins and lipids. The outer layer of the *C. albicans* cell wall also has fimbriae, which are composed of mostly mannoproteins and appear as a dense network of radially projecting fibrils (Chaffin *et al.*, 1998; Cannon and Chaffin, 1999). These fibrillar structures on the cell wall surface have been shown to play a role in the adhesion of *C. albicans* to a range of surfaces (Douglas, 1987). However, in this study fibrils were not observed on the surface of the yeast cell wall and this could have been due to some limitations to this study. For further information on this, refer to Section 4.9, Page 105. Mannoproteins are composed of both polysaccharide and protein. Proteins found at the external surface of the cell wall (i.e. adhesins and lectins) play an important role in adhesion to host surfaces (Castillo *et al.*, 2008).

Many techniques have been used to study the cell surface macromolecules of both yeast and hyphal forms of *C. albicans*. Most studies focused either on extraction of cells with sodium dodecyl sulphate (SDS) or dithiothreitol (DTT) or on digestion of the cell wall by zymolyase and the extracts analysed by crossed immuno-electrophoresis or SDS-polyacrylamide gel electrophoresis (PAGE) with Western blotting (Calderone and Braun, 1991). The method used in the present experiment involved exposing the *C. albicans* cells to the crude plant extract overnight. Thereafter treating the exposed cells with β -mercaptoethanol and then extracting the cell wall proteins by incubating the cells with zymolyase. The extracts were then analysed by SDS-PAGE.

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis separates proteins based solely on size therefore the molecular weight of a protein can be determined by this method. This type of electrophoresis involves the use of the detergent, sodium dodecyl sulphate (SDS), which gives all proteins the same charge and fully denatures the protein therefore all proteins will assume their primary form. Thus all proteins will move in the same direction when the gel is charged but rate of protein movement is dependant on their molecular weight. The percentage of a polyacrylamide gel is dependent on the amount of acrylamide in the mixture for example a higher amount of acrylamide results in a higher crosslinking percentage. Therefore high molecular weight proteins will have retarded rates of migration through a high percentage gel (Wilson and Walker, 1995).

When the bands on each gel were compared to the bands of the molecular weight marker, it could be observed that each percentage has a different molecular weight range. A 6% gel can separate proteins with molecular weights ranging from 50 kDa to 250 kDa. An 8% gel can separate proteins of sizes ranging between 20 kDa and 150 kDa. A 12% gel separates proteins whose sizes range between 10 kDa and 75 kDa. Three different percentages of gels were used since the molecular weights of the proteins extracted in the experiments were unknown.

After running the three gels, it was observed that the cell wall proteins had low molecular weights ranging between 9kDa to 56 kDa. There were approximately 20 bands seen on the various percentage gels. These proteins were present in the cell wall of *C. albicans* exposed to the plant extract as well as in unexposed cells. This suggests

that the plant extract may not have any effect on the adhesins tested in this study. All three strains showed similar results.

Many adhesins are known with molecular weights between 13 kDa and 650 kDa (Casanova *et al.*, 1992). However, only a few of these proteins have been identified that are thought to play a role in adherence. A low molecular weight protein with a size of 19 kDa was found on the hyphal cell wall only and was identified as an adhesin (Ponton and Jones, 1986). Glycoproteins that may act as adhesins with molecular weights ranging from 55 to 60 kDa (Fukayama and Calderone, 1991), 60 to 68 kDa (Casanova *et al.*, 1992), 80kDa (Casanova and Chaffin, 1991) and 165kDa (Fukayama and Calderone, 1991) have been identified. High molecular weight mannoproteins of sizes 180 kDa (Casanova *et al.*, 1989), 235-250 kDa (Ponton and Jones, 1986), and 260 kDa (Casanova *et al.*, 1989) have also been identified. All these cell wall components play an important role in adherence which is the initial stage of infection. In our study, cell wall proteins with molecular weights of 10 to 250 kDa were solely investigated therefore this study may have excluded the effect on the few known high molecular weight cell wall proteins.

Further research needs to be carried out to examine the effect of the plant extract on the high molecular weight proteins and identify them by Western blotting using antibodies that select for these proteins. This will further determine whether the proteins found in the control and treatments show any difference as well as their function in the infection cycle.

4.6 Plant chemicals with antimicrobial effect

Although this study did not investigate the chemicals responsible for the beneficial effects described here, an attempt was made to identify potential constituents which are known and may be responsible for the anti-*Candida* effect. Medicinal plants contain many chemicals that are responsible for different beneficial as well as harmful effects.

Phenols, phenolic acids, terpenoids and essential oils are active against bacteria, fungi, viruses and protozoa. Quinones have an antimicrobial effect and its target in the microbial cell could possibly be cell-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Flavones, flavonoids and flavonols are effective against a wide range of microorganisms. Its activity is probably due to its ability to complex with extracellular and soluble proteins as well as to complex with bacterial cell walls, which is similar to quinones. These chemicals may also disrupt microbial membranes. The antimicrobial action of tannins may be related to their ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins (Cowan, 1999).

Dodonaea viscosa var. *angustifolia* is known to contain flavonoids, reducing sugars, alkaloids, saponins, and tannins (Amabeoku *et al.*, 2001). One flavone, 5,7,4'-Trihydroxy-3,6-dimethoxyflavone, has been isolated from *D. viscosa* var. *angustifolia*, which is a major leaf flavonoid (van Heerden *et al.*, 2000). Flavonoids have antimicrobial action against a wide range of microorganisms. Their main target is the microbial cell wall and cell membrane since it can complex with extracellular and soluble proteins on the cell surface. The effect of *D. viscosa* var. *angustifolia* on the *C. albicans* cell wall and cell membrane shown in this study may have been due to

flavonoids present in the crude plant extract. Saponins have shown to inhibit hyphal formation in *Candida* species. One saponin, Methyl 4-O-Methyl- β -D-xylopyranoside has been isolated from *D. viscosa* var. *angustifolia* (Patel, 2011 Personal Communication). This saponin is similar to those isolated and identified from the Chinese Medicinal herb, *Tribulus terrestris* L. (Zhang *et al.*, 2006) which reduced germ tube formation by *C. albicans*. The plant extract of *D. viscosa* var. *angustifolia* inhibited germ tube formation in both planktonic cells and in cells within a biofilm form. Therefore in our study, flavonoids and saponins present in the leaves and twigs of *D. viscosa* var. *angustifolia* may have been responsible for the beneficial effects. Further research needs to be performed to identify the active ingredients and chemicals responsible for the effect of the plant extract on the ultrastructure of the *C. albicans* cell wall and biofilm formation.

4.7 *Dodonaea viscosa* var. *angustifolia* and the oral cavity

This study has shown that *D. viscosa* var. *angustifolia* affects *C. albicans* cells and the effect is different at different concentrations. A high percentage of immunocompromised individuals carry *C. albicans* in their mouth and frequently develop candidiasis. The *D. viscosa* var. *angustifolia* extract could be incorporated into a mouthrinse or into an oral gel prescribed to use regularly in the mouth; and on application, high concentrations would kill *C. albicans* cells therefore reduce the number of cells. Due to the constant flow of saliva in the mouth, the high concentration will not be maintained unless the substantivity of this plant is good and although the plant extract will become diluted due to saliva, at a low concentration it will still continue to act on the surviving *C. albicans* cells rendering them avirulent. The low concentration will reduce the adherence of *C. albicans* cells to oral epithelial

cells and prostheses. In addition, it will reduce hyphae formation which is also crucial in the pathogenesis of this organism. This study showed that this plant has a preventative and therapeutic potential against *C. albicans* and candidiasis. However, further research is necessary.

4.8 Future Research

- a. *In vivo* studies for the efficacy in different susceptible sites of infection is required. Candidiasis can occur in the mouth, vagina and systemically. It is therefore possible that the body fluids can render the plant extract inactive.
- b. Substantivity of this plant extract needs to be established. High substantivity would be beneficial particularly for use in the mouth and vagina where there are constant secretions of bodily fluids. Absorption of the active chemicals to the mucosa and slow release would be ideal as seen with chlorhexidine.
- c. Flavonoids, terpenoids, tannins and steroids were chemicals isolated from the *Dodonaea viscosa* plant extract shown to have an anticandidal effect (Khurram *et al.*, 2011). However, they need to be further identified and characterised. Once identified, it can be synthesised in a laboratory environment at low cost and it can be developed into a therapeutic agent.
- d. Often, plants show excellent beneficial effects in laboratory studies but they are toxic to human cells. Although consumption of *Dodonaea viscosa* by cattle causes poisoning when large quantities are ingested (Colodel *et al.*, 2003), this plant extract has shown to be non toxic in mice (Khalil *et al.*, 2006). Furthermore, the application of the plant extract to the skin of mice and rabbits showed no sign of toxicity (Teshome *et al.*, 2010). However, the plant extract

of *D. viscosa* var. *angustifolia* may be different therefore cytotoxicity of this plant extract needs to be established.

- e. Efficacy of this plant towards other *Candida species* and other oral pathogens can be explored since they coexist with these organisms within the oral cavity. A preliminary study on the effect of *D. viscosa* var. *angustifolia* on oral pathogens such as cariogenic bacteria and the causative organisms of periodontal diseases has been conducted (Naidoo, 2011 Personal Communication).

4.9 Technical difficulties and possible shortcomings

Transmission Electron Microscopy is a time consuming technique and the outcomes are technique dependent. Therefore optimization of the required method became necessary. The TEM at the Microscopy and Microanalysis Unit at Medical School, University of the Witwatersrand, Johannesburg is a very old piece of equipment with low magnification capacity. Therefore some of the cell wall observations such as cell surface fibrils were not possible to do.

On initial observation, it was seen that the *C. albicans* cells had various cell wall thicknesses in the control sample therefore grading of the cells became necessary. However, this was also a very lengthy process since 150 cells had to be counted for each strain.

Another possible limitation to this study could be the fact that only one geographical sample was studied. Plants sampled from other areas may have different chemical

constituents responsible for its mode of action hence its biological outcomes may be different.

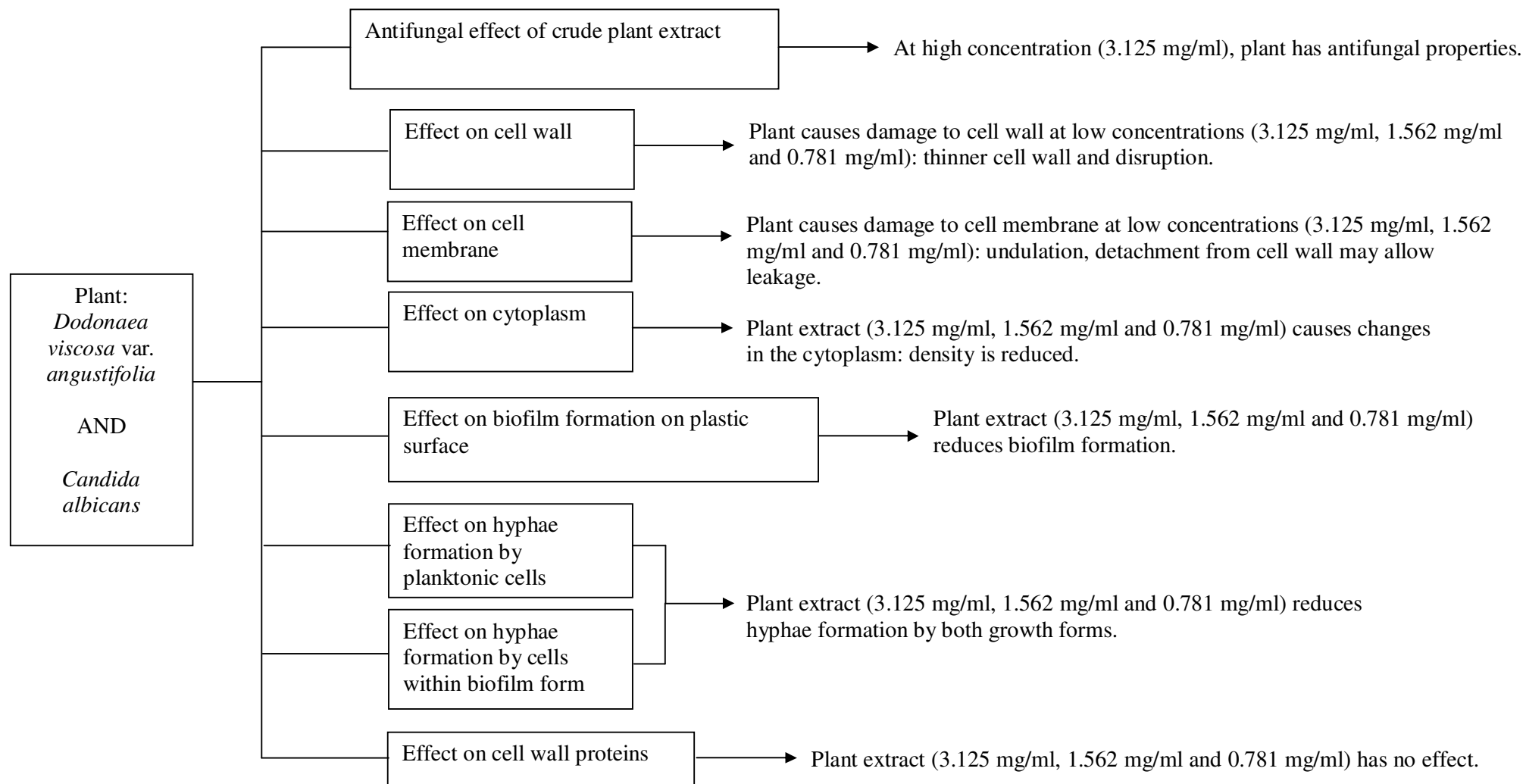


Figure 4.1: Diagram illustrating the summary of results of the effect of the crude plant extract of *Dodonaea viscosa* var. *angustifolia* on *Candida albicans*.

CHAPTER 5: CONCLUSION

This study showed that the crude extract of *D. viscosa* var. *angustifolia* at high concentrations (3.125 mg/ml) kills *C. albicans* and at low concentrations (1.562 mg/ml and 0.781 mg/ml) damages the cell wall of planktonic cells by making it thinner and causing disruption. It also damages the cell membrane by reducing tension, creating undulation, increasing permeability and allowing the cellular content to permeate out of the cell. At low concentrations (3.125 mg/ml, 1.562 mg/ml and 0.781 mg/ml), it inhibits biofilm formation and hyphae formation by planktonic cells as well as by cells within the biofilm form. The crude plant extract therefore prevents *C. albicans* from adhering to host cell surfaces and penetrating host tissue. However, *D. viscosa* var. *angustifolia* has no effect on some of the cell wall associated adhesin proteins that were investigated in this study. These results suggest that *D. viscosa* var. *angustifolia* reduces the number of *C. albicans* and reduces the virulence of this organism. Therefore it has a potential to be developed into an antifungal agent for the prevention and treatment of oral candidiasis.

CHAPTER 6: APPENDICES

Appendix A

Composition and preparation of Media, Buffers and Stains

Ammonium carbonate

Ammonium carbonate (Merck, South Africa)	1.89 g
Distilled water	1 L

The powder was dissolved and the pH adjusted to 8.64.

10% Ammonium persulphate

Ammonium persulphate (Sigma)	1 g
Distilled water	8 ml

These were mixed well by gentle shaking. The volume was adjusted to 10 ml with distilled water. The solution was stable at 4°C for two weeks.

1% (v/v) β -mercaptoethanol

β -mercaptoethanol (Merck, South Africa)	1 ml
Ammonium carbonate	100 ml

These were mixed well under a fume hood since β ME is very toxic.

Coomassie Blue staining solution

Coomassie Brilliant Blue R-250 (Sigma)	2.5 g
Ethanol (Merck, South Africa)	450 ml
Acetic acid (Merck, South Africa)	100 ml
Distilled water	400 ml

The volume was adjusted to 1 L with distilled water. The solution was stored at room temperature.

Coomassie Blue destaining solution

Methanol (Merck, South Africa)	450 ml
Acetic acid	100 ml
Distilled water	400 ml

The volume was adjusted to 1 L with distilled water and the solution stored at room temperature.

2.5% Gluteraldehyde

25% gluteraldehyde (Agar Scientific)	10 ml
Phosphate Buffered Saline (PBS) pH 7.3	90 ml

These were mixed well under a fume hood.

Lead citrate stain for Transmission electron microscope copper grids

Lead citrate (Agar Scientific)	0.25 g
Distilled water	25 ml
Concentrated NaOH (Merck, South Africa)	0.5 ml

These ingredients were mixed well and spun down for 10 minutes at 3000 rpm. Copper grids were stained for 2-3 minutes. Grids were then rinsed in dilute NaOH (1 drop/50 ml). Grids were rinsed 3X in distilled water (10 dips each). Grids were allowed to dry before viewing under TEM

1% Osmium tetroxide

4% osmium tetroxide (Agar Scientific)	2 ml
Phosphate Buffered Saline (PBS) pH 7.3	6 ml

These were mixed well under a fume hood. The solution was protected from light by placing it in a dark bottle.

Phosphate Buffered Saline pH 7.3 (Merck, South Africa)

Distilled water	500 ml
Sodium chloride	4.2 g
Sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$)	0.078 g
Sodium hydrogen phosphate (NaHPO_4)	0.64 g

These ingredients were dissolved and the solution was adjusted to pH 7.3. Thereafter, the solution was autoclaved at 121°C for 15 min.

0.6M Potassium chloride

Potassium chloride (Merck, South Africa)	44.64 g
Distilled water	1 L

These were mixed well.

Sabouraud Dextrose Agar

Sabouraud Agar (Merck, South Africa)	60 g
Distilled water	1 L

The ingredients were boiled whilst stirring until completely dissolved. Thereafter, the solution was autoclaved at 121°C for 15 min. The solution was allowed to cool rapidly to 40-45°C and mixed well. It was aseptically poured into sterile petri dishes.

Sabouraud Dextrose Broth

Sabouraud Broth (Merck, South Africa)	20 g
Distilled water	1 L

The ingredients were mixed well and the solution was autoclaved at 121°C for 15 min.

10% SDS

SDS (BioRad)	10 g
Distilled water	90 ml

These were mixed well in a fume hood by stirring gently. The volume was made up to 100 ml with distilled water and stored at room temperature.

20% SDS

SDS	20 g
Distilled water	80 ml

These were mixed well in a fume hood by stirring gently. The volume was made up to 100 ml with distilled water and stored at room temperature.

2X SDS-PAGE sample buffer (BioRad)

1.5M Tris (pH 6.8)	10 ml
20% SDS	6 ml
Glycerol	30 ml
β -mercaptoethanol	15 ml
Bromophenol blue	1.8 mg

These were mixed well in a fume hood and the volume was adjusted to 100 ml with distilled water. The solution was stored at -20°C.

Spurrs Epoxy Resin (Agar Scientific)

Standard Medium (Firm)	
ERL 4221	4.10 g
DER	1.43 g
NSA	5.90 g
DMAE (accelerator)	0.1 g

These were weighed out exactly in the order given and dissolved.

1% Toluene Blue stain (Agar Scientific)

Solution 1

Pyronin B (or Y)	0.2 g
Distilled water	20 ml

Solution 2

Toluidine	0.8 g
Borax	0.8 g
Distilled water	80 ml

One part of solution 1 was mixed with four parts of solution 2. This solution was then used to stain semi thin sections before ultra thin sections were cut for copper grids.

1.5M Tris pH 6.8

Tris base (Merck, South Africa)	27.23 g
Distilled water	80 ml

These ingredients were dissolved and the solution made up to a volume of 150 ml with distilled water. The pH was adjusted to 6.8 and the solution was stored at 4°C.

1.5M Tris pH 8.8

Tris base	27.23 g
Distilled water	80 ml

These ingredients were dissolved and the solution made up to a volume of 150 ml with distilled water. The pH was adjusted to 8.8 and the solution was stored at 4°C.

Uranyl acetate stain

Uranyl acetate (Agar Scientific)	1 g
Methanol	5 ml

The uranyl acetate powder was dissolved into the methanol making up a saturated solution. More powder was added if necessary. The solution was spun down for 10 minutes at 3000 rpm. Copper grids were stained for 3-5 minutes. Grids were rinsed 3X in 50% methanol (10 dips each). Grids were allowed to dry for 1 hour before staining with lead citrate.

Appendix B

Statistical Results

Figure B1: Frequency of cell wall thickness (frequency weights) by the different treatments of plant extract and water. Treatment 1: control, treatment 2: 3.125 mg/ml, treatment 3: 1.562 mg/ml and treatment 4: 0.781 mg/ml.

Treatment	Cell wall thickness			Total
	1+	2+	3+	
1	51	141	258	450
2	99	111	240	450
3	184	112	154	450
4	216	102	132	450
Total	550	466	784	1,800

Figure B2: One way Analysis of Variances test results for the effect of different concentrations of plant extract on the *Candida albicans* cell wall. Treatment 1: control, treatment 2: 3.125 mg/ml, treatment 3: 1.562 mg/ml and treatment 4: 0.781 mg/ml.

Treatment	Summary of cell wall			Obs.
	Mean	Std. Dev.	Freq.	
1	2.46	.69001791	450	450
2	2.3133333	.81031765	450	450
3	1.9333333	.86506047	450	450
4	1.8133333	.86031019	450	450
Total	2.13	.85124243	1800	1800

Source	Analysis of Variance			F	Prob > F
	SS	df	MS		
Between groups	126.66	3	42.22	64.43	0.0000
Within groups	1176.92	1796	.655300668		
Total	1303.58	1799	.724613674		

Bartlett's test for equal variances: $\chi^2(3) = 28.2272$ Prob> $\chi^2 = 0.000$

Figure B3: Pairwise comparison with Scheffe test results for changes caused by the different concentrations of plant extract on the *Candida albicans* cell wall. Treatment 1: control, treatment 2: 3.125 mg/ml, treatment 3: 1.562 mg/ml and treatment 4: 0.781 mg/ml.

Comparison of cell wall by Treatment (Scheffe)				
Row Mean- Col Mean	1	2	3	
2	-.146667 0.061			
3	-.526667 0.000	-.38 0.000		
4	-.646667 0.000	-.5 0.000	-.12 0.176	

Figure B4: Median test results for changes caused by the different concentrations of plant extract on the *Candida albicans* cell wall. Treatment 1: control, treatment 2: 3.125 mg/ml, treatment 3: 1.562 mg/ml and treatment 4: 0.781 mg/ml.

Greater than the median	Treatment				Total
	1	2	3	4	
no	192	210	296	318	1,016
yes	258	240	154	132	784
Total	450	450	450	450	1,800

Pearson chi2(3) = 105.2145 Pr = 0.000

Figure B5: Frequency of cell membrane changes (frequency weights) by the different treatments of plant extract and water. Treatment 1: control, treatment 2: 3.125 mg/ml, treatment 3: 1.562 mg/ml and treatment 4: 0.781 mg/ml.

Treatment	Cell membrane change			Total
	1+	2+	3+	
1	403	41	6	450
2	87	91	272	450
3	79	104	267	450
4	98	69	283	450
Total	667	305	828	1,800

Figure B6: One way Analysis of Variances test results for the effect of different concentrations of plant extract on the *Candida albicans* cell membrane. Treatment 1: 3.125 mg/ml, treatment 2: 1.562 mg/ml, treatment 3: 0.781 mg/ml.

Treatment	Summary of cell membrane			
	Mean	Std. Dev.	Freq.	Obs.
Controls	1.1177778	.36175081	450	450
1	2.4111111	.79382983	450	450
2	2.4177778	.77179942	450	450
3	2.4111111	.82411381	450	450
Total	2.0894444	.90720035	1800	1800

Source	Analysis of Variance			F	Prob > F
	SS	df	MS		
Between groups	566.495	3	188.831667	371.01	0.0000
Within groups	914.104444	1796	.50896684		
Total	1480.59944	1799	.823012476		

Bartlett's test for equal variances: $\chi^2(3) = 313.4867$ Prob> $\chi^2 = 0.000$

Figure B7: Pairwise comparison with Scheffe test results for changes caused by the different concentrations of plant extract on the *Candida albicans* cell membrane. Treatment 1: control, treatment 2: 3.125 mg/ml, treatment 3: 1.562 mg/ml and treatment 4: 0.781 mg/ml.

Comparison of cell membrane by Treatment (Scheffe)				
Row Mean- Col Mean	1	2	3	
2	1.29333 0.000			
3	1.3 0.000	.006667 0.999		
4	1.29333 0.000	0 1.000	-.006667 0.999	

Figure B8: Median test results for changes caused by the different concentrations of plant extract on the *Candida albicans* cell membrane. Treatment 1: 3.125 mg/ml, treatment 2: 1.562 mg/ml and treatment 3: 0.781 mg/ml.

Greater than the median	Treatment				Total
	Control	1	2	3	
no	444	178	183	167	972
yes	6	272	267	283	828
Total	450	450	450	450	1,800

Pearson $\chi^2(3) = 483.1097$ Pr = 0.000

Figure B9: One way Analysis of Variances results for the effect of different concentrations of plant extract on germ tube formation. Concentration 0: control, concentration 1: 3.125 mg/ml, concentration 2: 1.562 mg/ml and concentration 3: 0.781 mg/ml.

Summary of % germ tube			
conc	Mean	Std. Dev.	Freq.
0	45.555556	6.3069626	9
1	6.6666667	2.8284271	9
2	17.333333	5	9
3	33.555556	3.8441875	9
Total	25.777778	15.760912	36

Analysis of Variance					
Source	SS	df	MS	F	Prob > F
Between groups	7993.77778	3	2664.59259	121.73	0.0000
Within groups	700.44444	32	21.888889		
Total	8694.22222	35	248.406349		

Bartlett's test for equal variances: chi2(3) = 5.0883 Prob>chi2 = 0.165

Figure B10: Pairwise comparison with Scheffe test for the effect of different concentrations of plant extract on germ tube formation.

Comparison of % germ tube by conc (Scheffe)			
Row Mean- Col Mean	0	1	2
1	-38.8889		
	0.000		
2	-28.2222	10.6667	
	0.000	0.000	
3	-12	26.8889	16.2222
	0.000	0.000	0.000

Appendix C

Figure C1: Ethical clearance

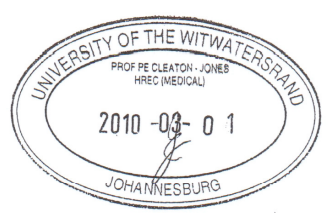
Human Research Ethics Committee (Medical) (formerly Committee for Research on Human Subjects (Medical)) Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708 Private Bag 3, Wits 2050, South Africa Ref: W-CJ-100301-1	University of the Witwatersrand, Johannesburg  01/03/2010
TO WHOM IT MAY CONCERN:	
Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).	
Investigators: Dr AM Patel, Serisha Naicker (Student No 0606058D)	
Project title: The effect of <i>Dodonaea viscosa</i> var. <i>angustifolia</i> on the ultrastructure of <i>Candida albicans</i> cell wall and biofilm formation.	
Reason: This is a wholly laboratory study using plant material and stock cultures isolated from project M000402. There are no humans involved.	
 Professor Peter Cleaton-Jones Chair: Human Research Ethics Committee (Medical) 	
copy: Anisa Keshav, Research Office, Senate House, Wits	

Figure C2: Random Sampling Grid (Reddy, 2001 Personal Communication)

Page 1 of 1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	8	0	9	4	2	5	2	5	8	2	4	7	1	3	4	7	7	4	3	3	3	6	2	0	1	8	9	7	2	1	3	4
2	3	5	6	3	2	1	9	8	8	2	1	1	9	0	4	5	2	6	1	8	2	7	5	1	2	6	2	7	1	0	9	5
3	1	3	3	0	6	3	3	1	3	7	5	3	9	6	9	3	8	7	3	8	6	8	1	5	1	5	3	8	8	5	4	3
4	3	5	6	5	0	0	1	6	2	2	4	3	6	4	3	2	4	7	9	6	6	0	9	5	5	2	8	3	1	8	2	0
5	7	8	5	0	5	9	2	6	5	5	8	8	7	3	1	1	2	1	9	2	4	5	4	5	3	5	3	0	5	5	8	9
6	4	4	9	0	5	4	1	7	9	7	2	7	6	1	5	3	5	9	0	1	4	8	7	8	9	9	8	0	9	8	7	7
7	6	6	4	5	9	1	0	4	9	3	1	8	8	8	1	9	7	5	3	7	2	7	8	5	9	3	7	3	2	4	4	5
8	3	6	2	6	5	9	9	5	1	2	1	5	9	7	5	3	9	2	2	3	5	6	5	8	2	9	4	4	2	8	9	9
9	4	6	6	5	4	8	2	0	7	5	5	4	0	6	1	2	9	6	8	9	4	2	5	1	9	1	3	8	1	7	0	9
10	6	4	9	8	7	5	1	9	0	4	7	4	7	8	1	8	6	8	3	2	9	6	8	3	9	8	7	2	4	0	9	0
11	6	7	2	2	9	8	6	9	9	3	6	1	7	8	7	5	4	8	8	8	1	3	1	5	9	6	7	9	8	8	3	4
12	9	7	4	8	5	9	3	2	5	1	1	5	2	7	2	1	0	0	3	3	9	3	0	3	9	7	1	3	4	0	1	2
13	5	6	4	1	1	4	1	7	1	4	1	9	7	4	3	4	8	1	6	5	7	3	6	8	1	2	1	8	5	0	3	9
14	7	4	4	4	9	2	0	0	8	8	4	0	5	8	8	2	4	3	9	8	3	9	0	4	9	1	9	9	9	3	3	6
15	8	2	7	9	3	0	1	9	4	6	7	2	3	7	4	3	3	9	7	9	4	6	8	9	9	0	2	1	6	9	9	0
16	0	1	6	1	7	6	1	7	1	0	2	4	2	3	8	7	2	8	9	1	6	6	7	7	1	5	8	5	2	4	8	2
17	7	3	8	8	9	7	5	9	7	5	5	5	6	8	2	4	9	9	7	7	2	0	0	8	5	5	9	6	9	7	4	0
18	7	8	3	0	4	7	1	4	3	6	9	5	2	9	1	9	1	8	0	4	4	0	4	4	1	0	3	4	2	5	9	7
19	9	8	8	7	4	2	1	6	6	5	2	6	4	5	3	5	8	4	3	0	5	2	7	0	9	8	0	5	0	7	8	8
20	1	2	6	1	2	5	1	6	8	5	6	9	2	3	1	0	3	9	3	9	8	7	0	3	9	8	4	1	0	3	5	3
21	3	9	4	7	4	9	3	7	7	6	3	4	2	5	4	3	6	2	3	9	7	4	5	5	2	0	5	5	7	7	9	5
22	4	5	5	0	8	1	0	3	1	2	5	0	2	3	0	4	1	1	3	8	9	7	8	8	9	1	4	4	4	5	2	6
23	1	3	4	4	9	6	9	7	2	3	8	3	6	9	7	6	6	2	5	1	4	2	0	1	2	0	3	8	6	5	5	2
24	8	9	7	6	5	8	2	3	8	4	8	7	0	4	5	0	3	1	0	8	9	1	6	6	2	7	1	7	7	6	0	1
25	7	7	1	0	9	9	4	3	6	9	7	8	8	2	7	3	9	7	1	4	9	7	0	0	1	5	6	6	2	8	8	9
26	8	9	5	9	6	0	0	8	8	4	4	2	2	2	8	2	1	5	2	4	2	5	1	7	5	8	1	8	0	0	8	1
27	7	9	4	1	2	3	1	2	2	4	3	1	6	7	0	2	9	9	8	4	3	4	6	9	3	0	8	5	4	7	6	2
28	2	2	8	4	0	8	9	6	9	1	0	7	5	5	4	2	7	3	1	9	3	7	8	2	1	0	6	8	9	5	7	4
29	9	5	9	4	7	4	1	8	9	3	6	5	6	0	4	5	1	1	8	3	5	9	1	8	9	5	9	9	1	1	4	3
30	4	6	1	3	8	5	4	9	6	3	6	9	3	2	0	8	5	1	0	9	9	6	8	0	1	1	6	8	6	1	3	3

1st No : Major Block

2nd No : Minor Block

From random # table, 1st # is major # block & 2nd is minor #

- Take 3 readings for a block; if no success move on.

- Ensure that you have 3 measurements in total

Figure C3: Random Number Table (Modified from Weibel, 1980)

1	2	1	2	1	2	1	2	1	2
3	4	3	4	3	4	3	4	3	4
1	2	1	2	1	2	1	2	1	2
3	4	3	4	3	4	3	4	3	4
5	6	5	6	5	6	5	6	5	6
7	8	7	8	7	8	7	8	7	8
5	6	5	6	5	6	5	6	5	6
7	8	7	8	7	8	7	8	7	8
9	0	9	0	9	0	9	0	9	0
1	2	1	2	1	2	1	2	1	2

B 36

CHAPTER 7: REFERENCES

- Akpan, A., Morgan, R., 2002. Oral Candidiasis. *Postgraduate Medical Journal* 78, 455-459.
- Al-Fattani, M.A., Douglas, L.J., 2006. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *Journal of Medical Microbiology* 55, 999-1008.
- Amabeoku, G.J., Eagles, P., Scott, G., Mayeng, I., Springfield, E., 2001. Analgesic and antipyretic effects of *Dodonaea angustifolia* and *Salvia africana-lutea*. *Journal of Ethnopharmacology* 75, 117-124.
- Anil, S., Ellepola, A.N.B., Samaranayake, L.P., 2001. The impact of chlorhexidine gluconate on the relative cell surface hydrophobicity of oral *Candida albicans*. *Oral Diseases* 7, 119-122.
- Asres, K., Bucar, F., Kartnig, T., Witvrouw, M., Pannecouque, C., De Clercq, E., 2001. Antiviral activity against Human Immunodeficiency Virus Type 1 (HIV-1) and Type 2 (HIV-2) of ethnobotanically selected Ethiopian medicinal plants. *Phytotherapy Research* 15, 62-69.
- Audus, K.L., Tavakoli-Saberi, M.R., Zheng, H., Boyce, E.N., 1992. Chlorhexidine effects on membrane lipid domains of human buccal epithelial cells. *Journal of Dental Research* 71, 1298-1303.
- Bachmann, S.P., VandeWalle, K., Ramage, G., Patterson, T.F., Wickes, B.L., Graybill, J.R., Lopez-Ribot, J.L., 2002. *In Vitro* Activity of Caspofungin against *Candida albicans* Biofilms. *Antimicrobial Agents and Chemotherapy* 46, 3591-3596.

- Baillie, G.S., Douglas, L.J., 1998. Effect of growth rate on resistance of *Candida albicans* biofilms to antifungal agents. *Antimicrobial Agents and Chemotherapy* 42, 1900-1905.
- Baillie, G.S., Douglas, L.J., 1999. Role of dimorphism in the development of *Candida albicans* biofilms. *Journal of Medical Microbiology* 48, 671-679.
- Bandara, H.M.H.N., Yau, J.Y.Y., Watt, R.M., Jin, L.J., Samaranayake, L.P., 2010. *Pseudomonas aeruginosa* inhibits *in vitro* *Candida* biofilm development. *BMC Microbiology* 10, 125-134.
- Banerjee, M., Thompson, D.S., Lazzell, A., Carlisle, P.L., Pierce, C., Monteagudo, C., Lopez-Ribot, J.L., Kadosh, D., 2008. *UME6*, a Novel Filament-specific Regulator of *Candida albicans* Hyphal Extension and Virulence. *Molecular Biology of the Cell* 19, 1354-1365.
- Barki, M., Koltin, Y., Yanko, M., Tamarkin, A., Rosenberg, M., 1993. Isolation of a *Candida albicans* DNA sequence conferring adhesion and aggregation on *Saccharomyces cerevisiae*. *Journal of Bacteriology* 175, 5683-5689.
- Barki, M., Koltin, Y., van Wetter, M., Rosenberg, M., 1994. A *Candida albicans* surface antigen mediating adhesion and autoaggregation in *Saccharomyces cerevisiae*. *Infection and Immunity* 62, 4107-4111.
- Beachey, E.H., 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *Journal of Infectious Diseases* 143, 325-345.
- Blignaut, E., Botes, M.E., Nieman, H.L., 1999. The treatment of oral candidiasis in a cohort of South African HIV/AIDS patients. *South African Dental Journal* 54, 605-608.

- Bradway, S.D., Levine, M.J., 1993. Do proline-rich proteins modulate a transglutaminase catalyzed mechanism of candidal adhesion? *Critical Reviews in Oral Biology and Medicine* 4, 293-299.
- Brawner, D.L., Cutler, J.E., 1986. Variability in Expression of Cell Surface Antigens of *Candida albicans* during Morphogenesis. *Infection and Immunity* 51, 337-343.
- Calderone, R.A., Braun, P.C., 1991. Adherence and Receptor Relationships of *Candida albicans*. *Microbiological Reviews* 55, 1-20.
- Calderone, R.A., 1993. Recognition between *Candida albicans* and host cells. *Trends in Microbiology* 1, 55-58.
- Cannon, R.D., Chaffin, W.L., 1999. Oral colonization by *Candida albicans*. *Critical Reviews in Oral Biology and Medicine* 10, 359-383.
- Cao, Y., Dai, B., Wang, Y., Huang, S., Xu, Y., Cao, Y., Gao, P., Zhu, Z., Jiang, Y., 2008. *In vitro* activity of baicalein against *Candida albicans* biofilms. *International Journal of Antimicrobial Agents* 32, 73-77.
- Casanova, M., Gil, M.L., Cardenoso, L., Martinez, J.P., Sentandreu, R., 1989. Identification of wall-specific antigens synthesized during germ tube formation by *Candida albicans*. *Infection and Immunity* 57, 262-271.
- Casanova, M., Chaffin, W.L., 1991. Cell wall glycoproteins of *Candida albicans* as released by different methods. *Journal of General Microbiology* 137, 1045-1051.
- Casanova, M., Lopez-Ribot, J.L., Martinez, J.P., Sentandreu, R., 1992. Characterization of cell wall proteins from yeast and mycelial cells of *Candida albicans* by labeling with biotin: Comparison of other techniques. *Infection and Immunity* 60, 4898-4906.

- Castillo, L., Calvo, E., Martinez, A.I., Ruiz-Herrera, J., Valentin, E., Lopez, J.A., Sentandreu, R., 2008. A study of the *Candida albicans* cell wall proteome. *Proteomics* 8, 3871-3881.
- Chaffin, W.L., Lopez-Ribot, J.L., Casanova, M., Gozalbo, D., Martinez, J.P., 1998. Cell Wall and Secreted Proteins of *Candida albicans*: Identification, Function, and Expression. *Microbiology and Molecular Biology Reviews* 62, 130-180.
- Chandra, J., Kuhn, D.M., Mukherjee, P.K., Hoyer, L.L., McCormick, T., Ghannoum, M.A., 2001. Biofilm formation by the fungal pathogen *Candida albicans*: Development, Architecture and Drug Resistance. *Journal of Bacteriology* 183, 5385-5394.
- Colodel, E.M., Traverso, S.D., Seitz, A.L., Correa, A., Oliveira, F.N., Driemeier, D., Gava, A., 2003. Spontaneous poisoning by *Dodonaea viscosa* (Sapindaceae) in cattle. *Veterinary and Human Toxicology* 45, 147-148.
- Cowan, M.M., 1999. Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews* 12, 564-582.
- Critchley, I.A., Douglas, L.J., 1987. Isolation and partial characterization of an adhesin from *Candida albicans*. *Journal of General Microbiology* 133, 629-636.
- Douglas, L.J., McCourtie, J., 1983. Effect of tunicamycin treatment on the adherence of *Candida albicans* to human buccal epithelial cells. *FEMS Microbial Letters* 16, 199-202.
- Douglas, L.J., 1987. Adhesion of *Candida* species to epithelial surfaces. *Critical Reviews in Microbiology* 15, 27-43.

- Douglas, L.J., 2002. Medical Importance of biofilms in *Candida* infections. *Revista Iberoamericana de Micologia* 19, 139-143.
- Ellepola, A.N.B., Samaranayake, L.P., 1998. The effect of limited exposure to antimycotics on the relative cell-surface hydrophobicity and the adhesion of oral *Candida albicans* to buccal epithelial cells. *Archives of Oral Biology* 43, 879-887.
- Ellepola, A.N.B., Samaranayake, L.P., 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, 166-171.
- Eloff, J.N., 1999. The antibacterial activity of 27 southern African members of the *Combretaceae*. *South African Journal of Science* 95, 148-152.
- Endo, E.H., Cortez, D.A.G., Ueda-Nakamura, T., Nakamura, C.V., Filho, B.P.D., 2010. Potent antifungal activity of extracts and pure compound isolated from pomegranate peels and synergism with fluconazole against *Candida albicans*. *Research in Microbiology* 161, 534-540.
- Epstein, J.B., Kimura, L.H., Menard, T.W., Truelove, E.L., Pearsall, N.N., 1982. Effects of specific antibodies on the interaction between the fungus *Candida albicans* and human oral mucosa. *Archives of Oral Biology* 27, 469-474.
- Epstein, J.B., Truelove, E.L., Izutzu, K.T., 1984. Oral candidiasis: Pathogenesis and Host defense. *Reviews of Infectious Diseases* 6, 96-106.
- Epstein, J.B., Vickars, L., Spinelli, J., Reece, D., 1992. Efficacy of chlorhexidine and nystatin rinses in prevention of oral complications in

leukemia and bone marrow transplantation. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics 73, 682-689.

- Fekete-Forgacs, K., Gyure, L., Lenkey, B., 2000. Changes of virulence factors accompanying the phenomenon of induced fluconazole resistance in *Candida albicans*. Mycoses 43, 273-281.
- Ferretti, G.A., Ash, R.C., Brown, A.T., Parr, M.D., Romond, E.H., Lillich, T.T., 1988. Control of oral mucositis and candidiasis in marrow transplantation: A prospective, double-blind trial of chlorhexidine digluconate oral rinse. Bone Marrow Transplantation 3, 483-493.
- Franzblau, S.G., Cross, C., 1986. Comparative *in vitro* antimicrobial activity of Chinese medicinal herbs. Journal of Ethnopharmacology 15, 279-288.
- Fu, Y., Rieg, G., Fonzi, W.A., Belanger, P.H., Edwards, J.R., J.E., Filler, S.G., 1998. Expression of the *Candida albicans* gene *ALS1* in *Saccharomyces cerevisiae* induces adherence to endothelial and epithelial cells. Infection and Immunity 66, 1783-1786.
- Fukayama, M., Calderone, R.A., 1991. Adherence of cell surface mutants of *Candida albicans* to buccal epithelial cells and analyses of the cell surface proteins of the mutants. Infection and Immunity 59, 1341-1345.
- Furletti, V.F., Teixeira, I.P., Obando-Pereda, G., Mardegan, R.C., Sartoratto, A., Figueira, G.M., Duarte, R.M.T., Rehder, V.L.G., Duarte, M.C.T., Hofling, J.F., 2011. Action of *Coriandrum sativum* L. Essential oil upon oral *Candida albicans* biofilm formation. Evidence-Based Complementary and Alternative Medicine, 1-9.

- Garcia-Sanchez, S., Aubert, S., Iraqui, I., Janbon, G., Ghigo, J.M., d'Enfert, C., 2004. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryotic Cell* 3, 536-545.
- Gaur, N.K., Klotz, S.A., 1997. Expression, cloning, and characterization of a *Candida albicans* gene, *ALA1* that confers adherence properties upon *Saccharomyces cerevisiae* for extracellular matrix proteins. *Infection and Immunity* 65, 5289-5294.
- Gaur, N.K., Klotz, S.A. Henderson, R.L., 1999. Overexpression of the *Candida albicans* *ALA1* gene in *Saccharomyces cerevisiae* results in aggregation following attachment to extracellular matrix proteins, adherence properties similar to those of *Candida albicans*. *Infection and Immunity* 67, 6040-6047.
- Gauwerky, K., Borelli, C., Korting, H.C., 2009. Targeting virulence: A new paradigm for antifungals. *Drug Discovery Today* 14, 214-222.
- Ghannoum, M.A., Abu El-Teen, K., Radwan, S.S., 1987. Blocking adherence of *Candida albicans* to buccal epithelial cells by yeast glycolipids, yeast wall lipids and lipids from epithelial cells. *Mycoses* 30, 371-378.
- Ghannoum, M.A., Rice, L.B., 1999. Antifungal Agents: Mode of Action, Mechanisms of Resistance, and Correlation of these mechanisms with bacterial resistance. *Clinical Microbiology Reviews* 12, 501-517.
- Giuliana, G., Pizzo, G., Milici, M.E., Musotto, G.C., Giangreco, R., 1997. *In vitro* Antifungal Properties of Mouthrinses Containing Antimicrobial agents. *Journal of Periodontology* 68, 729-733.

- Giuliana, G., Pizzo, G., Milici, M.E., Giangreco, R., 1999. *In vitro* activities of antimicrobial agents against *Candida* species. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics 87, 44-49.
- Guinet, R., Bruneau, S., 1988. Characterization of the fibrinogen binding factor from *Candida albicans* and *Candida tropicalis* in quantitative immunoelectrophoresis. Mycoses 31, 508-514.
- Hauman, C.H., Thompson, I.O., Theunissen, F., Wolfaardt, P., 1993. Oral carriage of *Candida* in healthy and HIV-seropositive persons. Oral Surgery Oral Medicine Oral Pathology 76, 570-572.
- Hawser, S.P., Baillie, G.S., Douglas, L.J., 1998. Production of extracellular matrix by *Candida albicans* biofilms. Journal of Medical Microbiology 47, 253-256.
- Haynes, K., 2001. Virulence in *Candida* species. Trends in Microbiology 9, 591-596.
- Hazen, K.C., 1989. Participation of yeast cell surface hydrophobicity in adherence of *Candida albicans* to human epithelial cells. Infection and Immunity 57, 1894-1900.
- Hazen, K.C., Hazen, B.W., 1992. Hydrophobic surface protein masking by the opportunistic fungal pathogen *Candida albicans*. Infection and Immunity 60, 1499-1508.
- Hernandez-Richter, T., Schardey, H.M., Lohlein, F., Fleischer, C.T., Walli, A.K., Boos, K.S., Schildberg, F.W., 2000. Binding Kinetics of Triclosan (Irgasan ®) to Alloplastic Vascular Grafts: An *in vitro* study. Annals of Vascular Surgery 14, 370-375.

- Hoyer, L.L., 2001. The ALS gene family of *Candida albicans*. Trends in Microbiology 9, 176-180.
- Ishida, K., Rodrigues, J.C.F., Ribeiro, M.D., Vila, T.V.M., de Souza, W., Urbina, J.A., Nakamura, C.V., Rozental S., 2009. Growth inhibition and ultrastructural alterations induced by $\Delta^{24(25)}$ - sterol methyltransferase inhibitors in *Candida* spp. isolates, including non-*albicans* organisms. BMC Microbiology 9, 1-12.
- Jayatilake, J.A.M.S., Samaranayake, Y.H., Samaranayake, L.P., 2005. An ultrastructural and a cytochemical study of candidal invasion of reconstituted human oral epithelium. Journal of Oral Pathological Medicine 34, 240-246.
- Jimenez-Lucho, V., Ginsburg, V., Kirvan, H.C., 1990. *Cryptococcus neoformans*, *Candida albicans*, and other fungi bind specifically to the glycosphingolipid lactosylceramide (Gal β 1-4Glc β 1-Cer), a possible adhesion receptor for yeast. Infection and Immunity 58, 2085-2090.
- Jin, Y., Samaranayake, L.P., Samaranayake, Y., Yip, H.K., 2004. Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. Archives of Oral Biology 49, 789-798.
- Jones, D.S., Gorman, S.P., McCafferty, D.F., Woolfson, A.D., 1991. The effects of three non-antibiotic, antimicrobial agents on the surface hydrophobicity of certain micro-organisms evaluated by different methods. Journal of Applied Biotechnology 71, 218-227.
- Jones, D.S., Schep, L.J., Shepherd, M.G., 1995. The effect of cetylpyridinium chloride (CPC) on the cell surface hydrophobicity and adherence of *Candida albicans* to human buccal epithelial cells *in vitro*. Pharmaceutical Research 12, 1896-1900.

- Kennedy, M.J., Rogers, A.L., Hanselman, L.R., Soll, D.R., Yancey, R.J. Jr., 1988. Variation in adhesion and cell surface hydrophobicity in *Candida albicans* white and opaque phenotypes. *Mycopathologia* 102, 146-156.
- Khalil, N.M., Sperotto, J.S., Manfron, M.P., 2006. Anti-inflammatory activity and acute toxicity of *Dodonaea viscosa*. *Fitoterapia* 77, 478-480.
- Khurram, M., Hameed, A., Amin, M.U., Gul, A., Ullah, N., Hassan, M., Qayum, A., Manzoor, W., 2011. Phytochemical screening and *in vitro* evaluation of anticandidal activity *Dodonaea viscosa* (L.) Jaeq. (Sapindaceae). *African Journal of Pharmacy and Pharmacology* 5, 1422-1426.
- Kimura, L.H., Pearsall, N.N., 1980. Relationship between germination of *Candida albicans* and increased adherence to human buccal epithelial cells. *Infection and Immunity* 28, 464-468.
- Kornev, N.R., Velichko, E.V., 1987. Distribution of epitheliocytes by the number of *Candida albicans* adherent to them and the assessment of the intensity of the adhesion in an *in vitro* test. *Zhurnal Mikrobiologii, Epidemiologii, Immunobiologii* 5, 28-32.
- Ku, T.S.N., Palanisamy, S.K.A., Lee, S.A., 2010. Susceptibility of *Candida albicans* biofilms to azithromycin, tigecycline and vancomycin and the interaction between tigecycline and antifungals. *International Journal of Antimicrobial Agents* 36, 441-446.
- Kwon-Chung, K.J., Lehman, D., Good, C., Magee, P.T., 1985. Genetic Evidence for Role of Extracellular Proteinase in Virulence of *Candida albicans*. *Infection and Immunity* 49, 571-575.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

- Leidich, S.D., Ibrahim, A.S., Fu, Y., Koul, A., Jessup, C., Vitullo, J., Fonzi, W., Mirbod, F., Nakashima, S., Nozawa, Y., Ghannoum, M.A., 1998. Cloning and disruption of *caPLB1*, a Phospholipase B gene involved in the pathogenicity of *Candida albicans*. The Journal of Biological Chemistry 278, 26078-26086.
- Lemar, K.M., Turner, M.P., Llyod, D., 2002. Garlic (*Allium sativum*) as an anti-*Candida* agent: a comparison of the efficacy of fresh garlic and freeze-dried extracts. Journal of Applied Microbiology 93, 398-405.
- MacDonald, F., Odds, F.C., 1983. Virulence for Mice of a Proteinase-secreting Strain of *Candida albicans* and a Proteinase-deficient Mutant. Journal of Microbiology 129, 431-438.
- Mackenzie, D.W.R., 1962. Serum tube identification of *Candida albicans*. Journal of Clinical Pathology 15, 563-565.
- MacNeill, S., Rindler, E., Walker, A., Brown, A.R., Cobb, C.M., 1997. Effects of tetracycline hydrochloride and chlorhexidine gluconate on *Candida albicans*: An *in vitro* study. Journal of Clinical Periodontology 24, 753-760.
- Marr, K.A., Lyons, C.N., Ha, K., Rustad, T.R., White, T.C., 2001. Inducible Azole Resistance associated with a heterogenous phenotype in *Candida albicans*. Antimicrobial Agents and Chemotherapy 45, 52-59.
- Marsh, P., Martin, M.V., 1999. Oral Microbiology 4th Edition. Elsevier Science Limited, Edinburgh, 8-9.
- Martin, M.V., Lamb, C.J., 1982. Frequency of *Candida albicans* serotypes in patients with denture-induced stomatitis and in normal denture wearers. Journal of Clinical Pathology 35, 888-891.

- Masoko, P., Picard, J., Eloff, J.N., 2005. Antifungal activities of six South African *Terminalia* species (*Combretaceae*). *Journal of Ethnopharmacology* 99, 301-308.
- Masoko, P., Picard, J., Eloff, J.N., 2007. The antifungal activity of twenty-four southern African *Combretum* species (*Combretaceae*). *South African Journal of Botany* 73, 173-183.
- McCourtie, J., Douglas, L.J., 1981. Relationship between cell surface composition of *Candida albicans* and adherence to acrylic after growth on different carbon sources. *Infection and Immunity* 32, 1234-1241.
- McCourtie, J., Douglas, L.J., 1984. Relationship between cell surface composition, adherence and virulence of *Candida albicans*. *Infection and Immunity* 45, 6-12.
- McCourtie, J., MacFarlane, T.W., Samaranayake, L.P., 1986. Effect of saliva and serum on the adherence of *Candida* species to chlorhexidine-treated denture acrylic. *Journal of Medical Microbiology* 21, 209-213.
- Miceli, M.H., Bernardo, S.M., Lee, S.A., 2009. *In vitro* analyses of the combination of high-dose doxycycline and antifungal agents against *Candida albicans* biofilms. *International Journal of Antimicrobial Agents* 34, 326-332.
- Mims, C., Dockrell, H., Goering, R., Roitt, I., Wakelin, D., Zuckerman, M., 2004. *Medical Microbiology* 3rd Edition. Mosby, Elsevier Limited, Philadelphia, 504.
- More, G., Tshikalange, T.E., Lall, N., Botha, F., Meyer, J.J., 2008. Antimicrobial activity of medicinal plants against oral microorganisms. *Journal of Ethnopharmacology* 119, 473-477.

- Motsei, M.L., Lindsey, K.L., van Staden, J., Jager, A.K., 2003. Screening of traditionally used South African plants for antifungal activity against *Candida albicans*. Journal of Ethnopharmacology 86, 235-241.
- Mukherjee, P.K., Sheehan, D.J., Hitchcock, C.A., Ghannoum, M.A., 2005. Combination treatment of invasive fungal infections. Clinical Microbiology Reviews 18, 163-194.
- Nakamura, C.V., Ishida, K., Faccin, L.C., Filho, B.P.D., Cortez, D.A.G., 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.
- Nisengard, R.J., Newman, M.G., 1994. Oral Microbiology and Immunology 2nd Edition. W.B. Saunders Company, Philadelphia, 295-297.
- Nobile, C.J., Nett, J.E., Andes, D.R., Mitchell, A.P., 2006. Function of *Candida albicans* adhesion Hwp1 in biofilm formation. Eukaryotic Cell 5, 1604-1610.
- Olsen, I., 1990. Oral adhesion of yeasts. Acta Odontologica Scandinavica 48, 45-53.
- Palombo, E.A., 2009. Traditional Medicinal Plant Extracts and Natural Products with activity against oral bacteria: Potential application in the prevention and treatment of oral diseases. Oxford University Press, 1-15.
- Patel, M., Shackleton, J.T., Coogan, M.M., 2006. Effect of antifungal treatment on the prevalence of yeasts in HIV-infected subjects. Journal of Medical Microbiology 55, 1279-1284.

- Patel, M., Coogan, M.M., 2008. Antifungal activity of the plant *Dodonaea viscosa* var. *angustifolia* on *Candida albicans* from HIV-infected patients. *Journal of Ethnopharmacology* 118, 173-176.
- 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, In *Antimicrobial Resistance in Developing countries*, 137-156. Edited by A.J. Sosa, D.K. Byarugaba, C.F. Amabile-Cuevas, P.R. Hsueh, S. Kariuki, I.N. Okeke. Springer New York Dordrecht Heidelberg London.
- Pitarch, A., Sanchez, M., Nombela, C., Gil, C., 2002. Sequential Fractionation and Two-dimensional Gel analysis unravels the complexity of the dimorphic fungus *Candida albicans* cell wall proteome. *Molecular and Cellular Proteomics* 1, 967-982.
- Ponton, J., Jones, J.M., 1986. Analysis of cell wall extracts of *Candida albicans* by sodium dodecyl sulphate – polyacrylamide gel electrophoresis and western blot techniques. *Infection and Immunity* 53, 565-572.
- Portela, M.B., Kneipp, L.F., Ribeiro de Souza, I.P., Holandino, C., Alviano, C.S., Meyer-Fernandes, J.R., de Araujo Soares, R.M., 2010. Ectophosphatase activity in *Candida albicans* influences fungal adhesion: study between HIV-positive and HIV-negative isolates. *Oral Diseases* 16, 431-437.
- Ramage, G., VandeWalle, K., Wickes, B.L., Lopez-Ribot, J.L., 2001. Characteristics of biofilm formation by *Candida albicans*. *Revista Iberoamericana de Micologia* 18, 163-170.

- Rani, M.S., Pippalla, R.S., Mohan, K., 2009. *Dodonaea viscosa* Linn. – An overview. Journal of Pharmaceutical Research and Health Care 1, 97-112.
- Ruhnke, M., Eigler, A., Tennagen, I., Geiseler, B., Engelmann, E., Trautmann, M., 1994. Emergence of Fluconazole-resistant strains of *Candida albicans* in patients with recurrent oropharyngeal candidosis and Human Immunodeficiency Virus infection. Journal of Clinical Microbiology 32, 2092-2098.
- Runyoro, D.K., Ngassapa, O.D., Matee, M.I., Joseph, C.C., Moshi, M.J., 2006a. Medicinal plants used by Tanzanian traditional healers in the management of *Candida* infections. Journal of Ethnopharmacology 106, 158-165.
- Runyoro, D.K., Matee, M.I., Ngassapa, O.D., Joseph, C.C., Mbwambo, Z.H., 2006b. Screening of Tanzanian medicinal plants for anti-*Candida* activity. BMC Complementary and Alternative Medicine 6, 11.
- Samaranayake, L.P., McCourtie, J., MacFarlane, T.W., 1980. Factors affecting the *in vitro* adherence of *Candida albicans* to acrylic surfaces. Archives of Oral Biology 25, 611-615.
- Samaranayake, L.P., MacFarlane, T.W., 1982a. Factors affecting the *in vitro* adherence of the fungal oral pathogen *Candida albicans* to epithelial cells of human origin. Archives of Oral Biology 27, 869-873.
- Samaranayake, L.P., MacFarlane, T.W., 1982b. The effect of dietary carbohydrates on the *in vitro* adhesion of *Candida albicans* to epithelial cells. Journal of Medical Microbiology 15, 511-517.
- Samaranayake, L.P., McLaughlin, L., MacFarlane, T., 1988. Adherence of *Candida* species to fibrin clots *in vitro*. Mycopathologia 102, 135-138.

- Samaranayake, L.P., 2002. Essential Microbiology for Dentistry. Harcourt Publishers Limited, London, 60-145.
- Samaranayake, Y.H., Samaranayake, L.P., Yau, J.Y.Y., Ellepola, A.N.B., Anil, S., Yeung, K.W.S., 2003. Adhesion and cell-surface-hydrophobicity of sequentially isolated genetic isotypes of *Candida albicans* in an HIV-infected Southern Chinese cohort. *Mycoses* 46, 375-383.
- Sandin, R.L., 1987. Studies on cell adhesion and concanavalin A-induced agglutination of *Candida albicans* after mannan extraction. *Journal of Medical Microbiology* 24, 145-150.
- 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.
- Schaller, M., Korting, H.C., Schafer, W., Bastert, J., Chen, W., Hube, B., 1999. Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Molecular Microbiology* 34, 169-180.
- Schmidt-Westhausen, A., Schiller, R.A., Pohle, H.D., Reichart, P., 1991. Oral *Candida* and Enterobacteriaceae in HIV-1 infection: correlation with clinical candidiasis and antimycotic therapy. *Journal of Oral Pathology and Medicine* 20, 469-472.
- Schwab, U., Milatovic, D., Braveny, I., 1997. Increased adherence of *Candida albicans* to buccal epithelial cells from patients with AIDS. *European Journal of Clinical Microbiology and Infectious Diseases* 16, 848-851.
- Silva, M.L.A., Coimbra, H.S., Pereira, A.C., Almeida, V.A., Lima, T.C., Costa, E.S., Vinholis, A.H.C., Royo, V.A., Silva, R., Filho, A.A.S., Cunha, W.R., Furtado, N.A.J.C., Carlos, C., Martins, H.G. Carvalho, T.C., Bastos,

- J.K., 2007. Evaluation of *Piper cubeba* extract, (-)-cubebin and its semi-synthetic derivatives against oral pathogens. *Phytotherapy Research* 21, 420-422.
- Silva, F., Ferreira, S., Duarte, A., Mendonca, 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*. Article in press.
 - Singleton, D.R., Masuoka, J., Hazen, K.C., 2001. Cloning and Analysis of a *Candida albicans* gene that affects cell surface hydrophobicity. *Journal of Bacteriology* 183, 3582-3588.
 - Skerl, K.G., Segal, E., Sreevalsan, T., Scheld, M., 1984. *In vitro* binding of *Candida albicans* yeast cells to human fibronectin. *Canadian Journal of Microbiology* 30, 221-227.
 - Spijkervet, F.K., van Saene, H.K., Panders, A.K., Vermey, A., van Saene, J.J., Mehta, D.M., Fidler, V., 1989. Effect of chlorhexidine rinsing on the oropharyngeal ecology in patients with head and neck cancer who have irradiation mucositis. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* 67, 154-161.
 - Staab, J.F., Ferrer, C.A., Sundstrom, P., 1996. Developmental expression of a tandemly repeated proline- and glutamine- amino acid motif on hyphal surfaces of *Candida albicans*. *The Journal of Biological Chemistry* 271, 6298-6305.
 - Sterer, N., 2006. Antimicrobial effect of mastic gum methanolic extract against *Porphyromonas gingivalis*. *Journal of Medicinal Food* 9, 290-292.

- Sudbery, P., Gow, N., Berman, J., 2004. The distinct morphogenic states of *Candida albicans*. Trends in Microbiology 12, 317-324.
- Sundstrom, P.M., Kenny, G.E., 1984. Characterization of antigens specific to the surface of germ tubes of *Candida albicans* by immunofluorescence. Infection and Immunity 43, 850-855.
- Sundstrom, P., 1999. Adhesins in *Candida albicans*. Current Opinion in Microbiology 2, 353-357.
- Tayel, A.A., Moussa, S., El-Tras, W.F., Knittel, D., Opwis, K., Schollmeyer, E., 2010. Anticandidal action of fungal chitosan against *Candida albicans*. International Journal of Biological Macromolecules 47, 454-457.
- Teanpaisan, R., Nittayananta, W., 1998. Prevalence of *Candida* species in AIDS patients and HIV-free subjects in Thailand. Journal of Oral Pathology and Medicine 27, 4-7.
- Teshome, K., Gebre-Mariam, T., Asres, K., Engidawork, E., 2010. Toxicity studies on dermal application of plant extract of *Dodonaea viscosa* used in Ethiopian traditional medicine. Phytotherapy Research 24, 60-69.
- Thurmond, J.M., Brown, A.T., Sims, R.E., Ferretti, G.A., Raybould, T.P., Lillich, T.T., Henslee, P.J., 1991. Oral *Candida albicans* in bone marrow transplant patients given chlorhexidine rinses: Occurrence and susceptibilities to the agent. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics 72, 291-295.
- Tronchin, G., Bouchara, J.P., Annaix, V., Robert, R., Senet, J.M., 1991. Fungal cell adhesion molecules in *Candida albicans*. European Journal of Epidemiology 7, 23-33.

- Tyagi, A.K., Malik, A., 2010. *In situ* SEM, TEM and AFM studies of the antimicrobial activity of lemon grass oil in liquid and vapour phase against *Candida albicans*. *Micron* 41, 797-805.
- van Heerden, F.R., Viljoen, A.M., van Wyk, B-E., 2000. The major flavonoid of *Dodonaea angustifolia*. *Fitoterapia* 71, 602-604.
- van Wyk, B.-E., Van Oudtshoorn, B., Gericke, N., 2002. Medicinal plants of South Africa. Briza Publications, Pretoria, 108-109.
- Velichko, E.V., Karaev, Z.O., 1987. Effect of antibiotics on *Candida* adhesion to epitheliocytes. *Antibiotics and Medical Biotechnology* 32, 360-362.
- Wahlin, Y.B., 1989. Effects of chlorhexidine mouthrinse on oral health in patients with acute leukemia. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* 68, 279-287.
- Weibel, E.R., 1980. *Stereological Methods Vol. 2: Theoretical Foundation*. Academic Press, London, 340.
- Weisdorf, D.J., Bostrom, B., Raether, D., Mattingly, M., Walker, P., Pihlstrom, B., Ferrieri, P., Haake, R., Goldman, A., Woods, W., 1989. Oropharyngeal mucositis complicating bone marrow transplantation: prognostic factors and the effect of chlorhexidine mouth rinse. *Bone Marrow Transplantation* 4, 89-95.
- Whiteway, M., Bachewich, C., 2007. Morphogenesis in *Candida albicans*. *Annual Reviews in Microbiology* 61, 529-553.
- Wilson, K., Walker, J.M., 1995. *Principles and Techniques of Practical Biochemistry*. Cambridge University Press, Cambridge, 434-436.
- Zhang, J-D., Xu, Z., Cao, Y-B., Chen, H-S., Yan, L., An, M-M., Gao, P-H., Wang, Y., Jia, X-M., Jiang, Y-Y., 2006. Antifungal activities and action

mechanisms of compounds from *Tribulus terrestris* L.. Journal of
Ethnopharmacology 103, 76-84.