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In vitro* biological activity of extracts and compounds from *Ptaeroxylon obliquum* (Thunb.) Radlk. against oral strains of *Candida albicans

CANDICE VAN WYK

**Thesis submitted to the Phytomedicine Programme
Department of Paraclinical Sciences,
Faculty of Veterinary Sciences, University of Pretoria,
for the fulfilment of the Degree Doctor of Philosophy**

**SUPERVISOR: DR FS BOTHA (PhD)
CO-SUPERVISOR: PROF JN ELOFF (DSc)**

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DECLARATION

I declare that this thesis entitled "*In vitro* biological activity of extracts and compounds from *Ptaeroxylon obliquum* (Thunb.) Radlk. against oral strains of *Candida albicans*", which I herewith submit to the University of Pretoria for a Doctor of Philosophy is my own original work, and has never been submitted for any academic award to any other institution of higher learning.

CANDICE VAN WYK

DATE

PAPERS PREPARED FROM THIS THESIS

1. Van Wyk C, Botha FS, Steenkamp V (2009). *In vitro* antimicrobial activity of medicinal plants against oral *Candida albicans* isolates. *International Journal of Biomedical and Pharmaceutical Sciences*, **3**, 26-30.
2. Van Wyk C, Steenkamp V (2011). Host factors affecting oral candidiasis. *Southern African Journal of Epidemiology and Infection*, **26(1)**, 18-21.
3. Van Wyk C, Botha FS, Steenkamp V, Eloff JN. The biological activity of seven medicinal plants used traditionally in the treatment of fungal infections. (Submitted to the South African Journal of Botany, October 2011).
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5. Van Wyk C, Botha FS, Bagla VP, Eloff JN. *In vitro* inhibitory effect of *Ptaeroxylon obliquum* (Ptaeroxylaceae) on adhesion of *Candida albicans* to human buccal epithelial cells (HBEC). (In final preparation for submission to the Journal of Ethnopharmacology).
6. Van Wyk C, Botha FS and Eloff JN. The anti-candidal activity and safety of fractions and compounds isolated from of *Ptaeroxylon obliquum* (Ptaeroxylaceae). (In final preparation for submission to the South African Dental Journal).

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2. Van Wyk C, Botha FS, Steenkamp V, Eloff JN. The effect of four medicinal plants on oral *Candida albicans* isolates. *South African Association of Botanists (SAAB) 38th Annual Conference, 15 – 18 January 2012, University of Pretoria.*
3. Van Wyk C, Botha FS, Bagla VP, Eloff JN. The *in vitro* inhibitory effect of *Ptaeroxylon obliquum* (Thunb.) Radlk. on adhesion of *Candida albicans* to human buccal epithelial cells (HBEC). *South African Association of Botanists (SAAB) 38th Annual Conference, 15 – 18 January 2012, University of Pretoria.*

CONFERENCE PRESENTATIONS

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3. Van Wyk C, Botha FS, Eloff JN. Antifungal activity of medicinal plants against oral *Candida albicans* isolates. Faculty Day, Faculty of Health Sciences, University of Pretoria, 30 and 31 August 2011.
4. Van Wyk C, Botha FS, Bagla VP. Ability of *Ptaeroxylon obliquum* (Thunb.) Radlk. to prevent adhesion of *Candida albicans* to human buccal epithelial cells. Microscopy Society of Southern Africa, 49th Annual Conference, MSSA 2011, 5-9 December 2011.
5. Van Wyk C, Botha FS, Steenkamp V, Eloff JN. The effect of four medicinal plants on oral *Candida albicans* isolates. *South African Association of Botanists (SAAB) 38th Annual Conference, 15 – 18 January 2012, University of Pretoria.*

6. Van Wyk C, Botha FS, Bagla VP, Eloff JN. The *in vitro* inhibitory effect of *Ptaeroxylon obliquum* (Thunb.) Radlk. on adhesion of *Candida albicans* to human buccal epithelial cells (HBEC). *South African Association of Botanists (SAAB) 38th Annual Conference, 15 – 18 January 2012, University of Pretoria.*

AWARDS

1. **Junior Researcher, Basic Sciences, First prize.** Faculty Day, Faculty of Health Sciences, University of Pretoria, 30 and 31 August 2011: Van Wyk C, Botha FS, Steenkamp V, Eloff JN. The biological activity of medicinal plants, active against *Candida albicans* isolates.
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LIST OF ABBREVIATIONS

ABTS	-	2,2'-azinobis-(3-ethyl benzothiazoline 6-sulfonic acid) diammonium salt
AIDS	-	Acquired Immunodeficiency Syndrome
ANOVA	-	Analysis of Variables
°C	-	degrees Celcius
CFU	-	colony forming units
DMSO	-	Dimethyl Sulfoxide
DPPH	-	1-1-diphenyl-2-picryl-hydrazyl
EIMS	-	Electron impact mass spectrometry
ETAC	-	ethyl acetate
g	-	gram
GMP	-	good manufacturing practices
h	-	hour/s
H ₂ O	-	water
HBEC	-	human buccal epithelial cells
HCA	-	<i>Harpephyllum caffrum</i> acetone extract
HIV	-	Human Immunodeficiency Virus
INT	-	<i>p</i> -iodonitro-tetrazolium violet
L	-	Litre
MEM	-	minimal essential medium
MFC	-	minimal fungicidal concentration
mg	-	milligram
MIC	-	minimal inhibitory concentration
min	-	minutes
ml	-	millilitre
mm	-	millimetre
mM	-	millimolar
MTT	-	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MN	-	mononuclear
MS	-	Mass Spectrometry
n	-	number
nm	-	nanometre

NMR	-	Nuclear Magnetic Resonance
NNFA	-	National Nutritional Foods Association
NSS	-	normal saline solution
p	-	probability
PBS	-	phosphate-buffered saline
PMN	-	polymorphonuclear
POA	-	<i>Ptaeroxylon obliquum</i> acetone extract
R _f	-	retardation factor
RMA	-	<i>Rapanea melanophloeos</i> acetone extract
rpm	-	rotations per minute
SAE	-	<i>Streblus asper</i> leaf ethanolic extract
SBA	-	<i>Sclerocarya birrea</i> acetone extract
SI	-	Selectivity index
TE	-	Trolox equivalence
TLC	-	thin layer chromatography
µg	-	microgram
µl	-	microlitre
µm	-	micrometre
UPPP	-	Phytomedicine Programme University of Pretoria
USP	-	United States Pharmacopoeia
UV	-	ultraviolet
WHO	-	World Health Organization

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ABSTRACT

In vitro* biological activity of extracts and compounds from *Ptaeroxylon obliquum* (Thunb.) Radlk. against oral strains of *Candida albicans

BY

CANDICE VAN WYK

Supervisor:

Dr FS Botha

BSc, BSc Hons (Microbiology), MSc (Microbiology) (North-West University, Potch), PhD (Pret)

Co-supervisor:

Prof JN Eloff

BSc Hons (Botany), MSc (Chemistry), DSc (Plant Biochemistry) (Potch)

External examiners:

Prof Dr F Bucar

Institute of Pharmaceutical Sciences, Department of Pharmacognosy
University of Graz, Austria

Prof Dr S Glasl-Tazreiter

Fakultät für Lebenswissenschaften, Department für Pharmakognosie
Universität Wien, Austria

Internal examiner:

Prof N Lall

Faculty of Natural and Agricultural Sciences, Department of Plant Science
University of Pretoria

Degree:

PhD

In most countries of subtropical Africa, fungal infections represent an increasing problem. *Candida albicans* is the most common fungal pathogen and has developed an extensive array of putative virulent mechanisms that allows successful colonization and infection of the host under suitable predisposing conditions.

Crude acetone, water and hexane extracts of eight plant species, used as traditional medicine to treat fungal related diseases were evaluated for antifungal activity against *C. albicans* standard strain (ATCC 10231) and six clinical isolates using a serial microplate dilution method. Antioxidant activity was also determined by using the TEAC and DPPH assays and cytotoxicity was determined against mouse fibroblast cells with a MTT assay.

MIC's below 1 mg/ml were observed for the acetone and water extracts of *Sclerocarya birrea* (stem-bark), as well as the acetone extracts of *Harpephyllum caffrum* (leaves), *Ptaeroxylon obliquum* (leaves) and *Rapanea melanophloeos* (leaves). Bioautography confirmed the presence of compounds with antifungal activity in extracts of *S. birrea* and *P. obliquum*. *S. birrea* extracts had the highest antioxidant activity and the lowest cytotoxicity. However, *P. obliquum* contained the highest number of active compounds against *C. albicans* standard strain (ATCC 10231) and clinical isolates. MIC data also indicated that *P. obliquum* was the most active of all the plant species tested against *C. albicans* standard strain (ATCC 10231) and clinical isolates. Therefore, *P. obliquum* was chosen for bioassay-guided fractionation and isolation of antifungal compounds from *P. obliquum* acetone leaf extract.

Two compounds were isolated from the most active fraction obtained from *P. obliquum* acetone leaf extract, using column chromatography with silica gel as the stationary phase.

Compound 1 and Compound 2 inhibited the growth of *C. albicans* standard strain (ATCC 10231) at MIC 0.004 mg/ml compared to amphotericin B, MIC 0.11 mg/ml. The cytotoxicity of the compounds was determined against mouse fibroblast cells using the MTT assay. Berberine was used as a positive control and was found to be toxic with a LC₅₀ of 9 04 µg/ml. The cytotoxicity of Compound 1 (against mouse fibroblast cells) was 0.002 µg/ml and that of Compound 2, 7.23 µg/ml.

Selectivity Index (SI) of Compound 1, against both *C. albicans* standard strain (ATCC 10231) and clinical isolates, was 0.0004 and for Compound 2 it was 1.8 against the standard strain (ATCC 10231) and >0.03 against the clinical isolates tested.

The structures of the compounds were determined by extensive nuclear magnetic resonance (NMR) techniques and chemical methods mainly by 1D NMR (^1H , ^{13}C and DEPT) and 2D NMR (HSQC, HMBC and COSY) and by comparison with the literature data. Compound 1 is an isomeric mixture of β -amyrin and lupeol. Compound 2 is a novel compound and was identified as:

8,11-Dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4H-pyrano[2,3-g] [1]benzoxepin-4-one 12-O-acetate.

Candidal adherence to human buccal epithelial cells (HBEC) is considered the critical initial step in the pathogenesis of oral candidiasis, which may eventually lead to a systemic infection, especially in immuno-compromised individuals. Therefore, it was important to determine the ability of extracts and isolated compounds on the adherence of *C. albicans* standard strain (ATCC 10231) and clinical isolates to HBEC cells. *P. obliquum* acetone leaf extract at concentrations of 15.6 mg/ml and 7.8 mg/ml inhibited the proliferation and hyphae formation of *C. albicans* and thus adhesion. Natural products inhibiting adhesion of *C. albicans* to the oral mucosa may be beneficial in managing oral candidiasis.

The high antifungal activity of the acetone leaf extract of *P. obliquum* indicates that it could be considered as a potential therapeutic agent against oral *Candida* infections. The therapeutic effects of this extract may be limited only to low concentrations to avoid cytotoxic reactions as the cytotoxicity was less favourable ($\text{LC}_{50} = 35.58 \mu\text{g/ml}$ against mouse fibroblast cells). However, as oral rinses are used topically and not swallowed the systemic effect may not be of critical importance.

Compound 2 was obtained as a pure novel compound namely: 8,11-Dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4*H*-pyrano[2,3-*g*][1]benzoxepin-4-one 12-*O*-acetate. This compound had a higher anti-candidal activity against *C. albicans* ATCC 10231, 0.004 mg/ml, compared to the activity of amphotericin B, 0.11 mg/ml. Considering this antifungal activity, as well as the fact that the cytotoxicity of Compound 2 ($LC_{50}=7.23 \mu\text{g/ml}$) was lower than that of amphotericin B ($LC_{50} = 1.46 \mu\text{g/ml}$), it will be of significant importance to investigate the development of this compound into a new oral hygiene and / or antifungal product.

CHAPTER 1: INTRODUCTION

In most countries of subtropical Africa, bacterial and fungal infections represent an increasing problem, particularly with patients suffering from severe immune deficiencies, such as AIDS (Atindehou *et al.*, 2002). Moreover, the validation of medicinal plant based therapies is imperative for African developing countries, as more than 70 percent of the population use such remedies (Pousset, 1994).

Although various studies in the vast and still growing literature on traditional medicine have reported the indigenous use of medicinal plants in the treatment of oral diseases, oral health-related problems have not been particularly singled out for comment (Tapsoba and Deschamps, 2006).

It is clear that reliance on plant products for the management of oral health-related problems is common around the world (Tapsoba and Deschamps, 2006). Publications are found on this topic not only in African countries such as Equatorial Guinea (Akendengue, 1992), Madagascar (Novy, 1997), and South Africa (Lin *et al.*, 1999) but also in other parts of the world including Nepal (Manandhar, 1998), Guatemala (Hunter and Arbona, 1995), and Palestine (Ali-Shtayeh *et al.*, 2000).

Medicinal plants have attracted considerable research attention as new sources of antimicrobial agents against *Candida* infections (Taweechaisupapong *et al.*, 2005). A wide variety of plant extracts have antimicrobial effects and anti-inflammatory properties (Kweifio-Okai, 1991; Matsuda *et al.*, 1997; Taweechaisupapong *et al.*, 2000a; Taweechaisupapong *et al.*, 2000b; Atindehou *et al.*, 2002; Mahasneh, 2002; Taweechaisupapong *et al.*, 2002a; Taweechaisupapong *et al.*, 2002b). Several herbal extracts have been added to some cosmetics and health-care preparations (Taweechaisupapong *et al.*, 2005).

Candida species are responsible for a wide range of systemic as well as superficial opportunistic infections (candidiasis), which are of particular importance amongst debilitated or immuno-compromised patients (Taweechaisupapong *et al.*, 2005). External candidiasis occurs most frequently in vaginal or oral mucosa (Cannon *et al.*,

1995; Williams *et al.*, 1997). Species of the genus *Candida* comprise part of the oral commensal microflora of healthy individuals (Taweechaisupapong *et al.*, 2005). Among them, *Candida albicans* is the most common pathogen of the group, being isolated intra-orally in 17 to 75 percent of healthy individuals (Arendorf and Walker, 1980; Bastiaan and Reade, 1982; Rindum *et al.*, 1994).

The adhesion of microbes to the host's mucosal surfaces is a major determinant of successful microbial colonization and subsequent infection, and its critical role in the pathogenesis of many fungal infections is well recognized (King *et al.*, 1980; Shibl, 1985; Fukazawa and Kagaya, 1997).

Various *in vitro* studies (Samaranayake and MacFarlane, 1981; Samaranayake and MacFarlane, 1982) and animal studies (McCourtie and Douglas, 1984; Calderone *et al.*, 1985) provide evidence for a relationship between the ability of *Candida* species to adhere to mucosal surfaces and their presence in infections. Therefore, candidal adherence to human buccal epithelial cells (HBEC) is considered the critical initial step in the pathogenesis of oral candidiasis, which may eventually lead to a systemic infection, especially in immuno-compromised individuals (Schafer-Korting *et al.*, 1996).

CHAPTER 2: AIM AND OBJECTIVES

2.1 Aim

To develop a product that can protect users against oral *Candida* infections.

2.2 Objectives

The broad objectives to attain the aim in this study were to:

- I. determine the *in vitro* antimicrobial activity of selected medicinal plants against normal and multiple resistant *Candida albicans* isolates;
- II. determine the number of antifungal compounds present in different plant extracts by bioautography;
- III. determine the antioxidant activity of different plant extracts;
- IV. determine the safety of different plant extracts to select the best species for in depth evaluation;
- V. isolate the antifungal compounds, from the best plant species selected, using bioassay-guided fractionation; and test the compounds for antimicrobial activity and cytotoxicity;
- VI. determine the ability of *C. albicans* standard strain (ATCC 10231) and clinical isolates to adhere to human buccal epithelial cells (control); as well as to determine the ability of *C. albicans* standard strain (ATCC 10231) and clinical isolates to adhere to human buccal epithelial cells in the presence of the selected plant species in order to evaluate the possibility that a useful product can be developed based on the results.

CHAPTER 3: LITERATURE REVIEW

3.1 Adhesion of *Candida albicans* in the oral cavity

Candida species are normal oral commensals. The transition of this innocuous commensal to the disease-causing "parasite" may be associated with the virulence attributes of the organism. It is generally accepted that the host factors are of critical importance in the development of the disease state and *Candida* species are strictly opportunistic pathogens which mainly cause disease when the host defences are inadequate (Samaranayake, 1990).

A diverse array of host factors (local host factors and systemic factors) has been implicated in the pathogenesis of oral candidiasis (Samaranayake, 1990). These local and systemic factors act in concert and the eventual outcome of these disease processes is frequently related to the superimposition of the local factors upon systemic factors or vice versa. The interactions between *Candida* species and the host are extremely complex, involving a myriad of mechanisms which can be discussed under three main headings:

- (i) local host factors,
- (ii) systemic factors, and
- (iii) iatrogenic factors.

(i) Local host factors that predispose to oral candidiasis

A. **Mucosal barrier**

In order to establish an infective process *Candida* species must necessarily adhere to a host surface and subsequently proliferate and penetrate the first line of host defence which is essentially the keratinized or non-keratinized oral mucosa. The proteins present in the keratinized or non-keratinized mucosal cells may themselves act as antifungals and retard candidal invasion. The hyphal elements of the yeasts are seldom seen within the viable cellular layers and one

can speculate that such proteins may limit the candidal invasion of the oral mucosa (Kashima *et al.*, 1989).

The epithelium of the oral mucosa may undergo either atrophy, hyperplasia or dysplasia and each of these changes may affect the efficacy of the barrier mechanism of the mucosa. The constant desquamation of the mucosa takes place at a rate greater than at which *Candida* species grow *in vivo*, thus being one reason why the superficial mucosae protect against *Candida* so well (Samaranayake, 1990). It is thought that if *Candida* does begin to penetrate superficial epithelia then a hyperplastic response may compensate for invasion (Sohnle and Kirkpatrick, 1978; Odds, 1988).

However, a specific means of attachment can prevent organisms from being sloughed off a surface by secretions, fluids and the movement of food and defecation (King *et al.*, 1980). Furthermore, attachment onto solid surfaces enables an organism to benefit from the enriched nutrient status that exists at solid-liquid interfaces (Marshall, 1976).

Evidence has confirmed that *Candida albicans* cell surface modulation occurs *in vivo* (De Benardis *et al.*, 1994). These surface changes may enable a commensal yeast strain to escape immune surveillance (Diamond, 1993) or adhere to different host receptors, thereby promoting candidiasis (Cannon *et al.*, 1995). Changes in surface protein glycosylation may expose hydrophobic protein structures at the cell surface, in turn affecting adherence properties (Hazen and Glee, 1994). Yeast cell surface changes may be brought about by *Candida*/host interactions. For example, adherence to human buccal epithelial cells induced the synthesis of new proteins in *C. albicans* and the expression of signal proteins (Bailey *et al.*, 1995). An understanding of adherence mechanisms, the signals they generate and the processes that they induce, may therefore lead to specific preventive treatments for individuals predisposed to candidiasis (Cannon *et al.*, 1995).

In vitro studies show that changing environmental conditions can induce morphogenesis of *C. albicans* (Odds, 1988) and that the cell surface of the hyphal form of *C. albicans* displays a number of proteins that are either absent or masked in the yeast form (Ollert and Calderone, 1990; Ponton *et al.*, 1993). The hyphal form also exhibits increased adherence properties (Kimura and Pearsall, 1980; Samaranayake and MacFarlane, 1982; Odds, 1994) which appear to correlate with increased expression of the 5 β 1-like fibronectin receptor in cells forming hyphae (Santoni *et al.*, 1994).

B. Saliva

A continuous flow of saliva is as important as a healthy mucosal barrier in preventing oral colonization by *Candida* species. The constant flushing action of saliva essentially removes the unattached or loosely attached *Candida* species from the oral cavity (Samaranayake, 1990). Furthermore, the secretory IgA component of saliva aggregates yeasts and assists in clearance and thus inhibits candidal adhesion to host surfaces (Challacombe, 1990; Samaranayake, 1990; Scully *et al.*, 1995). In addition saliva contains antifungal factors such as lysozyme, lactoperoxidase, lactoferrin and histidine-rich polypeptides, all of which may help to keep the oral candidal populations at bay (Samaranayake, 1990).

Saliva flow rate affects microbial clearance, and the important role saliva plays as a front-line host defence mechanism is exemplified in clinical situations where the quantity and the quality of the saliva is affected (Samaranayake, 1990; Cannon *et al.*, 1995).

In Sjögren's syndrome (primary/secondary) the salivary output is affected due to pathological changes in the salivary acini and recurrent oral candidal infections are commonly seen in these patients (MacFarlane and Mason, 1973). The oral carriage of *Candida* species and coliforms is significantly higher in patients with burning mouth syndrome and this may be either directly or indirectly related to reduce salivary gland function in this patient group (Lamey and Lamb, 1988; Samaranayake *et al.*, 1989).

A concomitant increase in oral carriage of *Candida* species has been demonstrated in patients on cytotoxic therapy and irradiation therapy where there is a reduction in salivary flow (Martin *et al.*, 1981; Samaranyake *et al.*, 1984; Samaranyake *et al.*, 1988). However, an intra-oral co-existence of *Candida* species and coliforms in significant proportions has been observed in these patients as well as in Sjögrens patients. Although coliforms are generally regarded as transient oral colonizers which are usually found in small numbers among oral commensals, in the above mentioned diseased states they constitute an integral component of resident commensals (Samaranyake *et al.*, 1989). The coliforms may further promote candidal colonization of epithelia, as the yeasts show preferential ability to co-adhere with coliforms (such as *Klebsiella* species) rather than to epithelial surfaces (Makrides and MacFarlane, 1982; Centeno *et al.*, 1983). Thus, the higher prevalence of yeasts observed in the above mentioned patient groups could be a secondary phenomenon consequent on primary colonization of the oral cavity by coliforms (Samaranyake, 1990).

Qualitative changes in saliva such as the concentration of salivary glucose may also influence the oral carriage of *Candida* species (Samaranyake, 1990). Glucose may be a prime factor in the predisposition of diabetics to candidiasis as has been shown by experiments *in vitro* which demonstrate poor or absence of growth of *Candida* species in saliva which, when supplemented with glucose, fosters the multiplication of yeasts (Knight and Fletcher, 1971; Samaranyake *et al.*, 1984; Samaranyake *et al.*, 1986).

Salivary pH is another factor which may influence oral candidal colonization. The normal pH of saliva ranges from 5.6 to 7.8. The incidence of yeasts is higher in acid saliva (Young *et al.*, 1951) and a relationship exists between high candidal colonization and low salivary pH on the dorsum of the tongue, under upper dentures, and in the oral cavity (Shipman, 1979; Arendorf and Walker, 1980; Samaranyake 1990). A possible mechanism by which low pH conditions may promote oral candidal colonization is the superior ability of yeasts to adhere to epithelia and denture acrylic surfaces at a low pH (of pH 2 to pH 4) (Samaranyake and MacFarlane, 1980; Samaranyake and MacFarlane, 1982).

Another is the aciduric and acidophilic nature of *Candida* species which thrive in a low pH milieu, as opposed to most oral commensals which may not survive such extremes of acidity (Odds, 1988). Thus, from the above discussion the important role saliva plays in defence against candidal invasion is clear.

C. Phagocytes

Phagocytes provide the second line of defence against invasive *Candida* infection (Cannon *et al.*, 1995). In the immune competent host, phagocytosis of yeast and hyphal forms of *C. albicans* that gain access to deeper tissues is achieved by neutrophils, eosinophils, and monocytes (Heimdahl and Nord, 1990). During acute inflammatory responses to *Candida* infection, neutrophils are predominately responsible for *Candida*-cidal activity. Both oxidative and non-oxidative mechanisms are involved in the intracellular killing of *Candida* species, but these are not always effective (Diamond, 1993). For maximum killing efficiency, granulocytes and macrophages require augmentation by cytokines such as interferon-gamma, granulocyte-macrophage colony-stimulating factor, and interleukin-1 and -2, produced by T-cells (Greenfield, 1992; Diamond, 1993). A significant factor in the pathogenicity of *C. albicans* is the ability of surface molecules, such as mannoproteins and complement receptors, to modulate phagocyte responses (Diamond, 1993). The importance of cell-mediated immunity in resistance to *Candida* infection is illustrated by the severe mucosal candidiasis seen as a result of T-cell dysfunction in AIDS patients and in many people with chronic mucocutaneous candidiasis (Odds, 1988). This contrast with neutropenic patients, who are at a greater risk of disseminated infection. Humeral immunity, in the form of serum antibodies, plays a lesser role in the defence against candidal infection (Cannon *et al.*, 1995).

D. Morphogenesis

C. albicans is a fungus that can grow in a number of morphological forms, ranging from yeast to hyphae (Cannon *et al.*, 1995). Pseudo-hyphal forms can also be observed in several other *Candida* species (Odds, 1988). There is a common belief that the hyphal form is invasive and pathogenic, while the yeast is the commensal non-pathogenic form. Evidence for this is questionable (Kerridge,

1993; Odds, 1994). Histopathological examination of candidiasis lesions indicates that hyphae are not always present (Kerridge, 1993; Odds, 1994; Cannon *et al.*, 1995). Hyphae are also capable of contact-sensing to epithelial cells (Sherwood *et al.* 1992). *C. albicans* hyphae incubated on perforated filters over agar plates grew through the pores and along grooves. This property could facilitate the penetration of some tissues. Certain *C. albicans* strains exhibit high-frequency switching of colony morphology when nutritionally stressed. This is accompanied by changed cellular morphology and, in some cases, by chromosomal translocation. High-frequency phenotypic switching can simultaneously affect expression of many potential virulence factors and may be a genetic mechanism that allows the asexual *C. albicans* to adapt to environmental change (Soll, 1992).

ii) Systemic factors which predispose to oral candidiasis

A. Immuno-compromised individuals

The compromised patient can be defined as an individual in whom one or more of the different mechanisms of protection against infection are impaired, rendering that person less resistant to infection (Warnock, 1991). The mechanisms that protect the human host against fungal infection are complicated, diverse and often interdependent. Protection against infection depends on a combination of non-specific and specific mechanisms. In the compromised patient, alterations in phagocytic or lymphocytic cell numbers or function are often the most critical factors predisposing to fungal infection, but defects in other elements of host protection are also important (Warnock, 1991).

Thus, the incidence of candidiasis in patients with diseases such as diabetes mellitus, leukaemia, AIDS, and cancer may be due to a decrease in some aspect of host immunity to such infections (Odds, 1988; Warnock, 1991). The high frequency of oral *Candida* carriage and candidiasis in immuno-compromised individuals emphasizes that a fully functional immune system is needed to prevent candidiasis (Hauma *et al.*, 1993).

Diabetes and oral candidiasis. It is well known that uncontrolled diabetes predisposes to superficial and systemic infections, and oral candidiasis in particular is thought to be more prevalent among these individuals (Samaranayake, 1990). However, the mechanism by which diabetes predisposes to high oral carriage of *Candida* species is not clear. It has been suggested that high salivary glucose levels in diabetics may favour the growth of yeasts (Knight and Fletcher, 1971; Odds *et al.*, 1987). Other investigators have been unable to show a relationship between glycaemic control and oral candidal carriage (Tapper-Jones *et al.*, 1981; Fisher *et al.*, 1987).

It has been reported that the adhesion of *Candida* species to buccal epithelial cells from diabetics is significantly greater than cells obtained from non-diabetic controls (Darwazeh *et al.*, 1990). When the adhesion of *C. albicans* to buccal epithelial cells from 50 diabetics and 50 control non-diabetics (age, sex and denture status matched) was compared, there was a 55 percent increase in candidal adhesion to the cells of the diabetics ($p < 0.001$). This implies that in diabetics, there may be intrinsic qualitative changes on the cell surface receptors modulating yeast adhesion (Darwazeh *et al.*, 1990; Samaranayake, 1990).

Another systemic host factor which may promote oral carriage of *Candida* species in diabetics is possible defects in *Candida*-cidal activity of neutrophils, particularly in the presence of glucose (Wilson and Reeves, 1986). Micro-vascular degeneration in capillaries within the lamina propria, as seen in diabetics with atrophic candidal glossitis, might promote candidal carriage and/or infection (Farman and Nutt, 1976).

It would appear that in addition to the foregoing systemic factors other local parameters such as the presence of dentures and salivary glucose levels may act in a cumulative manner upon the presence and density of *Candida* species in the mouths of diabetics. Thus, equal attention should be given to both local and systemic predisposing factors to suppress the candidal numbers and hence reduce the risk of oral candidiasis in diabetics (Samaranayake, 1990).

HIV and oral candidiasis. Oral opportunistic infections developing secondary to human immunodeficiency virus (HIV) infection have been reported from the early days of the epidemic and have been classified by both the EC-Clearinghouse and the World Health Organization (WHO) (Hodgson and Rachanis, 2002).

Oropharyngeal candidiasis is the most common fungal infection among patients infected with the human immunodeficiency virus (Coleman *et al.*, 1993; Lal and Chussid, 2005; Coogan *et al.*, 2006; Wingeter *et al.*, 2007). The frequency of yeasts in mouthwashes from HIV-positive patients presenting with oropharyngeal candidiasis were determined and *C. albicans* was the most frequent specie (93 percent) (Wingeter *et al.*, 2007). Colonization of the oral cavities of HIV-infected patients by the genus *Candida* were investigated and found to be the prevailing species (Menon *et al.*, 2001; Costa *et al.*, 2006).

C. albicans strains causing oral candidiasis in patients with HIV infection are identical to the commensal ones found in healthy individuals. After identification of *C. albicans* isolates using conventional methods and determination of carbohydrate assimilation profiles, serotyping was performed by slide agglutination with the Iatron IF6 serotyping system. Sensitivity of the isolates to 5-Fluorocytosine (5-FC) was assessed using the broth microdilution method. There was no difference in phenotype between HIV-positive and HIV-negative individuals. Serotype A was found to be dominant in strains isolated from both HIV-positive (34/40=85%) and HIV-negative (17/20=85%) study subjects (Woldeamanuel and Abate, 1998).

Candida dubliniensis is a yeast specie that has only recently been differentiated from *C. albicans* (Blignaut *et al.*, 2003). *C. dubliniensis* colonization was initially associated with human immunodeficiency virus (HIV)-positive individuals. Because of the large proportion of AIDS patients in South Africa, the generality of this association was tested by assessing the prevalence of *C. dubliniensis* colonization among 253 black HIV-positive individuals, 66 healthy black individuals, 22 white HIV-positive individuals, and 55 healthy white individuals in South Africa carrying germ tube-positive yeasts in their oral cavities. Molecular

fingerprinting with Ca3, a complex DNA fingerprinting probe specific for *C. albicans*, and Cd25, a complex DNA fingerprinting probe specific for *C. dubliniensis*, provides the first conclusive evidence of the existence of *C. dubliniensis* among South African clinical yeast isolates and reveals a higher prevalence of this species among white healthy individuals (16%) than among HIV-positive white individuals (9%), black healthy individuals (0%), and black HIV-positive individuals (1.5%). The authors concluded that in South Africa, *C. dubliniensis* carriage is influenced more by race than by HIV status (Blignaut *et al.*, 2003).

The prevalence of oral colonization with *Candida* species (*C. albicans*, *Candida stellatoidea*, *Candida krusei* and *Candida tropicalis*) in 28 HIV-seropositive and 28 healthy persons in a South African population were studied. *Candida* species were cultured from 75 percent and 68 percent of HIV-positive and control persons, respectively, with a significantly higher density carriage in the HIV-seropositive group. The higher density *Candida* species carriage in the HIV-positive group suggests that HIV seropositivity may lead to changes in the oral environment and in local immunologic reactivity thus allowing greater numbers of *Candida* species to colonize the oral cavity. This may explain the high incidence of oral candidiasis in HIV-seropositive patients while still in the early stages of the disease (Hauman *et al.*, 1993). Mycelial findings in smears of subjects from both groups without any clinical symptoms of candidiasis confirm the results of Torssander *et al.* (1987) who suggested that mycelial carriers may possibly develop oral candidiasis more frequently in cases of impaired host defence. Another explanation is that the mycelial forms may indicate a transition from commensalism to parasitism in these specific subjects, as Torssander *et al.* (1987) reported positive smears in orally symptomless immuno-compromised patients followed by the development of clinically manifested infections.

Biotype 1 isolates of *C. albicans* were the most frequently recovered phenotype, accounting for 65 percent and 55 percent of the HIV-seropositive and healthy control group, respectively (Hauman *et al.*, 1993). This correlates with results from

previous studies (Korting *et al.*, 1988; Francker *et al.*, 1990; Menon *et al.*, 2001; Costa *et al.*, 2006; Wingeter *et al.*, 2007).

Historically, blood CD4 cell numbers were the primary prognosticator for the development of oral candidiasis. However, statistic evaluation of the predictive role of HIV viral load vs CD4 cell counts revealed viral load to be a stronger predictor for oral candidiasis than CD4 cell count (Coogan *et al.*, 2006).

Oral candidiasis is one of the earliest indicators of the progression from HIV-seropositive status to the acquired immunodeficiency syndrome (AIDS) (Cannon *et al.*, 1995). Oral candidiasis already becomes clinically apparent in the prodromal stages of AIDS (Klein *et al.*, 1984) with more than 75 percent of infected patients presenting with candidiasis during the course of the disease (Farmon *et al.*, 1986). Esophageal candidiasis in AIDS patients can be extensive and these lesions can be a source of infection for other forms of oral candidiasis that are often seen in AIDS patients (Holmstrup and Samaranayake, 1990).

This high frequency of oral *Candida* carriage and candidiasis in HIV-seropositive and AIDS patients emphasizes that a fully functional immune system is needed to prevent candidiasis (Farmon *et al.*, 1986; Hauman *et al.*, 1993). It has also been proposed that no single factor appears to be responsible for the pathogenicity of *C. albicans*, but that a combination of different factors contributes at each stage of infection (Samaranayake, 1990; Cutler, 1991; Calderone, 1994; Matthews, 1994; Odds, 1994). However, dental professionals should be aware that local host factors influences not only the colonization but also the form of candidal infection that is likely to be established (Samaranayake 1990; Cannon *et al.*, 1995).

Salivary anti-candidal activity and salivary composition in stimulated whole saliva of 18 advanced HIV-infected patients were investigated and these values were compared to healthy controls. Stimulated whole saliva from HIV-infected patients showed decreased anti-*Candida* activity. The flow rate was reduced by 40 percent as compared with controls. For HIV-infected patients, the saliva concentrations of lactoferrin, secretory IgA and Cl⁻ were increased while the

secretion rate of lysozyme, total protein and K⁺ were reduced. The conclusion was that these alterations may have contributed to the increased incidence of oral candidal infections in HIV-seropositive patients (Lin *et al.*, 2001).

In certain clinical situations, therefore, oral candidiasis can be of diagnostic and prognostic value for patients who already have, or are at high risk of developing, serious underlying disease.

Leukaemia and lymphoma. Although the term “compromised patient” is often taken to mean an individual with serious impairment of immunological function, it should also be applied to the person in whom non-immunological mechanisms of protection are impaired (Warnock, 1991).

Fungal infection is a frequent problem in patients with malignant haematological conditions. This is not surprising since host protection against infection depends in major part on the cells that are affected in these disorders (Warnock, 1991). Each of these conditions is associated with characteristic defects of phagocytic or lymphocytic cell numbers or function and this helps to account for the particular infections encountered in each disorder (Hersch *et al.*, 1976). Treatment will further disturb the mechanism that protect the host, changing the nature of the infectious complications encountered (Warnock, 1991).

The most important factor predisposing the leukaemic patient to infection is the marked reduction in the number of normal circulating neutrophil polymorphonuclear (PMN) and mononuclear (MN) phagocytic cells. The neutrophils that remain have metabolic defects which result in impaired migration and microbicidal function. Treatment leads to profound neutrophil depletion and deficient T- and B-cell function, which persist until remission is attained. Long periods of profound neutrophil depletion have been identified as an important factor predisposing leukaemic patients to candidiasis (Odds, 1988; Warnock, 1991).

Systemic candidiasis associated with malignancies, particularly leukaemia's and lymphomas, is well documented (Odds, 1988). Arguably, less attention has been paid to oral candidiasis consequent on malignant disease. The incidence of oral candidiasis can vary widely in individuals with malignancies although, in general, this would appear to be greater than in healthy subjects (Samaranayake, 1990). The increased incidence of oral candidiasis seen in these individuals stems partly from therapeutic measures such as cytotoxic and immunosuppressive drugs and radiotherapy which are essential in the treatment of many malignancies, whilst concurrent administration of antibiotics is also relevant in this context (Samaranayake, 1990; Warnock, 1991).

B. Altered nutritional states

Nutritional factors act in concert with a number of other co-factors in the pathogenesis of oral candidiasis. Nutritional factors, such as iron and/or vitamin deficiency, have been investigated in relation to oral candidiasis and probably influence the disease process via systemic pathways (Samaranayake, 1986).

Iron. The association that exist between oral candidiasis and iron deficiency was described by Cawson (1963). Subsequently it was suggested that iron deficiency may result in persistent chronic mucocutaneous candidiasis which is difficult to eradicate as long as the iron deficiency remains (Higgs and Wells, 1972). A high prevalence of candidal infection was found in iron-deficient patients who had angular cheilitis and atrophic glossitis (Fletcher *et al.*, 1975). A relationship between angular cheilitis and the level of plasma iron was also observed in a group of denture wearers of whom 70 percent exhibited chronic atrophic candidiasis. In four of these patients angular lesions and oral candidiasis regressed when treated with systemic iron therapy alone (Rose, 1968).

Swabs were taken from the dorsal surfaces of 181 individuals' tongues, working or residing in the Kalahari National Gemsbok Park, to determine a possible association between oral yeasts and clinically observed oral lesions as well as other underlying conditions detectable by serum chemistry. Identification of yeasts was performed with a commercially available identification system,

namely the ATB 32C (Montalieu, Vercieu). Yeasts were isolated from 30.4 percent (n=55) of individuals, of whom 43.6 percent (n=24) had only *C. albicans*, 3.6 percent (n=2) had *C. albicans* together with other yeasts and 52.7 percent (n=29) had other yeasts. Many of these yeasts were not the commonly encountered isolates. The results of the study revealed that a significant association ($p < 0.02$) exists between yeasts (n=55) and low serum iron concentrations (n=50) (Blignaut *et al.*, 1995).

The literature indicates that iron deficiency may play an important role in oral carriage of *Candida* species and the pathogenesis of candidal infections. However, the mechanisms by which iron could influence the oral carriage of *Candida* species may be diverse. Iron deficiency may produce an impairment of iron-dependent enzyme systems thereby affecting the metabolism and, hence, the kinetics of the rapidly dividing oral epithelial cells (Rennie and MacDonald, 1982). Such alterations may result in an epithelial surface more conducive for the adhesion, growth and invasion of *Candida* species. In addition to epithelial affections, iron deficiency could substantially depress the cell-mediated immune response (Joynson *et al.*, 1972). Other general effects of iron deficiency which may bear a relationship to oral candidiasis include impaired phagocytosis and inadequate antibody production (Wilton and Lehner, 1981; Rennie *et al.*, 1984).

Vitamins. Few reports are available in the literature linking candidiasis to vitamin deficiency. However, as one effect of folate deficiency is widespread degenerative changes in the oral mucosa, it is possible that this may contribute to the pathogenesis of oral candidiasis by providing a less hostile surface for candidal colonization (Ferguson, 1975; Samaranayake, 1990). Nevertheless, the effect of vitamin deficiencies on the oral mucosa cannot be considered in isolation and must be viewed in conjunction with the systemic effects of the deficiency state.

iii) Iatrogenic factors that predispose the host to infection

A. Antibiotic therapy

Antibiotic treatment can cause *C. albicans* overgrowth in the oral cavity by eliminating competing micro-organisms and exposing additional sites suitable for colonization (Cannon *et al.*, 1995). Broad-spectrum antibiotic therapy, rather than narrow-spectrum antibiotics, is generally accepted as one of the more common iatrogenic factors which initiate oral candidiasis. The most common broad-spectrum drug associated with oral candidiasis is tetracycline (Samaranayake, 1990).

B. Corticosteroid therapy

Corticosteroids are one of the most common group of drugs used in modern medicine due to their potent anti-inflammatory and immunosuppressive properties (Samaranayake, 1990; Warnock, 1991). Consequently they can lower host resistance to infection and thus predispose individuals to systemic and superficial candidiasis (Samaranayake, 1990).

The impact of systemic steroid therapy on oral candidiasis would appear to be minimal compared to the effect of steroid aerosol inhalers (Marsh *et al.*, 1983; Samaranayake, 1990). The relationship between steroid inhalers and oral candidiasis was studied and in 5 out of 25 patients, provided with dexamethasone inhalers for asthma, oral candidiasis were present (Dennis and Itkin, 1964).

Since the introduction of the corticosteroid aerosol inhalations their use has dramatically increased, particularly for the treatment of less severe forms of asthma in children and adults (Samaranayake, 1990; Warnock, 1991). Beclomethasone dipropionate, betamethasone valerate, sodium cromoglycate and triamcinolone acetonide are all synthetic steroids which are commonly used for this purpose and a major side effect associated with the use thereof is oral and pharyngeal candidiasis. Generally the lesions are superficial in nature and present

as the atrophic or the pseudomembranous variety (Stead and Cooke, 1989; Samaranayake, 1990).

The putative mechanisms by which either inhaled or systemic steroids predispose to oral candidiasis have not been fully clarified, as yet. In man, treatment with these drugs leads to a marked reduction in neutrophil migration and this result in suppression of inflammation. Neutrophil phagocytic and microbicidal function is also impaired. Treatment with corticosteroids also leads to a profound, but transient reduction in the number of circulating T-cells; the number of B-cells are also reduced, but to a lesser extent (Cupps and Fauci, 1982). Although corticosteroids do not abolish lymphokine production by activated T- cells, the response of macrophages to these mediators is often reduced or abolished. The production of a number of macrophage products is also inhibited and this will affect T-cell function (Warnock, 1991). Patients treated with corticosteroids showed high levels of salivary glucose and this may also promote growth, proliferation and adhesion of *Candida* species (Knight and Fletcher, 1971).

C. Cytotoxic- and radiotherapy (Irradiation)

Irradiation has been shown to affect both specific and non-specific elements of host protection against infection. The epithelial barrier of the mouth and gastrointestinal track will often be damaged, but myelosuppression is often the major factor leading to infectious complications. Irradiation will also affect most aspects of T- and B-cell function, resulting in profound suppression of cell-mediated and humeral immunological reactions (Warnock, 1991).

Opportunistic oral candidiasis is commonly observed in patients who are on cytotoxic- and radiotherapy. *Candida* species are responsible for approximately one half of oral infections that arise during anti-leukaemia chemotherapy and for almost two thirds of those that occur in patients given anti-neoplastic drugs for solid tumours (Peterson and Sonis, 1983).

The prevalence of oral *Candida* species in longitudinal surveys during radiotherapy for oral and laryngeal cancer and during cytotoxic therapy for solid tumours of oral and perioral regions increased during therapy. The yeast infection persisted for at least 4 to 6 months after treatment (Martin *et al.*, 1981; Main *et al.*, 1984).

D. Cigarette/tobacco smoking and oral candidiasis

The mechanism(s) by which the prevalence and metabolism of *Candida* species in the oral cavity may be affected by cigarette or cigar smoke is uncertain. Smoking may lead to localized epithelial alterations which facilitate candidal colonization (Arendorf and Walker, 1980). An alternative hypothesis is that cigarette smoke contains nutritional factors for *C. albicans*. This has important implications as aromatic hydrocarbons contained in cigarette smoke may be converted by inducible enzyme systems present in *Candida* species to carcinogen end products. This together with the observation that *C. albicans* could catalyse the formation of *N*-nitrosobenzyl-methylamine may partly explain why candidal leucoplakia has a higher potential for malignant change than other leucoplakias (Samaranayake, 1990).

3.2 History of herbal medicine

Phytotherapy is the treatment and prevention of disease using plants, plant parts, and preparations made from them. Plants and plant parts that are used in the preparation of phytotherapeutic medicines contain a multitude of possible active chemical constituents. To isolate some of these ingredients can be a lifelong task, but with modern physiochemical technologies, various techniques have been proven to successfully isolate a great number of constituents in a plant. These isolated chemicals should in fact be classified as herbal or natural drugs, but the chemically or synthetically modified derivatives could not be termed as natural drugs. Unfortunately, many substances used for the synthesis of chemicals have very little relation to the original plant chemicals. The chemical constituents of the

plant should be studied to understand the composition of the plant (Weiss and Fintelmann, 2000). Of the 250 000 flowering plant species, only a small percentage have been studied up to date, therefore the opportunities for further investigations into plant species are enormous.

Although the term phytotherapy started with the French physician Henri Leclerc (1870 – 1955) in Paris, the origin of herbal medicine is much older and dates back to the times before Christ. The driving forces behind the transition of folk medicine to modern medicine owe some of the recognition to one of the founding fathers of German phytotherapy, Rudolf Fritz Weiss (1895 -1992). Important and renowned physicians of the 19th century used herbal medicines as a basis for prescription of medical drugs. The transition of the unspecific and unscientific methods of herbal medicines to the evolvement of a practice of phytotherapy, that is influenced by scientific methodology and tested for efficacy and safety, was necessary for the survival of phytotherapy in modern times (Weiss and Fintelmann, 2000).

Phytotherapy is sometimes regarded as a form of alternative medicine, grouped with other therapies such as homeopathy, acupuncture, biofeedback, colour therapy, music therapy, hypnotherapy, aromatherapy, ayurvedic medicine, or Bach flower remedies. These other therapies can be regarded as unscientific and erroneous, whereas phytotherapy no longer falls in that category. It is important to note that phytotherapy is a separate school of practice and must be clearly distinguished from homeopathy. Phytotherapy sometimes has clearly different treatment indications for specific plants than homeopathy (Weiss and Fintelmann, 2000).

Knowledge of phytotherapy should be incorporated into the basic and specialized training of medical and to some extent dental students but cannot be conveyed in just a purely theoretical form, practical experience should also form part of their training. Medical and dental students must become acquainted with medicinal herbs in the pre-clinical years and must receive an introduction to herbal medicine including phytochemistry, phytopharmacy, phytopharmacology

and phytotherapy. During the clinical years, practical application of these herbs could be taught and practiced (Weiss and Fintelmann, 2000).

The existence of the placebo phenomenon has been scientifically proven, but we are still unable to explain exactly how this effect works. Abraham Lincoln once said "You can fool some of the people all of the time, and all of the people some of the time, but you can't fool all of the people all of the time" (Weiss and Fintelmann, 2000). This could mean that if a drug or herb has been used for centuries, repeatedly asked for by patients and prescribed by doctors, one could probably assume that it is effective (Weiss and Fintelmann, 2000). Numerous plants have been proven to exhibit antibacterial properties and some have been tested to show limited or no toxic effects in the human body (Cowan, 1999).

About 70 percent of practitioners prescribe herbal medicines in Germany. The active part of a plant can usually be ascribed to a specific ingredient within the plant. Some of the active ingredients include the following substances: sugars and gums, glycosides and aglycones, amino acids, lectins, glyco-proteins, flavonoids, tannins, quinones, coumarins and alkaloids (Fetrow and Avila, 2001).

3.3 Some compounds isolated from plants

Phenolic compounds

Plants contain an abundance of phenolic compounds. These compounds include: flavonoids, flavones, catechols, anthocyanins and polymeric phenolic compounds. Phenolic compounds account for pharmacological and therapeutic effects of many medicinal plant species (Lovkova *et al.*, 1990).

The flavones, flavonoids and flavonols are phenolic structures and have been extensively researched due to their occurrence in oolong green teas and popular use in eastern countries (Fessenden, 1982; Toda *et al.*, 1989). Their chemical structure assists in complexing them with extracellular and soluble proteins and to bacterial cell walls (Tsuchiya *et al.*, 1996). They contain a mixture of catechin compounds and have shown inhibition *in vitro* against *Streptococcus mutans* and

other bacteria and micro-organisms (Sakanaka *et al.*, 1989; Sakanaka *et al.*, 1992).

Flavonoids have antioxidant activity which protects cells and tissue from injurious effects of free radicals (Sigh *et al.*, 2005). The compounds 2,3-digalloyl-4-(E)-caffeoyl-L-threonine and kaempferol 3-O- α -L-rhamnoside have been isolated from the leaves of *Cornus controversa*, a member of the family *Cornaceae* (Lee *et al.*, 2000).

Terpenoids

Terpenoids contribute tremendously to the pharmacological activity of many medicinal plants (Shai, 2007). They are a major class of physiologically active substances that includes more than 10 000 compounds. Depending on the number of isoprenoid groups (C₅H₈), monoterpenes, sesquiterpenes, diterpenes, tetraterpenes and polyterpenes are found in plants (Paseshnichenko, 1987; McGarvey and Croteau, 1995; Shai, 2007). In plants triterpenoids act as attractants for pollinators and seed dispensers, competitive phytotoxins, herbivore repellents and toxins (Harbone, 1991). Triterpenoids also function as hormones (gibberellins, abscisic acid), photosynthetic pigments (phytol, carotenoids) and electron carriers (ubiquinone, plastoquinone) (McGarvey and Croteau, 1995; Shai, 2007).

Pentacyclic triterpenes that had antimicrobial activity against *C. albicans*, *Escherichia coli*, *Mycobacterium fortuitum*, *Proteus vulgaris* and *Staphylococcus aureus* were isolated from members of the African *Combretaceae* (Katerere *et al.*, 2003). Betulinic acid, platonic acid and oleanolic acid isolated from leaves of *Syzigium claviform* had anti-HIV activity in the H9 lymphocyte cell line (Fujoka and Kashiwada, 1994). Triterpenes and their derivatives display activities such as cytotoxicity to tumour cell lines *in vitro* through the induction of apoptosis (Lee *et al.*, 1989; Shai, 2007).

Essential oils

Essential oils are complex mixtures of fragrant volatile substances, monoterpenes, sesquiterpenes, aromatic compounds and their derivatives (Shai, 2007). Bactericidal, astringent and anti-inflammatory effects of some plant species have been linked to the presence of essential oils (Lovkova *et al.*, 2001; Shai, 2007).

3.4 Manufacturing of herbal drugs

It cannot be emphasized strongly enough that herbs in their medicinal sense are drugs. Certain special interest groups continually emphasize their point of view that medicinal herbs are foods or dietary supplements, but scientifically, although not legally under current law, that is not the case. If they are used in the treatment (cure or mitigation) of disease or improvement of health (diagnosis or prevention of disease), they conform to the definition of the word *drug* (Foster and Tyler, 1999).

Restrictions on the collection of certain wild plants, and limit availability of plant resources, can increase the cost of production. Countries are forced to specialize in producing only certain types of phytomedicinal resources. Factors such as age, environmental conditions, temperature, rainfall, length of daylight, altitude, atmosphere, and soil, can affect the quality and concentration of active plant constituents. Drying the plant parts can take very long (depending on the technique of drying), and preventive measurements must ensure that the plant parts are thoroughly cleaned from any infestations before dried. To minimise microbial contamination, proper storage and preservation have to be in place to ensure quality of the product until the crude drug undergoes various processes before being transformed into a herbal pharmaceutical (Fetrow and Avila, 2001).

Plant parts that can be used, includes the following: the roots, bulbs, scales, rhizomes, tubers, bark, leaves, stems, flowers, fruit, and seeds. Gums and nectars can also be used. The method and form into which the plant will be prepared is also important. The different forms include: enemas, extracts, infusions, inhalations,

linctuses, liniments, lotions, mixtures, nasal drops, ointments, tinctures and snuffs. The most common methods of administering plant medicines are orally, sublingually, rectally, topically, nasally, smoking, steaming and bathing (Van Wyk *et al.*, 2009).

In 1995 the United States Pharmacopoeia (USP) commissioned an advisory panel on natural products; their goal was to establish standards and to develop information concerning herbal and dietary supplements. The National Nutritional Foods Association (NNFA) of the United States is an organization of representatives from several thousand manufacturers, retailers, suppliers, and distributors of natural products, health foods, and dietary supplements. The NNFA has accreditation programs that inspect members with manufacturing facilities to determine whether they meet NNFA-specified standards of good manufacturing practices (GMP). From 1999 the NNFA seal appeared on some dietary supplement products. The time-tested and labour-intensive process of investigation and development of any new pharmaceutical entity should never fall short of determining its risk-versus-benefit profile (Fetrow and Avila, 2001).

Registration of medicines in South Africa is regulated by Act 101 of 1965 (Medicines and Related Substances Act), that stipulates all medicines should be safe (non-toxic), efficacious and of good quality. However, some of the plants used as food or for medicinal purposes are potentially toxic, mutagenic and carcinogenic (Schimmer *et al.*, 1988; Higashimoto *et al.*, 1993; Kassie *et al.*, 1996). It would appear that a clear link between herbal medicines and toxic incidents applied to less than 0.1 percent of toxicity cases (Wolpert, 2001); however misidentification of the plant species, poor quality of the preparations, prolonged usage and the addition of toxic substances to plant derived remedies contribute significantly to the toxicity associated with plant extracts (Stewart and Steenkamp, 2000; Wolpert, 2001; Fennell *et al.*, 2004).

3.5 Drugs discovered from ethnobotanical leads

Substances derived from plants or their model derivatives constitute over 25 percent of all prescribed commercial drugs (Farnsworth, 1988; Fabricant and Farnsworth, 2001). Indigenous knowledge systems provide leads that largely guide the discovery of these drugs. The three types of plant-derived drugs are:

- 1) unmodified natural products where the ethnomedicinal use suggested clinical efficacy;
- 2) unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use and;
- 3) synthetic substances based on a natural product from a plant used in traditional medicine (Cox, 1994; Balunas and Kinghorn, 2005; Shai, 2007).

Examples of plant-derived commercial drugs are displayed in the table below:

Drug	Medicinal use	Plant source
Aspirin	Analgesic, anti-inflammatory	<i>Filipendula ulmaria</i>
Cocaine	Ophthalmic, anaesthetic	<i>Erythroxylum coca</i>
Colchicine	Gout	<i>Colchicum autumnale</i>
Emetine	Amoebic dysentery	<i>Psychotria ipecacuanha</i>
Eugenol	Toothache	<i>Syzygium aromaticum</i>
Morphine	Analgesic	<i>Papaver somniferum</i>
Scopolamine	Motion sickness	<i>Datura stramonium</i>
Vinblastine	Hodgkin's disease	<i>Catharanthus roseus</i>
Vincristine	Leukaemia	<i>Catharanthus roseus</i>
Quinine	Malaria prophylaxis	<i>Cinchona pubescens</i>

(Adapted from Fabricant and Farnsworth, 2001)

3.6 Treatment of oral candidiasis

3.6.1 Conventional therapy

Candidiasis has become a major public health problem as an opportunistic infection in patients being treated with chemotherapy and radiotherapy for cancer, and in patients who are immuno-compromised because of HIV infection

and AIDS. *C. albicans* is the most common fungal pathogen and has developed an extensive array of putative virulent mechanisms that allows successful colonization and infection of the host under suitable predisposing conditions (Runyoro *et al.*, 2006; Ship *et al.*, 2007).

Treatment of candidiasis is complicated by the emergence of strains of *Candida* that are resistant to the currently used antifungal agents (Perea *et al.*, 2001; Khan *et al.*, 2003). Patients are treated with the membrane-active polyenes nyastatin and amphotericin B, usually administered as a suspension or lozenges, while the ergosterol biosynthesis inhibitors (imidazoles and triazoles) are administered as tablets (miconazole, ketoconazole, and fluconazole), as a gel (miconazole), or as troches (clotrimazole) (Budtz-Jørgensen, 1990; Martin, 1990; Cannon *et al.*, 1995). These currently used antifungal agents are not only limited in number, but, many are in addition toxic (Mehta *et al.*, 2002; Ship *et al.*, 2007) and very costly (Salie *et al.*, 1996; Mehta *et al.*, 2002). Further, the social stigma associated with the HIV disease in many developing regions in Africa and Asia appears to modify the therapeutic strategies and management of fungal infections (Samaranayake *et al.*, 2002). Relapse of *Candida* infections is also very common and this increases the burden of managing this opportunistic infection (Debruyne, 1997). These factors prompt the need for development of new antifungal agents in order to widen the spectrum of activities against *Candida* species and combat strains expressing resistance to the available antifungals.

3.6.2 Herbal remedies

Despite the availability of different approaches for discovery of medicines, plants still remain one of the best reservoirs of new structural types and are also a valuable source of new bioactive compounds (Runyoro *et al.*, 2006). Many medicinal plants have demonstrable pharmacological properties, such as antimicrobial, anti-inflammatory and cytostatic properties and natural products have been used for medicinal purpose throughout the world for thousands of years (Patel and Coogan, 2008). It is estimated that up to a quarter of all prescriptions in industrialised countries contain one or more components derived from plants (Farnsworth, 1990).

In Africa and in many developing countries, medicinal plants are used in the treatment of various ailments and a large number of people depend on medicinal plants because they have no access to modern medicines (Elmi, 1991; Khan and Nkunya, 1991; Patel and Coogan, 2008). To prevent recurrence of oral candidiasis in patients from resource-poor countries; to reduce cost of treatment; and to prevent the development of antifungal resistance, oral rinsing with an antimicrobial agent is a suggestion. Lawsone methyl ether, isolated from *Rhinacanthus nasutus* (dainty spurs) leaves is a cost-effective mouthrinse with potent antifungal activity (Blignaut *et al.*, 2006).

The beneficial effect of *Dodonaea viscosa* var. *angustifolia* (hopbush) is well known (Van Wyk *et al.*, 2002). As far back as the 1700's a decoction of the leaves was accepted as an early remedy that is still used today for the treatment of oral infections (Van Wyk *et al.*, 2002; Patel and Coogan, 2008). The report that this plant extract has an analgesic effect is a further advantage because patients with oral candidiasis have painful mouths and experience difficulty with eating and swallowing (Amabeoku *et al.*, 2001). Another advantage is that it has antiviral activity against both HIV 1 and 2 and is non-toxic (Asres *et al.*, 2001). These properties suggest that the extract has the potential to be used as an effective mouth rinse for the prevention of recurrent oral candidiasis by reducing the number of *Candida* species in the mouth to an acceptable level (Patel and Coogan, 2008).

The flora of the Western Cape, known as Cape fynbos, forms part of the Cape Floral Kingdom which consists of 8550 species distributed over an area of 90 000 km² (Cowling *et al.*, 1992). The *Asteraceae* is the largest family in the fynbos biome; and most traditional medicines used by the indigenous communities are in fact derived from plants belonging to this family (Green, 1991; Cowling *et al.*, 1992). Many fynbos plants have also long been used as herbal remedies by descendants of the original inhabitants, the Khoi and San people (De Selincourt, 1992).

Three indigenous Asteraceae species investigated for *in vitro* antimicrobial activity demonstrated activity against the fungus *C. albicans*. These species include *Eriocephalus africanus* L. (Cape of Good Hope shrub), in which the activity resided in a lipophilic extract of the leaves, *Helichrysum crispum* (L.) D. Don (Hottentots bedding), in which a lipophilic extract of the stems caused the activity, and *Felicia erigeroides* DC. (Felicia) in which semi-polar extracts of all three plant organs produced the activity (Salie *et al.*, 1996). The authors concluded that these three plants, particularly *F. erigeroides* which is a perennial, may, therefore, serve as readily accessible and inexpensive alternative remedies for the treatment of fungal infections (Salie *et al.*, 1996).

In an attempt to find a traditional remedy to treat oral candidiasis, the plant extracts of *Allium sativum* (garlic), *Glycyrrhiza glabra* (licorice), *Polygala myrtifolia* (August/September bush), and *Tulbaghia violacea* (wild garlic) had the best activity against *C. albicans* standard strain (ATCC 10231) and two clinical isolates from a 5-month-old baby and an adult (Motsei *et al.*, 2003). Thin layer chromatography (TLC)-bioautography indicated several active compounds in *Allium sativum* and *Tulbaghia violacea* bulb extracts; one of the active compounds in both plants could tentatively be identified as allicin (Wagner and Bladt, 1996). Allicin has long been identified as an active compound in garlic with antimicrobial and antifungal properties. It is active against most Gram-positive and Gram-negative bacteria, as well as *C. albicans*. Allicin's main antimicrobial effect is due to its chemical reaction with thiol groups of various enzymes (Ankri and Mirelman, 1999). The inhibitory effect that garlic shows against *C. albicans* is attributed to the oxidation of essential protein thiol, causing inactivation of enzymes and subsequent microbial growth inhibition (Ghannoum, 1988). Saponins were identifiable in the aqueous extracts of *Polygala myrtifolia* and *Glycyrrhiza glabra* from the froth they produced. *Glycyrrhiza glabra* and *Polygala senega* (Seneca snakeroot) are also extensively used in Europe as treatment for oral candidiasis; both these plants contain saponins with antifungal activity (Bruneton, 1995).

The Phytomedicine Programme, University of Pretoria (UPPP), South Africa, conducts biological activity investigations on plant species collected randomly or through ethnomedicinal leads. Of the more than 350 species collected thus far, many species have shown promising activity with minimal inhibitory concentration (MIC) values of crude extracts ranging from 0.08-1.0 mg/ml against *C. albicans* (Shai, 2007). Seven of these species were collected, namely: *Curtisia dentata* (Cape lancewood, assegai); *Trichilia emetica* (Natal mahogany); *Kigelia africana* (sausage tree); *Terminalia sambesiaca*; *Vepris reflexa* (bushveld white-ironwood); *Terminalia phanerophlebia* (Lebombo cluster-leaf) and *Cussonia zuluensis* (cabbage tree) for further focused investigations of antifungal activity (Shai, 2007).

The acetone extract of *Terminalia* resulted in very low MIC values against some of the fungal species investigated (*Aspergillus fumigatus*; *C. albicans*, *Cryptococcus neoformans*; *Microsporum canis*; *Sporothrix schenckii*). Low MIC values were also obtained with all the extracts (hexane, dichloromethane and acetone) of *Terminalia phanerophlebia*. The total activity values against fungi resulting from *Terminalia sambesiaca* and *Terminalia phanerophlebia* were the highest. The lowest total activity was obtained with acetone and hexane extracts of *Kigelia africana*. *Curtisia dentata*, *Terminalia phanerophlebia* and *Terminalia sambesiaca* extracts had the best MIC values (0.02 mg/ml) against *M. canis* (Shai, 2007).

Low MIC values against *C. albicans* were observed with both the acetone and dichloromethane extracts of *Curtisia dentata*, with MIC values of 0.12 mg/ml and 0.15 mg/ml, respectively. *Terminalia phanerophlebia*, *Terminalia sambesiaca* and *Kigelia africana* extracts resulted in low MIC values against *C. albicans*. *Trichilia emetica*, *Vepris reflexa* and *Cussonia zuluensis* resulted in MIC values above 0.5 mg/ml against *C. albicans*. *Trichilia emetica* and *Vepris reflexa* were the least active. *Curtisia dentata* extracts were the only ones that resulted in high total activity against *C. albicans*. The acetone extracts of *Curtisia dentata* had the highest total activity (1083 ml/g) against *C. albicans* (Shai, 2007).

Oral candidiasis is popularly known among traditional healers as “*Utando mweupe wa mdomoni*”, a condition that has been known in Tanzania for a long time (Matee *et al.*, 1995). Plants from Tanzania that have proven antifungal activity against *C. albicans* and used to treat oral candidiasis include:

- Dried fruits of *Acacia nilotica* (black thorn tree);
- Saponin fraction from the mesocarp of *Balanites aegyptiaca* (simple thorned torch tree, Jericho balsam);
- Methanolic extract of *Cajanus cajan* (pigeon pea);
- Fruits, roots, latex and leaves of *Carica papaya* (papaya);
- Methanol extract of the dried bark of *Combretum molle* (velvet bush willow);
- Dried stem of *Dichrostachys cinerea* (Chinese lantern tree, Kalahari Christmas tree (South Africa), sicklebush);
- Methanol extract of dried root bark of *Harrisonia abyssinica*;
- Dried stem bark of *Ozoroa insignis* (tropical resin tree);
- Roots of *Salvadora persica* (toothbrush tree);
- Ethanolic extract of dried stem bark of *Sclerocarya birrea* (marula);
- Aqueous, dichloromethane and ethanol extracts of *Securidaca longepedunculata* (violet tree);
- Aqueous and methanol extracts of stem bark of *Ziziphus mucronata* (buffalo thorn) and stem bark of *Zanha africana* (velvet-fruited zanha).

Some of the plants were also active against other species of fungi including *C. neoformans*, one of the important pathogenic fungi in HIV/AIDS (Runyoro *et al.*, 2006).

The Ivory Coast, as many African countries, is severely affected by the AIDS pandemy and among HIV related opportunistic diseases, bacterial and fungal infections have a high morbidity rate (Atindehou *et al.*, 2002). It is thus important to find plants from the local flora able to treat such infections, as traditional medicine is mostly the single therapeutic alternative. Therefore, the antibacterial and antifungal potential of 115 plants used in Ivorian traditional medicine has been evaluated (Atindehou *et al.*, 2002). Plants were submitted to antifungal assays against *C. albicans* and *Cladosporium cucumerinum*, a human and plant pathogenic micro-organism, respectively, known to be good indicators of

antifungal activity (Rahalison *et al.*, 1991). Two species were particularly active against fungi: *Dioscorea minutiflora* (Ivory Coast wild yam) and *Erythrina vogelii* ('Ouossoupalie' à Fleurs rouges (French)/red flower tree). Interestingly, young tubers of *D. minutiflora* displayed a strong activity against *C. albicans* and *C. cucumerinum*, whereas older tubers did not show any antifungal properties. This could be due to the probable presence in young tubers of diosgenin heterosides, already reported in the West African *Dioscorea* species (Quigley, 1978).

Streblus asper Lour (Moraceae; toothbrush tree) is a medicinal plant, used for several pharmaceutical purposes. Its bark is used for relief of fever, dysentery, toothache and gingivitis (Gaitonde *et al.*, 1964); the leaf extract has demonstrated insecticidal activity towards mosquito larvae (Kritsaneepaiboon, 1989); and the twigs of the plant have been used as a "toothbrush" for strengthening teeth and gums (Lewis, 1980). The root has been applied to unhealthy ulcers, sinuses and locally as an antidote to snake bite; and the milky juice has been used as an antiseptic and astringent for chapped hands and cracked heels (Mukherjee and Roy, 1983).

In order to provide more information about the potential of *Streblus asper* leaf ethanolic extract (SAE) for its development as a natural hygiene product, the inhibitory effects of SAE on the *in vitro* adhesion of *C. albicans* to human buccal epithelial cells (HBEC) were investigated. The minimum concentration of SAE that significantly reduced adherence ($p < 0.05$) after one hour exposure was 15.6 mg/ml. However, there was a significant reduction ($p < 0.05$) of candidal adhesion to HBEC after one minute exposure to 125 mg/ml of SAE. Pre-treatment of either *Candida* or HBEC, or both, with 125 mg/ml of SAE for one hour resulted in reduced adherence. The SAE-treated *C. albicans* cells at concentrations of 125- and 250 mg/ml also showed 41 and 61 percent inhibition of germ tube formation, respectively, which might affect adherence (Taweechaisupapong *et al.*, 2005).

Similarly, exposure to sub-inhibitory concentrations of garlic extract (Ghannoum, 1990), date extract (Abu-Elteen, 2000), octenidine and pirtenidine (Ghannoum *et al.*, 1990), amphotericin B and nystatin (Macura, 1988; Abu-Elteen *et al.*, 1989) and

ketoconazole (King *et al.*, 1980; Sobel and Obedeanu, 1983) inhibited germination of *Candida* species and reduced attachment to human epithelial cells. Thus, the inhibition of germ tube formation by the SAE is important, since it is well known that germ tube and mycelial forms of *C. albicans* adhere more efficiently to host cells (Kimura and Pearsall, 1980; Sobel *et al.*, 1981; Hostetter, 1994; Pendrak and Klotz, 1995).

The observed suppression of germ tube formation elicited by *Streblus asper* leaf ethanolic extract (SAE) *in vitro*, may be related to the effect of this agent on the cell walls of *Candida* species (Taweechaisupapong *et al.*, 2005). The complex ultra-structural dynamics of the cell wall during the blastospore to hyphal phase transition, and considering that the cell wall of germ tube possesses stratification comparable to that of the blastospore wall, it is possible that SAE may affect the cell wall structure, thereby suppressing germ tube formation (Cassone *et al.*, 1973).

The mechanism responsible for inhibition of adherence by SAE remains undetermined, but it could include alterations to cell surface features that could mask the adhesions present on the yeast or on the receptors present on the buccal cells. Another possibility is that SAE may interfere with the synthesis of adhesins that may be involved in the adhesion process, or it may cause a mechanical distortion of the adhesins already present in the outer envelopes, thereby blocking adherence (Taweechaisupapong *et al.*, 2005).

The *in vitro* study demonstrated that sub-lethal concentrations of *Streblus asper* leaf ethanolic extract (SAE) modulate candidal colonization of the oral mucosa thereby suppressing the invasive potential of the pathogen (Taweechaisupapong *et al.*, 2005). Previous studies demonstrated that SAE possess antibacterial activity for dental caries associated bacteria and endo- and periodontal pathogens (Taweechaisupapong *et al.*, 2000a; Taweechaisupapong *et al.*, 2000b; Wongkham *et al.*, 2001; Taweechaisupapong *et al.*, 2002a). Moreover, mouthwash containing SAE also measurably improves gingival health (Taweechaisupapong *et al.*, 2002b). These advantages suggest the potential development of SAE as a natural product for oral hygiene.

CHAPTER 4: PRELIMINARY SCREENING OF SELECTED PLANT SPECIES

4.1 Plant species used in this study

Plant species (listed in Table 4.1) were selected based on their reported biological activities, recorded antifungal activity, recorded medicinal uses, scientific research carried out to date and availability of plant material. The Phytomedicine Programme, University of Pretoria (UPPP), South Africa, conducts biological activity investigations on plant species collected randomly or through ethnomedicinal leads. Of the more than 350 species collected thus far, many species have shown promising activity with minimal inhibitory concentration (MIC) values of crude extracts ranging from 0.08-1.0 mg/ml against *C. albicans*. Three of these species were included in this study, namely: *Harpephyllum caffrum*, *Ptaeroxylon obliquum* and *Rapanea melanophloeos* for further focused investigations of antifungal activity.

Table 4.1: Plant species used in this study

Species (Family)	Common Name	Parts used	Voucher number	References
<i>Bidens pilosa</i> L. (Asteraceae)	Black Jack	Whole plant	NH 1908	Jäger <i>et al.</i> , 1996; Motsei <i>et al.</i> , 2003
<i>Dichrostachys cinerea</i> (L.) Wight & Arn. Syn. (Fabaceae)	Sickle bush	Stem bark	NH 1871	Almagboul <i>et al.</i> , 1988; Motsei <i>et al.</i> , 2003
<i>Harpephyllum caffrum</i> Bernh. (Anacardiaceae)	Wild plum	Leaves	PRU 96170	UPPP, South Africa
<i>Ptaeroxylon obliquum</i> (Thunb.) Radlk. (Ptaeroxylaceae)	Sneezewood	Leaves	PRU 96709	UPPP, South Africa
<i>Rapanea melanophloeos</i> (L.) Mez. (Myrsinaceae)	Cape beech	Leaves	PRU 96711	UPPP, South Africa
<i>Sclerocarya birrea</i> (A. Rich) Hochst. Syn. (Anacardiaceae)	Marula	Leaves and Stem bark	NH 1910	Adoum <i>et al.</i> , 1997 ; Motsei <i>et al.</i> , 2003
<i>Securidaca longepedunculata</i> Fresen. (Polygalaceae)	Violet tree	Root bark	LT 0012	Taniguchi <i>et al.</i> , 1978; Desta, 1993; Motsei <i>et al.</i> , 2003
<i>Ziziphus mucronata</i> Willd. (Rhamnaceae)	Buffalo thorn	Root bark	NH 1909	Gundiza, 1986; Motsei <i>et al.</i> , 2003

All plants were authenticated by the National Herbarium of the South African National Biodiversity Institute (Pretoria). Voucher specimens of plants with numbers beginning with NH are lodged at the Soutpansbergenis Herbarium (Makhado, Limpopo) whereas the rest are deposited in the herbarium of the Department of Paraclinical Sciences, Faculty of Veterinary Sciences, and University of Pretoria, South Africa.

The plant material (species) was dried and ground to a fine powder (Ika Analytical Mill). This fine powder was then stored in brown bottles in a dark area, until all the extracts were prepared.

4.1.1 *Bidens pilosa* L.

Asteraceae

umhlabangubo (Xhosa, Zulu); amalenjane(Zulu); Black Jack (English); knapsekêrel (Afrikaans)



Figure 4.1: *Bidens pilosa* L. (Australian Naturopathic Network, 1998)

Botanical description: A weak annual herb (considered as a weed in some habitats) usually a metre or less in height, with spreading branches. The leaves are in opposite pairs, mostly divided but also undivided, soft and hairy (Australian Naturopathic Network, 1998). The individual flowers are borne summer to autumn; they are small, yellow and usually contain four to five white petals. The seeds are black and contain four to five black burs which cling to livestock and humans (Grubben and Denton, 2004).

Medicinal uses: The whole flowering plant can be used for: coughs, conjunctivitis, dysentery, haematuria, urethritis, cystitis, cloudy urine, benign prostatic hypertrophy, kidney stones, diarrhoea and respiratory infections (Australian Naturopathic Network, 1998).

Preparation and dosage: Infusions have been used as a tonic and stimulant. In Southeast Asia a decoction was drunk for coughs and the juice of leaves dropped into the eye for conjunctivitis. In Africa young shoots were chewed for the relief of rheumatism and a strong decoction of the leaves was drunk for any inflammation. A diffusion was used to treat dysentery and the fresh juice for earache (Australian Naturopathic Network, 1998).

Active ingredients: The plant is said to contain chalcone and aurone glycosides, volatile oil, acylated okanin glycosides (in the leaves), gallic and oxalic acid and phenolic astringents (Australian Naturopathic Network, 1998).

Pharmacological effects: The plant is said to be an astringent, anti-haemorrhagic, styptic, urogenital system tonic (diuretic, kidney and bladder tonic, anti-inflammatory), and a mucous membrane tonic throughout the body (Australian Naturopathic Network, 1998).

Distribution: *B. pilosa* is widespread around the world. It grows in disturbed areas or waste ground; cracks in pavements and walls.

4.1.2 *Dichrostachys cinerea* (L.) Wight & Arn. Syn.

Fabaceae

uGagane (Zulu); morêtsê (Northern Sotho); sickle bush (English); sekelbos (Afrikaans)



Figure 4.2: *Dichrostachys cinerea* (L.) Wight & Arn. Syn. (Venter and Venter, 1996)

Botanical description: The sickle bush is a semi-deciduous to deciduous tree up to seven metres tall with an open crown. On young branches the bark is green and hairy but dark grey-brown and longitudinally fissured on older branches and stems, but smooth on spines formed from modified side-shoots (Venter and Venter, 1996). The leaves are twice-compound ending in two leaflets with 20-27 pairs of leaflets each, glands are prominent on leaf stalk and rachis. The flowers are borne in pendulous, 40 mm to 50 mm long, from October to February. The flowers are two-coloured spikes; the upper part is pink and consisting of staminodes, and the lower part yellow made up of fertile flowers. Fruit is a cluster of non-splitting, contorted pods up to 100 x 15 mm, and borne from May to September (Venter and Venter, 1996).

Medicinal uses: The inner bark is very tough and is used as a remedy for toothache and stomach troubles. The powdered bark is used as a local application for all kinds of skin conditions. Scorpion stings and snake bites are treated with the roots (Venter and Venter, 1996).

Preparation and dosage: Decoctions of the stem bark is used for toothache and stomach troubles. Chewed roots are placed on scorpion stings and snake bites (Venter and Venter, 1996).

Distribution: The tree is widely distributed throughout the African continent, from Ethiopia in the north to KwaZulu-Natal in the south (Venter and Venter, 1996).

4.1.3 *Harpephyllum caffrum* Bernh.

Anacardiaceae

umgwenya (Xhosa, Zulu); mothêkêlê (Northern Sotho); wild plum (English); wildepruim (Afrikaans)



Plum-like fruits of *Harpephyllum caffrum*



Leaves and flowers of *Harpephyllum caffrum*



Pounded bark of *Harpephyllum caffrum*

Figure 4.3: *Harpephyllum caffrum* Bernh. (Van Wyk et al., 2002)

Botanical description: A large evergreen tree reaching a height of up to 15 metres (Palmer and Pitman, 1972; Coates Palgrave, 1977). In old specimens the bark is rough and dark brown (Van Wyk et al., 2002). Dark green, shiny leaves are divided into several leaflets, each of which is distinctly asymmetrical. The flowers are very small, whitish or yellowish, and male and female flowers occur on separate trees (Van Wyk et al., 2002). The sour edible fruits are bright red and plum-like (Palmer and Pitman, 1972; Coates Palgrave, 1977).

Medicinal uses: Decoctions of the bark are used as blood purifiers or emetics (Watt and Breyer-Brandwijk, 1962; Pujol, 1990). It may also be used for facial saunas and skin washes, and to treat skin problems such as acne and eczema

(Pujol, 1990). Powdered burnt bark is applied to scarifications to treat sprains and fractures (Hutchings, 1996).

Preparation and dosage: Powdered bark is boiled in water and taken daily. Only one or two small wine glasses of the preparation are taken, larger doses will induce vomiting (Pujol, 1990).

Active ingredients: Chemical constituents of *Harpephyllum* are poorly known, but the presence of polyphenolic compounds and flavonoids have been reported. These include organic acids, such as protocatechuic acid, and flavonols, such as Kaempferol (El Sherbeiny and El Ansari, 1976; Van Wyk *et al.*, 2002).

Pharmacological effects: Benefits derived from the use of this traditional medicine may be related to the phenolic compounds (El Sherbeiny and El Ansari, 1976; Van Wyk *et al.*, 2002).

Distribution: The wild plum is restricted to Southern Africa and has become a popular garden tree (Palmer and Pitman, 1972; Von Breitenbach, 1986; Van Wyk *et al.*, 2002).

4.1.4 *Ptaeroxylon obliquum* (Thunb.) Radlk.

Ptaeroxylaceae

umthathe (Xhosa, Zulu); sneezewood (English); nieshout (Afrikaans)



Bark of *Ptaeroxylon obliquum*



Leaves and fruit capsules of *Ptaeroxylon obliquum*



Flowers of *Ptaeroxylon obliquum*

Figure 4.4: *Ptaeroxylon obliquum* (Thunb.) Radlk. (Van Wyk *et al.*, 2002)

Botanical description: Varies in size from a shrub to a large tree of about 15 to 20 metres in height (Palmer and Pitman, 1972; Coates Palgrave, 1977; Van Wyk, 1995; Van Wyk *et al.*, 2002). The bark becomes rough and flaky in old specimens, and is pale greyish-brown to dark grey. Leaves have up to eight pairs of leaflets, each of which are distinctly asymmetrical in shape (Van Wyk *et al.*, 2002). The flowers are small, pale yellow, borne in dense clusters; and male and female flowers occur on different trees. The fruits are oblong capsules, each releasing two winged seeds when ripe (Palmer and Pitman, 1972; Coates Palgrave, 1977; Van Wyk, 1995; Van Wyk *et al.*, 2002).

Medicinal uses: To relieve headache, the powdered wood is used as a snuff (Watt and Breyer-Brandwijk, 1962; Hutchings and Van Staden, 1994). The bark is used for the treatment of rheumatism and arthritis (Pujol, 1990). Infusions of the powdered wood are taken for the treatment of rheumatism and heart disease (Watt and Breyer-Brandwijk, 1962).

Preparation and dosage: Wood is powdered and used as a snuff, or decoctions and infusions of the wood or bark are taken (Watt and Breyer-Brandwijk, 1962; Pujol, 1990; Van Wyk *et al.*, 2002).

Active ingredients: The leaves contain perforatin A (Van Wyk *et al.*, 2002). The wood is chemically highly complex and contains numerous unusual chromones and other phenolic compounds. Examples are ptaeroxylone and umtatin (Dean and Taylor, 1966; Dictionary of Natural Products on CD-ROM, release 4:2, 1996; Van Wyk *et al.*, 2002).

Pharmacological effects: Sneezing is induced presumably by the chromones in the wood. Perforatin A has antihypertensive effects (Van Wyk *et al.*, 2002).

Distribution: Sneezewood grows naturally along the eastern coastal parts of South Africa and northwards to the Northern Province (Von Breitenbach, 1986; Van Wyk *et al.*, 2002).

4.1.5 *Rapanea melanophloeos* (L.) Mez.

Myrsinaceae

Isiqalaba-sehlathi (Xhosa, Zulu); isiqwane-sehlathi (Xhosa); umaphipha (Zulu)
Cape beech (English); Kaapse boekenhout (Afrikaans)



Bark, leaves and fruits of *Rapanea melanophloeos*



Flowers of *Rapanea melanophloeos*

Figure 4.5: *Rapanea melanophloeos* (L.) Mez. (Van Wyk *et al.*, 2002)

Botanical description: Medium sized tree of about five to ten metres in height (Palmer and Pitman, 1972; Coates Palgrave, 1977; Van Wyk *et al.*, 2002). The bark is grey, and can sometimes be dotted with small diamond-shaped spots on the raised areas (Van Wyk *et al.*, 2002). The leaves are oblong in shape, 100 mm long and narrowing towards their bases. Small greenish flowers are borne densely clustered along the stems, followed by small, round, purple berries of about five millimetres in diameter.

Medicinal uses: The Zulu uses decoctions of the bark as expectorants and emetics (Watt and Breyer-Brandwijk, 1962), and also for muscular pain, stomach disorders and to strengthen the heart (Watt and Breyer-Brandwijk, 1962; Pujol, 1990; Hutchings, 1996; Van Wyk *et al.*, 2002).

Preparation and dosage: Decoctions or infusions of the bark are taken or the roots may be eaten (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Van Wyk *et al.*, 2002).

Active ingredients: *Rapanea* bark is known to contain tannins (Watt and Breyer-Brandwijk, 1962). Tri-terpenoid saponins, such as sakurasosaponin, have been isolated from the leaves (Van Wyk *et al.*, 2002).

Pharmacological effects: Nothing appears to be known about the pharmacological activities of *Rapanea* bark (Van Wyk *et al.*, 2002).

Distribution: The tree grows naturally in the afro-montane region and along the east coast of South Africa (Von Breitenbach, 1986).

4.1.6 *Sclerocarya birrea* (A. Rich) Hochst. Syn.

Anacardiaceae

umganu (Zulu); morula (Northern Sotho); marula (English); maroela (Afrikaans)



Figure 4.6: *Sclerocarya birrea* (A. Rich) Hochst. Syn. (Van Wyk *et al.*, 2002)

Botanical description: The marula is a medium-sized, single-stemmed tree of up to 15 metres in height. The rough bark is flaky, with a mottled appearance due to contrasting grey and pale brown patches. The leaves are divided into ten or more pairs of leaflets, each about 60 mm long, dark green above, much paler below, with the tip abruptly narrowing to a sharp point (Van Wyk *et al.*, 2002). The flowers are borne in small, oblong clusters. Male and female flowers occur separately, usually but not always on separate trees. The flowers are small, with red sepals and yellow petals (Van Wyk *et al.*, 2002). Large, rounded, slightly flattened fruits of about 30 mm in diameter are borne in profusion in late summer to mid-winter (Palmer and Pitman, 1972; Coates Palgrave, 1977).

Medicinal uses: The bark, which is believed to be of value in combatting fever, is used in South Africa to treat diarrhoea, dysentery, unspecified stomach problems and in the treatment of malaria (Watt and Breyer-Brandwijk, 1962; Hutchings, 1989; Hutchings, 1996). It is also used as a general tonic (Pujol, 1990). Chewing the fresh leaves and swallowing the astringent juice will help with indigestion. Numerous other traditional uses have been recorded (Watt and Breyer-Brandwijk, 1962; Palmer and Pitman, 1972; Coates Palgrave, 1977; Hutchings, 1989; Hutchings, 1996).

Preparation and dosage: Decoctions of the bark or roots are taken orally or as enemas (Watt and Breyer-Brandwijk, 1962; Hutchings, 1989; Pujol, 1990; Hutchings, 1996).

Active ingredients: The bark contains procyanidins (Galvez *et al.*, 1993) and the plant is also said to contain gallotannins, flavonoids and catechins (Watt and Breyer-Brandwijk, 1962; Iwu, 1993).

Pharmacological effects: The bark has an astringent taste and the antidiarrheal effects have been experimentally linked to procyanidins (Galvez *et al.*, 1993).

Distribution: The tree is widely distributed throughout the African continent. In southern Africa, only the subspecies *caffra* is found (Coates Palgrave, 1977).

4.1.7 *Securidaca longepedunculata* Fresen.

Polygalaceae

mpesu (Venda); violet tree (English); krinkhout (Afrikaans)

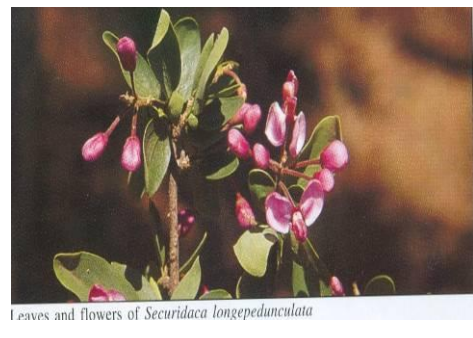


Figure 4.7: *Securidaca longepedunculata* Fresen. (Van Wyk *et al.*, 2002)

Botanical description: This is a small, erect tree of up to six metres in height, with a pale grey, smooth bark (Palmer and Pitman, 1972; Coates Palgrave, 1977). The plant has a very distinctive appearance and is not likely to be confused with any others. The more or less hairless, oblong leaves are variable in size and shape, about 30 mm long and crowded towards the stem tips. In early summer, clusters of attractive pink to purple flowers are produced. Each flower is about 10 mm long and is borne on a long, slender stalk. The fruit is a round nut with a single large, curved wing (Palmer and Pitman, 1972; Coates Palgrave, 1977).

Medicinal uses: This is perhaps one of the most popular of all traditional medicines in Africa and has been used for almost every conceivable ailment (Watt and Breyer-Brandwijk, 1962). In South Africa, the roots are used for coughs and chest complaints, rheumatism, toothache and headache (Watt and Breyer-Brandwijk, 1962). Externally, it has been applied to wounds and sores and for the relief of rheumatism (Watt and Breyer-Brandwijk, 1962).

Preparation and dosage: Decoctions are taken for chest complaints, while the roots are chewed to relieve toothache. A hot water poultice of the roots is said to give symptomatic relief of rheumatism, while powdered root or wood scrapings are rubbed into scarifications on the forehead to treat headaches (Watt and Breyer-Brandwijk, 1962).

Active ingredients: The volatile oil of the roots contains large amounts of methyl salicylate, better known as wintergreen oil. The plant also produces various sapogenins, including presenegenin, the toxic indole alkaloid securinine and some ergot alkaloids (Dictionary of Natural Products on CD-ROM, release 4:2 (1996)).

Pharmacological effects: The presence of salicylates may partly explain the recorded uses of *S. longepedunculata*. Chemically similar plants (such as queen-of-the-meadow, *Filipendula ulmaria*) are traditionally used in Europe for the symptomatic treatment of minor pain in the joints, fever, headaches and toothache (Bruneton, 1995). Methyl salicylate is a toxic counter-irritant which

penetrates the skin and underlying tissue to act as an anti-inflammatory (Merck, 1989). Drugs containing this compound are also ingredients of oral hygiene products. Presenegenin is associated with plant medicines which are traditionally used for the symptomatic treatment of coughs (Bruneton, 1995).

Distribution: The tree is widely distributed in tropical Africa and occurs in the North-West and Northern provinces of South Africa (Palmer and Pitman, 1972; Coates Palgrave, 1977; Von Breitenbach, 1986).

4.1.8 *Ziziphus mucronata* Willd.

Rhamnaceae

mokgalo (North Sotho, Tswana); umphafa (Xhosa, Zulu); umlahlankosi (Zulu); buffalo-thorn (English); blinkblaar-wag-'n-bietjie (Afrikaans)



Figure 4.8: *Ziziphus mucronata* Willd. (Van Wyk *et al.*, 2002)

Botanical description: This widely used medicinal plant is a small to medium-sized tree, mostly about five metres in height but sometimes up to ten metres or more (Palmer and Pitman, 1972; Coates Palgrave, 1977; Van Wyk, 1995). It has a wide, spreading crown and rough, greyish-brown bark. Sharp thorns are usually present on the twigs. They are paired - the one straight, the other curved (Van Wyk *et al.*, 2002). The leaves are bright green and shiny above, slightly paler beneath, with three main veins arising from the base and with the margins toothed in the upper half. Small yellowish-green flowers are borne in clusters above each leaf. The fruits are small, rounded berries of about 10 mm in diameter, which become reddish-brown when mature (Van Wyk *et al.*, 2002).

Medicinal uses: Warm bark infusions (sometimes with roots or leaves added) are used as expectorants (also as emetics) in cough and chest problems, while root infusions are popular as a remedy for diarrhoea and dysentery (Watt and Breyer-Brandwijk, 1962; Rood, 1994; Makgakga, 1995; Hutchings, 1996). Decoctions of roots and leaves (or chewed leaves) are applied externally to boils, sores and glandular swellings, not only to promote healing but also for pain relief (Watt and Breyer-Brandwijk, 1962; Rood, 1994; Makgakga, 1995; Hutchings, 1996).

Preparation and dosage: Warm infusions of roots, bark or leaves are taken orally as tea or decoctions are used topically to treat painful sores, boils and swellings (Van Wyk *et al.*, 2002).

Active ingredients: Several alkaloids, commonly referred to as peptide alkaloids, are known from *Ziziphus* species (Dictionary of Natural Products on CD-ROM, release 4:2, 1996). Mucronine D is a typical example (Van Wyk *et al.*, 2002).

Pharmacological effects: The strong sedative effects of *Z. vulgaris* and *Z. jujuba* (the sources of two Chinese phytomedicines) are due to the presence of franguloline (also known as sanjoinine A) (Bruneton, 1995). This alkaloid is structurally closely related to some of the alkaloids extracted from *Z. mucronata* (Van Wyk *et al.*, 2002).

Distribution: The buffalo-thorn is one of the most widely distributed of all South African trees (Palmer and Pitman, 1972; Coates Palgrave, 1977)

4.2 Objective

The objective of this part of the study was to investigate anti-candidal activity of the selected eight plant species, by means of the disc diffusion and serial microplate dilution assays as the methods of choice for screening.

4.3 Materials and Methods

4.3.1 Fungal cultures

Candida albicans standard strain (ATCC 10231) and clinical isolates (obtained from the Department of Microbiology, National Health Laboratory Services, Pretoria, South Africa) were maintained on Sabouraud dextrose agar at 4°C to prevent overgrowing and morphological changes.

Subcultures were freshly prepared before use. Inocula of the fungal cultures were prepared from the 24 h cultures.

4.3.1.1 Preparation of inocula for disc diffusion assay

Microbiological inocula were prepared by transferring colonies from freshly prepared subcultures to sterile saline (0.85 percent) until a turbidity of MacFarland Standard 0.5 was reached.

4.3.1.2 Preparation of inocula for broth micro-dilution assay

Inocula for use in the broth micro-dilution assay was prepared by transferring colonies from freshly prepared subcultures to Sabouraud dextrose broth until a turbidity of MacFarland Standard one was reached.

4.3.2 Disc diffusion

Diffusion of the extract on a disc in agar in a Petri dish requires no sophisticated equipment and has been the method of choice in many studies. The disc diffusion assay as described by Bauer *et al.* (1966) was used to identify active extracts. Although this technique is commonly used for most antibiotics, examining plant extracts containing unknown compounds using this method is associated with problems. Eloff (1998b) writes that this assay is affected by agar type, salt concentration, incubation temperature and the molecular size of the test compound(s).

4.3.2.1 Extraction

Distilled water, acetone or hexane (Merck) was used as extractants. One gram of powdered plant material were extracted with 10 ml of appropriate solvent, lightly shaken, sonicated for 30 min and allowed to stand for 24 h in the refrigerator (4°C). The extracts were then centrifuged at 4000 x g for 15 minutes. The supernatant was retained and filtered through 0.22 µm syringe filters (Millipore). Extracts were stored at -18°C to limit chemical decomposition. Yields of the extracts were determined gravimetrically using one millilitre (1 ml) of the extract (described in greater detail in Section 4.3.2.2).

4.3.2.2 Determination of yields

Yields were determined gravimetrically:

1. Weigh glass dish.
2. Place 500 µl of the plant extract in the pre-weighed glass dish.
3. Dry extract overnight.
4. Weigh glass dish containing dry plant extract and calculate the difference.
The difference is the yield per 500 µl of the plant extract.
5. Yields were determined in duplicate for each plant extract.

4.3.2.3 Disc diffusion assay

1. Three sterile filter paper discs (Whatmann, 10 mm) were impregnated with 200 µl of the respective plant extract on the first occasion and 300 µl on the second occasion.
2. These discs were dried to ensure that no solvent remained on the discs.
3. 23 ml of Mueller-Hinton Agar was poured in each Petri dish, on a completely horizontal surface to ensure that all plates were prepared in a standardized fashion.
4. 100 µl of the specific culture suspension (5×10^5 CFU/ml) was spread on the surface of each plate, which created a lawn of microbial growth.
5. The extract impregnated filter paper discs were placed on the inoculated Agar plates.

6. For positive control, antibiotic discs (amphotericin B, 10 µg (Mast diagnostics)) were placed on similarly inoculated plates. A negative control was prepared by using the respective solvent.
7. Plates were incubated at 37°C for 24 h.
8. Antimicrobial activity was expressed as the mean diameter of the zone of inhibition (mm) around the disc and was measured at three places.
9. The assay was performed in duplicate.

4.3.3 Minimal inhibitory concentration determination

Minimal inhibitory concentration (MIC) was evaluated on plant extracts that showed antimicrobial activity in the disc diffusion assay using the microplate dilution method developed by Eloff (1998b), with modifications for antifungal activity assay by Masoko *et al.* (2005). The serial microplate dilution method also allows for the testing of relatively large number of extracts simultaneously (Eloff, 1998b). MIC values were regarded as the lowest concentrations of the extract that inhibited the growth of *C. albicans* standard strain (ATCC 10231) and clinical isolates. Total activity values were calculated as described by Eloff (2004). The total activity (ml) of the extracts was calculated as the total mass (mg) of the extract divided by the MIC value (mg/ml). Total activity value indicates the volume to which the extract can be diluted and still inhibit the growth of microbial cells (Eloff, 2004).

4.3.3.1 Minimal inhibitory concentration assay

A. Preparation of crude plant extracts

Distilled water, acetone or hexane (Merck) was used as extractants. Three grams (3 g) of powdered plant material were extracted with 30 ml of the appropriate solvent, lightly shaken, sonicated for 30 min and allowed to stand for 24 h in the refrigerator (4°C). The extracts were then centrifuged at 4000 x g for 15 min. The supernatant was retained and filtered through 0.45 µm and then through 0.22 µm syringe filters (Millipore); and decanted into pre-weighed labelled containers.

Hexane extracts were evaporated to dryness in an Air flow cabinet (Labotec, SA) overnight. Aqueous extracts were freeze-dried; and acetone extracts were stored at -18°C to limit chemical decomposition.

B. **Broth micro-dilution assay**

The assay was initiated by pouring sterile water aliquots (100 µl) into the wells of a 96-well microtitre plate. Exactly 100 µl of a 16 mg/ml plant extract (hexane, acetone or distilled water) was added in row A and mixed using a micropipette. From row A 100 µl was aspirated and added into row B and mixed. The procedure was repeated until all the wells were filled. An additional 100 µl in row H was discarded. 100 µl of *C. albicans* standard strain (ATCC 10231) or clinical isolates (transferred to Sabouraud dextrose broth until a turbidity of MacFarlane Standard 1 = 4×10^7 CFU was reached) was added to the wells of the microtitre plate. 50 µl of a 0.2 mg/ml aqueous solution of *p*-iodonitro-tetrazolium violet (INT, Sigma) was added to each well. The plates were incubated at 35°C for 24 h. Inhibition of microbial growth was indicated by the failure of the well to change colour; uninhibited growth was a pink colour. Amphotericin B was used as a positive control; acetone, hexane and distilled water were used as negative controls. The experiments were performed in triplicate and on two separate occasions.

4.3.4 Minimal fungicidal concentration determination

The minimal fungicidal concentration (MFC) values were determined as the lowest concentration of the extract that inhibited 100 percent growth of *C. albicans* standard strain (ATCC 10231) and clinical isolates.

4.3.4.1 Minimal fungicidal concentration assay

The assay was initiated by pouring fresh sterile water aliquots (100 µl) into the wells of a microtitre plate. Exactly 50 µl of the suspensions from the wells, which did not show any growth after incubation during MIC assays, were added to the wells. 50 µl of a 0.2 mg/ml aqueous solution of INT (Sigma) was added to each well. These suspensions were re-incubated at 35°C for 24 h.

4.4 Results and Discussion

4.4.1 Disc diffusion

Zones of inhibition around each disc were measured using a calliper. The results are presented in Table 4.2. Antimicrobial activity was expressed as the mean diameter of the zone of inhibition (mm) around the disc and was measured in three places. The assay was performed in triplicate. The disc diffusion assay was not performed on the *H. caffrum* (leaf), *P. obliquum* (leaf) and *R. melanophloeos* (leaf) water/acetone extracts as these plant species have previously been screened for antifungal activity by the Phytomedicine Programme, University of Pretoria (UPPP), South Africa. Amphotericin B (Table 4.2) was used as positive control and the respected solvents (water, acetone and hexane) as negative control.

4.4.2 Minimal inhibitory and minimal fungicidal concentrations

The acetone and/or water extracts of all eight plant species showed anti-candidal activity against the *C. albicans* standard strain (ATCC 1023) and clinical isolates tested (Tables 4.3; 4.4). The antifungal compound, amphotericin B, inhibited the growth of all the strains tested (Table 4.5). Only the hexane extract of *Bidens pilosa* (whole plant) (MIC=2.00 mg/ml) showed anti-candidal activity against *C. albicans* standard strain (ATCC 10231). Although aqueous extracts inhibited *Candida* growth, the acetone extracts had the lowest MIC values. However, the effectivity of the water extracts is worth noting since traditional medicine in South Africa is mainly prepared as decoctions, infusions, syrups or tinctures, taken orally (Van Wyk *et al.*, 2002; Van Wyk *et al.*, 2009).

Inhibition at concentrations <1 mg/ml, against the *C. albicans* strains tested, was observed for the acetone extracts of *B. pilosa* (whole plant), *H. caffrum* (leaves), *P. obliquum* (leaves), *R. melanophloeos* (leaves) and *S. birrea* (stem bark) as well as the aqueous extracts of *B. pilosa* (whole plant) and *S. birrea* (stem bark) (Table 4.3). Motsei *et al.* (2003) found much less activity with *B. pilosa* aqueous extract

against *C. albicans* clinical isolates from a 5-month-old baby (25 mg/ml) and an adult (25 mg/ml), and a standard strain of *C. albicans* ATCC 10231 (25 mg/ml). In fact, of the twenty-four South African medicinal plants and 273 extracts evaluated, 211 (77 percent) had MIC values as high of even higher than the highest concentration tested (8.35 mg/ml). This indicates that the tested plants were much less active than those in the current study, or that the technique they used was not as sensitive as the tetrazolium violet technique that was used in the current study.

Previous findings for antifungal activity of the plants tested in this study, confirming the results, have been reported for the aqueous extracts of *Z. mucronata* stem-bark (Gundiza, 1986), *S. longepedunculata* root (Desta, 1993), *H. caffrum* bark (Buwa and Van Staden, 2006), methanolic/ethanolic extracts of *S. birrea* root/stem-bark (Eloff, 2001; Hamza *et al.*, 2006; Runyoro *et al.*, 2006). No antifungal activity has previously been reported for the extracts of *R. melanophloeos* (Hamza *et al.*, 2006, Steenkamp *et al.*, 2007), which may explain the weak activity evidenced in this study.

Table 4.2: Disc diffusion

Plant	Solvent	* Zone of Inhibition (mm)	
		200 μ l	300 μ l
<i>Bidens pilosa</i>	Water	2.31	4.52
	Acetone	-	-
	Hexane	-	-
<i>Dichrostachys cinerea</i>	Water	1.14	-
	Acetone	-	-
	Hexane	1.23	-
<i>Sclerocarya birrea</i>	Water	1.86	2.11
	Acetone	2.85	1.22
	Hexane	-	-
<i>Securidaca longepedunculata</i>	Water	-	-
	Acetone	-	-
	Hexane	1.13	-
<i>Ziziphus mucronata</i>	Water	0.86	-
	Acetone	-	-
	Hexane	0.83	-
Control:			
Amphotericin B		5.73	7.43

* Mean diameter of the zone of inhibition (mm)

- No zone of inhibition

Table 4.3: Minimal inhibitory concentrations for the plant extracts investigated

Plant	Plant part	Solvent	*MIC (mg/ml) <i>Candida albicans</i>						
			ATCC	Clinical isolates					
			10231	M0824	M0825	M0826	1051604	1051608	1051255
<i>Bidens pilosa</i>	Whole plant	Water	1.50	0.50	2.00	0.50	0.23	0.25	2.00
		Acetone	2.00	1.25	2.00	0.50	0.62	0.25	2.00
		Hexane	2.00	nd	nd	nd	nd	nd	nd
<i>Dichrostachys cinerea</i>	Stem bark	Water	nd	nd	nd	nd	nd	nd	nd
		Acetone	4.00	nd	nd	nd	nd	nd	nd
		Hexane	nd	nd	nd	nd	nd	nd	nd
<i>Harpephyllum caffrum</i>	Leaves	Water	2.00	2.00	2.00	2.00	2.00	2.00	2.00
		Acetone	1.00	2.00	1.00	0.50	0.50	0.50	1.00
<i>Ptaeroxylon obliquum</i>	Leaves	Water	2.00	2.00	2.00	2.00	2.00	2.00	2.00
		Acetone	0.50	0.50	0.50	0.50	0.50	0.50	0.50
<i>Rapanea melanophloeos</i>	Leaves	Water	2.00	2.00	2.00	2.00	1.00	2.00	2.00
		Acetone	2.00	1.00	2.00	0.50	1.00	2.00	2.00
<i>Sclerocarya birrea</i>	Stem bark	Water	3.00	1.25	2.00	0.50	1.25	0.50	2.00
		Acetone	0.25	1.25	0.13	0.50	0.75	0.13	0.13
		Hexane	nd	nd	nd	nd	nd	nd	nd
	Leaves	Water	>2.00	>2.00	>2.00	>2.00	>2.00	>2.00	>2.00
		Acetone	1.50	1.50	0.75	2.00	2.00	>2.00	2.00
		Hexane	nd	nd	nd	nd	nd	nd	nd
<i>Securidaca longepedunculata</i>	Root bark	Water	nd	nd	nd	nd	nd	nd	nd
		Acetone	4.00	nd	nd	nd	nd	nd	nd
		Hexane	nd	nd	nd	nd	nd	nd	nd
<i>Ziziphus mucronata</i>	Root bark	Water	4.00	nd	nd	nd	nd	nd	nd
		Acetone	4.00	nd	nd	nd	nd	nd	nd
		Hexane	nd	nd	nd	nd	nd	nd	nd

*MIC: Minimal inhibitory concentration representing the mean value
 nd: MIC not determined since the crude plant extract had such a high MIC with ATCC 10231 and showed no zone of inhibition when using the disc-diffusion assay

Table 4.4: Minimal fungicidal concentrations for the plant extracts investigated

Plant	Plant part	Solvent	*MFC (mg/ml) <i>Candida albicans</i>						
			ATCC	Clinical isolates					
			10231	M0824	M0825	M0826	1051604	1051608	1051255
<i>Bidens pilosa</i>	Whole plant	Water	2.00	1.00	2.00	1.00	1.00	1.00	2.00
		Acetone	2.00	2.00	2.00	1.00	1.00	1.00	2.00
		Hexane	2.00	nd	nd	nd	nd	nd	nd
<i>Dichrostachys cinerea</i>	Stem bark	Water	nd	nd	nd	nd	nd	nd	nd
		Acetone	4.00	nd	nd	nd	nd	nd	nd
		Hexane	nd	nd	nd	nd	nd	nd	nd
<i>Harpephyllum caffrum</i>	Leaves	Water	2.00	2.00	2.00	2.00	2.00	2.00	2.00
		Acetone	2.00	2.00	2.00	0.50	0.50	0.50	2.00
<i>Ptaeroxylon obliquum</i>	Leaves	Water	2.00	2.00	2.00	2.00	2.00	2.00	2.00
		Acetone	1.00	0.50	1.00	0.50	0.50	0.50	1.00
<i>Rapanea melanophloeos</i>	Leaves	Water	2.00	2.00	2.00	2.00	1.00	2.00	2.00
		Acetone	2.00	1.00	2.00	0.50	1.00	2.00	2.00
<i>Sclerocarya birrea</i>	Stem bark	Water	3.00	4.00	2.00	1.00	2.00	1.00	2.00
		Acetone	2.12	4.00	0.50	1.00	2.00	1.00	0.50
		Hexane	nd	nd	nd	nd	nd	nd	nd
	Leaves	Water	>2.00	>2.00	>2.00	>2.00	>2.00	>2.00	>2.00
		Acetone	1.50	1.50	0.75	2.00	2.00	>2.00	2.00
		Hexane	nd	nd	nd	nd	nd	nd	nd
<i>Securidaca longepedunculata</i>	Root bark	Water	nd	nd	nd	nd	nd	nd	nd
		Acetone	4.00	nd	nd	nd	nd	nd	nd
		Hexane	nd	nd	nd	nd	nd	nd	nd
<i>Ziziphus mucronata</i>	Root bark	Water	4.00	nd	nd	nd	nd	nd	nd
		Acetone	4.00	nd	nd	nd	nd	nd	nd
		Hexane	nd	nd	nd	nd	nd	nd	nd

*MFC: Minimal fungicidal concentration representing the mean value

nd: MFC not determined; no antimicrobial activity was present

Table 4.5: Minimal inhibitory and minimal fungicidal concentrations for amphotericin B

	<i>Candida albicans</i>						
	ATCC	Clinical isolates					
	10231	M0824	M0825	M0826	1051604	1051608	1051255
*MIC (mg/ml)	0.11	0.03	0.03	0.06	0.06	0.11	0.03
*MFC (mg/ml)	0.11	0.11	0.06	0.11	0.06	0.11	0.23

*MIC: Minimal inhibitory concentration representing the mean value

*MFC: Minimal fungicidal concentration representing the mean value

Table 4.6: Total activity values (ml) for the plant extracts investigated against *Candida albicans* standard strain (ATCC 10231) and six clinical isolates

Plant	Plant part	Solvent	Total activity values (ml/mg)						
			ATCC	Clinical isolates					
			10231	M0824	M0825	M0826	1051604	1051608	1051255
<i>Bidens pilosa</i>	Whole plant	Water	126	379	95	379	824	758	95
		Acetone	12	18	12	46	37	92	12
		Hexane	14	-	-	-	-	-	-
<i>Dichrostachys cinerea</i>	Stem bark	Acetone	51	-	-	-	-	-	-
<i>Harpephyllum caffrum</i>	Leaves	Water	92	92	92	92	92	92	92
		Acetone	143	71	143	286	286	286	143
<i>Ptaeroxylon obliquum</i>	Leaves	Water	119	119	119	119	119	119	119
		Acetone	323	323	323	323	323	323	323
<i>Rapanea melanophloeos</i>	Leaves	Water	127	127	127	127	255	127	127
		Acetone	70	140	70	280	140	70	70
<i>Sclerocarya birrea</i>	Stem bark	Water	44	107	67	266	107	266	67
		Acetone	158	32	304	79	53	304	304
	Leaves	Water	68	68	68	68	68	68	68
		Acetone	17	17	33	13	13	13	13
<i>Securidaca longepedunculata</i>	Root bark	Acetone	7	-	-	-	-	-	-
<i>Ziziphus mucronata</i>	Root bark	Water	22	-	-	-	-	-	-
		Acetone	11	-	-	-	-	-	-

4.5 Conclusion

All the plant species examined had some activity against the fungal test organisms in this study. *H. caffrum*, *P. obliquum*, *R. melanophloeos* and *S. birrea* were the most active of the plant species tested against *C. albicans* standard strain (ATCC 10231) and clinical isolates. These results also imply that the extracts contain compounds with therapeutic potential against *C. albicans*. *D. cinerea*, *S. longepedunculata* and *Z. mucronata* extracts were the least sensitive against the fungal test organisms. The stem bark of *S. birrea* resulted in lower MIC values compared to the leaves (Table 4.3).

Following the finding that *H. caffrum* (leaves), *P. obliquum* (leaves), *R. melanophloeos* (leaves) and *S. birrea* (stem bark) had the lowest MIC values against *C. albicans* standard strain (ATCC 10231) and clinical isolates tested; the next step involved bioautography analysis from the acetone extracts of these species.

CHAPTER 5: BIOAUTOGRAPHY

5.1 Introduction

Bioautography is regarded as the most efficient method for activity-guided isolation of components (Hostettmann *et al.*, 2000). Bioautography combines thin layer chromatography (TLC) with a bioassay *in situ* and allows the localization of the active compounds in a complex plant extract (Shai, 2007). Cultures of bacteria or fungi are sprayed on developed TLC plates from which the eluents have been removed and incubated for a specific duration in humid chambers. Zones of inhibition are visualized by the detection of dehydrogenase activity with a tetrazolium salt. A reference chromatogram stained with a suitable reagent provides information about the nature of the active component, such as relative polarity (Shai, 2007).

5.2 Objective

The objective of this part of the study was to locate the active compounds in the most active acetone plant extracts identified after MIC and MFC (Section 4.3.3 and Section 4.3.4 respectively) namely: *Harpephyllum caffrum* (leaves); *Ptaeroxylon obliquum* (leaves); *Rapanea melanophloeos* (leaves) and *Sclerocarya birrea* (stem bark).

5.3 Bioautography procedure

For bioautography analysis thin layer chromatography (TLC) plates (10 x 10 cm) were loaded with 10 μ l (10 mg/ml) of the extracts and eluted in the three different mobile eluting solvent systems of varying polarity: developed in the Phytomedicine Programme Laboratory, namely: ethyl acetate/methanol/ water [EMW] [10:1.35:1] (polar/neutral); chloroform/ethyl acetate/formic acid [CEF] [10:8:2] (intermediate polarity/acidic); benzene/ethanol/ ammonia [BEA] [18:2:0.2] (non-polar/basic) (Kotze and Eloff, 2002). Duplicate TLC plates were

visualized under ultraviolet (UV) light (366 nm) and sprayed with vanillin spray reagent (0.1 g vanillin dissolved in 28 ml methanol and 1 ml sulphuric acid added) and served as reference plates (Fig. 5.1).

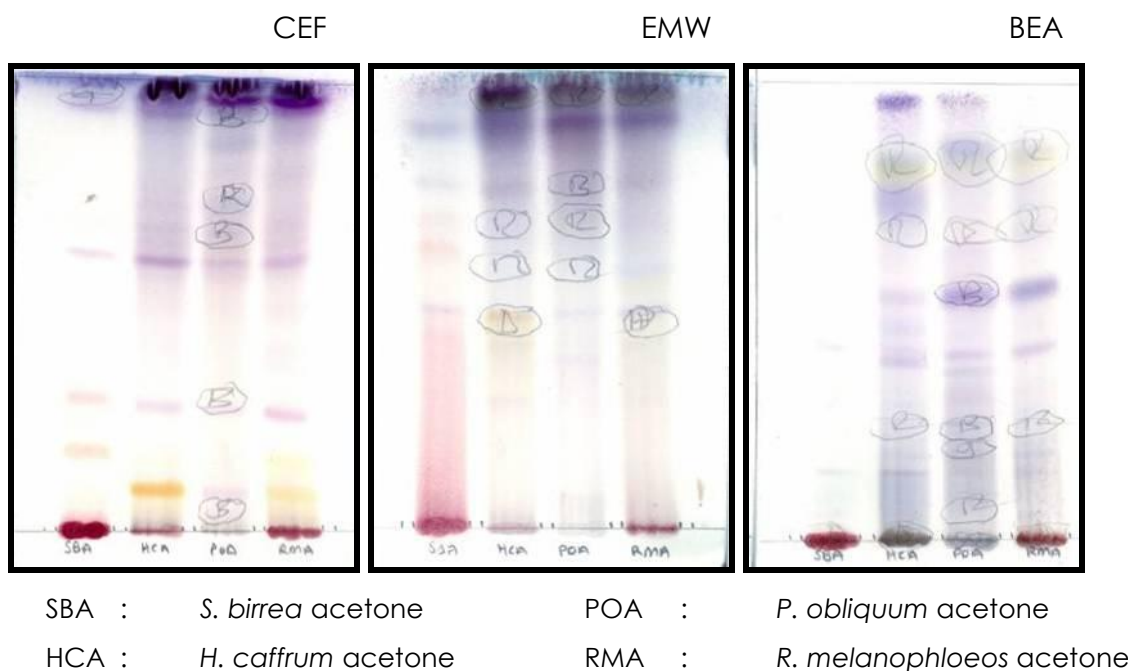


Figure 5.1: TLC Reference plates

The chromatograms were dried for 5 days at room temperature to remove the eluent solvent systems. Developed TLC plates were sprayed with a concentrated suspension of the test organism (*C. albicans* standard strain (ATCC 10231) and clinical isolates) prepared in Sabouraud dextrose broth in a Biosafety Class II cabinet (Labotec, SA). The sprayed plates were placed in a humid chamber (100 percent relative humidity) and incubated overnight at 25°C. Plates were then sprayed with a 2 mg/ml *p*-iodonitro-tetrazolium violet (INT, Sigma) solution and further incubated at 25°C until a purple-red colour change was evident. Plates with white spots at bands where reduction of INT to formazan did not occur, were tightly sealed in transparent plastic bags and retardation factor (R_f) values of inhibitory zones recorded.

The Bioautography procedure was repeated on three separate occasions.

5.4 Results and Discussion

The compounds whose retardation factor (R_f) values are listed in Table 5.1 were identified as the active constituents that displayed growth inhibition of *C. albicans* standard strain (ATCC 10231) and clinical isolates tested. The acetone extracts of *H. caffrum* and *R. melanophloeos* leaves had no visible activity against *C. albicans* standard strain and/or clinical isolates tested as evidenced by the absence of clear zones on TLC bioautograms (Fig. 5.2).

The acetone extracts of *P. obliquum* had the highest number of active compounds against the opportunistic pathogen *C. albicans* and clinical isolates (Fig. 5.2). The active components in the acetone extract of the leaves of *P. obliquum* range from non-polar to intermediate polarity in nature.

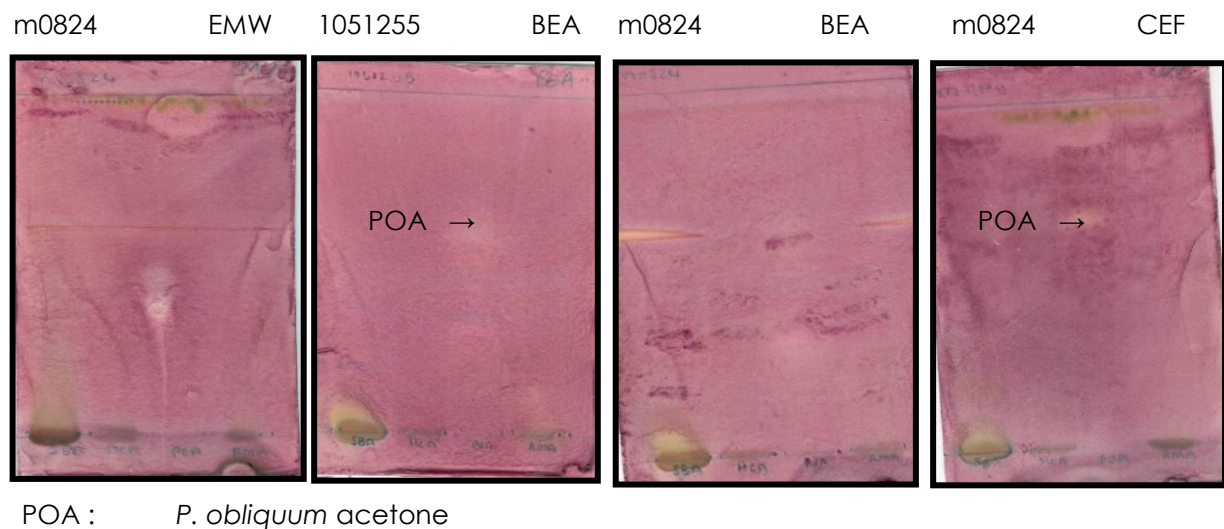


Figure 5.2: TLC bioautograms

Table 5.1: The R_f values of active compounds from acetone leaf and stem bark extracts of different plant species against *C. albicans* standard strain and six clinical isolates

Plant species	Plant part	Eluting Solvent system	<i>Candida albicans</i>						
			ATCC	Clinical isolates					
			10231	M0824	M0825	M0826	1051604	1051608	1051255
<i>Harpephyllum caffrum</i>	Leaves	EMW	-	-	-	-	-	-	-
		CEF	-	-	-	-	-	-	-
		BEA	-	-	-	-	-	-	-
<i>Ptaeroxylon obliquum</i>	Leaves	EMW	-	-	-	-	-	-	0.88
		CEF	0.88	-	-	-	-	0.88	0.83
		BEA	0.66	-	-	0.65	-	0.68	0.60
<i>Rapanea melanophloeos</i>	Leaves	EMW	-	-	-	-	-	-	-
		CEF	-	-	-	-	-	-	-
		BEA	-	-	-	-	-	-	-
<i>Sclerocarya birrea</i>	Stem bark	EMW	-	0.06	-	-	-	0.02	-
		CEF	0.04	-	-	0.03	-	-	0.17
		BEA	-	0.05	-	-	-	0.05	0.05

R_f : retardation factor

EMW : ethyl acetate/methanol/water

CEF : chloroform/ethyl acetate/formic acid

BEA : benzene/ethanol/ammonia

- : no visible activity

Bioassay-guided isolation has resulted in the identification of the responsible antifungal agents in *S. birrea*. These include procyanidins (Galvez *et al.*, 1993; Van Wyk *et al.*, 2009), gallotannins and catechins (Watt and Breyer-Brandwijk, 1962; Iwu, 1993; Van Wyk *et al.*, 2009). Bark yields 3.5 – 20.5 percent tannin, 10.7 percent tanning matter and traces of alkaloids (Watt and Breyer-Brandwijk, 1962; Eloff 2001). Chemical constituents of *P. obliquum* are poorly known, but the presence of unusual chromones and other phenolic compounds (such as ptaeroxylone and umtatin) in the wood (Dean and Taylor, 1966; Dictionary of Natural Products, 2008; Van Wyk *et al.*, 2009) and perforatin A from the leaves have been reported. Perforatin A has antihypertensive effects (Langenhoven *et al.*, 1988; Van Wyk *et al.*, 2009). *H. caffrum* contains a large amount of phenolic compounds which are reported to be responsible for the antifungal activity (El Sherbeiny and El Ansari, 1976). From the leaves of *R. melanophloeos*, saponins such as sakurasosaponin have been isolated which contain antifungal activity (Ohtani *et al.*, 1993).

5.5 Conclusion

The results indicate that *P. obliquum* had the highest number of active compounds against the fungal test organisms. *S. birrea* had the second highest number of active compounds against the fungal test organisms. These results correlate with the MIC and MFC values (Section 4.4.2; Tables 4.3, 4.4) where *P. obliquum* had lower MIC values against the fungal test organisms compared to *S. birrea*. *H. caffrum* and *R. melanophloeos* had no visible activity against the fungal test organisms as evidenced by the absence of clear zones on TLC bioautograms. These extracts were also less sensitive against the fungal test organisms. (Section 4.4.2; Tables 4.3, 4.4) compared to *P. obliquum* and *S. birrea* with MIC values ranging between 1.00 mg/ml to 2.00 mg/ml. The fact that no zones of inhibition were encountered although there was some activity in the extracts may possibly be due to volatility of the active compound(s) or to synergism between different compounds that are not active once separated.

Some medicinal plants contain free radical scavengers such as polyphenols, flavonoids and phenolic compounds (Khalaf *et. al.*, 2008), and these antioxidants may reduce oxidative stress, thereby stimulating the immune process. Although *H. caffrum* and *R. melanophloeos* extracts did not have any active compounds by bioautography and have been used traditionally to treat infections, the efficacy in traditional use may be because the plant extracts lead to an increase in the immune modulatory status of the host.

Following this finding, the next step involved detecting antioxidant activity in acetone extracts of *H. caffrum* (leaves); *P. obliquum* (leaves); *R. melanophloeos* (leaves) and *S. birrea* (stem bark).

CHAPTER 6: ANTIOXIDANT ACTIVITY

6.1 Introduction

Antioxidants are substances which have the capability to neutralize free radicals (superoxide, hydroxyl radicals and nitric oxide) and other reactive species (hydrogen peroxide, hypochloric acid and proxynitrite) produced during aerobic metabolism in the body that can cause oxidative damage of amino acids, lipids, proteins and DNA (Iuliano *et al.*, 1997; Fang *et al.*, 2002; Vimal *et al.*, 2009). Free radicals have the ability to react with and damage many structures in the body and are involved in various related physiological processes and diseases such as ageing, cancer and atherosclerosis (Fikel and Holbrook, 2000; Senthil *et al.*, 2004; Rackova *et al.*, 2007; Vimal *et al.*, 2009).

Natural antioxidants of plant origin are responsible for inhibiting or preventing the deleterious consequences of oxidative stress (Khalaf *et al.*, 2008). Medicinal plants contain free radical scavengers such as polyphenols, flavonoids and phenolic compounds (Khalaf *et al.*, 2008), and may be used as nutraceuticals and phytochemicals as they have significant impact on the status of human health and disease prevention (Noguchi and Nikki, 2000; Vimal *et al.*, 2009).

6.2 Objective

The objective of this part of the study was to detect antioxidant activity in acetone plant extracts of *Harpephyllum caffrum* (leaves); *Ptaeroxylon obliquum* (leaves); *Rapanea melanophloeos* (leaves) and *Sclerocarya birrea* (stem bark).

6.3 Materials and Methods

6.3.1 Qualitative evaluation

The qualitative antioxidant activity of selected plant species was carried out using the method of Deby and Margotteaux (1970). Thin layer chromatography (TLC)

plates (10x10 cm) were loaded with 10 µl (10 mg/ml) of the extracts and eluted in the three different mobile eluting solvent systems of varying polarity, developed in the Phytomedicine Programme Laboratory, namely: ethyl acetate/ methanol/ water [EMW] [10:1.35:1] (polar/neutral); chloroform/ethyl acetate/formic acid [CEF] [10:8:2] (intermediate polarity/acidic); benzene/ethanol/ammonia [BEA] [18:2:0.2] (non-polar/basic) (Kotze and Eloff, 2002).

For the determination of antioxidant activity, developed TLC plates were sprayed with 0.2% 1-1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma®) in methanol as indicator. A positive reaction was indicated by the appearance of a yellow spot against a purple background.

6.3.2 DPPH Radical scavenging activity

The effect of the extracts on the DPPH radical was estimated using the method of Liyana-Pathirana and Shahidi (2005), with minor modifications. A solution of 0.135 mM DPPH in methanol was prepared and 185 µl of this solution was mixed with 15 µl of varying concentrations of the extract in a 96-well plate. After 15 min of incubation in the dark, the absorbance of the mixture was determined at 570 nm using a microplate reader. Trolox was used as the reference antioxidant compound and the result expressed as Trolox equivalence (TE). TE is defined as the ratio of the sample slope to the standard slope and is thus unit less.

6.3.3 TEAC assay

The assay was performed according to the method of Re *et al.* (1999), with minor modifications. The assay involves the production of the ABTS^{•+} through the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, which is stored in the dark for at least 16h at 4°C. Into a 96-well plate was pipetted: 20 µl standard or sample at varying concentrations and 200 µl ABTS^{•+}. The absorbance of the mixture was determined at 405 nm using a microplate reader. Trolox was used as the reference antioxidant compound and the result expressed as Trolox equivalence (TE).

6.3.4 Determination of total flavonoids

Total flavonoid content was determined using the method of Ordonez *et al.* (2006), with modifications. Into a 96-well plate was pipetted: 20 µl of the standard (rutin) or sample, 20 µl sodium nitrate (3%), 20 µl aluminium trichloride (1%) and 100 µl 2M sodium hydroxide. Sample (20 µl) and 140 µl water served as background control to eliminate phytochemical absorbance interference. The absorbance was measured at 570 nm (Hewlett Packard UV-VIS spectrophotometer). Total flavonoid content was calculated as rutin equivalence (mg/g).

6.3.5 Determination of total phenolics

Total phenolic content was determined using the modified Folin-Ciocalteu method of Wolfe *et al.* (2003). Into a 96-well plate was pipetted: 20 µl standard (gallic acid) or sample, 60 µl 0.3M Folin-Ciocalteu reagent and 100 µl 3% sodium carbonate. The mixture was incubated for 2 h. The absorbance was measured at 630 nm (Hewlett Packard UV-VIS spectrophotometer). Total phenolic content is expressed as mg/g gallic acid equivalence.

6.4 Results and Discussion

6.4.1 Qualitative evaluation

All the plant extracts tested showed antioxidant activity when eluted in the three different mobile eluting solvent systems mentioned in Section 6.3. The acetone stem bark extract of *S. birrea* showed the highest antioxidant activity when eluted in the three different mobile eluting solvent systems. The antioxidant activity of *H. caffrum*, *P. obliquum* and *R. melanophloeos* did not move from the base (Fig. 6.1).

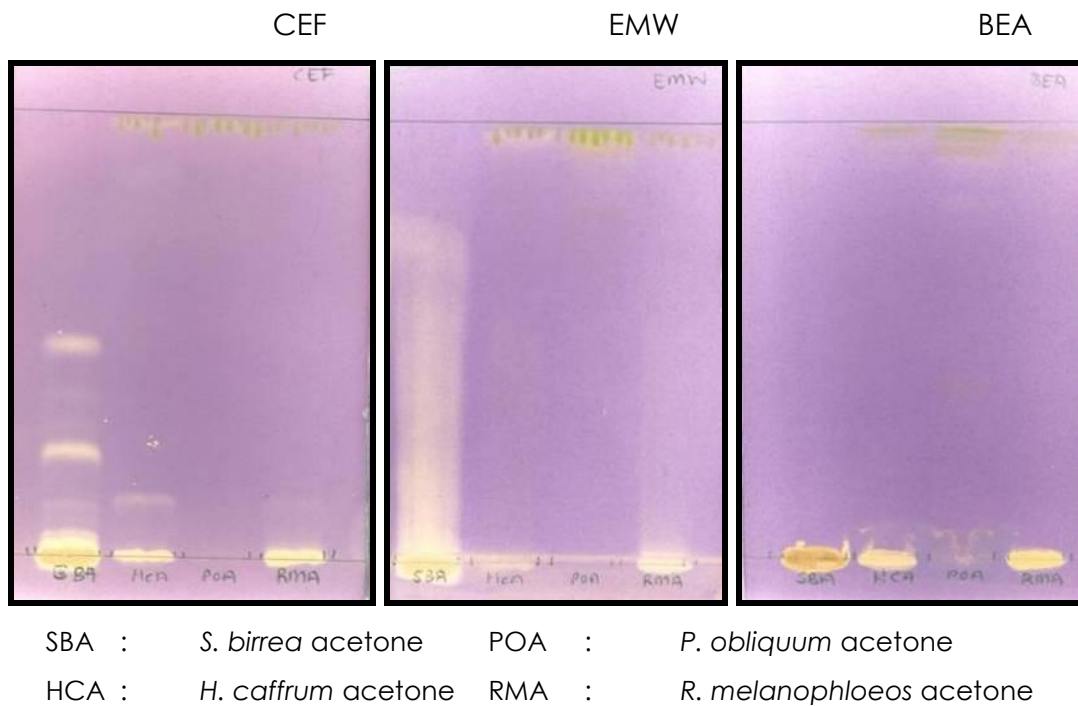


Figure 6.1: Antioxidant activity of *S. birrea*

To make the antioxidant activity of *H. caffrum*, *P. obliquum* and *R. melanophloeos* move from the base (Fig. 6.2); three different mobile eluting solvent systems were used as those described in Section 6.3. These mobile eluting solvent systems are also developed in the Phytomedicine Programme Laboratory, namely: ethyl acetate/methanol/water/acetic acid [EMWA] [10:1.35:1:2.5] (polar/neutral) ethyl acetate/water/formic acid/acetic acid [FAWE] [70:20:3:2] (polar/neutral) and butanol/acetic acid/water [BAW] [4:1:5] (polar/neutral) (Kotze and Eloff, 2002).

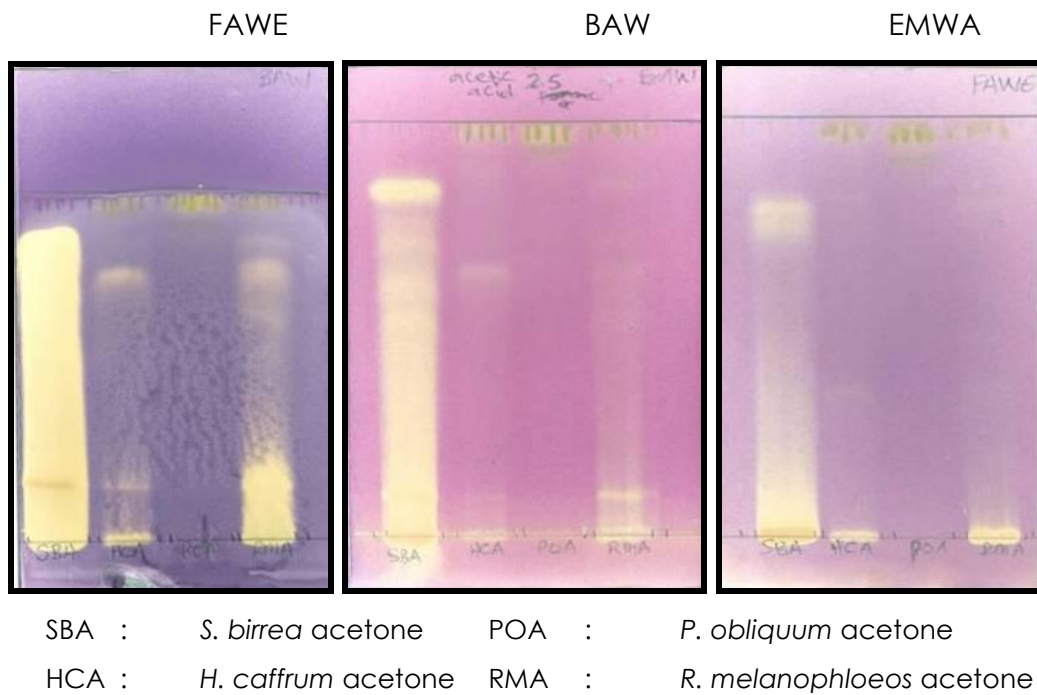


Figure 6.2: Antioxidant activity of *H. caffrum*, *P. obliquum*, *R. melanophloeos* and *S. birrea*

6.4.2 DPPH Radical scavenging activity, TEAC assay, determination of total flavonoids and total phenolics

S. birrea had the highest antioxidant activity of the four plant species tested (Table 6.1). High antioxidant activity has previously been reported for this plant by other authors (Masoko *et al.*, 2008; Moya *et al.*, 2010). Polyphenols and flavonoids are said to be responsible for the antioxidant activity in *S. birrea* (Braca *et al.*, 2003). This activity may be ascribed to the high polyphenolic content detected in the extracts (Table 6.1).

Table 6.1: Antioxidant and polyphenolic content of the plants containing significant antifungal activity

Plant	Antioxidant activity		Polyphenolic content	
	DPPH ^a	TEAC ^a	TPC ^b	TFC ^c
<i>S. birrea</i>	1.23 ± 0.03	1.05 ± 0.08	4.05 ± 0.76	36.08 ± 1.47
<i>H. caffrum</i>	0.22 ± 0.01	0.33 ± 0.02	0.65 ± 0.05	14.88 ± 0.66
<i>R. melanophloeos</i>	0.03 ± 0.00	0.06 ± 0.00	0.27 ± 0.01	5.88 ± 0.24
<i>P. obliquum</i>	0.02 ± 0.00	0.03 ± 0.00	0.48 ± 0.04	5.11 ± 0.41

^a: Trolox equivalence

^b: Gallic acid equivalence (mg/g)

^c: Rutin equivalence (mg/g)

Chemical constituents of *H. caffrum* are poorly known, but the presence of polyphenolic compounds and flavonoids have been reported. These include organic acids, such as protocatechuic acid, and flavonols such as kaempferol (El Sherbeiny and El Ansari, 1976; Van Wyk *et al.*, 2009). The responsible antioxidant agents in *P. obliquum* and *R. melanophloeos* are not known. No antifungal compounds were detected in the bioautography of the *H. caffrum* extract (Section 5.4). The fact that *H. caffrum* extracts had the second highest antioxidant activity may explain the traditional use to combat infection indirectly via stimulating the host immune response and not by a direct antifungal effect.

6.5 Conclusion

All four plant species tested contained compounds that have antioxidant activity, (Sections 6.4.1; 6.4.2) as well as anti-candidal compounds (Sections 6.4.1, 6.4.2; Tables 4.3, 4.4). In order to develop a natural product that can protect users against oral *Candida* infections, it is therefore of utmost importance to test the safety of plant extracts. Testing of the cytotoxicity of plant extracts involves exposure of human or mammalian cells to specified concentrations of the test substances or mixture. After incubation for specific periods, the cells viability is

determined using various methods. Therefore, the next step involved determining the cytotoxicity of dried acetone extracts of *H. caffrum*; *P. obliquum*; *R. melanophloeos* and *S. birrea* reconstituted in dimethyl sulfoxide (DMSO) against mouse fibroblast cells.

CHAPTER 7: CYTOTOXICITY

7.1 Introduction

Users of traditional medicines accept that these preparations are safe for human consumption and that medicinal plants are "pure and natural" which equates to harmless (Street *et al.*, 2008). Some plants have chemical defences enabling them to deter, stun, poison or kill threatening species, therefore one cannot assume that plant extracts are inevitably safe (Gurib-Fakim, 2006). Some plants used for medicinal purposes or as food are potentially toxic, mutagenic and carcinogenic (Schimmer *et al.*, 1988; Higashimoto *et al.*, 1993; Kassie *et al.*, 1996). Misidentification of plant species, poor quality of the preparations, prolonged usage and addition of toxic substances to plant derived remedies contribute significantly to the toxicity associated with plant extracts (Stewart and Steenkamp, 2000; Wolpert, 2001; Fennell *et al.*, 2004). It is therefore of utmost importance to test the safety of plant extracts. Testing of the cytotoxicity of plant extracts and the isolated compounds involves exposure of human or mammalian cells to specified concentrations of the test substance or mixture. After incubation for specific periods the cell viability is determined using various methods (Mosmann, 1983).

7.2 Objective

The objective of this part of the study was to determine the cytotoxicity of dried acetone extracts of *Harpephyllum caffrum* (leaves); *Ptaeroxylon obliquum* (leaves); *Rapanea melanophloeos* (leaves) and *Sclerocarya birrea* (stem bark) using the MTT [3-(4,5-dimethylthiazolyl)2,5-diphenyltetrazolium bromide] reduction assay.

7.3 Material and Methods

The MTT assay was used to determine the cytotoxicity of dried acetone extracts of *H. caffrum* (leaves); *P. obliquum* (leaves); *R. melanophloeos* (leaves) and *S. birrea* (stem bark) reconstituted in dimethyl sulfoxide (DMSO) against mouse fibroblast cells (obtained from the Department of Plant Science, Faculty of Natural and Agricultural Sciences, University of Pretoria, South Africa).

The MTT assay is widely used for measuring cell proliferation and cytotoxicity (Shai, 2007). MTT (yellow) is reduced into a formazan (purple) by viable cells. The colour intensity of the formazan produced, which is directly proportional to the number of viable cells, is measured using a spectrophotometer (Mosmann, 1983; Shai, 2007).

Cells were maintained in minimal essential medium (MEM) (Highveld Biological, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cultures for the assay were prepared from confluent monolayer cells and seeded at a density of 1×10^4 cells per ml in a 96 well microtitre plate and incubated overnight at 37°C in a 5% CO₂ atmosphere. Dried crude plant extracts (100 mg) were reconstituted in 1 ml of DMSO and serial 10-fold dilutions of each extract were prepared in growth medium. The growth medium on sub-confluent monolayer cells grown overnight in microtitre plates was removed and cells were exposed to 200 µl of the extracts at different concentrations and incubated again at 37°C for 5 days. Viability of cells was determined using the tetrazolium-based colorimetric assay (MTT assay) described by Mossmann (1983). Before the addition of MTT, the extract-containing medium on the cells was replaced with fresh culture medium to exclude reduction of the tetrazolium compound by plant extracts. The assay is based on mitochondrial dehydrogenase activity which is assessed by the reductive cleavage of the tetrazolium salt MTT [3-(4,5-dimethylthiazolyl)2,5-diphenyltetrazolium bromide] due to the succinic dehydrogenase present in living cells to yield a purple formazan dye. The cells were incubated with 30 µl of 5 mg/ml MTT in phosphate-buffered saline (PBS) for four hours. Thereafter, medium was removed and cells washed

with PBS. DMSO (50 µl) was added to the cells and absorbance was measured using a Versamax microplate reader at 570 nm. Berberine chloride (Sigma) was used as a positive control, wells containing only cells acted as the negative control and a solvent control was also included. The percentage cell viability following the addition of varying concentrations of the extracts in relation to controls was calculated. The LC₅₀ values were calculated (using SoftMax Pro4.8 Software) as the concentration of plant extracts resulting in 50 percent reduction of absorbance compared to untreated cells. Tests were carried out in quadruplicate and each experiment was repeated three times.

7.4 Results and Discussion

The cytotoxicity of dried acetone extracts of *H. caffrum* (leaves); *P. obliquum* (leaves); *R. melanophloeos* (leaves) and *S. birrea* (stem bark) was determined against mouse fibroblast cells using the MTT assay. The results are presented in Figures 7.1, 7.2, 7.3 and 7.4. Berberine was used as a positive control and was found to be toxic with a LC₅₀ of 9.04 µg/ml (Fig. 7.5). *H. caffrum* was more toxic than berberine, with a LC₅₀ of 2.07 µg/ml (Fig. 7.1). *P. obliquum*, with a LC₅₀ of 35.58 µg/ml (Fig. 7.2), was less toxic than berberine and *H. caffrum* but more toxic than *R. melanophloeos* (LC₅₀ = 434.50 µg/ml) (Fig. 7.3) and *S. birrea* (LC₅₀ > 1000 µg/ml) (Fig. 7.4). *S. birrea* has been reported to have cytotoxic effects in Vero cells (McGaw *et al.*, 2007) and to reduce cell viability in renal epithelial cell lines (Gondwe *et al.*, 2008). No reports regarding cytotoxicity could be obtained for the other plant extracts investigated.

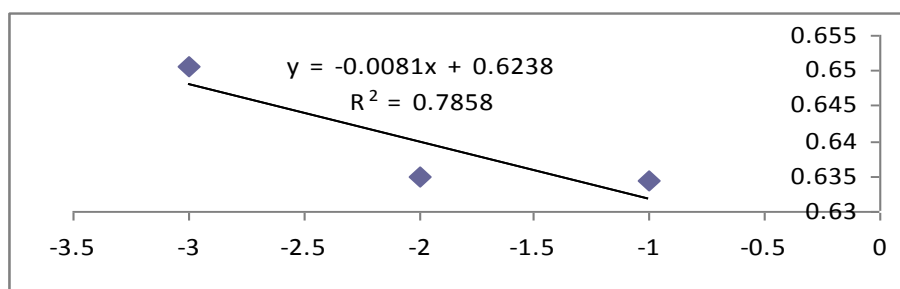


Figure 7.1: Cytotoxicity of *H. caffrum* (LC₅₀ = 2.07 µg/ml) against mouse fibroblast cells

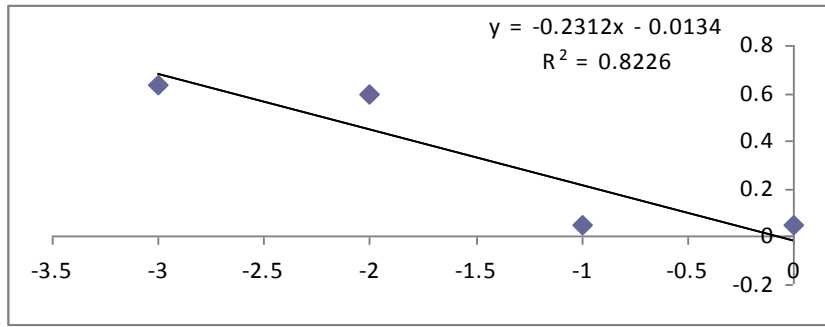


Figure 7.2: Cytotoxicity of *P. obliquum* ($LC_{50} = 35.58 \mu\text{g/ml}$) against mouse fibroblast cells

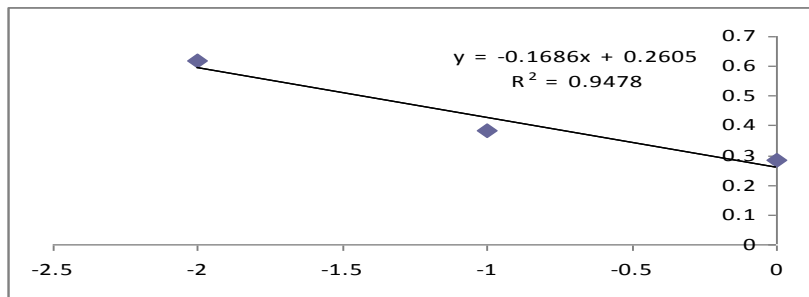


Figure 7.3: Cytotoxicity of *R. melanophloeos* ($LC_{50} = 434.50 \mu\text{g/ml}$) against mouse fibroblast cells

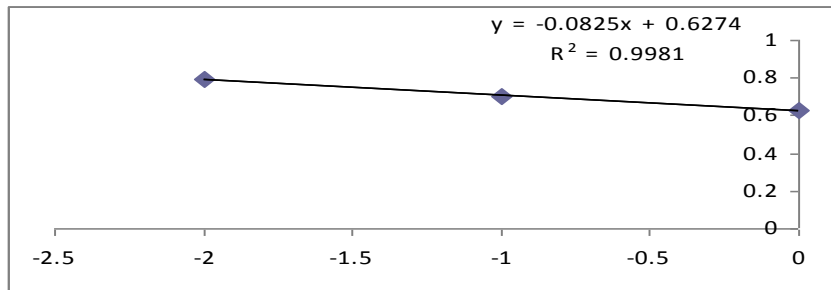


Figure 7.4: Cytotoxicity of *S. birrea* ($LC_{50} > 1000 \mu\text{g/ml}$) against mouse fibroblast cells

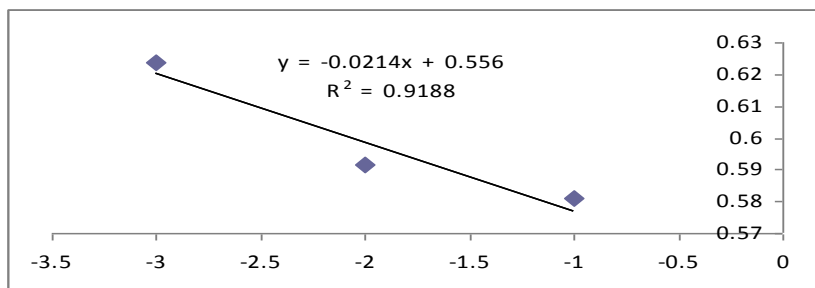


Figure 7.5: Cytotoxicity of berberine ($LC_{50} = 9.04 \mu\text{g/ml}$) against mouse fibroblast cells

Selective activity of the extracts was calculated as follows:

$$\text{Selectivity index (SI)} = \text{LC}_{50} \text{ against mouse fibroblast cells/MIC}$$

The selectivity index is calculated to determine the relationship of activity of a test product to its cytotoxic concentration; the higher the number the better the product. The selectivity index obtained for *S. birrea* was by far the highest compared to *H. caffrum*, *P. obliquum* and *R. melanophloeos* (Table 7.1). It would mean that *S. birrea* results in high inhibition of fungal growth with relative lower toxicity to host cells. Though *H. caffrum*, *P. obliquum* and *R. melanophloeos* resulted in relatively low selectivity indexes, the selectivity index (SI) of *R. melanophloeos* was, on average, six times better against *C. albicans* standard strain (ATCC 10231) and clinical isolates than the SI of *P. obliquum*. The selectivity index of *P. obliquum* was, on average, 28 times better against *C. albicans* standard strain (ATCC 10231) and clinical isolates than the SI of *H. caffrum* (Table 7.1).

Table 7.1: Selectivity index (SI) and LC₅₀ values (mg/ml) of acetone leaf extracts of *H. caffrum*, *P. obliquum* and *R. melanophloeos* and acetone stem bark extract of *S. birrea* against *C. albicans* standard strain (ATCC 10231) and clinical isolates

Acetone extracts	LC ₅₀	<i>Candida albicans</i>							Average
		ATCC	Clinical isolates						
		10231	M0824	M0825	M0826	1051604	1051608	1051255	
<i>H. caffrum</i> (leaf)	0.002	0.001	0.001	0.001	0.004	0.004	0.004	0.001	0.002
<i>P. obliquum</i> (leaf)	0.036	0.036	0.071	0.036	0.071	0.071	0.071	0.036	0.056
<i>R. melanophloeos</i> (leaf)	0.434	0.217	0.435	0.217	0.869	0.435	0.217	0.217	0.372
<i>S. birrea</i> (stem bark)	>1000	>4.000	>0.800	>7.692	>2.000	>1.333	>7.692	>7.692	>4.458

7.5 Conclusion

The data suggests that the antifungal activity of *H. caffrum*, *P. obliquum* and *R. melanophloeos* might be as a result of general metabolic toxins, therefore the low selectivity indices.

However, the results of the current study indicate that *P. obliquum* has the highest number of active compounds against *C. albicans* standard strain (ATCC 10231) and clinical isolates. The results also indicate that *H. caffrum* and *R. melanophloeos* had no visible activity against the fungal test organisms as evidenced by the absence of clear zones on TLC bioautograms (Section 5.4; Table 5.1). Minimal inhibitory concentration (MIC) data also indicates that *P. obliquum* is the most active of all the plant species tested against *C. albicans* standard strain (ATCC 10231) and clinical isolates (Section 4.4; Table 4.2).

Plants utilize the concept of synergy to combat microbial infections by producing small molecule antibiotics, which work together to fight off infection. These compounds are often isolated and developed into chemical drugs for human consumption (Hemaiswarya, *et al.*, 2008). If a plant exhibits high antimicrobial activity, it may be worth isolating the compounds that contribute to the activity of the plant.

As *P. obliquum* had high activity against the fungal test organisms, with a relatively low selectivity index indicative of a general metabolic toxin, the next step involved bioassay-guided fractionation of *P. obliquum* acetone leaf extract.

CHAPTER 8: BIOASSAY-GUIDED FRACTIONATION OF *Ptaeroxylon obliquum* (Thunb.) Radlk. ACETONE LEAF EXTRACT

8.1 Introduction

The active ingredients (chemical compounds) in leaves, roots or bark may be quite different – one part may be very toxic and another part may be harmless. It is therefore very rare that the whole plant will be used for medicinal purposes. Most medicinal plants are used primarily for their leaves and/or twigs, their stem-bark, their underground parts and wood itself is only rarely used (Van Wyk *et al.*, 2009). It is reported that from *P. obliquum* only the wood (Watt and Breyer-Brandwijk, 1962; Hutchings and Van Staden, 1994; Van Wyk *et al.*, 2009) or bark (Hutchings *et al.*, 1996; Van Wyk *et al.*, 2009) itself is used for medicinal purposes. Therefore, reports concerning the isolation of compounds from *P. obliquum* leaves are scanty. The wood contains chromones and other phenolic compounds such as ptaeroxylone and umtatin (Dean and Taylor, 1966; Dictionary of Natural Products, 2008; Van Wyk *et al.*, 2009); the leaves contain perforatin A. Perforatin A has antihypertensive effects (Langenhoven *et al.*, 1988; Dictionary of Natural Products, 2008; Van Wyk *et al.*, 2009).

8.2 Objectives

The objectives of this part of the study were to:

- i) obtain fractions from the dried acetone leaf extract of *P. obliquum* by means of solvent-solvent fractionation;
- ii) locate the active compounds in the solvent-solvent fractions by means of bioautography and TLC-fingerprinting;
- iii) determine the minimal inhibitory concentration (MIC) of the solvent-solvent fractions, obtained from *P. obliquum* acetone leaf extract, after 12 and 24 hours;
- iv) determine the cytotoxicity of the solvent-solvent fractions, obtained from *P. obliquum* acetone leaf extract, using the MTT reduction assay.

8.3 Materials and Methods

8.3.1 Plant material

Leaves of *P. obliquum* were harvested from the Pretoria Botanical Gardens, South Africa, on 17 November 2009. The leaves were collected in loosely woven orange bags and dried in the dark at room temperature. The dried leaves were ground to powder using a Macsalab mill (Model 200 Lab).

8.3.2 Bulk extraction

In a bulk extraction, powdered leaves (1000 g) of *P. obliquum* were extracted with acetone at room temperature. The filtrate was collected by passing the mixture through Whatman 185 mm filter paper using a Büchner funnel. The filtrate was concentrated under reduced pressure using a Büchi Rotavapor R-114 at temperatures not exceeding 50°C. The dried extract was weighed before solvent-solvent fractionation was carried out. Approximately 79.52 g of extract was obtained from 1000 g of dried pulverized leaves.

8.3.3 Solvent-solvent fractionation

The solvent-solvent extraction/fractionation of plant extracts protocol developed by the National Cancer Institute was modified and used to obtain solvent-solvent fractions from *P. obliquum* acetone leaf extract (Suffness and Douros, 1979; Eloff, 1998c).

8.3.3.1 Acetone extraction

- I. The plant material was extracted by dissolving a 10:1 (ml:g) of leaf material in acetone in a container with a closable lid and placed on the shaking machine for 30 minutes.
- II. The solution was left to settle and the clear liquid decanted into a clean, marked new container through filter paper. The process was repeated three times and the filtered solutions combined into one container.

- III. The contents was placed in a round bottom flask and evaporated under reduced pressure using a Büchi Rotavapor R-114 rotating at 100 rpm in a water bath at 50°C.
- IV. The flask was removed from the water just before all the content have evaporated (to prevent heat inactivation or change); kept under vacuum above water level; a small volume of water added to the content and the last traces of acetone removed with the Rotavapor.

8.3.3.2 Chloroform-water fraction

- I. Equal parts of water/chloroform were added to the content in the round bottom flask and after mixing well the content were poured into a separation funnel.
- II. The components separated with the water fraction on the top and the chloroform fraction at the bottom.

8.3.3.3 Hexane fraction

- I. The heavier chloroform fraction (Section 8.3.3.2) was evaporated to dryness using a Büchi Rotavapor R-114 under reduced pressure, rotating at 100 rpm in a water bath at 50°C.
- II. The flask was removed from the water just before all the contents have evaporated.
- III. Equal parts of hexane/chloroform were added to the content in the round bottom flask and after mixing well the content were poured into a separation funnel.
- IV. The components separated with the hexane fraction on the top and the chloroform fraction at the bottom.

8.3.3.4 Ethyl acetate fraction

- I. The heavier chloroform fraction (Section 8.3.3.3) was evaporated to dryness using a Büchi Rotavapor R-114 under reduced pressure, rotating at 100 rpm in a water bath at 50°C.
- II. The flask was removed from the water just before all the content has evaporated.
- III. Equal parts of ethyl acetate and chloroform were added to the content in the round bottom flask and after mixing well the content were poured into a separation funnel.
- IV. The components separated with the ethyl acetate fraction on the top and the chloroform fraction at the bottom.

8.3.4 Thin layer chromatography (TLC)

After solvent-solvent fractionation, solutions of 10 mg/ml of each fraction were prepared in acetone. A sonicator was used to enhance the solubility of each fraction. For each fraction, 10 µl aliquots (100 µg) were loaded on aluminium-backed TLC plates (Merck Silica F₂₅₄ plates) and developed in various mobile phases of varying polarities. Hexane: ethyl acetate [70:30]; hexane : ethyl acetate [50:50] and chloroform : methanol [95:5] were used as solvent systems. Duplicate TLC plates were visualised under ultraviolet (UV) light and visible spots were circled. For visualization of non-fluorescing spots, plates were sprayed with vanillin spray reagent (0.1 g vanillin dissolved in 28 ml methanol and 1 ml sulphuric acid added) and heated at 100°C for 5 min. Plates were scanned, using a HP Scanjet 5470c scanner, immediately after heating to record the chromatograms.

8.3.5 Bioautography procedure

The chromatograms were dried for 5 days at room temperature to remove the eluent solvent systems. Developed TLC plates were sprayed with a concentrated suspension of the fungal test organism (*C. albicans* standard strain (ATCC 10231) and clinical isolates) prepared in Sabouraud dextrose broth in a Biosafety Class II cabinet (Labotec, SA). The sprayed plates were placed in a humid chamber (100 percent relative humidity) and incubated overnight at 25°C. Plates were then

sprayed with a 2 mg/ml *p*-iodonitro-tetrazolium violet (INT, Sigma) solution and incubated at 25°C until a purple-red colour change was evident. Plates with white bands where reduction of INT to formazan did not occur were tightly sealed in transparent plastic bags and retardation factor (R_f) values of inhibitory zones recorded.

The bioautography procedure was repeated on two separate occasions.

8.3.6 Minimal inhibitory concentration (MIC)

8.3.6.1 Fungal cultures

Candida albicans standard strain (ATCC 10231) and clinical isolates (obtained from the Department of Microbiology, National Health Laboratory Services, Pretoria, South Africa) were maintained on Sabouraud dextrose agar at 4°C to prevent overgrowing and morphological changes.

Subcultures were freshly prepared before use. Inocula were prepared from the 24 h cultures by transferring colonies from the freshly prepared subcultures to Sabouraud dextrose broth.

8.3.6.2 Minimal inhibitory concentration (MIC) determination

To determine the MIC values, the microplate dilution method developed by Eloff (1998b) with modifications for antifungal activity assay by Masoko *et al.* (2005) was used.

The assay was initiated by pouring sterile water aliquots (100 μ l) into the wells of a 96-well microtitre plate. Exactly 100 μ l of a 10 mg/ml fraction (chloroform, hexane, water, ethyl acetate) was added in row A and mixed using a micropipette. From row A 100 μ l was aspirated and added into row B and mixed. The procedure was repeated until all the wells were filled. An additional 100 μ l in row H was discarded. 100 μ l of *C. albicans* standard strain (ATCC 10231) or clinical isolates (transferred to Sabouraud dextrose broth until a turbidity of MacFarlane

Standard 1 = 4×10^7 CFU was reached) was added to the wells of the microtitre plate. 50 μ l of a 0.2 mg/ml aqueous solution of p-iodonitro-tetrazolium violet (INT, Sigma) was added to each well. The plates were incubated at 35°C for 12 h and 24 h. Inhibition of microbial growth was indicated by the failure of the well to change colour; uninhibited growth was a pink colour. Amphotericin B was used as a positive control; acetone was used as a negative control. The experiment was performed in triplicate and on two separate occasions.

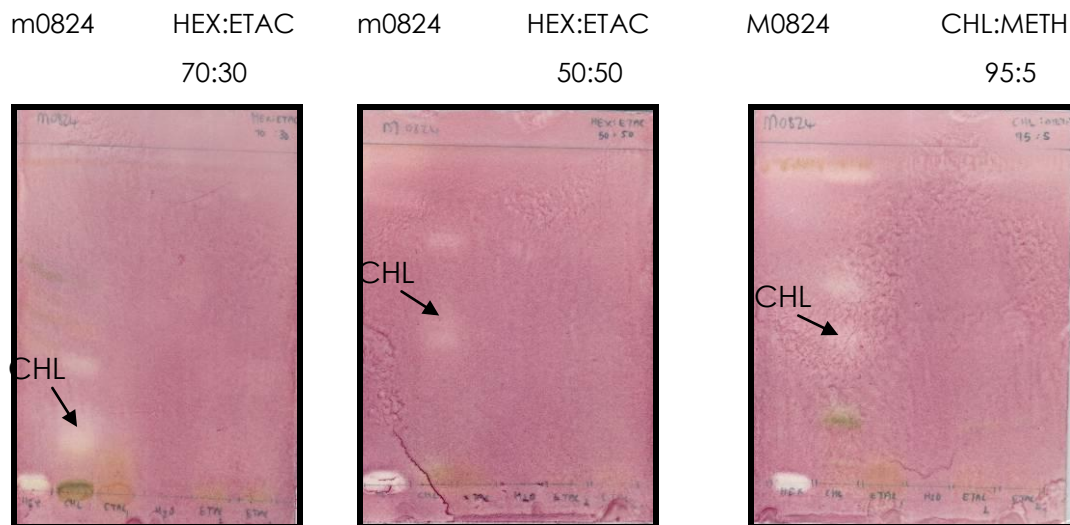
8.3.7 Cytotoxicity of the solvent-solvent fractions

The MTT [3-(4,5-dimethylthiazolyl)2,5-diphenyltetrazolium bromide] reduction assay of Mosmann (1983) was used for measuring cell proliferation and cytotoxicity (described in greater detail in Section 7.3). MTT (yellow) is reduced into a formazan (purple) by viable cells. The colour intensity of the formazan produced, which is directly proportional to the number of viable cells, is measured using a spectrophotometer (Mosmann, 1983).

8.4 Results and Discussion

8.4.1 Bioautography

The compounds whose retardation factor (R_f) values are listed in Table 8.1 were identified as the active constituents that displayed growth inhibition of the *C. albicans* standard strain (ATCC 10231) and clinical isolates tested. The chloroform-fraction had the highest number of compounds against the opportunistic pathogen *C. albicans* and clinical isolates (Fig. 8.1).



HEX : hexane

ETAC : ethyl acetate

CHL : chloroform

METH : methanol

Figure 8.1: TLC bioautograms

The water and ethyl acetate (ETAC₂ and ETAC₂₁) fractions had no visible activity against the test organisms as evidenced by the absence of clear zones on TLC bioautograms. The hexane fraction was active against all the strains tested. However, for the hexane fraction a R_f value of mostly zero was recorded. This suggests that the chemical constituent in the hexane fraction is highly polar in nature.

Table 8.1: The R_f values of active compounds from the solvent-solvent fractions obtained from *P. obliquum* acetone leaf extract against *C. albicans* standard strain (ATCC 10231) and clinical isolates

Fractions	Eluting solvent system	<i>Candida albicans</i>						
		ATCC	Clinical isolates					
		10231	M0824	M0825	1051604	1051608	1051255	
Chloroform	HEX : ETAC 50 : 50	0.65	0.70	0.72	0.91	0.69	0.75	
		0.49	0.43	0.56	0.76	0.49	0.41	
		0.38	0.04	0.44	0.43	0.40	-	
	HEX : ETAC 70 : 30	0.44	0.38	0.38	-	0.38	-	
		-	0.13	0.15	-	0.15	-	
	CHL : METH 95 : 5	0.89	0.90	0.92	-	-	-	
		0.57	0.57	0.57	-	0.59	0.54	
		0.43	0.43	0.43	0.49	0.44	0.43	
		0.22	0.19	0.25	-	-	-	
	Hexane	HEX : ETAC 50 : 50	0	0.84 0	0.03	0	0	0.02
		HEX : ETAC 70 : 30	0	0	0	0	0	0.01
		CHL : METH 95 : 5	0	0.93 0	0.92 0.03	0	0	0
ETAC ₁	HEX : ETAC 50 : 50	0.19	-	0.2	0.19	-	0.18	
	HEX : ETAC 70 : 30	-	-	-	-	-	-	
	CHL : METH 95 : 5	-	-	-	-	-	-	
ETAC ₂	HEX : ETAC 50 : 50	-	-	-	-	-	-	
	HEX : ETAC 70 : 30	-	-	-	-	-	-	
	CHL : METH 95 : 5	-	-	-	-	-	-	
ETAC ₂₁	HEX : ETAC 50 : 50	-	-	-	-	-	-	
	HEX : ETAC 70 : 30	-	-	-	-	-	-	
	CHL : METH 95 : 5	-	-	-	-	-	-	
H ₂ O	HEX : ETAC 50 : 50	-	-	-	-	-	-	
	HEX : ETAC 70 : 30	-	-	-	-	-	-	
	CHL : METH 95 : 5	-	-	-	-	-	-	

R_f : retardation factor

HEX : hexane

ETAC : ethyl acetate

ETAC₁ : ethyl acetate (9/2/2010)

ETAC₂ : ethyl acetate (10/2/2010)

ETAC₂₁ : ethyl acetate (10/2/2010)

CHL : chloroform

METH : methanol

H₂O : water

- : no visible activity

8.4.2 Minimal inhibitory concentration (MIC)

All the fractions showed anti-candidal activity against the *C. albicans* standard strain and clinical isolates tested (Table 8.2). Inhibition at concentrations <1 mg/ml, against the *C. albicans* standard strain (ATCC 10231) tested, was observed after 12 h for the hexane (0.63 mg/ml), chloroform (0.63 mg/ml), ETAC₁ (0.63 mg/ml) and ETAC₂ (0.63 mg/ml) fractions. The antifungal compound, Amphotericin B, inhibited the growth of *C. albicans* standard strain (ATCC 10231) and clinical isolates (Table 8.2).

Even at the highest concentrations tested (2.50 mg/ml), the water fraction did not inhibit the growth of *C. albicans* standard strain (ATCC 10231) and clinical isolates (Table 8.2). These results support the results of Kotze and Eloff (2002) that water extracts generally do not have antimicrobial activity. Acetone, the negative control, did not inhibit the growth of any of the strains tested.

To investigate the extreme variation in MIC values of some fractions and amphotericin B with time of incubation (12 h and 24 h); wells were re-inoculated onto Sabouraud dextrose agar plates and re-incubated at 35°C for 24 h (Table 8.3). Thus, the cidal/static effects of the fractions and/or amphotericin B on *C. albicans* standard strain (ATCC 10231) and clinical isolates could be determined.

Table 8.2: Minimal inhibitory concentrations for the solvent-solvent fractions and amphotericin B

Fractions/ Amphotericin B	Time of incubation (h)	*MIC (mg/ml) <i>Candida albicans</i>						
		ATCC	Clinical isolates					
		10231	M0824	M0825	M0826	1051604	1051608	1051255
Hexane	12	0.63	0.16	0.08	0.63	0.31	0.63	0.63
	24	1.25	1.25	0.31	0.63	1.25	1.25	1.25
Chloroform	12	0.63	0.16	0.08	0.31	0.31	0.63	0.31
	24	1.25	0.16	0.16	0.63	1.25	1.25	1.25
ETAC ₁	12	0.63	0.16	0.16	1.25	1.25	2.50	0.31
	24	2.50	0.31	0.31	2.50	>2.50	>2.50	>2.50
H ₂ O	12	>2.50	0.31	0.63	>2.50	>2.50	>2.50	>2.50
	24	>2.50	>2.50	2.50	>2.50	>2.50	>2.50	>2.50
ETAC ₂	12	0.63	0.31	0.16	1.25	1.25	2.50	1.25
	24	2.50	0.31	0.31	1.25	1.25	>2.50	2.50
ETAC ₂₁	12	1.25	0.31	0.16	1.25	1.25	1.25	1.25
	24	2.50	0.31	1.25	1.25	2.50	2.50	2.50
Amphotericin B	12	0.11	0.03	0.03	0.06	0.06	0.11	0.03
	24	0.11	0.11	0.06	0.11	0.06	0.11	0.23

*MIC : Minimal inhibitory concentration representing the mean value h : hours

H₂O : water

ETAC₁ : ethyl acetate (9/2/2010)

ETAC₂ : ethyl acetate (10/2/2010)

ETAC₂₁ : ethyl acetate (10/2/2010)

Table 8.3: Effect of the fractions/amphotericin B, re-inoculated and re-incubated, on the *C. albicans* strains tested

<i>C. albicans</i> strains	Fractions/ Amphotericin B	*MIC		Growth after re- inoculation and re-incubation	Cidal/static effects
		12 h	24 h		
ATCC 10231	ETAC ₁	0.63	2.50	no	cidal
	ETAC ₂	0.63	2.50	yes	static
M0824	Hexane	0.16	1.25	yes	static
	H ₂ O	0.31	>2.50	yes	static
	Amphotericin B	0.03	0.11	yes	static
M0825	Hexane	0.08	0.31	no	cidal
	H ₂ O	0.63	>2.50	yes	static
	ETAC ₂₁	0.16	1.25	no	cidal
1051604	Hexane	0.31	1.25	yes	static
	Chloroform	0.31	1.25	yes	static
	ETAC ₁	1.25	>2.50	yes	static
1051255	Chloroform	0.31	1.25	yes	static
	ETAC ₁	0.31	>2.50	yes	static
	Amphotericin B	0.03	0.23	yes	static

*MIC : Minimal inhibitory concentration representing the mean value h : hours

ETAC₁ : ethyl acetate (9/2/2010)

H₂O : water

ETAC₂ : ethyl acetate (10/2/2010)

ETAC₂₁ : ethyl acetate (10/2/2010)

8.4.3 Cytotoxicity of the solvent-solvent fractions

The cytotoxicity of the solvent-solvent fractions, obtained from *P. obliquum* acetone leaf extract, was determined against mouse fibroblast cells using the MTT assay. The results are presented in Figures 8.2, 8.3, 8.4, 8.5, 8.6 and 8.7 and in Table 8.4. Berberine was used as a positive control and was found to be toxic with a LC₅₀ of 9.04 µg/ml (Fig. 8.8).

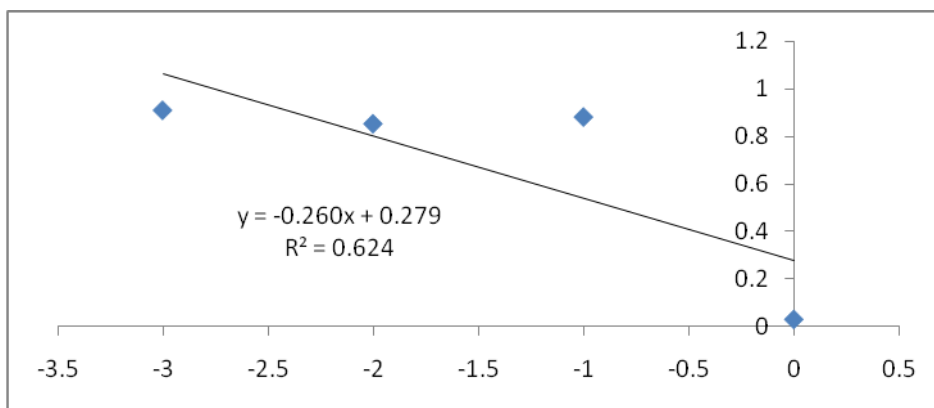


Figure 8.2: Cytotoxicity of the hexane-fraction (LC₅₀ = 211.99 µg/ml) against mouse fibroblast cells

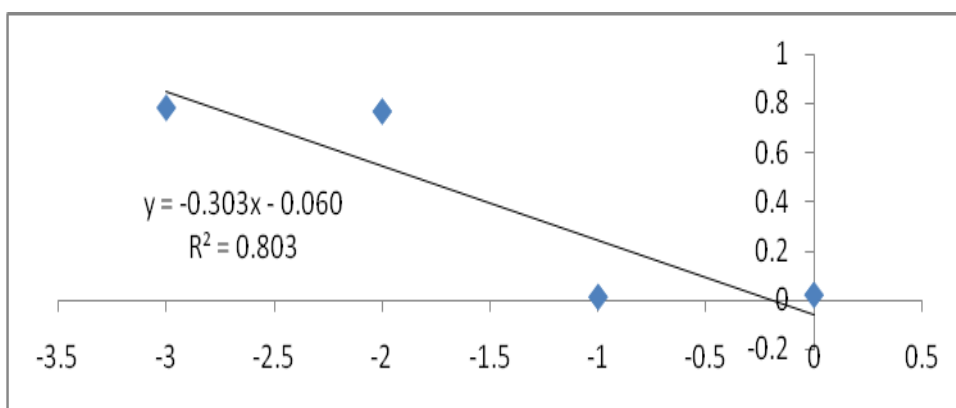


Figure 8.3: Cytotoxicity of the chloroform-fraction (LC₅₀ = 28.64 µg/ml) against mouse fibroblast cells

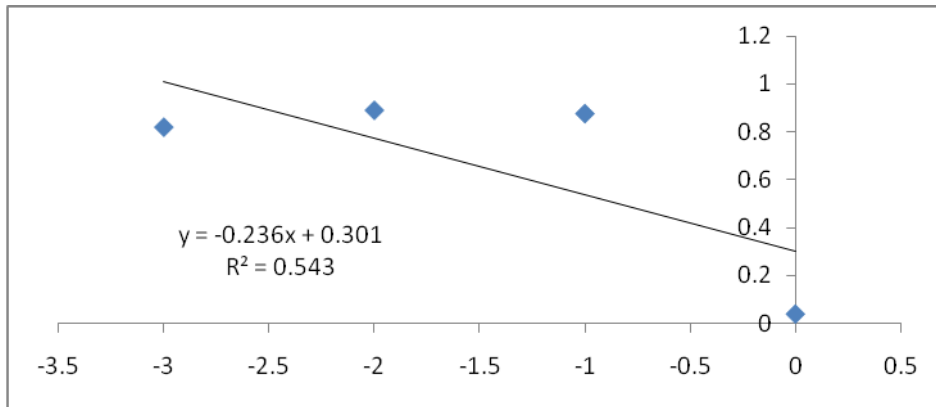


Figure 8.4: Cytotoxicity of the ETAC₁-fraction (LC₅₀ = 229.69 µg/ml) against mouse fibroblast cells

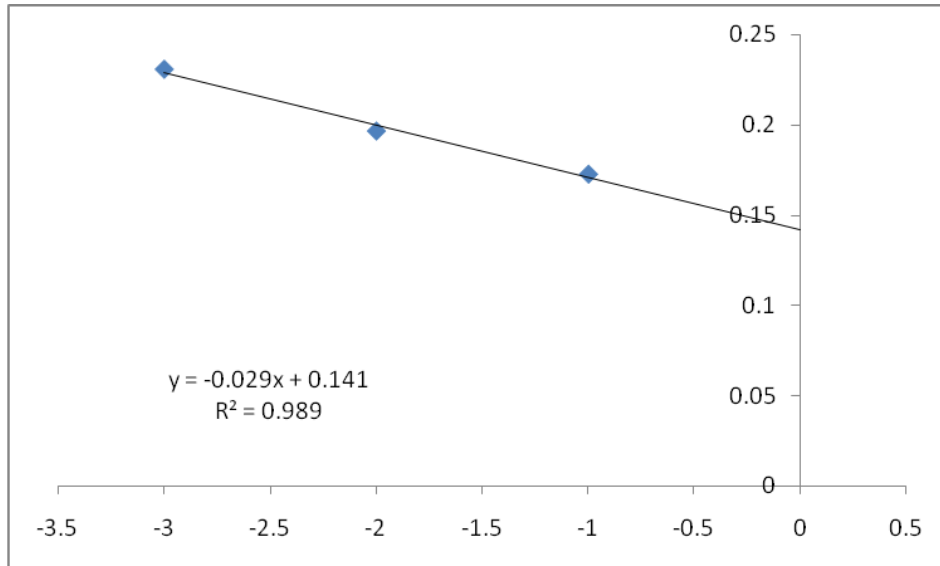


Figure 8.5: Cytotoxicity of the H₂O-fraction (LC₅₀ = 0.08 µg/ml) against mouse fibroblast cells

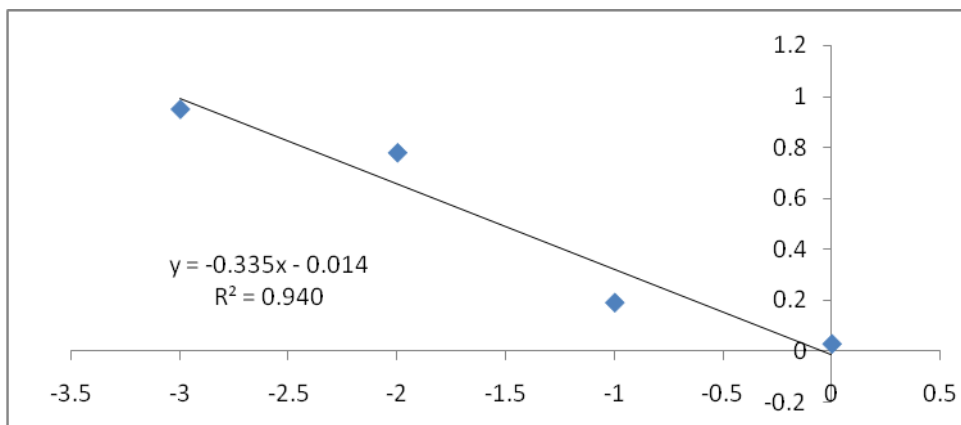


Figure 8.6: Cytotoxicity of the ETAC₂-fraction (LC₅₀ = 39.69 µg/ml) against mouse fibroblast cells

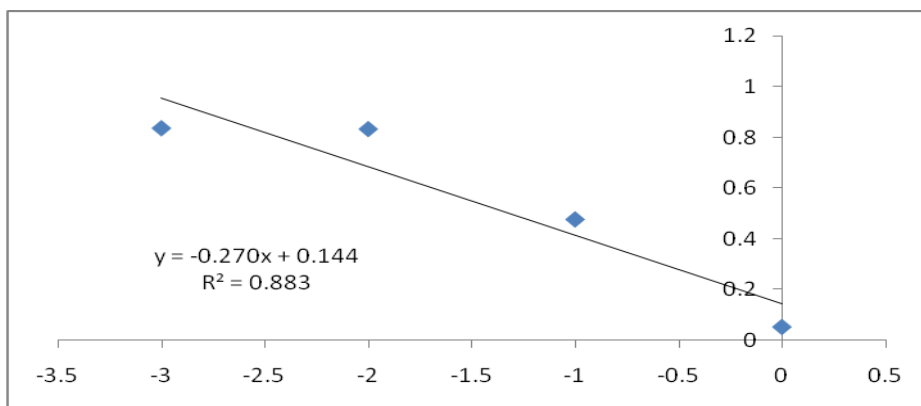


Figure 8.7: Cytotoxicity of the ETAC₂₁-fraction (LC₅₀ = 71.27 µg/ml) against mouse fibroblast cells

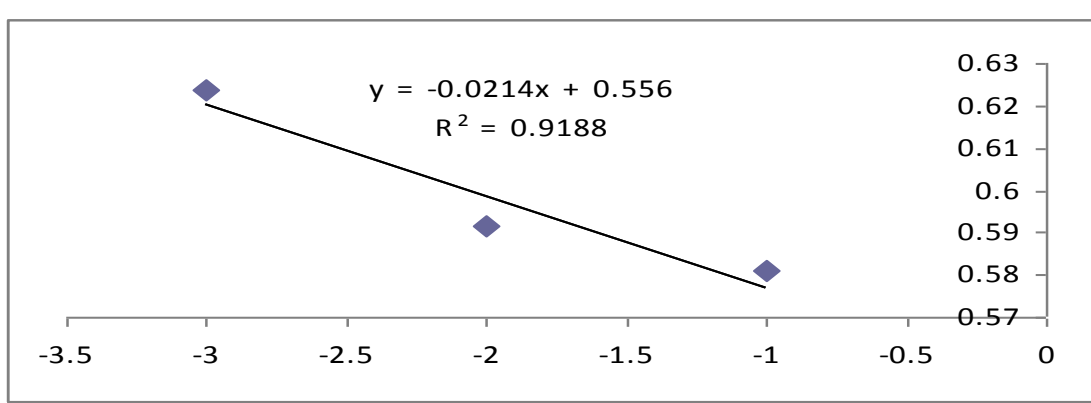


Figure 8.8: Cytotoxicity of berberine (LC₅₀ = 9.04 µg/ml) against mouse fibroblast cells

The selectivity index is the ratio of the efficacy against the pathogen relative to the toxicity to the cells. It relates to the degree to which the observed activity of a substance can be attributed to the toxicity of that substance. A high value indicates good efficacy and low toxicity. A value lower than one, indicates that the treatment is more harmful to cells than to the pathogen. The selectivity indices for the solvent-solvent fractions were calculated by dividing the LC₅₀ in mg/ml by the MIC in mg/ml. The selectivity indices were substantially lower than one with all fractions (Table 8.4).

The selectivity index (Table 8.4) was the highest for the EATC₁-fraction. The selectivity index of the hexane- and ETAC₁-fraction was, on average, four times

better against *C. albicans* standard strain (ATCC 10231) and clinical isolates than that resulting from the chloroform-fraction (Table 8.4).

The data (Table 8.4) suggests that the antifungal activity of the solvent-solvent fractions, obtained from *P. obliquum* acetone leaf extract, may be related to a general metabolic toxin leading to the low selectivity indices.

Table 8.4: Selectivity index (SI) and LC₅₀ values (mg/ml) of the solvent-solvent fractions, obtained from *P. obliquum* acetone leaf extract, against *C. albicans* standard strain (ATCC 10231) and clinical isolates

Fractions	LC ₅₀	Fractions	<i>Candida albicans</i>							Average
			ATCC	Clinical isolates						
			10231	M0824	M0825	M0826	1051604	1051608	1051255	
Hexane	0.212	Hexane	0.169	0.169	0.683	0.336	0.169	0.169	0.169	0.266
Chloroform	0.029	Chloroform	0.022	0.179	0.179	0.045	0.022	0.022	0.022	0.070
ETAC ₁	0.229	ETAC ₁	0.091	0.740	0.740	0.091	>0.091	>0.091	>0.091	0.276
H ₂ O	0.00	H ₂ O	0	0	0	0	0	0	0	0
ETAC ₂	0.039	ETAC ₂	0.015	0.128	0.128	0.031	0.031	>0.015	0.015	0.015
ETAC ₂₁	0.071	ETAC ₂₁	0.028	0.229	0.057	0.057	0.028	0.028	0.028	0.065

H₂O : water

ETAC₁ : ethyl acetate (9/2/2010)

ETAC₂ : ethyl acetate (10/2/2010)

ETAC₂₁ : ethyl acetate (10/2/2010)

8.5 Conclusion

The results of the current study indicate that the chloroform-fraction has the highest number of active compounds against *C. albicans* standard strain (ATCC 10231) and clinical isolates (Fig. 8.1; Table 8.1). Minimal inhibitory concentration (MIC) data also indicates that the chloroform-fraction is the most active of all the solvent-solvent fractions against the *C. albicans* standard strain (ATCC 10231) and clinical isolates tested (Table 8.2).

The low selectivity indices of the fractions indicate that there is hardly any chance that these could be used for therapeutic purposes (Table 8.4). It has to be taken into account that cellular toxicity does not necessarily equate to whole animal

toxicity because factors such as changes in the gut, bioavailability issues may lead to large differences. If extracts are to be used as an oral rinse against *Candida* with short term exposure it may still be possible to use it.

It is not necessarily the compounds that are responsible for the anti-candidal effect that are toxic to the cells. It may therefore be worth isolating the compounds that contribute to the activity. If some of these compounds are active against *Candida* and not toxic to human cells it may lead to an oral hygiene product that can protect patients against oral *Candida* infections.

Following this finding, the next step therefore involves the bioassay-guided isolation of the antifungal compounds from the chloroform-fraction, obtained from *P. obliquum* acetone leaf extract.

CHAPTER 9: BIOASSAY-GUIDED ISOLATION OF THE ANTIFUNGAL COMPOUNDS FROM THE CHLOROFORM-FRACTION OBTAINED FROM *P. obliquum* (Thunb.) Radlk. ACETONE LEAF EXTRACT

9.1 Introduction

Ptaeroxylaceae is a small family comprising two genera, *Ptaeroxylon*, and *Cedrelopsis*. *Ptaeroxylon* is a monotypic genus comprising only the southern African species, *Ptaeroxylon obliquum*; whereas *Cedrelopsis* is a genus limited to Madagascar and contains several species (Mulholland *et al.*, 2000). *Ptaeroxylon* and *Cedrelopsis* are grouped and placed in the Ptaeroxylaceae family, based on the structure of the secondary xylem and their similar pollen morphology (Styles and Pennington, 1975). *P. obliquum* contains a wide variety of simple and prenylated 6,7-dioxygenated coumarins and 5,7-dioxygenated prenylated chromones (Dean and Taylor, 1966; Mulholland *et al.*, 2000). An ethyl acetate extract of ground *P. obliquum* heartwood contains the chromones peucenin, found previously in the roots of *Peucedanum ostruthium* Koch (Umbelliferae); desoxykarenin and karenin; as well as isomeric coumarins 7-O-(3,3-dimethylallyl) scopoletin, nieshoutin and nieshoutol; and the ubiquitous β -sistosterol (McCabe *et al.*, 1967). A re-investigation of the bark of *P. obliquum* yielded the unusual aromadendrane diterpenoid, cneorubin X (Mulholland *et al.*, 2000). The isolated limonoids cedmilin and cedmilinol most closely resemble those reported previously from the Cneoraceae, for example, cneorin K from *Neochamaela pulverulenta* (Mondon *et al.*, 1978). The Cneoraceae also produced prenylated coumarins and cneorubin X has previously been isolated from *Cneorum tricoccon* (Trautmann *et al.*, 1980). Thus, chemical analysis indicates that the Ptaeroxylaceae may be closely related to the Cneoraceae family.

However, little is known in the literature concerning the isolation of compounds from *P. obliquum* leaves. The leaves are reported to contain Perforatin A which

has antihypertensive effects (Langenhoven *et al.*, 1988; Dictionary of Natural Products, 2008; Van Wyk *et al.*, 2009).

9.2 Objective

The objective of this part of the study was to isolate active antifungal compounds (against *C. albicans* standard strain (ATCC 10231) and clinical isolates), obtained from *P. obliquum* acetone leaf extract, identified as the most active solvent-solvent fraction after bioautography and minimal inhibitory concentration (MIC) determination.

9.3 Materials and Methods

9.3.1 Selection of stationary phase

Column chromatography using silica gel (Merck, Silica Gel 60) as the stationary phase was chosen for separation of compounds for the following reasons:

- I. silica gel was previously used in the Phytomedicine laboratory for isolation of compounds with a high degree of success (Martini and Eloff, 1998);
- II. separation on silica gel represents one of the cheapest methods for isolation of compounds;
- III. silica gel is readily accessible and preparation of separating systems is simple and quick; and
- IV. eluent system polarity can be varied to adjust elution of active compounds.

9.3.2 Group separation (Column I)

Silica gel (800 g) was mixed with hexane to form a homogenous suspension / slurry and stirred using a stirring rod to remove bubbles. The silica gel slurry was poured into a glass column (10 cm diameter and 50 cm length) whose outlet was plugged with cotton wool to retain the gel in the column. The solvent was allowed to flow out of the column opening to allow the gel to settle.

The chloroform-fraction (extract) sample was prepared by dissolving 15 g of the extract in 100 ml of ethyl acetate. To the solution, 30 g of silica was added, and mixed by stirring with a glass rod. The mixture was allowed to dry at room temperature under a stream of air for approximately 5 hours. The dried silica gel-extract mixture was carefully layered on the column gel bed. For elution hexane was used as the first mobile phase with the polarity increasing by 10% increments of ethyl acetate. For each eluent mixture 1.5 L volumes were used and 500 ml fractions collected in glass beakers. The 22 collected fractions were concentrated using a Büchi R-114 Rotavapor. TLC was used to analyse fractions, and those with similar chemical components were combined.

9.3.3 Combination of fractions from Column I (chloroform extract/fraction)

The antimicrobial activities of the fractions were assayed using bioautography as the method of choice with *C. albicans* standard strain (ATCC 10231) and clinical isolates as fungal test organisms. In most instances active compounds were found in more than one fraction. These fractions were combined to maximise the level of active compounds in order to obtain a high yield of the compounds.

9.3.4 Isolation of compound C1 (Column II)

A glass column with length of 50 cm and diameter of 2 cm was used for fractionation. Silica gel (150 g) was mixed with hexane by stirring and the slurry poured into the column whose bottom opening was plugged with cotton wool. The solvent was allowed to drip out of the silica gel to settle and establish a column bed. The sample was prepared by mixing 2 g of the fraction with 4 g silica gel in 60 ml of ethyl acetate. The mixture was stirred vigorously and dried under a stream of air. The dried sample was loaded onto the gel in the column. After a series of separations of the fraction on TLC plates in different solvent mixtures to obtain a system resulting in good resolution, hexane:ethyl acetate [70:30] was selected as the solvent system. The loaded column was eluted using

hexane:ethyl acetate [70:30] mixture with 50 ml fraction volumes collected at 5 ml/min.

9.3.5 Isolation of compound C2 (Column III)

Fractions containing similar antifungal compounds were combined and the mixture used for isolation of compound C2. A glass column with a length of 40 cm and diameter of 4 cm was used for fractionation of the mixture. Silica gel (200 g) was mixed with hexane by stirring and the slurry poured into the column whose bottom opening was plugged with cotton wool. The solvent was allowed to drip out to settle the gel and establish a column bed. The sample was prepared by mixing 3 g of the fraction mixture with 6 g silica gel in 90 ml ethyl acetate. The mixture was stirred vigorously and dried under a stream of air. The dried sample was then loaded onto the gel bed and eluted with hexane:ethyl acetate [50:50] mixture with polarity increased by 10% increments of ethyl acetate. Fifty ml fraction was collected at a flow rate of 5 ml/min.

9.4 Results and Discussion

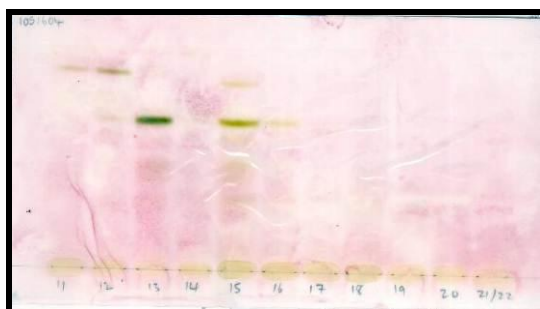
From Column I, 22 fractions were collected and analysed on TLC plates. The fractions containing compounds with R_f corresponding to the R_f values of bioautography assay were combined (Table 9.1). These fractions were tested for antifungal activity (Fig. 9.1). Active fractions were identified for isolation of anti-candidal compounds. The purification of the compounds was achieved by repeated column chromatography until a single spot was obtained from each compound using three different mobile phases to develop the chromatograms.

Table 9.1: The R_f values of active fractions against *C. albicans* and two clinical isolates

Eluting Solvent System	Fractions	<i>C. albicans</i> isolates	
		1051604	1051255
HEX:ETAC 70:30	3	-	0.89
	5	-	0.890
	7	-	0.3
	8	-	0.3
	9	-	1.0
	10	-	1.0
HEX:ETAC 50:50	19	0.3	0.45
	20	0.3	0.45

R_f : retardation factor
 HEX : hexane
 ETAC : ethyl acetate
 - : no visible activity

1051604



1051255



1051255



Figure 9.1: TLC bioautograms showing activity of fractions against *C. albicans* isolates

Two compounds were isolated using column chromatography with silica gel as the stationary phase.

9.5 Conclusion

Two active antifungal compounds were isolated using silica gel as the stationary phase in column chromatography, following the principles of bioassay-guided fractionation. This is the first report of isolation and identification of compounds with antifungal activity from *P. obliquum* leaves.

Following this result, the next step involved determining the biological activity of the isolated compounds, and to determine the structures of the two isolated compounds.

CHAPTER 10: BIOLOGICAL ACTIVITY OF THE ISOLATED COMPOUNDS AND STRUCTURE ELUCIDATION

10.1 Introduction

Two compounds were isolated from the most active fraction obtained from *Ptaeroxylon obliquum* acetone leaf extract, using column chromatography with silica gel as the stationary phase. In the available literature, very little information about compounds isolated from *P. obliquum* leaves was found. The leaves contain perforatin A which has anti-hypertensive effects (Langenhoven *et al.*, 1988; Dictionary of Natural Products, 2008; Van Wyk *et al.*, 2009).

10.2 Objectives

The objectives of this part of the study were to:

- I. determine the antifungal activity and cytotoxicity of the isolated compounds;
- II. elucidate the structures of the two isolated compounds, obtained from *P. obliquum* acetone leaf extract.

10.3 Materials and Methods

10.3.1 Fungal cultures

Candida albicans standard strain (ATCC 10231) and clinical isolates (obtained from the Department of Microbiology, National Health Laboratory Services, Pretoria, South Africa) were maintained on Sabouraud dextrose agar at 4°C to prevent overgrowing and morphological changes.

Subcultures were freshly prepared on Sabouraud dextrose agar before use. Inocula were prepared from the 24 h cultures.

10.3.2 Minimal inhibitory concentration (MIC) determination

To determine the MIC values, the microplate dilution method developed by Eloff (1998b) with modifications for antifungal activity assay by Masoko *et al.* (2005) was used.

The assay was initiated by pouring sterile water aliquots (100 µl) into the wells of a 96-well microtitre plate. Exactly 100 µl of the 1 mg/ml compound was added in row A and mixed using a micropipette. From row A 100 µl was aspirated and added into row B and mixed. The procedure was repeated until all the wells were filled. An additional 100 µl in row H was discarded. 100 µl of *C. albicans* standard strain (ATCC 10231) or clinical isolates (transferred to Sabouraud dextrose broth until a turbidity of MacFarlane Standard 1 = 4×10^7 CFU was reached) was added to the wells of the microtitre plate. 50 µl of a 0.2 mg/ml aqueous solution of *p*-iodonitro-tetrazolium violet (INT, Sigma) was added to each well. The plates were incubated at 35°C for 12 h and 24 h. Inhibition of microbial growth was indicated by the failure of the well to change colour; uninhibited growth was a pink colour. Amphotericin B was used as a positive control; acetone was used as a negative control. The experiment was performed in triplicate and on two separate occasions.

10.3.3 Cytotoxicity

The MTT [3-(4,5-dimethylthiazolyl)2,5-diphenyltetrazolium bromide] reduction assay of Mosmann (1983) was used for measuring cell proliferation and cytotoxicity (described in greater detail in Section 7.3). MTT (yellow) is reduced into a formazan (purple) by viable cells. The colour intensity of the formazan produced, which is directly proportional to the number of viable cells, is measured using a spectrophotometer (Mosmann, 1983).

10.3.4 Structure elucidation

The structures of the compounds were determined by extensive nuclear magnetic resonance (NMR) techniques and chemical methods mainly by 1D NMR (^1H , ^{13}C and DEPT) and 2D NMR (HSQC, HMBC and COSY) and by

comparison with the literature data. About 1 mg of each compound was submitted for spectrometric analysis at the Department of Chemistry, University of Pretoria. Electron impact mass spectrometry (EMS) was used to analyse the isolated compounds.

10.4 Results and Discussion

10.4.1 Minimal inhibitory concentration

Both compounds had anti-candidal activity against the *C. albicans* standard strain (ATCC 10231) and clinical isolates tested (Table 10.1). Amphotericin B inhibited the growth of *C. albicans* standard strain (ATCC 10231) and clinical isolates (Table 10.1)

Compound 1 and Compound 2 inhibited the growth of *C. albicans* standard strain (ATCC 10231) at a much lower value (0.004 mg/ml) as compared to amphotericin B (0.11 mg/ml) (Table 10.1). There appeared to be a remarkable interaction in the activity of Compound 2 and amphotericin B with the different isolates. In the ATCC strain Compound 2 was much more active, but against the clinical isolates it was generally much less active.

Table 10.1: Minimal inhibitory concentrations for the Compounds and amphotericin B tested

Compounds / Amphotericin B	Time of incubation (h)	*MIC (mg/ml) <i>Candida albicans</i>						
		ATCC	Clinical Isolates					
		10231	M0824	M0825	M0826	1051604	1051608	1051255
Compound 1	12	0.004	>0.25	>0.25	>0.25	>0.25	0.004	>0.25
	24	0.004	>0.25	>0.25	>0.25	>0.25	0.25	>0.25
Compound 2	12	0.004	>0.25	>0.25	>0.25	>0.25	0.008	>0.25
	24	0.004	>0.25	>0.25	>0.25	>0.25	0.25	>0.25
Amphotericin B	12	0.11	0.03	0.03	0.06	0.06	0.11	0.03
	24	0.11	0.11	0.06	0.11	0.06	0.11	0.23

* MIC: Minimal inhibitory concentration representing the mean value h: hours

10.4.2 Cytotoxicity

The cytotoxicity of the compounds was determined against mouse fibroblast cells using the MTT assay. The results are presented in Figures 10.1 and 10.2. Berberine was used as a positive control and was found to be toxic with a LC_{50} of 9.04 $\mu\text{g/ml}$ (Fig. 10.3). Amphotericin B was found to be toxic with a $LC_{50} = 1.46 \mu\text{g/ml}$ against mouse fibroblast cells (Fig. 10.4). The cytotoxicity of Compound 2 ($LC_{50} = 7.23 \mu\text{g/ml}$) was lower than that of amphotericin B ($LC_{50} = 1.46 \mu\text{g/ml}$) (Fig. 10.2, 10.4).

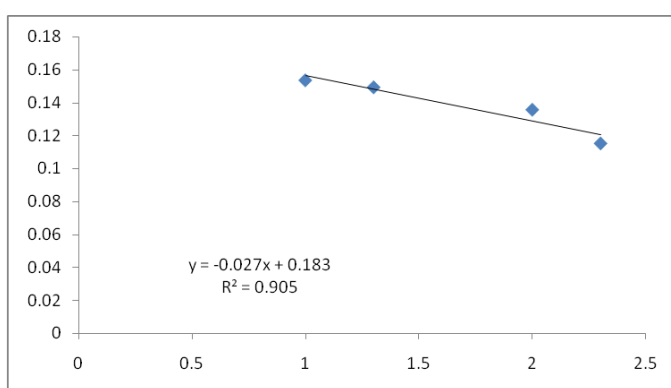


Figure 10.1: Cytotoxicity of Compound 1 ($LC_{50} = 0.001 \mu\text{g/ml}$) against mouse fibroblast cells

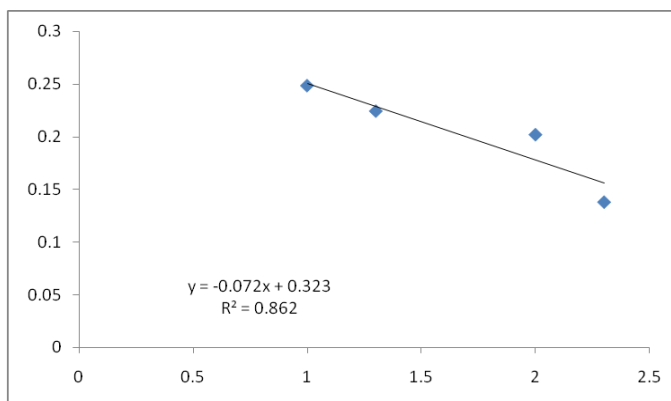


Figure 10.2: Cytotoxicity of Compound 2 ($LC_{50} = 7.23 \mu\text{g/ml}$) against mouse fibroblast cells

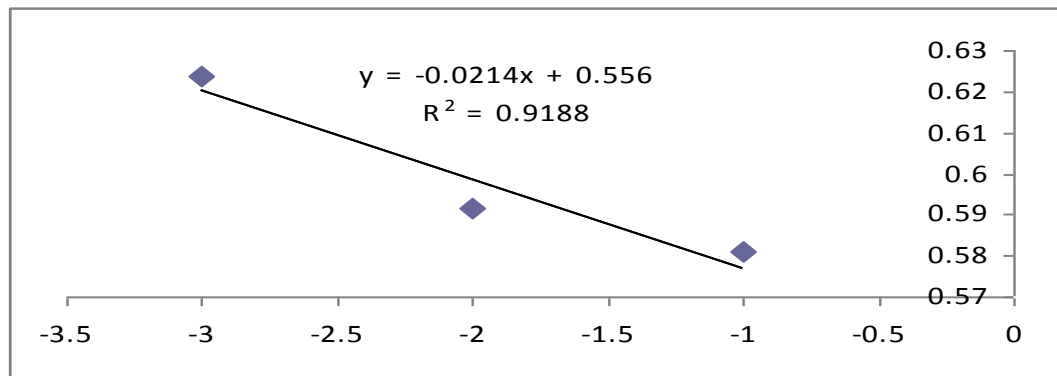


Figure 10.3: Cytotoxicity of berberine (LC₅₀ = 9.04 µg/ml) against mouse fibroblast cells

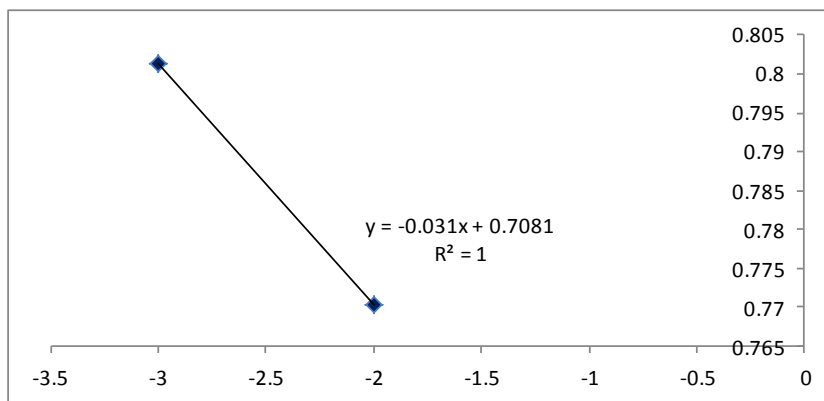


Figure 10.4: Cytotoxicity of amphotericin B (LC₅₀ = 1.46 µg/ml) against mouse fibroblast cells

Selective activity of the compounds was calculated as follows:

$$\text{Selectivity index (SI)} = \text{LC}_{50} \text{ against mouse fibroblast cells} / \text{MIC in same units}$$

The selectivity index is calculated to determine the relationship of activity of a test product to its cytotoxic concentration, the higher the number the safer the product. The selectivity index obtained for Compound 2 was by far the highest compared to Compound 1 (Table 10.2). It would mean that Compound 2 results in high inhibition of fungal growth with relative lower toxicity to host cells. Unfortunately only in the case of the *C. albicans* standard strain (ATCC 10231) was the value higher than one and would the product be safe to use.

Table 10.2: Selectivity index (SI) of the isolated Compounds against *C. albicans* standard strain (ATCC 10231) and clinical isolates

Compounds	<i>Candida albicans</i>							Average
	ATCC	Clinical isolates						
	10231	M0824	M0825	M0826	1051604	1051608	1051255	
Compound 1	0.0003	>0.000	>0.000	>0.000	>0.000	0.000	>0.000	0.000
Compound 2	1.808	>0.029	>0.029	>0.029	>0.029	0.029	>0.029	0.283

10.4.3 Structure elucidation

Compound 1 was obtained as isomeric mixture; the TLC fingerprint indicated unresolved single spot with three different mobile phases. However, the 1D and 2D NMR spectra exhibited chemical shift characteristic of both olean-12-ene and lupene. ¹³C NMR (DMSO): 145.20 (C-13, olean), 121.72 (C-12, olean); 150.97 (C-20, lup); 109.31 (C-29, lup); 41 (C-18, olean), 48.30 (C-18, lup), 27.97 (C-19, olean), 48.00 (C-19, lup) (Table 10.3).

From the HMBC, methyl group (C-29) resonance at δ H 1.62 (δ C 19.28) showed correlation to a methine at δ C 48.29 (C-19), methylene carbon resonance at 109.31 and unsaturated carbon resonance at δ C 150.97 (C-20). These correlations are characteristic feature of lupeol (Fig. 10.5).

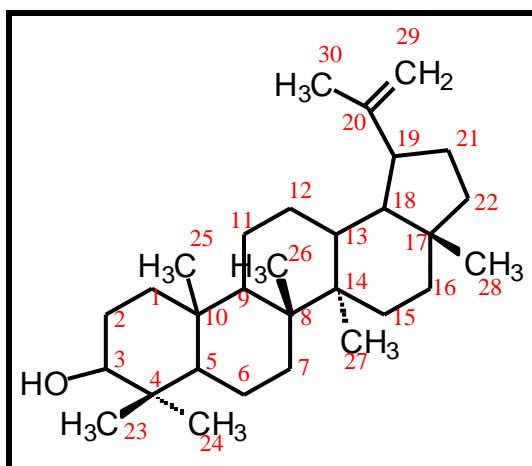


Figure 10.5: The structure of Compound 1.1 (lupeol) isolated from *P. obliquum* leaves

Methyl group (C-27) resonance at δ H 1.05 (δ C 26.93) showed correlation to a methylene at δ C 27 (C-15), two quaternary carbon resonance at 39.79 (C-8) and 41.71 (C-14), and unsaturated carbon resonance at δ C 145.20 (C-13). These correlations are characteristic feature of β -amyrin (Fig. 10.6).

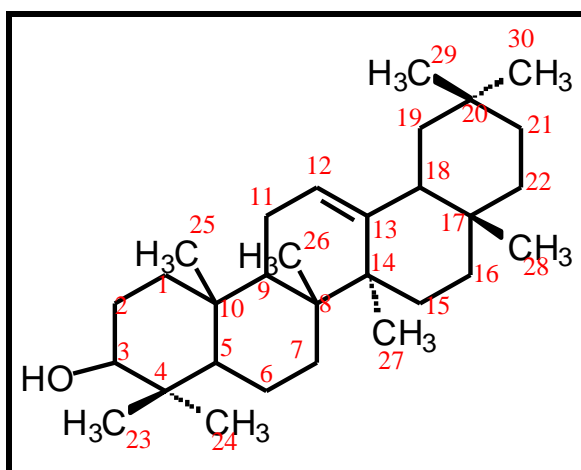


Figure 10.6: The structure of Compound 1.2 (β -amyrin) isolated from *P. obliquum* leaves

The methyl group (C-23) resonance at δ H 0.98 (δ C 27.00) showed correlation to a methyl group resonance at δ C 18.36 (C-24), methine carbon resonance at 55.16 (C-5) and 79.00 (C-3). The methyl group (C-24) resonance at δ H 0.79 (δ C 18.38) showed correlation to a methylene at δ C 27.00 (C-23), methine carbon resonance at 55.16 (C-5) and 79.00 (C-3). These correlations are characteristic feature of both β -amyrin and lupeol. Comparing the data with the literature values (Table 10.3), the two compounds in the mixture were identified to be β -amyrin and lupeol (Mahato and Kundu, 1994).

Table 10.3: ^{13}C NMR data of Compound 1.1 (lupeol) and Compound 1.2 (β -amyrin) compared with literature data (Mahato and Kudu, 1994)

Carbon position	β -amyrin (red colour represent the lupeol)		
	DEPT	C-13	Literature
1	CH ₂	38.81	38.7
2	CH ₂	27.46	27.2
3	CH	79.02	79.0
4	C	40.01	38.6
5	CH	55.40	55.3
6	CH ₂	18.61	18.5
7	CH ₂	32.88	32.8
8	CH	38.81	38.80
9	CH	47.85	47.7
10	C	37.35	35.6
11	CH ₂	23.76	23.6
12	CH (CH ₂)	121.93 (27.40)	121.8 (27.50)
13	C (CH)	145.42 (38.78)	145.10 (39.0)
14	C	41.95	41.80
15	CH ₂	26.21	26.2
16	CH ₂	27.16	27.0
17	C	32.72	32.50
18	CH	47.45	47.40
19	CH ₂ (CH)	47.05 (48.29)	46.90 (48.10)
20	C (C)	31.31 (150.97)	31.30 (151.1)
21	CH ₂	34.96	34.8
22	CH ₂	37.18	37.20
23	CH ₃	28.32 (26.92)	28.20
24	CH ₃	15.71 (18.36)	15.5
25	CH ₃	15.81	15.60
26	CH ₃	17.03	16.9
27	CH ₃	26.22	26.0
28	CH ₃	28.64	28.40
29	CH ₃ (CH ₂)	33.56 (109.31)	33.30 (109.5)
30	CH ₃	23.75 (19.28)	23.70 (19.5)

10.4.3.1 Lupeol and β -amyirin

Lupeol and β -amyirin are both pentacyclic triterpenes (de las Heras *et al.*, 2003) that have been reported as anti-inflammatory agents (Safayhi *et al.*, 1997; Safayhi and Sailer, 1997; Holanda Pinto *et al.*, 2008). The triterpenic family of compounds to which these two isolated compounds belong, have been reported to possess antifungal and antibacterial activity. For instance, ursolic acid was identified as one of the active components in rosemary, claimed to inhibit growth of some food-associated bacteria and yeasts (Collins and Charles, 1987). Ursolic acid and its derivatives inhibited the growth of *Staphylococcus aureus* and *Microsporium lenosum* (Zeletova, 1986).

The triterpene, α - and β -amyirin, was isolated as a mixture (1:2) from the crude resin of *Protium heptaphyllum* and evaluated for antifungal properties against *Candida albicans* (ATCC 18804); *Candida Krusei*, *Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata* (Johann *et al.*, 2007). Esterification of α - and β -amyirin with a variety of acyl chlorides provided a series of analogue derivatives. Among the 15 derivatives, α - and β -amyirin formiate and α - and β -amyirin acetate were the most active, inhibiting all the *Candida* species tested in concentrations that ranged from 30 $\mu\text{g/ml}$ to 250 $\mu\text{g/ml}$.

Minimal inhibitory concentration against *C. albicans* was: 125 $\mu\text{g/ml}$ (α - and β -amyirin); 60 $\mu\text{g/ml}$ (α - and β -formiate) and 250 $\mu\text{g/ml}$ (α - and β -acetate) (Johann *et al.*, 2007). These results support those of the current study where β -amyirin showed appreciable activity against *C. albicans* standard strain (ATCC 10231) (0.004 mg/ml) and six clinical isolates tested (Table 10.1).

α - and β -amyirin isolated from *Eugenia umbelliflora* were tested *in vitro* against a panel of standard and clinical isolates of human fungal pathogens (dermatophytes and opportunistic saprobes) and were found to be inactive against dermatophytes (*Epidermophyton floccosum*, *Microsporium canis*, *Microsporium gypseum*, *Trichophyton rubrum* and *Trichophyton mentagrophytes*) up to the maximum concentrations tested: 1000 $\mu\text{g/ml}$ (Machado *et al.*, 2009).

Tested against *Mycobacterium tuberculosis*, lupeol did not show any antibacterial activity (Chaiyadej *et al.*, 2004; Suksamrarn *et al.*, 2006; Gallo and Sarachine, 2009). In another investigation, lupeol was inactive against three bacteria species but revealed MIC's of 63 µg/ml against *Enterococcus faecalis* (Shai *et al.*, 2008). Lupeol was also inactive against *S. aureus*, *Salmonella typhi*, *Vibrio cholera*, *Escherichia coli*, *Shigella* spp. batch 0.57 (*S. dysentery*; *S. flexneri*, *S. sonnei* and *S. boydii*) displaying MICs >200 µg/ml (Mathabe *et al.*, 2008). However, lupeol showed significant zones of inhibition in the cultures of 18 hospital strains of the Gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumonia* at a concentration of 30 µg/ml (Ahamed *et al.*, 2007). Zones of inhibition was also observed in *P. aeruginosa*, *S. typhi* and *E. coli* cultures using lupeol acid-impregnated disks at a concentration of 10 mg/ml (Lutta *et al.*, 2008), while lupeol acetate did not display any activity against Gram-negative bacteria and fungi, but displayed a strong antimicrobial effect against Gram-positive bacteria (Freire *et al.*, 2002).

As an antifungal agent lupeol showed effects similar to its antibacterial activities concerning the effectiveness. Lupeol displayed moderate zones of inhibition in *Aspergillus niger*, *Aspergillus flavus*, *Rhizoctonia phaseoli* and *Penicillium chrysogenum* cultures at 1 mg/disc (Singh and Singh, 2003) while *A. niger* was significantly inhibited by 20(29)-lupene-3β-isoferulate at 0.01 mg/ml (Lall *et al.*, 2006), confirming that stronger inhibition can be reached when C-3 position is esterified. However, Nguyen *et al.* (2007) synthesized several ester derivatives from lupeol in the C-3 position (COMe, COCHMe₂, COP_n, COCH:CHP_n), which only yielded weak antimicrobial compounds.

In the current study lupeol showed appreciable activity against *C. albicans* standard strain (ATCC 10231) (0.004 mg/ml) and six clinical isolates tested (Table 10.1), but low selective activity against the fungal test organisms (Table 10.2). These results may suggest a cytotoxic effect for lupeol (LC₅₀ = 0.001481 µg/ml) (Fig. 10.2) against mouse fibroblast cells. The results of the current study differ from those of Shai *et al.* (2008) where lupeol failed to display appreciable activity against *C. albicans* (250 µg/ml), but demonstrated high and selective activity

against *Sporothrix schenckii* and *Micosporum canis*, 12 µg/ml and 16 µg/ml respectively. The authors explained those activities based on lupeols' cytotoxic LC₅₀ value (89.5 µg/ml) against monkey kidney (Vero) cells, suggesting a cytostatic action for lupeol (Shai *et al.*, 2008). Additionally, lupeol was inactive against *Cryptococcus neoformans*, *Cladosporium cladosporioides* and *Cladosporium sphaerospermum* (Marqui *et al.*, 2008).

10.4.4 Novel compound

Compound 2 was obtained as a pure compound; the TLC fingerprint indicated unresolved single spot with three different mobile phases. 1D and 2D NMR spectra exhibited chemical shift characteristic of a novel compound. The compound was described as 8,11-Dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4H-pyrano[2,3-g][1]benzoxepin-4-one 12-O-acetate (Fig.10.7); the 12-O-acetate derivative of eranthin. The linear isomer of eranthin O-acetate has been reported, but is not the same compound as the novel compound described in this study.

Comparison of the new compound's ¹H NMR spectrum with the limited data reported by Epe *et al.* (1981) clearly shows that the two compounds are different. The eranthin-12-O- β-D-glycoside has also been described (Junior, 1979), but is once again not the same compound than the one described in this study. The syntheses of the isomeric oxepinochromone eranthin, which differ from the novel compound, were also described (Bruder *et al.*, 2010).

Formula: C₁₇H₁₆O₆

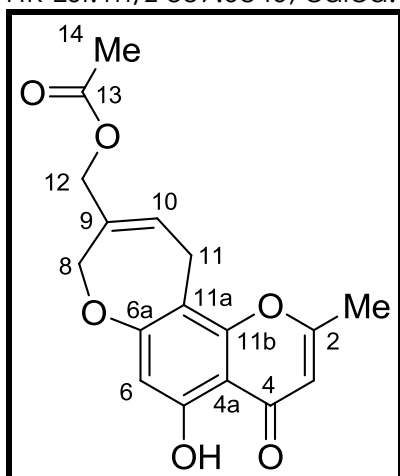
 HR ESI: *m/z* 339.0840; calcd. for C₁₇H₁₆O₆Na: 339.0845.


Figure 10.7: Structure of Compound 2 (novel compound) isolated from *P. obliquum* leaves: 8,11-Dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4Hpyrano[2,3-g][1]benzoxepin-4-one 12-O-acetate

Table 10.4: ¹³C NMR data of Compound 2 in CDCl₃ (for vleg1437 Candice 2) Bruker Avance III-400 spectrometer (Vleggaar, 2011)

Atom	Atom	δ _c	δ _H	HMBC (H→C)
1	2	167.14 S		–
2	3	108.66 D	3 5.99 q (<i>J</i> 0.7)	C-2, C-4, C-4a, 2-CH ₃
3	4	182.68 S	–	–
4	4a	106.72 S	–	–
5	5	155.79 S	–	–
6	6	99.29 D	6.47 s	C-4, C-6a, C-5, C-11a, C-4a
7	6a	164.39 S	–	–
8	8	71.04 T	4 4.61 tt (<i>J</i> 1.6, 1.6)	C-6a, C-9, C-10, C-12, C-11
9	9	133.23 S	–	–
10	10	128.30 D	2 6.02 tt (<i>J</i> 5.5, 1.2)	C-11a, C-8, C-12, C-15
11	11	21.14 T	6 3.52 d (<i>J</i> 5.6, 1.2, 1.6)	C-6a, C-11b, C-9, C-10, C-11a
12	11a	115.82 S	–	–
13	11-b	158.06 S	–	–
14	12	66.45 T	5 4.41 br s	C-9, C-10, C-8, C-13
15	2-Me	20.78 Q	7 2.30 d (<i>J</i> 0.7)	C-2, C-3
16	5-OH	–	12.94 br s	–
17	13	170.61 S	–	–
	14	20.42 Q	8 2.01	C-13

In the current study the novel compound showed appreciable activity against *C. albicans* standard strain (ATCC 10231) (0.004 mg/ml) and six clinical isolates tested (Table 10.1), but low selective activity against the fungal test organisms (Table 10.2). These results may suggest a cytotoxic effect for 8,11-Dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4*H*-pyrano[2,3-*g*][1]benzoxepin-4-one 12-*O*-acetate (LC₅₀ = 7.23 µg/ml) (Fig. 10.2) against mouse fibroblast cells.

10.5 Conclusion

In this study the triterpenic compounds and novel compound tested, inhibited the growth of *C. albicans* standard strain (ATCC 10231) and clinical isolates with low MIC values (Table 10.1). Using bioautography the inhibition of growth of the fungal test species was clearly demonstrated, as indicated by clear zones on TLC chromatograms (Fig. 9.1; Section 9.4). The activity observed with the isolated compounds further validates the traditional use of *P. obliquum* for treating fungal infections. The antifungal activity of lupeol; β-amyrin and 8,11-Dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4*H*-pyrano[2,3-*g*][1]benzoxepin-4-one 12-*O*-acetate are comparable with amphotericin B (Table 10.1). This is the first report of isolation and identification of compounds with antifungal activity from *P. obliquum* leaves.

Only 8,11-Dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4*H*-pyrano[2,3-*g*][1]benzoxepin-4-one 12-*O*-acetate appeared safe enough to use as an anti-candidal agent. This compound was much more active against the ATCC 10231 *C. albicans* strain than amphotericin B. This is a novel structure for an antifungal compound and changes to the chemical structure may increase the activity and reduce the toxicity of the compound. It may be worthwhile to isolate some more of this compound and test its activities against other fungi.

CHAPTER 11: ADHESION OF *Candida albicans* TO HUMAN BUCCAL EPITHELIAL CELLS (HBEC) IN THE PRESENCE OF *Ptaeroxylon obliquum* ACETONE LEAF EXTRACT

11.1 Introduction

Candidal adherence to human buccal epithelial cells (HBEC) is considered the critical initial step in the pathogenesis of oral candidiasis, which may eventually lead to a systemic infection, especially in immuno-compromised individuals (Schafer-Korting *et al.*, 1996). The adherence of *Candida* to HBEC is a factor which influences the balance of oral clearance mechanisms, mucosal colonization and development of clinical signs of candidiasis. Furthermore candidal adherence to the oral mucosa is intimately associated with all common forms of oral candidiasis (Taweechaisupapong *et al.*, 2005).

Ptaeroxylon obliquum (Thunb.) Radlk. is a medicinal plant used for several therapeutic purposes. For example, the powdered wood is used as a snuff to relieve headache and infusions of the powdered wood are taken for the treatment of rheumatism and heart disease (Watt and Breyer-Brandwijk, 1962). The bark is used for the treatment of rheumatism and arthritis (Pujol, 1990).

In the current study it was demonstrated that *P. obliquum* acetone leaf extract had antifungal activity towards *C. albicans* standard strain (ATCC 10231) and clinical isolates. In order to provide more information about the potential of *P. obliquum* acetone leaf extract for its development as a natural oral hygiene product, the inhibitory effects of *P. obliquum* acetone leaf extract on the *in vitro* adhesion of *C. albicans* standard strain (ATCC 10231) and two clinical isolates to human buccal epithelial cells (HBEC) was investigated.

11.2 Objectives

The objectives of this part of the study were to:

- I. determine the ability of *C. albicans* standard strain (ATCC 10231) and two clinical isolates to adhere to human buccal epithelial cells (control); and
- II. determine the ability of *C. albicans* standard strain (ATCC 10231) and two clinical isolates to adhere to HBEC in the presence of *P. obliquum* acetone leaf extract.

11.3 Materials and Methods

11.3.1 Subjects

Buccal epithelial cells were taken from 20 healthy subjects (student volunteers) between 20- and 40 years old with no known co-existing disease or not taking any medication.

Ethical approval was obtained from the Ethics Committee of the Faculty of Health Sciences of the University of Pretoria, South Africa, and all individuals gave informed consent prior to their participation in this study.

11.3.2 Preparation of yeast and buccal epithelial cells (Modified method of Taweechaisupapong *et al.*, 2005)

Yeast cells

C. albicans standard strain (ATCC 10231) and clinical isolates were maintained on Sabouraud-dextrose agar and grown in the yeast phase in Sabouraud-dextrose broth for this study. The organisms were washed three times in normal saline (NSS) by centrifugation at 5000 rpm for 10 minutes and finally suspended in NSS to 4×10^7 cells/ml (MacFarlane standard1).

Epithelial cells

Human buccal epithelial cells (HBEC) were collected from healthy individual subjects by gently scraping the cheek mucosa with a sterile wooden spatula. To minimize individual variations, HBEC from the twenty healthy individuals were pooled in 10 ml of sterile NSS (Sandin *et al.*, 1987). The pooled HBEC suspension was washed three times in NSS by centrifugation at 5000 rpm for 10 minutes to eliminate debris and loosely attached micro-organisms. Washed epithelial cells were confirmed to have no attached yeast before the adherence test. The HBEC was then re-suspended in NSS to a concentration of 9×10^5 cells/ml (by haemocytometer) and used immediately for the adhesion assay. The cells were collected at the same time of day on each occasion to minimize any possible effect of diurnal variations of HBEC on yeast adherence.

11.3.3 Preparation of the antifungal agent

The antifungal agent amphotericin B (Mast diagnostics) was used as the positive control and acetone as negative control. Minimal inhibitory concentration (MIC) values of *C. albicans* standard strain (ATCC 10231) and clinical isolates were determined by using the microplate dilution method developed by Eloff (1998b), with modifications for antifungal activity assay by Masoko *et al.* (2005). MIC values were regarded as the lowest concentration of amphotericin B that inhibited the growth of *C. albicans* standard strain (ATCC 10231) and clinical isolates. The minimal fungicidal concentration (MFC) values were determined as the lowest concentration of amphotericin B that inhibited 100 percent growth of *C. albicans* standard strain (ATCC 10231) and clinical isolates. The subcidal concentration values for amphotericin B towards *C. albicans* standard strain (ATCC 10231) and clinical isolates were the concentration slightly below the MFC of the drug. The MIC, MFC and subcidal concentration values for amphotericin B obtained in the present study are presented in Table 11.1.

Table 11.1: MIC, MFC and subcidal concentrations for amphotericin B

	<i>Candida albicans</i>		
	ATCC	Clinical isolates	
	10231	M0824	1051604
*MIC (mg/ml)	0.11	0.03	0.06
*MFC (mg/ml)	0.11	0.11	0.06
Subcidal concentration	0.11	0.06	0.06

*MIC : Minimal inhibitory concentration representing the mean value

*MFC : Minimal fungicidal concentration representing the mean value

11.3.4 Preparation of *P. obliquum* acetone leaf extract

Leaves of *P. obliquum* were harvested from the Pretoria National Botanical Gardens and voucher specimens were authenticated by the Pretoria National Herbarium of the South African National Biodiversity Institute. The leaves were collected in loosely woven orange bags and dried in the dark at room temperature. The dried leaves were ground to powder using a Macsalab mill (Model 200 Lab). In a bulk extraction, powdered leaves (1000 g) of *P. obliquum* were extracted with acetone at room temperature. Approximately 79.52 g of extract was obtained from 1000 g of dried pulverized leaves. 250 mg/ml *P. obliquum* acetone leaf extract was used as the starting material for the subsequent study.

11.3.5 Adhesion assay of *C. albicans* to HBEC after exposure to *P. obliquum* acetone leaf extract for one hour

Adhesion of *C. albicans* standard strain (ATCC 10231) and clinical isolates to HBEC was determined as follows:

- I. 0.2 ml of *P. obliquum* acetone leaf extract was serially two-fold diluted with normal saline (NSS) in tubes.
- II. Tubes containing 0.2 ml of amphotericin B and NSS served as positive and negative controls, respectively.
- III. 0.1 ml of HBEC and 0.1 ml of the yeast suspension prepared as described in Section 11.3.2 were added to each tube, mixed gently and incubated at 37°C for 1 hour with gentle agitation.

- IV. The suspension was then diluted in 1 ml of sterile NSS.
- V. The cells were filtered through a 12 µm pore size polycarbonate filter (Millipore), and washed twice with 15 ml of sterile NSS to remove unattached fungi. Each filter was carefully removed with forceps and placed on a glass slide with the HBEC against the glass surface. After 30 s, the filter was gently removed; leaving the HBEC adhered to the glass slide.
- VI. The slides were air-dried, fixed with methanol and stained with Gram's dye. The number of yeast cells adhering to 100 HBEC was determined by counting in a light microscopy (Nikon Trans microscope) at x 400 magnification and photographed with a Nikon digital camera DXM 1200 F.

The adhesion assay is a modification of a method previously described by Kimura and Pearsall (1978) and by Taweechaisupapong *et al.* (2005).

The numbers of fungi adhering per 100 human buccal epithelial cells were counted and the percentage reduction was determined as follows: crude plant extract concentration divided by the control (yeast+HBEC) x100 minus 100 =% reduction. The effect that *C. albicans* had on the structure of cells HBEC was also observed.

Each assay was performed with duplicate determinations. All slides were coded, images stored on CD, computer analysed and read without prior knowledge of treatments (blinded).

11.4 Results and Discussion

Calculations according to the modified method of Taweechaisupapong *et al.*, (2005) indicate that *P. obliquum* acetone extract at a concentration ≤ 250 mg/ml is capable of suppressing candidal adhesion to human buccal epithelial cells (HBEC) (Fig. 11.4) as effectively as subcidal concentrations of amphotericin B (Tables 11.2, 11.3, 11.4). The adhesion values and the percentage reduction in the adhesion of *C. albicans* and two clinical isolates to HBEC, following a 1-h exposure to various concentrations of *P. obliquum* acetone extract are presented in Tables 11.2, 11.3, 11.4. If the LC₅₀ values are determined using graphs (Fig. 11.1, 11.2,

11.3), it is evident that 50% adhesion of *C. albicans* cells is inhibited by concentration between 5 and 20 mg/ml, with a flattening off at concentrations above 20 mg/ml. Therefore it can be assumed that a much lower concentration of the extract can be used in a product to prevent candidal adhesion.

Table 11.2: Adhesion of *C. albicans* ATCC 10231 to human buccal epithelial cells following a 1-h exposure to various concentrations of *P. obliquum* acetone extract (mg/ml)

	Control (Y+ HBEC)	Amphotericin B (0,11 mg/ml)	Concentration of <i>P. obliquum</i> acetone extract (mg/ml)					
			7.8	15.6	31.2	62.5	125	250
Number of yeast cells per 100 HBEC	177	109	18	16	4	5	5	0
Reduction in adherence %	0	38.42	9.83	90.96	97.74	97.18	97.18	100

Y: yeast HBEC: human buccal epithelial cells

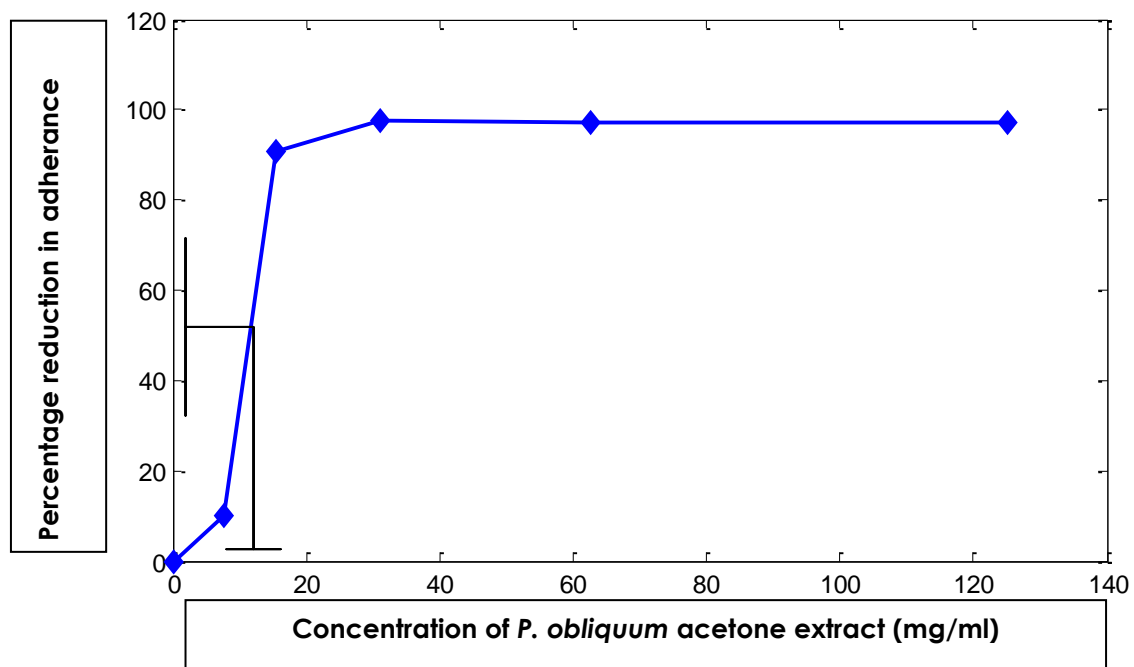


Figure 11.1: Adhesion at LC₅₀ of *C. albicans* ATCC 10231 to human buccal epithelial cells following a 1-h exposure to various concentrations of *P. obliquum* acetone extract (mg/ml)

Table 11.3: Adhesion of *C. albicans* M0824 to human buccal epithelial cells following a 1-h exposure to various concentrations of *P. obliquum* acetone extract (mg/ml)

	Control (Y+ HBEC)	Amphotericin B (0,06 mg/ml)	Concentration of <i>P. obliquum</i> acetone extract (mg/ml)					
			7.8	15.6	31.2	62.5	125	250
Number of yeast per 100 HBEC	182	12	124	35	25	23	0	0
Reduction in adherence %	0	93.41	31.86	80.76	86.26	87.36	100	100

Y: yeast HBEC: human buccal epithelial cells

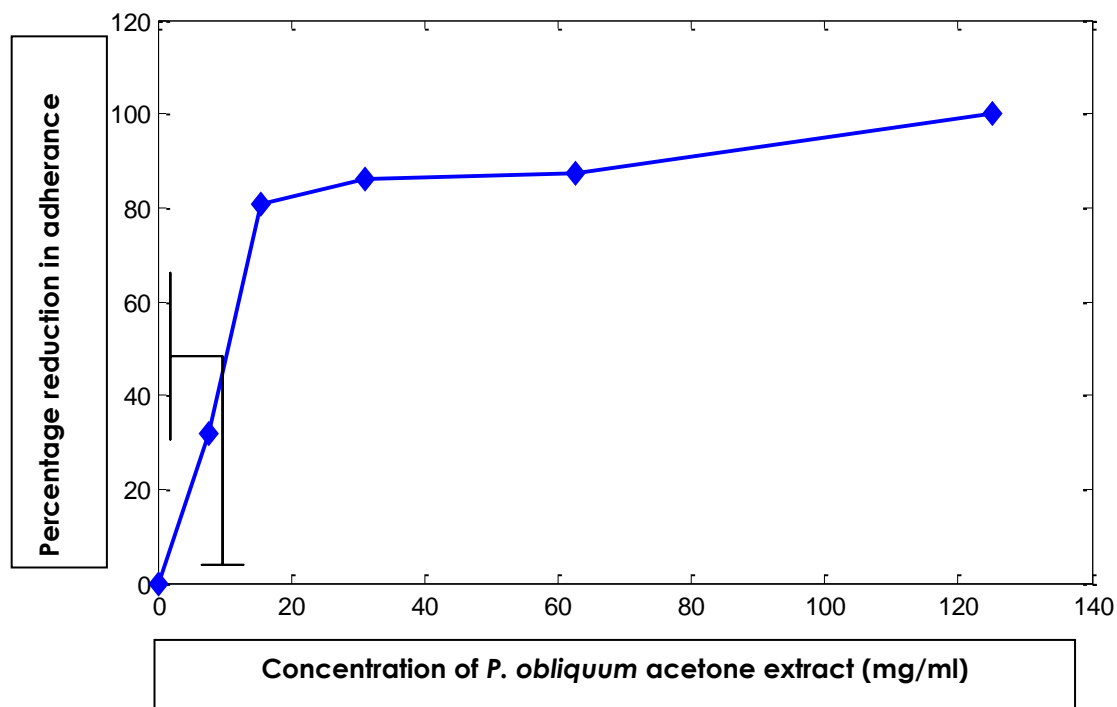


Figure 11.2: Adhesion at LC₅₀ of *C. albicans* M0824 to human buccal epithelial cells following a 1-h exposure to various concentrations of *P. obliquum* acetone extract (mg/ml)

Table 11.4: Adhesion of *C. albicans* 1051604 to human buccal epithelial cells following a 1-h exposure to various concentrations of *P. obliquum* acetone extract (mg/ml)

	Control (Y+ HBEC)	Amphotericin B (0,06 mg/ml)	Concentration of <i>P. obliquum</i> acetone extract (mg/ml)					
			7.8	15.6	31.2	62.5	125	250
Number of yeast per 100 HBEC	202	10	29	13	4	2	1	0
Reduction in adherence %	0	95.05	85.64	93.56	98.02	99.00	99.58	100

Y: yeast HBEC: human buccal epithelial cells

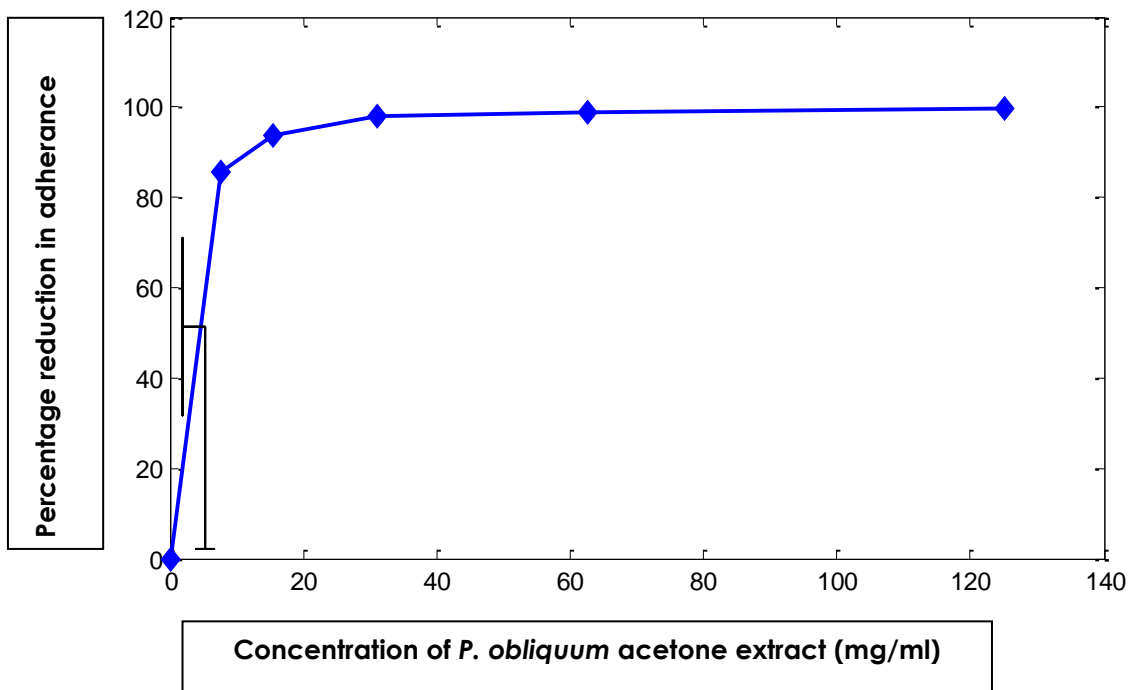


Figure 11.3: Adhesion at LC₅₀ of *C. albicans* 1051604 to human buccal epithelial cells following a 1-h exposure to various concentrations of *P. obliquum* acetone extract (mg/ml)

The development of the microscopy techniques and computer programmes, used in this study, with which the images can be studied made it possible to observe the effects the yeast cells have on human buccal epithelial cells (HBEC) (Fig. 11.5). The percentage reduction in adherence can be correlated to the destruction of the epithelial cells where the growth of the *C. albicans* cells was not inhibited by *P. obliquum* acetone extract.

Visual evaluation of HBEC revealed that in the presence of amphotericin B (control) at 0.06 mg/ml the *C. albicans* cells were budding and forming hyphae (Fig. 11.6, 11.7). This was observed in both the attached and un-attached *C. albicans* cells. The same was observed at lower concentrations of *P. obliquum* acetone extract (Fig. 11.8, 11.9). At these lower concentrations, and in the presence of amphotericin B, the *C. albicans* cells attack the HBEC and completely destroyed the epithelial cells (Fig. 11.10, 11.11). It is well known that mycelial forms of *C. albicans* adhere more efficiently to host cells than do yeast form cells (Kimura and Pearsall, 1980; Pendrak and Klotz, 1995). It was also observed in the current study, that where high numbers of *C. albicans* cells attached to an epithelial cell, the yeast cells started to destroy the nucleus and structure of the epithelial cell (Fig. 11.12, 11.13). This correlates with a high incidence of candidiasis in the oral cavity where the epithelial cells are destroyed by the attachment of the yeast cells (Samaranayake, 1990; Samaranayake *et al.*, 1994; Taweechaisupapong *et al.*, 2005).

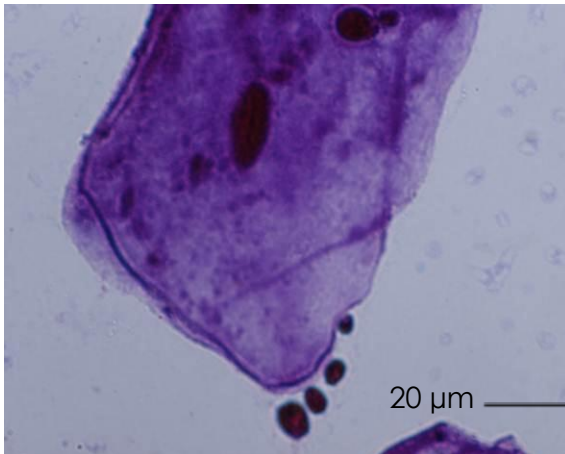


Figure 11.4: *P. obliquum* acetone extract suppressing candidal adhesion to human buccal epithelial cells (HBEC)

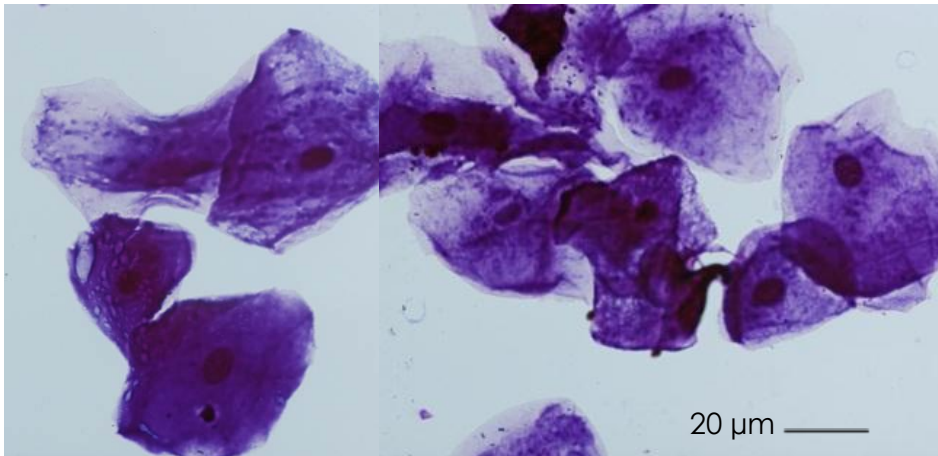


Figure 11.5: Normal human buccal epithelial cells (HBEC)

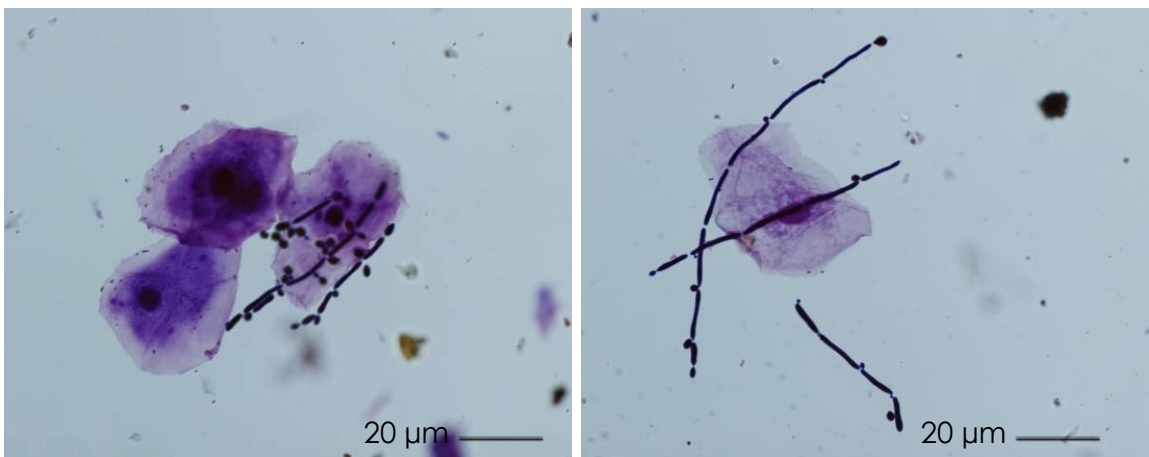


Figure 11.6, 11.7: In the presence of amphotericin B (control) the *C. albicans* cells were attaching, budding and forming hyphae

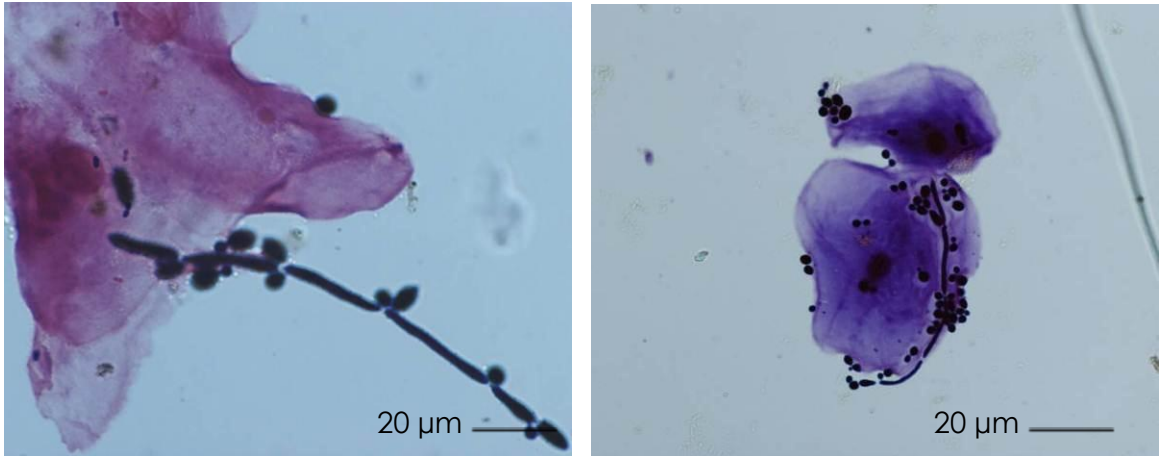


Figure 11.8, 11.9: In the presence of lower concentrations of *P. obliquum* acetone extract the *C. albicans* cells were attaching, budding and forming hyphae

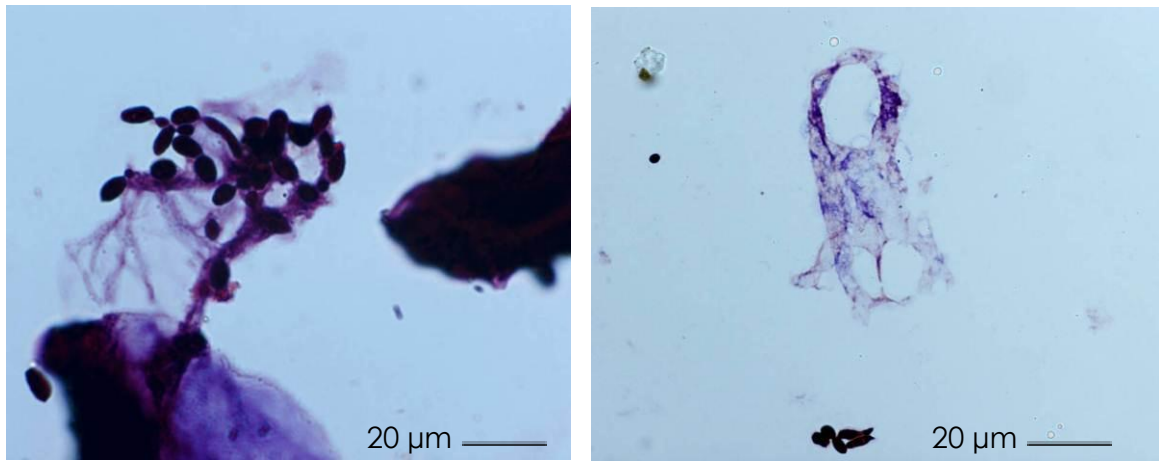


Figure 11.10, 11.11: At lower concentrations of *P. obliquum* acetone extract, and in the presence of amphotericin B, the *C. albicans* cells attack the HBEC and completely destroyed the epithelial cells

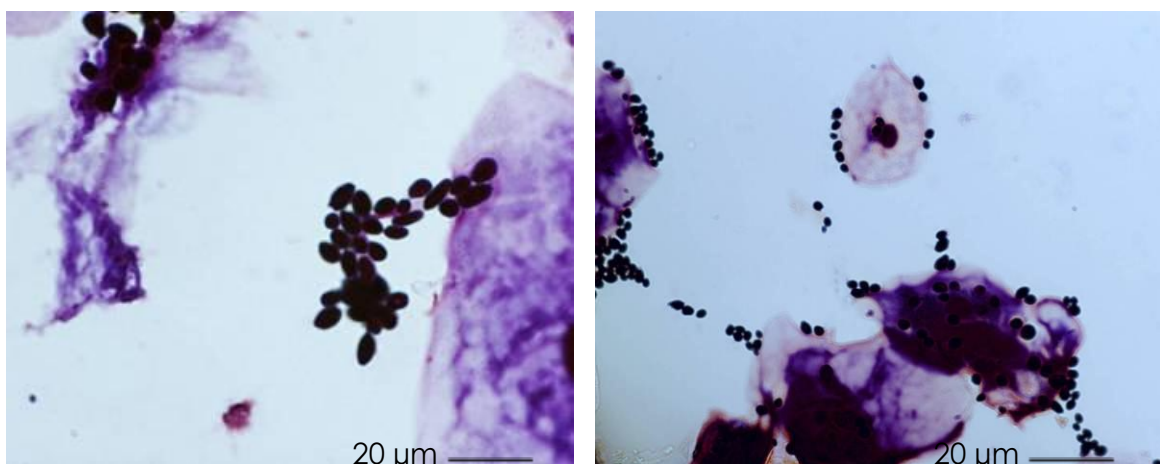


Figure 11.12, 11.13: At lower concentrations of *P. obliquum* acetone extract high numbers of *C. albicans* cells attached to an epithelial cell and were able to destroy the nucleus and structure of the epithelial cell

The observed suppression of candidal adherence to HBEC in the presence of *P. obliquum* acetone extract suggests the potential development of a natural oral hygiene product. The concentration at which 100% suppression occurred was probably too high to be of practical use, but a concentration of c. 20 mg/ml lead to c. 90% reduction of adhesion. Based on the toxicity of the acetone extracts (Section 7.4) this may not be a feasible practical application. Unfortunately there was not sufficient quantity of Compound 2, isolated from *P. obliquum* acetone leaf extract, available to evaluate the effect on adhesion.

11.5 Conclusion

After this microscopy study it is a concern that the concentration of human buccal epithelial cells (HBEC) that are affected by the attachment of the *C. albicans* cells are not a true reflection of the destructive effect of the yeast cells, because in some cases the HBEC were completely destroyed by the yeast cells and only a "ghost skeleton" was visible on the images. If a microscope with a low resolution is used this observation may be overlooked.

CHAPTER 12: GENERAL DISCUSSION AND CONCLUSION

12.1 Introduction

The aim of this study was to develop a product that can protect users against oral *Candida* infections. A number of objectives were identified which will be discussed below:

- I. To determine the *in vitro* antimicrobial activity of selected medicinal plants against normal and multiple resistant *Candida albicans* isolates.

The *in vitro* anti-candidal activity of eight medicinal plants, *Bidens pilosa*, *Dichrostachys cinerea*, *Harpephyllum caffrum*, *Ptaeroxylon obliquum*, *Rapanea melanophloeos*, *Sclerocarya birrea*, *Securidaca longepedunculata* and *Ziziphus mucronata* was determined against *Candida albicans* standard strain (ATCC 10231) and six clinical isolates. Inhibition at concentrations <1 mg/ml, against the *C. albicans* strains tested, was observed for the acetone extracts of *B. pilosa* (whole plant), *H. caffrum* (leaves), *P. obliquum* (leaves), *R. melanophloeos* (leaves) and *S. birrea* (stem bark), as well as the aqueous extracts of *B. pilosa* (whole plant) and *S. birrea* (stem bark). *H. caffrum*, *P. obliquum*, *R. melanophloeos* and *S. birrea* were the most active of the plant species tested against *C. albicans* standard strain (ATCC 10231) and clinical isolates.

- II. To determine the number of antifungal compounds present in different plant extracts by bioautography.

Three active compounds of *P. obliquum* and two of *S. birrea* were located by means of bioautography. Although *H. caffrum* and *R. melanophloeos* did not have any active compounds by bioautography and have been used traditionally to treat infections, it may be that the plant extracts lead to an increase in the immune modulatory status of the host.

III. To determine the antioxidant activity of different plant extracts.

Antioxidant activity was detected in acetone extracts of *H. caffrum* (leaves), *P. obliquum* (leaves), *R. melanophloeos* (leaves) and *S. birrea* (stem bark) by using the method of Deby and Margotteaux (1970); as well as by DPPH Radical scavenging activity, TEAC assay, determination of total flavonoids and total phenolics.

All four plant species tested contain chemical constituents that have antioxidant activity. *S. birrea* had the highest antioxidant activity of the four plant species tested. High antioxidant activity has previously been reported for this plant. Chemical constituents of *H. caffrum* are rare. The responsible antioxidant agents in *P. obliquum* and *R. melanophloeos* are not known.

IV. To determine the safety of different plant extracts to select the best species for in depth evaluation.

In order to develop a natural oral hygiene product that can protect patients against oral *Candida* infections, the safety of *H. caffrum*, *P. obliquum*, *R. melanophloeos* and *S. birrea* was determined by reconstituting dried acetone extracts of these plants in dimethyl sulfoxide (DMSO) against mouse fibroblast cells using the MTT assay (Mosman, 1983). *H. caffrum* ($LC_{50}=2.07 \mu\text{g/ml}$), *P. obliquum* ($LC_{50}=35.58 \mu\text{g/ml}$), *R. melanophloeos* ($LC_{50}=434.50 \mu\text{g/ml}$) and *S. birrea* ($LC_{50} >1000 \mu\text{g/ml}$) were toxic against mouse fibroblast cells. *S. birrea* has been reported to have cytotoxic effects. No reports regarding cytotoxicity could be obtained for *H. caffrum*, *P. obliquum* and *R. melanophloeos*.

- V. To isolate the antifungal compounds, from the best plant species selected, using bioassay-guided fractionation; and to test the compounds for antimicrobial activity and cytotoxicity.

As *P. obliquum* had the highest activity against *C. albicans* standard strain (ATCC 10231) and clinical isolates tested, the highest number of active compounds against the fungal test organisms and a relatively low selectivity index, the next step involved the bioassay-guided fractionation of *P. obliquum* acetone leaf extract. Six solvent-solvent fractions were obtained from *P. obliquum* acetone leaf extract and the chloroform-fraction had the highest number of active compounds against *C. albicans* standard strain (ATCC 10231) and clinical isolates. Two active antifungal compounds were isolated from the chloroform-fraction using silica gel as the stationary phase in column chromatography, following the principles of bioassay-guided fractionation. This is the first report of isolation and identification of compounds with antifungal activity from *P. obliquum* leaves. Compound 1 was obtained as isomeric mixture of lupeol and β -amyryn that are both pentacyclic triterpenes. Based on the cytotoxicity data of lupeol and β -amyryn, the therapeutic effects of this compound may be limited only to low concentrations.

Compound 2 was obtained as a pure compound; the TLC fingerprint indicated unresolved single spot with three different mobile phases. 1D and 2D NMR spectra exhibited chemical shift characteristic of a novel compound. The compound was described as 8,11-Dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4H-pyrano[2,3-g][1]benzoxepin-4-one 12-O-acetate.

- VI. To determine the ability of *C. albicans* standard strain (ATCC 10231) and clinical isolates to adhere to human buccal epithelial cells (control), as well as to determine the ability of *C. albicans* standard strain (ATCC 10231) and clinical isolates to adhere to human buccal epithelial cells in the presence of the selected plant species in order to evaluate the possibility that a useful product can be developed based on the results.

The results of the study indicate that *P. obliquum* acetone extract at a concentration ≤ 250 mg/ml is capable of suppressing candidal adhesion to human buccal epithelial cells (HBEC) as effectively as subcidal concentrations of amphotericin B. If the LC₅₀ values are determined using graphs (Fig. 11.1, 11.2, 11.3) it is evident that 50% adhesion of *C. albicans* cells are inhibited by concentration between 5 and 20 mg/ml, with a flattening off at concentrations above 20 mg/ml. Therefore it can be assumed that a much lower concentration of the extract can be used in a product to prevent candidal adhesion. The observed suppression of candidal adherence to HBEC in the presence of *P. obliquum* acetone extract, as well as the high antifungal activity of the acetone leaf extract of *P. obliquum* indicates that it could be considered as a potential topical therapeutic agent against oral *Candida* infections. However, because of the cytotoxicity of *P. obliquum* acetone leaf extract, we should limit the exposure time of HBEC *in vitro* to this extract. As oral rinses are used topically and not swallowed the systemic effect may not be of critical importance. Natural products inhibiting adhesion of *C. albicans* to the oral mucosa may be beneficial in managing oral candidiasis.

12.2 Recommendations for future work

- I. Based on the cytotoxicity data of *P. obliquum* acetone leaf extract, lupeol and β -amyryn, the therapeutic effects of this extract and compound may be limited only to low concentrations. Further investigation to ascertain the safety of the extract and compound to treat candidiasis are required.
- II. The cytotoxicity data in this study was only obtained with mouse fibroblast cells, and thus studies with several human cell lines may further indicate the safety of the compounds and extract of *P. obliquum* as treatment regiments against oral candidiasis.
- III. It would therefore be worthwhile to invest human and financial resources for intensified screening of medicinal plants with the realistic hope of finding potential antifungal agents and cheap alternative medicines. The focus must not only be to isolate compounds, but also to investigate the efficacy of the extracts as these would be cheaper and more freely available.

CHAPTER 13: REFERENCES

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ADDENDUM

1D and 2D NMR spectra analysis of isolated compounds