CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Introduction

Candidiasis is a common opportunistic infection that occurs in immuno-compromised individuals, usually caused by *Candida albicans*. Candidiasis is known be a marker of HIV infection. *C. albicans* is the common pathogenic species because it expresses higher levels of putative virulence factors compared to other *Candida* species. It is known to cause superficial (minor) infections such as oral candidiasis as well as severe systematic infections. Antifungal drugs are available that can successfully treat candidiasis. However, it has been discovered that *C. albicans* is developing resistance to the drugs used currently, they have side effects and are costly. Therefore, there is a compelling need for the development of new antifungal drugs that can successfully treat many fungal infections including oral candidiasis with minimal effect on the host and available at a cheaper cost. The approach to antifungal drug development can be to either target the fungal pathogen (kill) or target its virulence factors.

Medicinal plants have been used as primary health care in many parts of the world especially in rural areas of developing countries and many diseases have been reported to be treated by medicinal plants (Payyappallimana, 2010). Therefore, they can serve as the natural source for the discovery and development of new drugs. A plant called *Punica granatum Linn* commonly known as pomegranate is famous worldwide for its high antioxidant contents and antimicrobial properties on pathogens including oral pathogens (Abdollahzadeh *et al*., 2011). Studies have shown that extracts or purified compounds from different parts of the pomegranate plant have shown antifungal, antibacterial, antiviral and antidiarrhoeal properties (Endo *et al*., 2010). High concentrations of *P. granatum* have shown anti-candida activity but it is difficult

to maintain these concentrations in the oral cavity due to the constant salivary flow. Therefore, this study investigated the antifungal effect of the peel and seeds of *P. granatum* and the effect of the peel on the selected virulence properties of *C. albicans.*

1Literature review

1.1 *Candida albicans*

C. albicans is a polymorphic fungi and a normal colonizer in mucosal sites such as the gut, vagina and the oral cavity of many healthy individuals along with many other non-pathogenic fungi and harmless bacteria (Naglik *et al*., 2011; Mayer *et al*., 2013). The presence of *C. albicans* does not necessarily indicate infection by this organism (Naglik *et al*., 2011).

C. albicans can reside in the mouth of many healthy individuals for many years without them even being aware of its presence because it shows no clinical symptoms under normal oral environmental conditions (Naglik *et al*., 2011; Mayer *et al.*, 2013). In the oral cavity, a change from the harmless commensal state to a pathogenic state can occur even with the slightest environmental change (Williams and Lewis, 2011; Naglik *et al*., 2011). It is an opportunistic pathogen. Therefore, it takes advantage of disturbed oral environmental conditions such as compromised immunity as a result of HIV related infections, xerostomia, diabetes, prolonged use of antibiotics, unclean dentures and a mere poor oral hygiene (Odds, 1988; Colderone and Fonzi, 2001; McCarthy, 1992).

C. albicans has the ability to colonize, invade and multiply within the host oral cavity as well as in other body organs (Coronado-Castellote and Jiménez-Soriano, 2013). It is the most medically important *Candida* spp. as it is commonly isolated from most hospitalized patients (McCullough *et al*., 1996; Negri *et al*., 2010). It accounts for the highest mortality rate (20-40 %) caused by pathogenic fungi (Tripathi *et al*., 2014). Many contributing factors are re-

sponsible for the pathogenicity of *C. albicans* (Sweet *et al*., 1995). A clear understanding of conditions in which *C. albicans* colonizes, invade and cause infections in the host, is crucial in therapy development and has previously been investigated by researchers (Gow *et al*., 2011; Kabir *et al*., 2012).

1.1.1 Morphology and cultural characteristics

C. albicans grows as spherical to oval budding yeast cells 3-5 µm x 5-10 µm in size. These are called blastospores and they also form elongated, filamentous cells joined end to end called pseudohyphae. Cultures grow on Sabouraud medium as creamy-white, flat or hemi-spherical shaped colonies (Figure 1.1) with a beer-like aroma (Samaranayake, 2002).

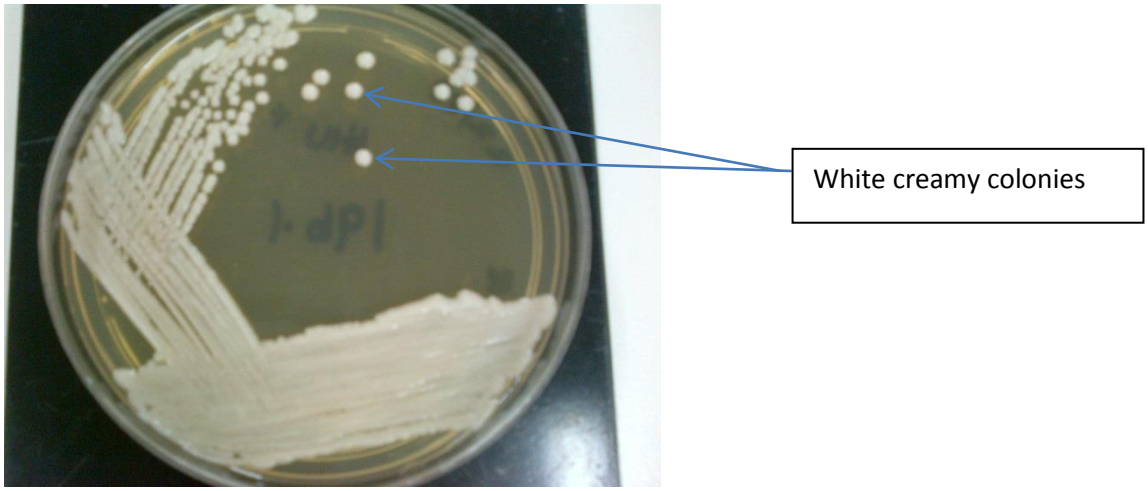

Figure 1.1 *C. albicans* colonies on Sabouraud agar

1.1.2 Cell wall and Morphogenesis

The fungal cell is quite a complex structure composed of glucans, chitin and many other proteins (Adams, 2004). This complex dynamic structure is not stable but constantly changing when the cell grows and during morphogenetic stages (Adams, 2004). The

C. albicans cell wall is composed of cell wall proteins and carbohydrates which are not found in humans and this increases the target points by immunity as well as therapeutic drugs. Cell shape and structure is provided by the inner layer of the cell called the ß - 1, 3 glucan and the skeletal polysaccharide, chitin (Gow *et al*., 2011). *C. albicans* cell wall structure is shown in Figure 1.2. Chitin of the *C. albicans* cell wall usually contributes ~ 2% of the dry weight of the cell wall whereas ß - 1, 3 glucan and ß - 1, 6 glucan contribute a greater percentage (20 and 40 %) respectively (Gow *et al*., 2011). However, cell wall composition varies within fungi species (Adams, 2004). The cell wall plays an important role in the pathogenesis of C. *albicans* such as interacting (adherence to host cells) with the host cell (Gozalbo *et al*., 2004).

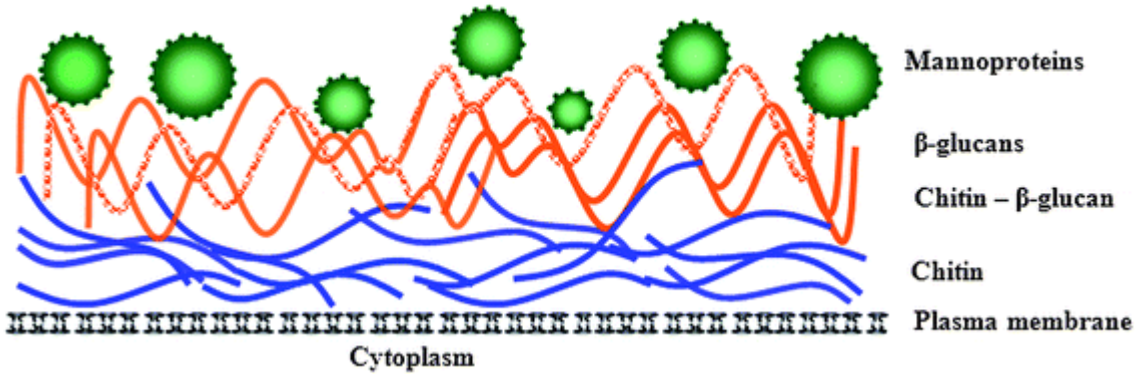

Figure 1.2 Cell wall structure of *C. albicans* (Ovchinnikova *et al*., 2012)

1.1.3 Virulence factors

Pathogenicity of *C. albicans* relies on successful production of virulence factors. Virulence factors are responsible for the transformation of *C. albicans* to a disease causing organism (pathogen) and makes it possible for *C. albicans* to survive, persist within the host and cause disease when the opportunity arises (Naglik *et al*., 2011; Rossoni *et al*., 2013). In addition, virulence factors assist *C. albicans* in adherence to oral tissues, colonize and escape host defense actions (Rossoni *et al*., 2013). In order for *C. albicans* to infect the host, it first has to react to the environmental changes and switch from the usual unicellular form into an inva-

sive, multicellular filamentous form (Mayer *et al*., 2013). Some of the important factors are adherence to epithelial cell surfaces, germ tube/ hyphal formation and production of hydrolytic enzymes (Mayer *et al*., 2013). Phospholipase, protease and lipase are some of the hydrolytic enzymes *C. albicans* can produce as some of its virulence factors. Phospholipase and protease are considered to be associated with the invasive capacity of *C. albicans* (Pinto *et al*., 2008). These two enzymes have been reported to be produced in high quantities in HIV infected patients, indicating enzymatic importance in the pathogenicity of *C. albicans* (Sweet *et al*., 1995).

Due to virulence factors, *C. albicans* is capable of causing two major types of human infections (superficial and systematic) which are classified according to disease severity. Superficial infections are localized infections such as oral candidiasis. Systematic infections are more severe and often life threatening as they penetrate deeper organs and tissues (Martin *et al*., 2011; Kabir *et al*., 2012; Mayer *et al*., 2013). A compromised host defence system can lead to severe systematic infections (Endo *et al*., 2010) including candidemia.

C. albicans isolated from HIV positive patients have been found to be more virulent than strains isolated from HIV negative patients. Sweet *et al.,* (1995) showed that *C. albicans* isolates from HIV- infected and AIDS patients have enhanced adherence ability to epithelial cells which is the initial stage of infection. Densely packed candida cells (*in vivo*) in HIV infected individuals can lead to depletion of nutrients resulting in a morphological switch which also plays an important role in fungus pathogenesis (Gallagher *et al*., 1992; Sweet *et al*., 1995). Similar results were reported by Jain *et al.,* (2010) who suggested that the presence of the predisposition may be responsible for this change. In addition, *C. albicans* isolated from HIV patients show high levels of proteinase activity which is responsible for the tissue

damage during infection (Ollert *et al*., 1995). De Bernardis *et al.*, (1996) reported similar results suggesting that the increase in the proteinase production occurs before the patient enters the HIV symptomatic stage. Increased phospholipase and haemolytic activities have also been noted in the 210 *C. albicans* strains isolated from HIV positive patients and this increase was seen in the colonizing strains as well as in the infecting strains (Mane *et al*., 2012).

1.1.3.1 Adherence

Adhesion is among many factors that contribute to the pathogenicity of *C. albicans* (Mathews, 1994). Tissue colonization has to occur before invasion can take place. Therefore, adherence of *C. albicans* to host cells is the most critical step in the colonization and infection process. *C. albicans* has the highest adherence ability among the candida species (Matthews, 1994). The adherence is initiated by the yeast form *in vitro* but it is uncertain as to whether the same is reflected *in vivo* (Naglik *et al*., 2011). Adherence of *C. albicans* to epithelial cells is a complex process and has been addressed by many studies. *C. albicans* possess proteins called adhesins that initiate attachments to other microorganisms and epithelial cells. Several adhesive proteins of *C. albicans* have been identified (Sheppard *et al*., 2004). Agglutinin-like sequence (ALS) proteins are the best studied adhesins of *C. albicans* and the ALS family consist of ALS 1-7 and ALS 9 (Meyer *et al*., 2013). The N-terminal of all ALS contains substrate binding proteins (Rauceo *et al*., 2006). The use of a heterologous system for ALS protein expression by Sheppard *et al*, 2004 demonstrated diverse adhesion as well as invasive functions of the ALS proteins (Sheppard *et al*., 2004).

C. albicans gene disruption studies have shown that ALS1p expression (heterologous) mediates binding to human vascular endothelial cells as well as epithelial cells (Fu *et al*., 2002). Agglutinin-like sequence protein (ALS3) is particularly adhesion specific (Naglik *et al*., 2011). Some of the adhesins are expressed only on the germ tubes of *C. albicans* (Mathews,

1994). The hyphal wall protein 1 abbreviated HWP 1, has an important role in the epithelial attachment process (Naglik *et al*., 2011). Hyphal wall protein 1 serves as a substrate for mammalian transglutaminases and this reaction may covalently link *C. albicans* to the host cell (Williams and Lewis, 2011).

1.1.3.2 Germ tube or hyphae formation

C. albicans, unlike many candida species, does not have a life cycle but rather exists as a polymorphic form meaning it can either grow as an ovoid-shaped budding yeast, as elongated ellipsoid cells with constrictions at the tip called septa commonly known as pseudohyphae or as a parallel walled true hyphae (Berman and Sudbery, 2002; Gow *et al*., 2011). Most pathogenic fungi are dimorphic, meaning they can interchange between the yeast and germ tube morphological states. Such interchange is often associated with the fungal growth or invasion within the host (Gow *et al*., 2002). The ability of *C. albicans* to switch (reversibly) from the yeast to germ tube form may help it adapt to different environmental conditions, both as a commensal or pathogenic organisms (Mathews, 1994). When *C. albicans* comes in contact with the host cell, yeast-to-hyphal transition is triggered. Germ tubes secrete hydrolases which assist *C. albicans* to actively penetrate into the host cell (Mayer *et al*., 2013) and therefore, this enzyme is considered as one of the virulence factors. In addition, germ tubes allow internal persistence by preventing *C. albicans* from being washed away by flushing with body secretions such as salivary flow (Martin *et al*., 2011).

1.1.3.3 Phospholipase production

Hydrolytic enzymes secreted by *C. albicans* also play an important role in the invasion process of the host tissues and most of these enzymes are secreted extracellularly (Mayer *et al*., 2013). The three classes of secreted hydrolases expressed by the *C. albicans,* are Secreted

Aspartic protease (Saps), Phospholipase and Lipase of which Saps and phospholipase are the main representatives of the core enzyme (Da Costa *et al*., 2012). These enzymes rearrange components of the host cell membranes causing a dysfunction resulting in cell death (Coutinho, 2009). Hydrolytic enzymes also break down large substrates into smaller potions that can easily be transported into the fungal cell as a nutritional source (Chaffin, 2008).

Phospholipids and protease enzymes are involved in the membrane disruption process that takes place during invasion of the host cell (Naglik *et al*., 2011). Non-candida species of a similar *C. abicans* structure is unable to produce phospholipase, indicating the importance of phospholipase in *C. albicans* pathogenicity. Its production leads to improper function of the cell or rupturing of the cell membrane which is necessary for the organism's adherence to the host cell (De Luca *et al*., 2012). Phospholipase can effectively cleave host phospholipids and destabilize the membrane which results in cell lysis.

Surface-located acid phosphatases of *C. albicans* may also play an important role in the pathogenesis of the organism. *C. albicans* isolated from the HIV positive patients have shown ecto-phosphatase activity which results in higher adhesion to epithelial cells. This suggests that the activity of acidic surface phosphatases may play a role in the early stages of infection hence allowing for successful colonization (Portela *et al*., 2010)

1.1.3.4 Aspartic Proteases (Saps) production

Among the hydrolytic enzymes extracellular Saps are among the few gene products that have shown a direct contribution to the pathogenicity of *C. albicans.* Secreted Aspartic protease breaks down or distorts the host's cell membranes allowing adherence of the pathogenic organism, with subsequent host tissue colonization and invasion (Höffling *et al*., 2011; Reddy *et al*., 2012). All proteinases are known to catalyze and break down peptide bonds (CO-CH) of proteins, but the mechanism of action may differ between different proteinases (Barrett and Rawlings, 1991). *C. albicans* possess a large number of proteinases and the Saps gene family may be expressed in different morphological forms.

The relationship between production of proteinase and the production of hyphae as well as other putative virulence factors of *C. albicans* have been investigated (Mardegan *et al*., 2006). Under a Saps-inducing setting in the laboratory, the majorly expressed *C. albicans* proteinase gene is SAP2 while SAP1 and SAP2 were found to be expressed in other phenotypic strains (Hube *et al*., 1994). Secreted Aspartic proteases 4-6 expression was observed during hyphal formation at neutral pH (Hube *et al*., 1994). Studies have investigated oral *C. albicans* strains associated with different types of clinical infections for proteinase activity and found it to correlate with virulence. *C. abicans* with high quantities of Saps were found to have increased adherence ability to buccal epithelial cells compared to the

C. albicans with reduced Saps activity, indicating correlation between Saps production and adherence (Mardegan *et al*., 2006). Secreted Aspartic proteases have also been found to take part in switching and promotion of hyphal growth, possess keratinolytic activity that can both serve to facilitate initial penetration of keratinized cells and providing a valuable source of nitrogen during colonization (Odds *et al*., 1994).

1.1.3.5 Lipase production

Lipase is produced naturally by many organisms such as fungi, bacteria and eukaryotes (Nesbit and Gunasekaran, 1993; Rekha *et al*., 2012). Opportunistic fungi such as candida are known to secrete extracellular lipases (Stehr *et al*.,2003). Studies on the role of lipase as a virulence factor of *C. albicans* has shown that it contributes to morphological transition, colonization as well as penetration to the host epithelial cells (Schofield *et al*.,2005). Another role of extracellular lipases is to enhance adhesion, which is regarded as a vital step in the pathogenesis of *C. albicans* (Stehr *et al*.,2003). In addition, lipase has a cytotoxic characteristic on host tissue which may contribute to the pathogenicity (Parage *et al*.,2009). Lipase hydrolyses ester bonds of triacylglycerols present in the cell wall to yield glycerol and fatty acids (Figure 1.3) in opportunistic yeasts, which they then use as substrates (Park *et al*., 2013). The release of fatty acids may enhance *C. albicans* adherence to host surfaces, thus serving as an additional advantage (Bhat *et al*., 2011).

C. albicans possess a large lipase gene family which is regulated differently depending on *in vitro* conditions (Stehr *et al*., 2003). The extracellular activity of these lipase encoding genes may play a major role in colonization of this fungus and ultimately resulting in infection (Schofield *et al*., 2005). Ten lipases have been found in *C. albicans* (LIP1-LIP10), of which only LIP 4 and LIP 5 have been characterized (Hube *et al*., 2000, Roustan *et al*., 2005). Stehr *et al.*, 2004) analyzed the expression of the *C. albicans* lipase gene family during experimental infection and in patient samples. They found that the gene expression profile of lipase is dependent on the infection stage (Stehr *et al*.,2004). Lipase is the least studied hydrolytic enzyme in *C. albicans* compared to proteinase and phospholipase and therefore, not much information is available in the literature (Gácser *et al*., 2007; Bhat *et al*., 2011). It has been

suggested by Stehr *et al.*, (2003) that prevention of lipases in pathogenesis could be important for development of new drugs.

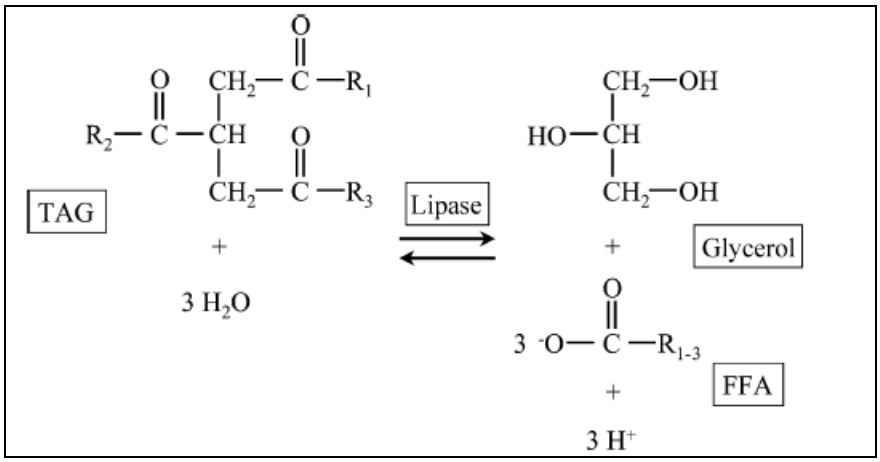

Figure 1.3 Lipase and breakdown of cell wall lipids (Stehr *et al.*, 2003).

1.1.3.6 Endocytosis

Candida invades epithelial cells by two distinctive mechanisms, either by endocytosis or by active penetration. In induced endocytosis *C. albicans* expresses invasins on the cell surface that mediates binding ligands which trigger engulfment of the fungal cell into the host cell. Two invasins ALS3 and Ssal (member of the HSP70 family) are involved in inducing endocytosis through a Clathrin-dependent mechanism. Furthermore, micropinocytes have also been applied in *C. albicans* induced endocytosis (Naglik *et al.*, 2011).

1.2 Candidiasis

Candidiasis is a yeast infection caused by candida. Among other candida species that cause this infection, *C. albicans* is the most common species. Candidiasis also called, thrush, can occur in the oral cavity (oral candidiasis), vagina (vaginal candidiasis) and in the blood

stream (candidemia). Candidemia is a serious illness that commonly occurs in hospitalized patients. Immunocompromised individuals such as HIV infected, cancer patients on treatment and transplant patients are prone to this infection. In addition, diabetic people, persons on corticosteroids and antibiotics can also develop candidiasis. Mycoses is a major problem in medical care centers due to the increased number of immune-deficient patients (Tsai *et al*., 2013).

1.2.1 Oral candidiasis

Oral candidiasis is a fungal infection found in the oral cavity caused by candida species (Akpan and Morgan, 2002). Although it is primarily caused by *C. albicans*, non-*albicans* candida such as *C. glabrata*, *C. parapsilosis* and *C. krusei* have been identified from oral lesions as well as from oral cavities of patients with oral candidiasis. HIV infected individuals frequently experiences oral candidiasis as an initial manifestation (Mathews, 1994).

The lesions appear as a white creamy substance on the tongue or mucosal surfaces of immune-compromised individuals (Motsei *et al*., 2003). If lesions remain untreated, chewing and swallowing of food may become difficult. In addition, untreated infection may disseminate and cause esophageal candidiasis or systemic illness. Other factors that may cause oral candidiasis are poor oral hygiene, prolonged use of antibiotics, cancer treatment, diabetes and poor oral hygiene (Sweet *et al*., 1995; Akpan and Morgan, 2002; Naglik *et al*., 2011).

The acute form of infection includes oral-pharyngeal candidiasis (OPC) which is highly common in Acquired Immunodeficiency Syndrome (AIDS) patients and its appearance is considered to be a marker of advanced disease (Klein *et al*., 1984). The chronic form occurs in cases of broad spectrum usage of antimicrobial agents where the oral bacterial population

is lowered resulting in *C. albicans* overgrowth (Williams *et al*., 2011). In addition, chronic oral candidiasis, also called, chronic mucocutaneous candidiasis is often associated with dental enamel dysplasia, thymoma, endocrinopathies and autoimmune diseases (Kirkpatrick,1989). Chronic oral candidiasis is not a common disorder (Cawson, 1966) but it may occur in denture wearers as denture stomatitis, or during inhaled corticosteroid therapy (Kirkpatrick,1989).

Candida–associated lesions include denture related stomatitis which occurs in denture wearers. In this case the denture is the major contributing factor particularly when the denture is not properly cleaned. Under such conditions the stagnant areas above the upper fitting surface of the denture provides a suitable environment for candida growth (Williams *et al*., 2011).

1.2.2 Treatment of oral candidiasis

1.2.2.1 Antifungal agents

There are four classes of drugs available for the invasive fungal infections in humans. They are polyenes, fluorinated pyrimidines, azoles and echnocandins. The grouping of these drugs is determined by their mode of action (Vanden Bossche,1997). Drugs interfere with the process of the cell synthesis and cell function in which they prevent cell synthesis, disturb the function of the membrane barrier or inhibit the synthesis of ergosterol (Vanden Bossche, 1997). The polyenes target the membranes containing ergosterol, disturbing the channel through which cell components such as potassium ions leak, resulting in the death of the cell (Vanden Bossche, 1997; White *et al*., 1998).

However, there are few antifungal drugs available to treat candida infections due to the availability, expense and side effects of such drugs. Azoles and polyenes are the most commonly used drugs to treat candidiasis (Miao *et al*., 2012) whereas echinocandins offer viable alternatives to conventional treatment, particularly in well-defined clinical settings such as invasive candidiasis and candidaemia (Bal, 2010). Nevertheless, *C. albicans* has developed resistance to these drugs (Gozalbo *et al*., 2004). Three mechanisms of azole resistance in *C. albicans* have been described (Kontoyiannis and Lewis, 2002). They are: the reduction of azole drug accumulation through an active efflux pump, over expression or alteration of the 14α- sterol-demethalase site which is encoded by EGR11 and function loss downstream in the ergosterol pathway (Kontoyiannis and Lewis, 2002) mutation. Apart from that, the polyenes are poorly absorbed through the gut causing a further limitation in the treatment of candida infections (Golzalbo *et al*., 2004).

The use of echinocandin drugs is limited by their large molecular size that dictates the need for intravenous administration and are their expense which is a problem in developing countries (Samie *et al*., 2010; Bal, 2010; Armstrong-James *et al*., 2014). However, fluconazole drugs have been used for the treatment of invasive fungal infections especially those caused by *C. albicans* (Mathews, 1994;Sanglard *et al*., 1998; Morschihhäuser, 2002). Fluconazole is cheaper and less toxic than echinocandin drugs (Armstrong-James *et al*., 2014). *C. albicans* has developed resistance against fluconazole, more commonly in HIV infected patients where fluconazole treatment is taken for longer time periods (Ramesh *et al*., 2011). Tripathi *et al*., (2014) have suggested that *C. albicans* resistance may be caused by mutations of the targeted enzymes. Studies have also shown that mutations affecting flucytosine are the major causes of the drug resistance (Dodgson *et al*., 2004). The prolonged use of drugs allows the organisms to develop resistant mechanisms such as mutations and efflux pumps, hence protecting

the fungal cell from taking in the drug, making the drug ineffective (Sanglard and Odds, 2002).

1.2.2.2 Combination therapy

The lack of availability of new classes of drugs or different molecular attack points has led to consideration of drug combinations as an alternative therapy (Endo *et al*., 2010). Interactions of drugs are described as indifferent, synergistic, antagonistic or additive (Lewis and Kontoyiannis, 2001). The advantage of drug combination therapy is the greater effect (synergy) of the antifungal agents compared to an individual drug. Drug combinations have been used for resistant *C. albicans* strains (Liu *et al*., 2014), however, this does not solve the drug resistance problem because of the eukaryotic cell nature of fungi. Chaturvedi *et al.*, (2011) tested the effect of two drug combinations (*in vitro*) on several candida species including *C. albicans,* and found that some of the fungi that were known to be resistant to azole or echinocandin drugs alone, were susceptible to the combination of the two drugs., However, has been stated that development of standardized drug combination testing methods was a problem and can be expensive (Chaturvedi *et al.*, 2011).

1.2.2.3 Alternative approach

The increase in fungal infections and in antifungal drug resistance has led to a demand in the search for alternative therapy that can be effective, less harmful as well as cost effective such as natural products for example (Tripathi *et al*., 2014). Antifungal therapy is limited compared to bacterial therapy choices. The restriction to new antifungal drug development is mostly due to the eukaryotic cell nature of fungi which is very similar to the human (host) cells. The pathogen cell targets are limited as it is difficult creating a therapeutic agent that would attack the organism without harming the host. (Mathews, 1994; Gozalbo *et al*., 2004).

A novel approach to fungal therapy has been suggested, where the virulence of *C. albicans* can be targeted (Mehra *et al*., 2012). This would be ideal in cases where the causative organism is a normal flora, such as *C. albicans* in the oral cavity (Gauwerky *et al*., 2009). Virulence factors such as germ tube formation, adherence ability and production of tissue damaging hydrolytic enzymes can be the possible targets of such new drugs.

1.3 Medicinal plants

The increasing rate of drug resistance of *C. albicans* and other fungi has led to screening of natural and synthetic products as possible alternative therapy (Harvey, 2008). Medicinal plants have been used for many years across the world for numerous diseases including oral candidiasis, especially in developing countries where hospitals are inaccessible and treatment affordability is still a problem. (van Wyk *et al*., 2009; Olila *et al*., 2001; Samie *et al*., 2010). An advantage of natural products is the ease of absorption compared to synthetic drugs, affordability and low toxicity (Lewis and Elvin-Lewis, 1995; Harvey, 2008).

South African medicinal plants have been screened for antimicrobial effects on *C. albicans*. Motsei *et al.,* (2003) screened twenty four plants of which only four were effective at high concentrations (lowest minimum inhibitory concentration was 0.56 mg/ml). Samie *et al*., (2010) also tested selected South African plants from the rural area called Venda, for antifungal activity against a few fungal organisms including *C. albicans.* Most of the plants tested showed potential antifungal properties. *Punica granatum L* has also been explored for its antimicrobial properties.

1.3.1 *Punica granatum L*

Punica granatum L (*Punicaceae*) commonly known as pomegranate (Figure 1.4) originated in the Middle East but is now cultivated all over the world including South Africa. It belongs to the plant family Punicaceae and *Punic*a is the sole genus in the family (ITIS, 2006). It grows to about 12-16 feet high with many thin branches. Many parts of the pomegranate plant have been used as an alternative to medicine. It is not only used as antimicrobial agent but also to treat or prevent many other conditions such as Alzheimer's, cancer, cardiovascular disease and diabetes (Bhandari, 2012). Pomegranate fruit is edible and all parts have been found to have potential antimicrobial properties (Endo *et al*., 2010; Abdollahzadeh *et al*., 2011). The fruit can be divided into the seed, peel, and juice. The fruit extracts contains high tannins or polyphenols which has some anti *C. albicans* activity (Endo *et al*., 2010). Methanolic root extract of *P. granatum* has also shown antibacterial and anti *C. albicans* effects (Duraipandiyan *et al*., 2006). In recent years *P. granatum* has gained interest in dentistry where causative agents of dental caries, dental plaque and periodontal diseases have been targeted (Narayan *et al*., 2014).

Figure 1.4 Small tree of *Punican granatum* (Pomagranate), (2015, March 13). Retrieved from http://www.todayprimetimes.com)

1.3.1.1 Pomegranate peel

Dahham *et al.*, (2010) tested antifungal and antibacterial properties of pomegranate peel, seed, juice and whole fruit extracts on selected fungi and bacteria. The results showed that all the parts were effective, however, the peel extracts had higher antimicrobial activity (Dahham *et al*., 2010). Mahmood *et al.*, (2010) investigated antimicrobial activity of pomegranate fruit against *Escherichia coli, Salmonella typhi* and *C. albicans*. The results showed that at high concentrations (200 to 700 mg/ml) pomegranate had antimicrobial effects against all the test organisms. The peel of pomegranate fruit has also been tested against oral pathogens (Abdollahzadeh *et al*., 2011), showing that at high concentrations (8 mg/ml) pomegranate peel can kill *S. mutans*, however, these extracts failed to show any anti *C. albicans* and *Actinomyces viscosus* activity. In contrast, Vasconcelos *et al.*, (2003 and 2006) reported that the gel form of pomegranate peel prevents adherence of *C. albicans* to glass and it would be beneficial to patients with denture stomatitis (Vasconcelos *et al*., 2003 and 2006). However, the results were observational and the organisms were not quantified. Pai *et al.* (2010) has shown some antifungal activity of aqueous peel extract (18.8 mm diameter of zone of inhibition). Chemical composition differs within pomegranates depending on the cultivar, climate, maturity, storage conditions and region of growth (Poyrazoğlu *et al*., 2002), which could be responsible for the contrasting results from different regions. There are no studies available in South Africa that shows antifungal activities of local cultivars of *P. granatum*.

1.3.1.2 Pomegranate seed

Pomegranate seeds are contained in a sack filled with liquid called pomegranate juice which is covered with a leathery pericarp or peel (Jurenka, 2008). Seeds are rich in polysaccharides, vitamins, minerals, polyphenols, oils, polysaturated fatty acids and sugars (Miguel *et al*., 2004; Sadeghi *et al*., 2009). The seed oil are mainly composed of sterols and punic acid

(Bandari, 2012; Narayan *et al*., 2014) with more than 80% being conjugated fatty acids (Hora *et al*., 2003). Seed extracts have antioxidant properties (Sadeghi *et al*., 2009). Not much is known about the antimicrobial properties of seed extracts. Dahhan *et al.*, (2010) evaluated antimicrobial effects of red and white seed extracts against *Bacillus cereus*, *B. subtilis*, *Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Aspergillus niger, Mucor indicus, Penicilium citrinum, Rhizopus oryzae* and *Trichoderma reesei* using an agar well technique. *Staphyloccus aureus* and *Aspergillus niger* were the best inhibited organisms with the zone diameters measuring 19 mm and 12 mm respectively. This suggests that pomegranate seed has a potential to be used as an antimicrobial agent. However, the antifungal effect of seed extracts against unicellular fungi such as *C. albicans* has not been tested (Dahham *et al*., 2010).

The above studies show that peel extracts of *P. granatum* has weak antifungal activity with high Minimum Inhibitory Concentrations (MIC) values. These concentrations are difficult to maintain in an oral cavity which is constantly flooded with saliva, but this diluted extract may have some effect on the causative organisms. A novel approach to therapy has been suggested, where the virulence of *C. albicans* can be targeted. This would be ideal where the causative organism is a normal flora, such as *C. albicans* in the oral cavity (Gauwerky *et al*., 2009). Virulence factors such as germ tube- and biofilm formation and/or production of tissue damaging enzymes can be possible targets of such new drugs. A medicinal plant such as *Dodenaea viscosa var.angustifolia* has shown to be effective against virulence properties of *C. albicans* (Patel *et al*., 2009). The effect of sub-inhibitory concentrations of crude extract of *P. granatum* peel on the virulence properties of *C. albicans* has not been studied.

1.4 Aim

The aim of this study was to investigate the antifungal effect of peel and seed extracts of the *Punica granatum* (pomegranate) fruit on the fungal organism, *C. albicans*, isolated from HIV positive and HIV negative patient's oral cavities, and furthermore to investigate the effect of peel extracts on the virulence factors of *C.albicans.*

1.5 Objectives

- To prepare crude extracts of the seed and peel of *P. granatum* using methanol, di-chloromethane(DCM)/methanol, ethanol, acetone, hexane, ethyl acetate and water.
- To obtain MICs and Minimum Fungicidal Concentrations (MFCs) of seed and peel extracts against *C. albicans* isolated from the oral cavities of HIV positive and HIV negative patients.
- To study the effect of the sub inhibitory concentration of the selected extract on germ tube formation, adherence ability, and the production of proteinase, phospholipase and lipase by *C. albicans*.
- To compare the results of the effect of these extracts on the pathogenic characteristics of *C. albicans* isolated from HIV positive and HIV negative patients.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cultures and Inocula

Twenty *C. albicans* cultures, 10 isolated from HIV positive and 10 isolated from HIV negative patient's oral cavities were obtained from the Oral Microbiology Laboratory, of the University of The Witwatersrand. Samples were collected from HIV positive and HIV negative patients under ethical clearance number: M000402. Cultures were grown on Sabouraud dextrose agar (SAB) and incubated for 48 hours at 37^{o}C. Fresh cultures were used for each experiment. For each experiment, inoculum was prepared using 10 ml of Sabouraud broth. The turbidity was adjusted to 0.5 Mc Farlands Standard obtaining 10^{5} cells/ml solution.

2.2 Plant material and preparation of extracts

Pomegranate fruits, from a cultivar called Wonderful were purchased from Zanddrift farm in Cape Town. Peel and seeds were separated, dried at room temperature in a shady area and pulverized using a grinder (Figures 2.1a, 2.1b and 2.1c). Extracts were prepared using, methanol, DCM/methanol, ethanol, hexane, acetone ethyl acetate, and water as described by Eloff, (1999). One gram of the peel or seed powder was transferred into a McCartney bottle and 10 ml of the respective solvent was added. The mixture was vortexed for 10 minutes and centrifuged for 5 minutes at 10000 rpm. The supernatant was transferred into pre-weighed 250 ml beakers. This procedure was repeated in triplicate for both the seed or peel extract, using each one of the 6 mentioned solvents. The extracts were dried in a cold air stream and stored at 4^{o}C until required. Fresh extracts were prepared as required.

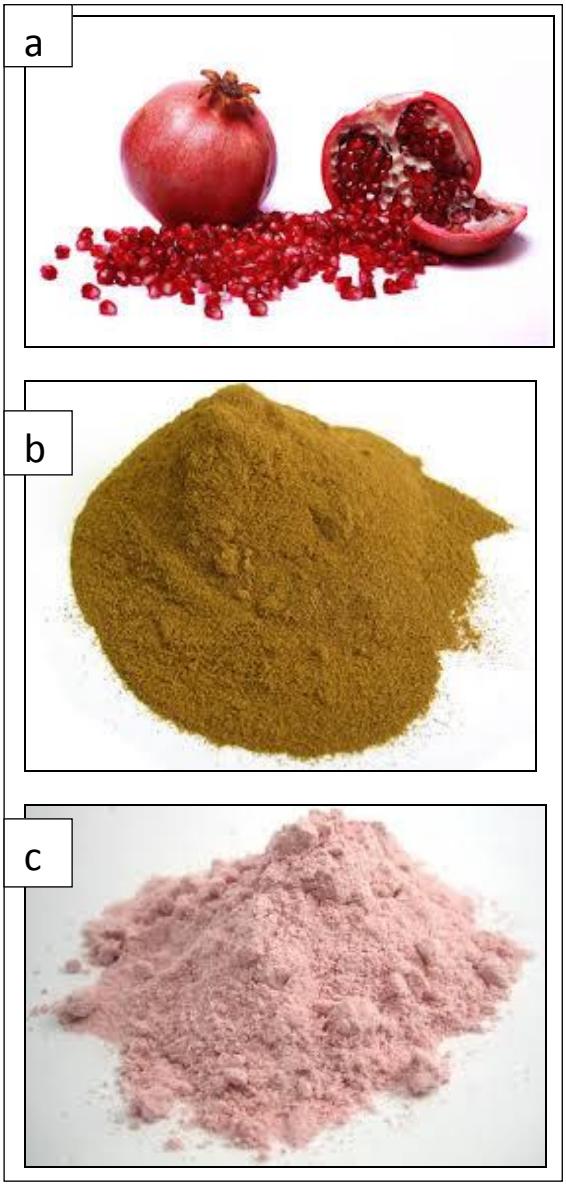

Figure 2.1 ***Punica granatum*** **(pomegranate): a) Fresh fruit,Parenting healthy babies. (2015, March 13). Retrieved from http://www.parentinghealthybabies.org, b) dried and pulverized peel. (2015, March 13). Retrieved from http:// www.puraorganic.org, c) dried and pulverized seeds. (2015,March 13). Retrieved from http:// www.nuts.com.**

2.3 Minimal Inhibitory concentration (MIC) and Minimum Fungicidal concentration (MFC)

Dehydrated peel and seed extracts were reconstituted with DMSO to obtain a final concentrations of 50 mg/ml. DMSO was used as a solvent because it can dissolve most natural chemicals. Minimal inhibitory concentration experiments were performed using a micro dilution technique described by Eloff, (1998). Extracts were diluted (double dilution) using SAB broth and 100 µl of each dilution were transferred into microtitre plate wells. Chlorhexidine and water were included in each experiment as positive and negative controls respectively. DMSO was also used as a vehicle control. Wells were inoculated with 100 µl of *C. albicans* inocula containing approximately 10^5 colony forming units (CFU)/ml organisms. The final concentrations of the extract into the wells were ranging from 25 mg/ml to 0.2 mg/ml. Microtitre plates were incubated at 37^{o}C for 48 hrs. A 5 µl aliquot of culture from each well were cultured onto SAB agar to obtain MFC results. These SAB agar plates were incubated at 37^{o}C for 48 hrs. Last concentration with no growth was taken as the MFC value. A 5 µl aliquot of a 0.2% solution of iodonitrozolium was added into each well, incubated for 24 hrs at 37^{o}C and a colour change from yellow to red was observed for viable growing cultures. The last concentration with no colour change was taken as the MIC value. All the MIC and MFC experiments were repeated 3 times. The concentration just lower than the MFC was selected asthe sub-inhibitory concentration and was subsequently used for the adherence, germ tube formation, phospholipase, proteinase and lipase assays.

2.4 Adherence assay

The study procedure and purpose was clearly explained to all voluntary participants in the study and a signature was obtained on the consent forms. Healthy donors (non-*C. albicans* carriers) were screened for *C. albicans,* by swabbing the oral buccal mucosa and tongue with

a sterile swab,and streaking a SAB plate which is then incubated for 2 days at 37ºC in order to culture the organism. A person with no growth of *C. albicans* was considered a *C. albicans* non-carrier. Cells were then collected from the donors by gently rubbing the oral mucosal surface of the cheek with a sterile swab and suspending it into 15 ml of sterile distilled water. The collection was done early in the morning before they had breakfast to avoid the presence of food particles. Buccal epithelial cells were collected from two *C. albicans* non-carriers and the cells were pooled together. Freshly collected cells were used for each experiment. Buccal epithelial cells were washed three times with sterile distilled water and resuspended into 2 ml sterile distilled water. The epithelial cells were counted with a haemocytometer and adjusted to 10^5 cells/ml (Patel *et al*., 2009).

The adhesion assay was performed using a technique described by Ghannoum *et al*, (1986). Fifteen millilitre of Sabouraud broth containing 3.13 mg/ml of methanol peel extract was inoculated with 10^6CFU of test culture and incubated at 37ºC for 2 hrs while shaking at 60 rpm. Water was used as a control which was also inoculated with 10^6 CFUof test culture and incubated at 37ºC for 2 hrs while shaking at 60 rpm. After incubation, peel extract treated yeast cells and control yeast cells were washed three times with sterile water and resuspended into 2 ml distilled water. The *C. albicans* count was standardized to 10^7cells/ml, using a heamocytometer. Two milliliter of treated yeast cells and 2 ml of epithelial cells were mixed and incubated at 37ºC for 3 hrs while shaking at 60 rpm. After incubation the epithelial cells were separated from non-adherent yeast cells using a 20 µm pore nylon filter. The epithelial cells were then washed twice with distilled water to remove non-adherent cells and resuspended in 2 ml sterile distilled water. Slides were prepared from the suspension and the cells stained with the gram staining technique (Figure 2.2). Three slides were prepared from each sample and results recorded in triplicate. This procedure was done for each of 20 isolates. The num-

ber of yeast cells adherent to 100 epithelial cells were recorded. The results of the tests were compared to the control using the Wilcoxon rank-sum (Mann-Whitney) test. The counts of the *C. albicans* isolated from the HIV positive patients were compared to the counts of isolates from the HIV negative patients using the two sample Student's t-test.

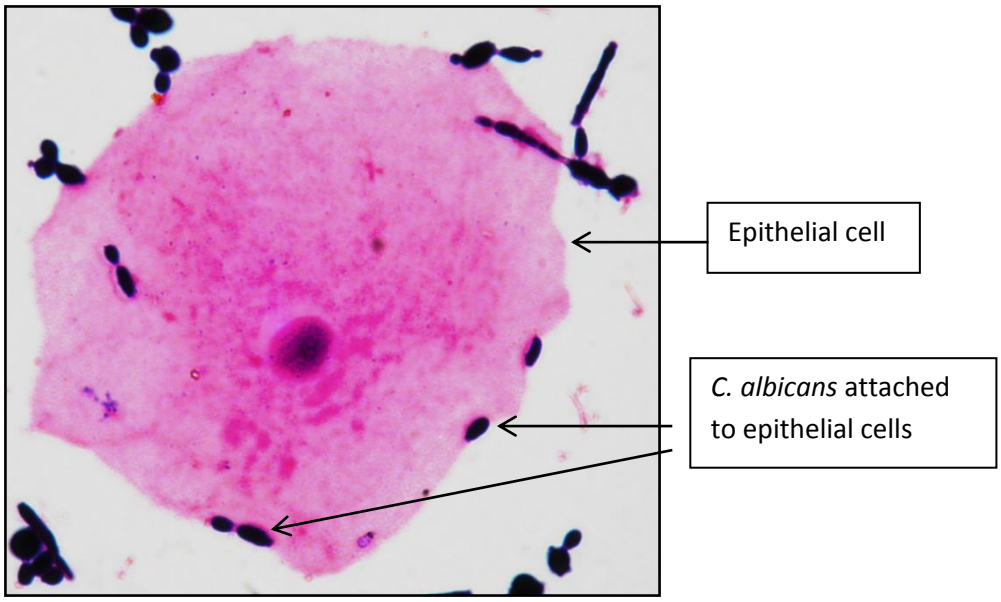

Figure 2.2 Light microscopy picture showing *C. albicans* attached to epithelial cells at 40 x magnification.

2.5 Germ tube (GT) assay

Germ tube formation was investigated using a technique described by Mackenzie, (1962) with minor modifications. Inoculum of *C. albicans* was prepared using sterile distilled water. Turbidity was adjusted to Mc Farlands Standards 2. Five hundred microliters of sterile horse serum containing 3.13 mg/ml of the methanol peel extract was inoculated with 100 µl of inoculum. A negative control containing water instead of plant extract was also included. Tubes were aerobically incubated for 2½ hrs, at 37°C. After incubation, the mixture was transferred

into Eppendorf tubes and centrifuged for 5 minutes, the supernatant was removed and the cells were reconstituted in 500 µl distilled water. A drop of suspension was placed on a clean microscope slide using a capillary tube, covered with a coverslip and examined under a light microscope using the 10 x and 40 x objective lenses for detecting the production of germ tubes. Cells with a germ tube at least twice the length of the cell was considered positive. One hundred yeast cells were observed and the number of cells with germ tubes was recorded. The number of germ tubes produced by *C. albicans* exposed to the peel extract was compared to the negative control by using the Wilcoxon rank-sum (Mann-Whitney) test. In addition, The GT results of the *C. albicans* isolated from the oral cavities of HIV positive patients were compared to the isolates from the HIV negative patients using the two sample Student's t-test.

2.6 Phospholipase assay

C. albicans isolates were screened for extracellular phospholipase activity (Ghannoum, 2000). Many isolates were continually screened until the required number of isolates was reached (10 isolates from HIV positive and 10 isolates from HIV negative patients). Isolates were screened by growing each one on egg yolk agar and observing a development of a zone of precipitation. Phospholipase assays were performed using a technique described by Patel *et al.*, (2009). Fifteen milliliters of SAB containing 3.13 mg/ml methanol plant extract was inoculated with 10^6 CFU of the test organism and incubated at 37°C for two hrs while shaking at 60 rpm. The negative control containing water instead of plant extract was also included in each test. After incubation the cells were washed three times and resuspended in 0.5 ml sterile distilled water. Ten microliters of yeast suspension was placed on sterile filter paper discs placed on egg yolk agar medium. Plates were incubated for 4 days at 37°C. The colony diameter and the zone of precipitation were measured and recorded. Phospholipase activity

(Pz) was calculated by dividing the colony diameter by the diameter of the precipitation zone (Figure 2.3). Two measurements were taken from two different sides and an average was calculated. The *C. albicans* control strain (ATCC 90028) was included in this study as a positive control and *C. parapsilosis* (ATCC 22019) as a negative control. Pz values of plant extract exposed *C. albicans* were compared to the negative, water, control using the Wilcoxon rank-sum (Mann-Whitney) test. In addition, Pz results of the *C. albicans* isolated from the oral cavities of HIV positive patients were compared to the isolates from the HIV negative patients using the two sample Student's t-test.

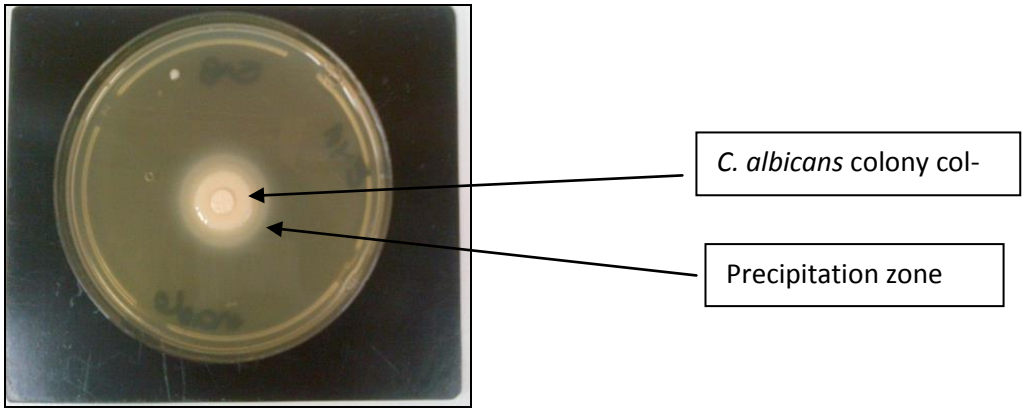

Figure 2.3 Phospholipase activity on egg yolk agar media.

2.7 Proteinase assay

The proteinase assay was undertaken using the method described by Sugita *et al.*, (2002).

C. albicans isolates were screened for the proteinase production using Bovine Serum Albumin (BSA) agar. Cultures were plated out on agar plates and incubated for 1 week at 37^{o}C. Plates were stained (Figure 2.4). Cultures with a light colour zone around the colonies were considered proteinase positive strains. Ten isolates from HIV positive patients and 10 from HIV negative that produced proteinase were selected for the study.

Fifteen millilitre of SAB broth containing 3.13 mg/ml of methanol plant extract was inoculated with 10^6 CFU of the test organism and incubated at 37°C for two hours while shaking at 60 rpm. Control containing water instead of plant extract was also included in each test. After incubation the cells were washed three times and resuspended into 0.5 ml sterile distilled water. Ten microliters of yeast suspension was placed on sterile filter paper discs placed on BSA agar plates and plates were allowed to dry before incubation at 37°C for a week. After incubation plates were examined for growth and diameter of the colony around the disc was measured. The plates were then stained by flooding them with coomasie blue staining solution and kept at room temperature for 20 minutes. The staining solution was decanted. The plates were destained by flooding them with destaining solution three times and each time allowed to stand for 20 minutes and once with tap water for 20 minutes. The light colour zone that developed around the colony was measured. Proteinase activity (Pr) values were calculated by dividing the diameter of the colony with the precipitation zone (Figure 2.4). Two measurements were taken from two different sides and an average was calculated. *C. albicans* control strain (ATCC 90028) was included in this study as a positive control and *C. parapsilosis* (ATCC 22019) as a negative control. *C. albicans* control strain (ATCC 90028) always produce proteinase and *C. parapsilosis* do not produce proteinase. Pr values of plant extract exposed *C. albicans* was compared to the control using the Wilcoxon rank-sum (Mann-Whitney) test. In addition, Pr results of the *C. albicans* isolated from the oral cavities of HIV positive patients were compared to the isolates from the HIV negative patients using the two sample Student's t-test.

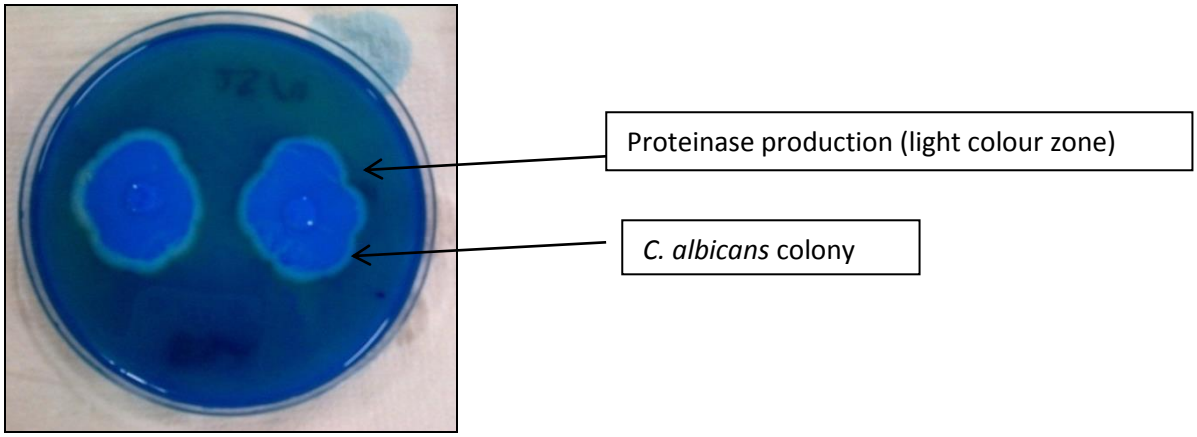

Figure 2.4 Proteinase activity on a bovine serum albumin agar (BSA) plate

2.8 Lipase assay

C. albicans strains isolated from the oral cavities of HIV positive and HIV negative patients were screened for the production of lipase by culturing them on SAB agar supplemented with 1% tributyrin. A clear zone around the colonies was considered positive. Ten strains of *C. albicans* isolated from HIV positive and 10 from HIV negative patients that produced lipase were selected for the experiments. Lipase assays were performed as described previously with modifications (Ramesh *et al*., 2011). Fifteen millilitre of SAB broth containing 3.13 mg/ml of methanol plant extract was inoculated with 10^6 CFU of the test organism and incubated at 37°C for two hours while shaking at 60 rpm. The negative control containing water instead of plant extract was also included in each test. After incubation the cells were washed three times and resuspended into 0.5 ml sterile distilled water. Ten microliters of yeast suspension was placed on sterile filter paper discs placed on SAB agar plates supplemented with 1% tributyrin, allowed to dry and incubated at 37°C for 2 days. The colony diameter and the clear zone were measured and recorded (Figure 2.5). Lipase activity (Pl) was calculated by dividing the colony diameter by the diameter of the clear zone. Two measurements were taken from two different sides and an average was calculated. A *C. albicans* control strain (ATCC 90028) was included in this study as a positive control and *C. parapsilosis* (ATCC

22019) as a negative control. Lipase values of plant extract exposed *C. albicans* were compared to the control using Wilcoxon rank-sum (Mann-Whitney) test. In addition, results of the *C. albicans* isolated from the oral cavities of HIV positive patients were compared to the isolates from the HIV negative patients using the two sample Student's t-test.

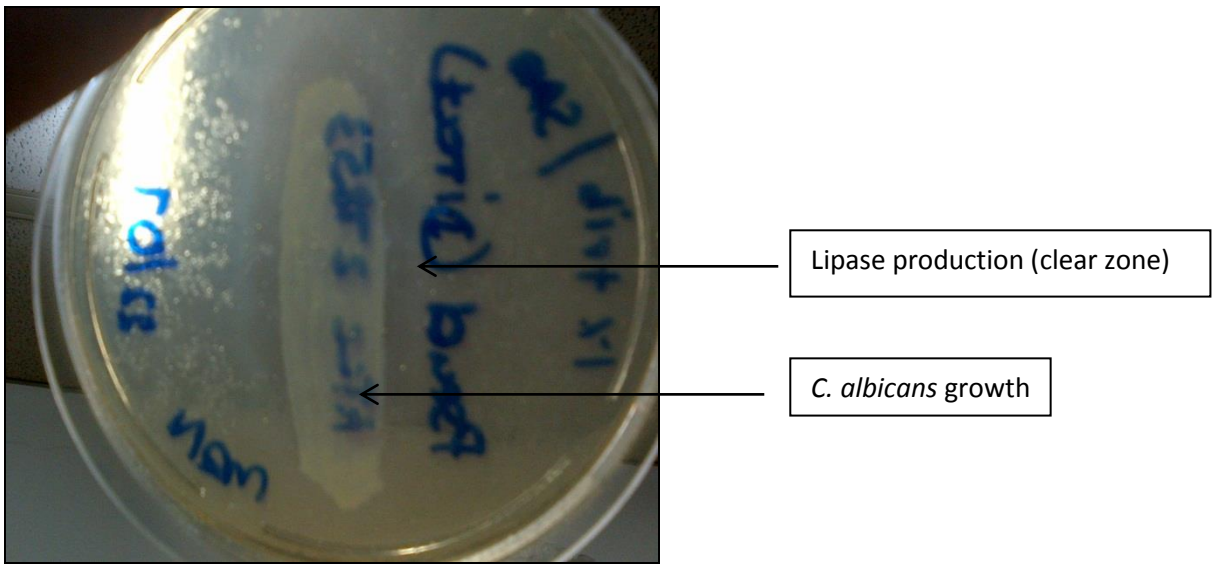

Figure 2.5 Lipase production on 1% tributyrin enriched Sabouraud agar.

The complete study design is shown in the flow diagram labelled Figure 2.6.

2.9 Statistical Analysis

A statistician was consulted to determine the number of strains to be used for each experiment to allow for adequate degree of freedom using the appropriate statistical techniques. Statistical significance was set at $p < 0.05$. All the results were analysed using the Stata 10 software (StataCorp. College station, Texas).

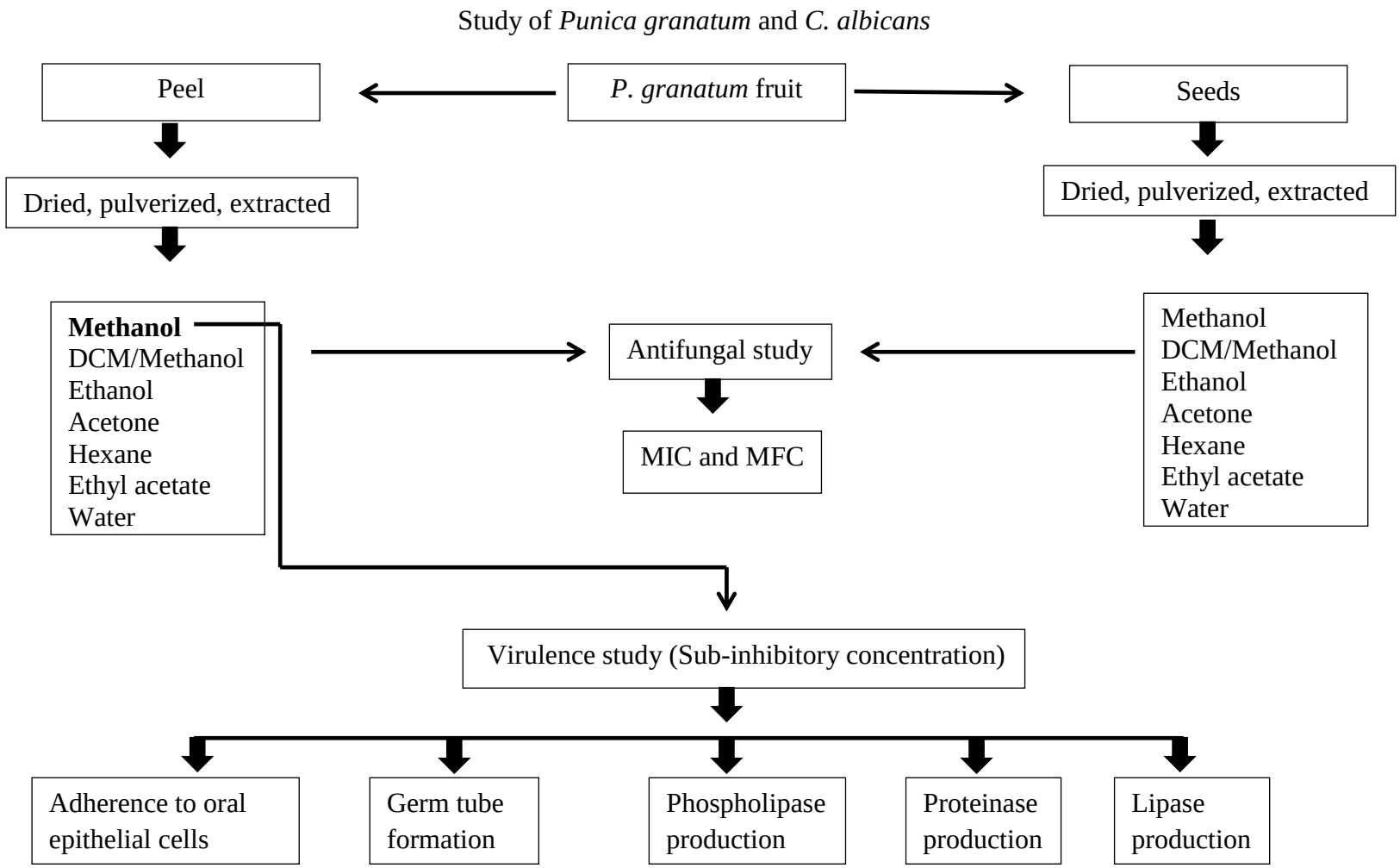

Figure 2.6 Study design

CHAPTER 3: RESULTS

3.1 *Punica granatum* (pomegranate) crude peel and seed extract yield

Crude extracts of pomegranate peel and seed were obtained using methanol, DCM/methanol, acetone, ethanol, hexane, ethyl acetate and water. All extracts were used for the MIC/MFC experiments. The crude extract yield of dried pomegranate peel and seeds are shown in Tables 3.1 and 3.2 respectively.

For the fruit peel methanol proved to be the best solvent for the crude extraction delivering a yield of 0.67g/1g of dry powder , followed by water (0.54g/1g of dry powder) as solvent. However, the yields from seeds were poor with both methanol as well as water used as solvents for the crude extraction (highest yield 0.2g/1g of powder).

Table 3.1 The dry weight of peel extracts of *Punica granatum*

Repeats		Yield per 1 gram of dried peel powder (extract in gram)					
	Methanol	**DCM /Methanol**	**Ethanol**	**Hexane**	**Acetone**	**Ethyl acetate**	**Water**
1	0.65	0.29	0.03	0.02	0.04	0.01	0.50
2	0.66	0.33	0.04	0.02	0.07	0.02	0.50
3	0.66	0.38	0.04	0.02	0.08	0.03	0.52
4	0.68	0.39	0.06	0.02	0.08	0.04	0.60
5	0.72	0.39	0.07	0.03	0.08	0.06	0.60
Average yield	**0.67**	**0.36**	**0.05**	**0.02**	**0.07**	**0.03**	**0.54**

Table 3.2 The dry weight of seed extracts of *Punica granatum*

Repeats		Yield per 1 gram of dried seed powder (extract in gram)					
	Methanol	**DCM /Methanol**	**Ethanol**	**Hexane**	**Acetone**	**Ethyl acetate**	**Water**
1	0.09	0.16	0.11	0.13	0.15	0.18	0.15
2	0.11	0.18	0.15	0.13	0.16	0.18	0.15
3	0.12	0.18	0.16	0.18	0.17	0.18	0.15
4	0.12	0.20	0.16	0.20	0.19	0.20	0.17
5	0.14	0.21	0.16	0.20	0.19	0.20	0.20
Average yield	**0.12**	**0.19**	**0.15**	**0.20**	**0.17**	**0.19**	**0.16**

3.2 Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentrations (MFC) of crude peel extract

The results of the MIC and MFC of the peel extracts are shown in Tables 3.3 to 3.6. The MIC and MFC values of peel extract against *C. albicans* isolated from HIV positive patients are shown in Table 3.3, the HIV negative patients in Table 3.4 and the summary of the results for HIV positive and HIV negative patients in Tables 3.5 and 3.6 respectively. Extracts obtained using all the test solvents showed antifungal properties. The MIC and MFC values of

the ethanol, acetone, hexane, ethyl acetate and water against all the test isolates was 1.56 mg/ml. DCM/methanol showed slightly higher MIC and MFC values of 3.13 mg/ml. The methanol extract showed the highest MIC and MFC values of 6.25 mg/ml. The lowest MIC/MFC was 0.39 mg/ml obtained with the ethyl acetate solvent (Table 3.4a). Chlorhexidine persistantly killed the test organisms and there was always organism growth in the negative control (water). Interestingly, DMSO had no effect on the *C. albicans* at the concentration used in the study.

No difference was found in the MIC and MFC values between the strains of *C. albicans* isolated from the HIV positive and HIV negative patients.

Although MFC values were expected to be higher than MIC for all the peel extracts, the MIC and MFC values were similar except with DCM/Methanol extract against C25 (Table 3.3a).

Table 3.3: MIC and MFC of pomegranate peel extracts (PLE) against *C. albicans* isolated from HIV positive patients.

Isolates	Repeats	Solvents - MIC and MFC (mg/ml)													
		Methanol		DCM/ Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
274	1	6.25	6.25	3.13	3.13	1.56	1.56	3.13	3.13	3.13	3.13	1.56	1.56	0.78	0.78
	2	6.25	6.25	3.13	3.13	1.56	1.56	3.13	3.13	3.13	3.13	1.56	1.56	0.78	0.78
	3	6.25	3.13	3.13	1.56	1.56	1.56	6.25	1.56	3.13	3.13	3.13	1.56	1.56	0.78
	Median	**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**	**0.78**	**0.78**
275	1	6.25	6.25	3.13	31.3	1.56	1.56	0.78	1.56	3.13	3.13	3.13	3.13	1.56	1.56
	2	6.25	6.25	3.13	3.13	1.56	1.56	0.78	1.56	3.13	3.13	3.13	3.13	1.56	1.56
	3	6.25	6.25	3.13	3.13	1.56	1.56	0.78	1.56	3.13	3.13	3.13	3.13	1.56	1.56
	Median	**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**0.78**	**1.56**	**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**
276	1	3.13	6.25	3.13	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	6.25	3.13	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	1.56	6.25	6.25	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**3.13**	**6.25**	**3.13**	**1.56**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
277	1	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	0.78	0.78	1.56	1.56
	2	6.25	6.25	1.56	3.13	1.56	1.56	1.56	1.56	1.56	1.56	0.78	0.78	1.56	1.56
	3	6.25	6.25	1.56	3.13	1.56	1.56	1.56	1.56	1.56	1.56	0.78	0.78	1.56	1.56
	Median	**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**0.78**	**0.78**	**1.56**	**1.56**
279	1	6.25	6.25	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	6.25	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	6.25	6.25	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**

DMSO had no effect on the *C. albicans* at the concentration used in the study

Table 3.3a: MIC and MFC of pomegranate peel extracts (PLE) against *C. albicans* isolated from HIV positive patients (continue).

Isolates	Repeats	Solvents - MIC and MFC (mg/ml)													
		Methanol		DCM/ Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C2	1	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
C3	1	6.25	6.25	3.13	3.13	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	6.25	3.13	3.13	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56	1.56	1.56
	3	3.13	6.25	3.13	3.13	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**0.78**	**0.78**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
C5	1	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
C13	1	3.13	3.13	1.56	1.56	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	3.13	1.56	1.56	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	156	1.56	1.56	1.56
	Median	**3.13**	**3.13**	**1.56**	**1.56**	**6.25**	**6.25**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	1.56	1.56
C25	1	6.25	6.25	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	6.25	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	6.25	6.25	3.13	0.78	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
Combined median		**6.25**	**6.25**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**

DMSO had no effect on the *C. albicans* at the concentration used in the study

Table 3.4: MIC and MFC of pomegranate peel extracts (PLE) against *C. albicans* isolated from HIV negative patients.

Isolates	Repeats	Solvents - MIC and MFC (mg/ml)													
		Methanol		**DCM/ Methanol**		**Ethanol**		**Acetone**		**Hexane**		**Ethyl Acetate**		**Water**	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
N2	1	3.13	6.25	6.25	6.25	1.56	1.56	1.56	1.56	3.13	3.13	3.13	3.13	1.56	1.56
	2	3.13	6.25	6.25	6.25	1.56	1.56	1.56	1.56	3.13	3.13	3.13	3.13	1.56	1.56
	3	6.25	6.25	6.25	6.25	1.56	1.56	3.13	1.56	3.13	3.13	1.56	3.13	1.56	1.56
	Median	**3.13**	**6.25**	**6.25**	**6.25**	**1.56**	**1.56**	**1.56**	**1.56**	**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**
N3	1	6.25	6.25	1.56	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	6.25	1.56	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	6.25	6.25	1.56	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**1.56**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N8	1	3.13	12.5	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	12.5	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	3.13	12.5	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**3.13**	**12.5**	**6.25**	**6.25**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N9	1	6.25	12.5	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	12.5	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	12.5	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**12.5**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N16	1	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**

DMSO had no effect on the *C. albicans* at the concentration used in the study

Table 3.4a: MIC and MFC of pomegranate peel extracts (PLE) against *C. albicans* isolated from HIV negative patients (continue).

Isolates	Repeats	Solvents - MIC and MFC (mg/ml)													
		Methanol		DCM/ Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
N11	1	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56
	2	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56
	3	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56
	Median	**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**0.39**	**0.39**	**1.56**	**1.56**
N33	1	6.25	6.25	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56
	2	6.25	6.25	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56
	3	6.25	6.25	6.25	6.25	1.56	1.56	1.56	156	1.56	1.56	0.39	0.39	1.56	1.56
	Median	**6.25**	**6.25**	**6.25**	**6.25**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**0.39**	**0.39**	**1.56**	**1.56**
N35	1	3.13	3.13	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	3.13	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	3.13	3.13	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**3.13**	**3.13**	**6.25**	**6.25**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N43	1	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N47	1	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56
	2	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56
	3	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56
	Median	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**0.39**	**0.39**	**1.56**	**1.56**
Combined median		**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**

DMSO had no effect on the *C. albicans* at the concentration used in the study

Table 3.5 Summary of Median MIC and MFC of crude peel extract (PLE) against *C. albicans* isolated from the HIV positive patients.

Isolates	Solvents – Median MIC and MFC (mg/ml)													
	Methanol		DCM/Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
274	6.25	6.25	3.13	3.13	1.56	1.56	3.13	3.13	3.13	3.13	1.56	1.56	0.78	0.78
275	6.25	6.25	3.13	3.13	1.56	1.56	3.13	3.13	3.13	3.13	3.13	3.13	1.56	1.56
276	3.13	6.25	3.13	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
277	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	0.78	0.78
279	6.25	6.25	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
C2	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
C3	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56
C5	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
C13	3.13	3.13	1.56	1.56	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
C25	6.25	6.25	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56

Table 3.6 Summary of Median MIC and MFC of crude peel extract (PLE) against *C. albicans* isolated from the HIV negative patients.

Isolates	Solvents – Median MIC and MFC (mg/ml)													
	Methanol		DCM/Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
N2	3.13	6.26	6.25	6.25	1.56	1.56	1.56	1.56	3.13	3.13	3.13	3.13	1.56	1.56
N3	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N8	3.13	6.25	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N9	6.25	12.5	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N11	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56
N16	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N33	6.25	6.25	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56
N35	3.13	3.13	6.25	6.25	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N43	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N47	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	0.39	0.39	1.56	1.56

3.3 Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentrations (MFC) of crude seed extract

The results of the MIC and MFC of the peel extracts are shown in Tables 3.7 to 3.10. The MIC and MFC values of seed extract against *C. albicans* isolated from HIV positive patients are shown in Table 3.7, HIV negative patients in Table 3.8 and the summary of the results for HIV positive and HIV negative patients in Tables 3.9 and 3.10 respectively in Table 3.9. All the test solvents showed antifungal properties. The median MIC and MFC values of the ethanol, acetone, hexane and water against all the test isolates was 1.56 mg/ml. DCM/methanol showed slightly higher MIC and MFC values of 3.13 mg/ml. In addition, methanol and ethyl acetate showed slightly higher MIC and MFC against isolates from HIV positive patients. The methanol extract showed the highest MIC and MFC values of 6.25 mg/ml against strains of *C. albicans* isolated from HIV positive patients. The lowest MIC/MFC was 0.78 mg/ml.

No difference was found in the MIC and MFC values between the strains of *C. albicans* isolated from the HIV positive and HIV negative patients.

Although MFC values were expected to be higher than MIC, for all the seed extracts, the MIC and MFC values were similar.

Table 3.7: MIC and MFC of pomegranate seed extracts against *C. albicans* isolated from HIV positive patients.

Isolates	Repeats	Solvents - MIC and MFC (mg/ml)													
		Methanol		DCM/ Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
274	1	6.25	6.25	3.13	6.25	1.56	1.56	0.78	0.78	3.13	1.56	1.56	1.56	3.13	3.13
	2	6.25	6.25	3.13	6.25	1.56	1.56	0.78	0.78	3.13	6.25	1.56	1.56	3.13	3.13
	3	6.25	6.25	3.13	6.25	1.56	1.56	0.78	0.78	3.13	6.25	0.78	1.56	3.13	3.13
	Median	**6.25**	**6.25**	**3.13**	**6.25**	**1.56**	**1.56**	**0.78**	**0.78**	**3.13**	**6.25**	**1.56**	**1.56**	**3.13**	**3.13**
275	1	6.25	6.25	0.78	0.78	1.56	1.56	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56
	2	6.25	6.25	0.78	0.78	1.56	1.56	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56
	3	6.25	6.26	0.78	0.78	1.56	1.56	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**0.78**	**0.78**	**1.56**	**1.56**	**1.56**	**1.56**	**0.78**	**0.78**	**1.56**	**1.56**	**1.56**	**1.56**
276	1	6.25	6.25	1.56	1.56	3.13	3.13	1.56	3.13	1.56	1.56	3.13	6.25	1.56	1.56
	2	6.25	6.25	1.56	1.56	3.13	3.13	1.56	3.13	1.56	1.56	3.13	6.25	1.56	1.56
	3	6.25	6.25	1.56	1.56	3.13	3.13	1.56	3.13	1.56	1.56	3.13	6.25	1.56	1.56
	Median	**6.25**	**6.25**	**1.56**	**1.56**	**3.13**	**3.13**	**1.56**	**3.13**	**1.56**	**1.56**	**3.13**	**6.25**	**1.56**	**1.56**
277	1	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	3.13	3.13	1.56	3.13	1.56	1.56
	2	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	3.13	3.13	1.56	3.13	1.56	1.56
	3	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	3.13	3.13	3.13	1.56	1.56
	Median	**6.25**	**6.35**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**3.13**	**3.13**	**1.56**	**3.13**	**1.56**	**1.56**
279	1	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	3.13	6.25	3.13	3.13	3.13	3.13
	2	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	3.13	6.25	3.13	3.13	3.13	3.13
	3	31.3	3.13	3.13	3.13	1.56	1.56	1.56	1.56	3.13	6.25	3.13	3.13	3.13	3.13
	Median	**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**3.13**	**6.25**	**3.13**	**3.13**	**3.13**	**3.13**

DMSO had no effect on the *C. albicans* at the concentration used in the study

Table 3.7a: MIC and MFC of pomegranate seed extracts against *C. albicans* isolated from HIV positive patients (continue).

Isolates	Repeats	Solvents - MIC and MFC (mg/ml)													
		Methanol		DCM/ Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C2	1	6.25	6.25	1.56	1.56	0.78	0.78	1.56	1.56	3.13	3.13	3.13	3.13	0.78	0.78
	2	6.25	6.25	1.56	1.56	0.78	0.78	1.56	1.56	3.13	3.13	3.13	3.13	0.78	0.78
	3	6.25	6.25	1.56	1.56	0.78	0.78	1.56	1.56	1.56	3.13	3.13	3.13	0.78	0.78
	Median	**6.25**	**6.25**	**1.56**	**1.56**	**0.78**	**0.78**	**1.56**	**1.56**	**3.13**	**3.13**	**3.13**	**3.13**	**0.78**	**0.78**
C3	1	6.25	6.25	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56
	2	6.25	6.25	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56
	3	6.25	6.25	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56
	Median	**6.25**	**6.25**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**3.13**	**3.13**	**1.56**	**1.56**
C5	1	6.25	6.25	6.25	6.25	1.56	1.56	0.39	0.39	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	6.25	6.25	6.25	1.56	1.56	0.39	0.39	1.56	1.56	1.56	1.56	1.56	1.56
	3	6.25	6.25	6.25	6.26	1.56	1.56	0.39	0.39	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**6.25**	**6.25**	**1.56**	**1.56**	**0.39**	**0.39**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
C13	1	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	6.25	3.13	1.56	1.56
	2	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	6.25	3.13	1.56	1.56
	3	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56
	Median	**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**6.25**	**3.13**	**1.56**	**1.56**
C25	1	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56
	2	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56
	3	6.25	6.26	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56
	Median	**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**3.13**	**3.13**	**1.56**	**1.56**
Combined median		**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**3.13**	**3.13**	**1.56**	**1.56**

DMSO had no effect on the *C. albicans* at the concentration used in the study

Table 3.8: MIC and MFC of pomegranate seed extracts against *C. albicans* isolated from HIV negative patients.

Isolates	Repeats	Solvents - MIC and MFC (mg/ml)													
		Methanol		DCM/ Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
N2	1	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	1.56	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N3	1	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N8	1	6.25	6.25	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
	2	6.25	6.25	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
	3	6.25	6.25	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**6.25**	**6.25**	**1.56**	**1.56**	**1.56**	**1.56**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N9	1	3.13	3.13	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	3.13	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
	3	3.13	3.13	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N16	1	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**

DMSO had no effect on the *C. albicans* at the concentration used in the study

Table 3.8a: MIC and MFC of pomegranate seed extracts against *C. albicans* isolated from HIV negative patients (continue).

Isolates	Repeats	Solvents - MIC and MFC (mg/ml)													
		Methanol		DCM/ Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
N11	1	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N33	1	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N35	1	1.56	1.56	3.13	3.13	3.13	3.13	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56
	2	1.56	1.56	3.13	3.13	3.13	3.13	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56
	2	1.56	1.56	3.13	3.13	3.13	3.13	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56
	Median	**1.56**	**1.56**	**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**	**0.78**	**0.78**	**1.56**	**1.56**	**1.56**	**1.56**
N43	1	1.56	1.56	3.13	3.13	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
	2	1.56	1.56	3.13	3.13	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
	3	1.56	1.56	3.13	3.13	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**1.56**	**1.56**	**3.13**	**3.13**	**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
N47	1	3.13	3.13	3.13	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	2	3.13	3.13	3.13	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	3	3.13	3.13	3.13	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56
	Median	**3.13**	**3.13**	**3.13**	**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**
Combined median		**3.13**	**3.13**	**3.13**	**3.13**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**	**1.56**

DMSO had no effect on the *C. albicans* at the concentration used in the study

Table 3.9 Summary of Median MIC and MFC of crude seed extract against *C. albicans* isolated from the HIV positive patients.

Isolates	Solvents – Median MIC and MFC (mg/ml)													
	Methanol		DCM/Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
274	6.25	6.25	3.13	6.25	1.56	1.56	0.78	0.78	3.13	6.25	1.56	1.56	3.13	3.13
275	6.25	6.25	0.78	0.78	1.56	1.56	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56
276	6.25	6.25	1.56	1.56	3.13	3.13	1.56	3.13	1.56	1.56	3.13	6.25	1.56	1.56
277	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	3.13	3.13	1.56	3.13	1.56	1.56
279	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	3.13	6.25	3.13	3.13	3.13	3.13
C2	6.25	6.25	1.56	1.56	0.78	0.78	1.56	1.56	3.13	3.13	3.13	3.13	0.78	0.78
C3	6.25	6.25	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56
C5	6.25	6.25	6.25	6.25	1.56	1.56	0.39	0.39	1.56	1.56	1.56	1.56	1.56	1.56
C13	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	3.13	1.56	1.56
C25	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56

Table 3.10 Summary of Median MIC and MFC of crude seed extract against *C. albicans* isolated from the HIV negative patients.

Isolates	Solvents – Median MIC and MFC (mg/ml)													
	Methanol		DCM/Methanol		Ethanol		Acetone		Hexane		Ethyl Acetate		Water	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
N2	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N3	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N8	6.25	6.25	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
N9	3.13	3.13	1.56	1.56	1.56	1.56	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
N11	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N16	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N33	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
N35	1.56	1.56	3.13	3.13	3.13	3.13	1.56	1.56	0.78	0.78	1.56	1.56	1.56	1.56
N43	1.56	1.56	3.13	3.13	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56
N47	3.13	3.13	3.13	3.13	3.13	3.13	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56

3.4 Effect of *P. granatum* fruit peel on the adherence of *C. albicans* isolated from HIV positive and HIV negative patients to epithelial cells

The results of the effect of *P. granatum* fruit peel on the adherence of *C. albicans* isolated from HIV positive and HIV negative patients to epithelial cells are shown in the Table 3.11 and Figure 3.1. All the test isolates showed adherence property to the oral epithelial cells. In the *C. albicans* isolated from HIV positive patients, adherence was reduced from 331 to 321 cells (3% reduction) by the plant extract. This reduction was not significant with a p value of > 0.05. Similarly, in the *C. albicans* isolated from HIV negative patients, the adherence was reduced from 242.9 to 213.3 cells (12.2% reduction)by the plant extract. This reduction was also not significant with a p value of > 0.05. In addition, the reduction in the adherence was not significantly different between the *C. albicans* isolates from HIV positive and HIV negative patients ($p \geq 0.05$).

Table 3.11 Effect of the sub-inhibitory concentration of methanol peel extract (PLE) of *P. granatum* on the adherence of *C. albicans* isolated from the HIV positive and HIV negative patients.

HIV positive				**HIV negative**			
Isolates	**Repeats**	**No. of *C. albicans***		**Isolates**	**Repeats**	**No. of *C. albicans***	
		Control	**PLE**			**Control**	**PLE**
274	1	271	54	N2	1	139	103
	2	260	104		2	74	93
	3	218	61		3	130	109
	Mean	**250**	**73**		**Mean**	**114**	**102**
275	1	240	97	N3	1	163	60
	2	212	85		2	115	89
	3	241	109		3	122	31
	Mean	**231**	**97**		**Mean**	**133**	**60**
276	1	61	77	N8	1	483	547
	2	78	69		2	457	297
	3	100	87		3	400	511
	Mean	**80**	**78**		**Mean**	**447**	**452**
277	1	1271	854	N9	1	261	225
	2	1360	799		2	313	188
	3	1119	988		3	440	102
	Mean	**1250**	**880**		**Mean**	**338**	**172**
279	1	172	928	N16	1	80	379
	2	145	861		2	132	421
	3	176	1038		3	99	451
	Mean	**164**	**942**		**Mean**	**104**	**417**
C2	1	302	249	N11	1	99	196
	2	228	302		2	60	88
	3	324	293		3	55	82
	Mean	**285**	**281**		**Mean**	**71**	**122**
C3	1	277	246	N33	1	80	91
	2	329	299		2	84	104
	3	344	270		3	76	171
	Mean	**317**	**272**		**Mean**	**80**	**98**
C5	1	409	108	N35	1	429	229
	2	183	127		2	545	273
	3	230	151		3	537	238
	Mean	**274**	**129**		**Mean**	**504**	**247**
C13	1	264	224	N43	1	93	65
	2	156	216		2	145	166
	3	204	178		3	125	85
	Mean	**208**	**206**		**Mean**	**121**	**105**
C25	1	251	267	N47	1	472	345
	2	282	256		2	600	326
	3	235	248		3	481	335
	Mean	**256**	**257**		**Mean**	**518**	**335**
Combined mean±SD		**331±322.2**	**321.5±312.2**			**242.9±184.3**	**213.3±144.9**

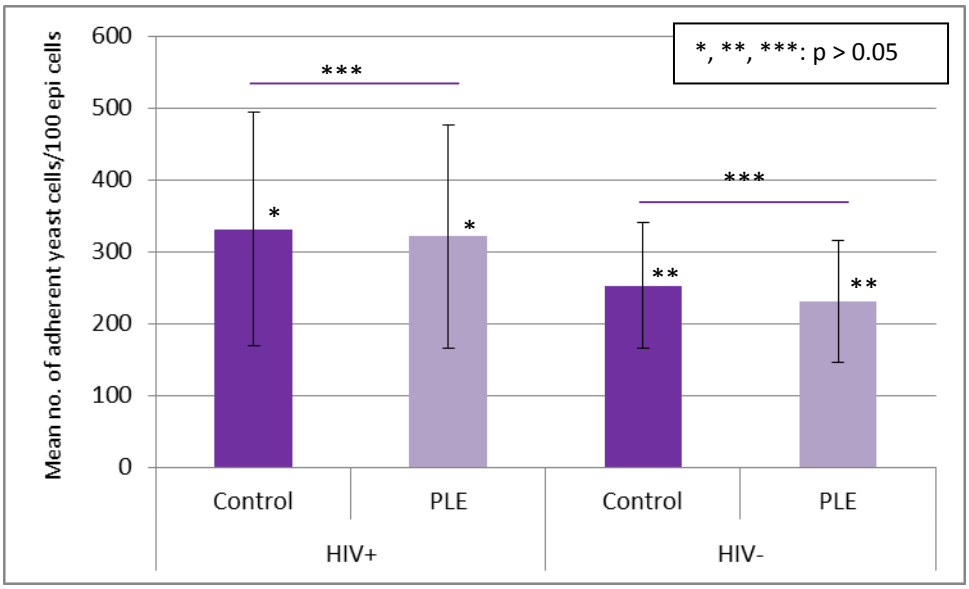

Figure 3.1 Effect of the sub-inhibitory concentration of methanol peel extract (PLE) of *P. granatum* on the adherence of *C. albicans* isolated from the HIV positive and HIV negative patients (summary). Water was used as a negative control. *,** and ***: $p \geq 0.05$

3.5 Effect of *P. granatum* fruit peel on the germ tube formation by *C. albicans* isolated from HIV positive and HIV negative patients

The results of the effect of *P. granatum* fruit peel on the germ tube formation by *C. albicans* isolated from HIV positive and HIV negative patients are shown in the Table 3.12 and Figure 3.2. All the test isolates produced germ tubes in the presence of horse serum within 2.5 hours. In the *C. albicans* isolated from HIV positive patients, germ tube formation was reduced from 68.7 to 51.1 cells (26% reduction) by the plant extract. This reduction was significant with a p value of < 0.01. Similarly, in the *C. albicans* isolated from HIV negative patients, germ tube formation was reduced from 72.4 to 38.4 cells (34% reduction) by the plant extract. This reduction was also significant with a p value of < 0.01. However, the reduction in the germ tube formation was not significantly different between the isolates from HIV positive and HIV negative patients ($p > 0.05$).

Table 3.12 Effect of the sub-inhibitory concentration of methanol peel extract (PLE) of *P. granatum* on the germ tube (GT) formation by *C. albicans* isolated from the HIV positive and HIV negative patients.

HIV positive				**HIV negative**			
Isolates	**Repeats**	**No. of cells with GT***		**Isolates**	**Repeats**	**No. of cells with GT***	
		Control	**PLE**			**Control**	**PLE**
274	1 2 3	88 93 92	34 54 36	N2	1 2 3	29 54 46	0 0 0
	Mean	**91**	**42**		**Mean**	**43**	**0**
275	1 2 3	67 66 74	62 57 60	N3	1 2 3	69 81 76	0 0 0
	Mean	**69**	**60**		**Mean**	**75**	**0**
276	1 2 3	2 2 1	1 3 5	N8	1 2 3	93 90 96	42 70 50
	Mean	**2**	**5**		**Mean**	**93**	**54**
277	1 2 3	1 82 86	80 79 76	N9	1 2 3	99 91 92	0 0 2
	Mean	**56**	**78**		**Mean**	**94**	**1**
279	1 2 3	92 99 98	87 82 87	N16	1 2 3	88 75 91	67 54 46
	Mean	**96**	**85**		**Mean**	**85**	**56**
C2	1 2 3	66 88 92	65 64 84	N11	1 2 3	77 64 58	60 75 80
	Mean	**82**	**71**		**Mean**	**66**	**72**
C3	1 2 3	67 58 63	64 85 76	N33	1 2 3	39 57 51	37 60 55
	Mean	**63**	**75**		**Mean**	**49**	**51**
C5	1 2 3	72 72 76	5 14 5	N35	1 2 3	52 72 86	85 40 69
	Mean	**73**	**8**		**Mean**	**70**	**65**
C13	1 2 3	71 72 84	35 32 27	N43	1 2 3	64 88 70	0 2 10
	Mean	76	31		Mean	74	4
C25	1 2 3	81 73 83	57 53 65	N47	1 2 3	74 79 72	83 81 85
	Mean	**79**	**58**		**Mean**	**75**	**83**
Combined mean±SD		**68.7±28.8**	**51.1±28.7**			**72.4±18.0**	**38.4±33.4**

*Number of *C. albicans* with germ tube formation was measured per total of 100 cells.

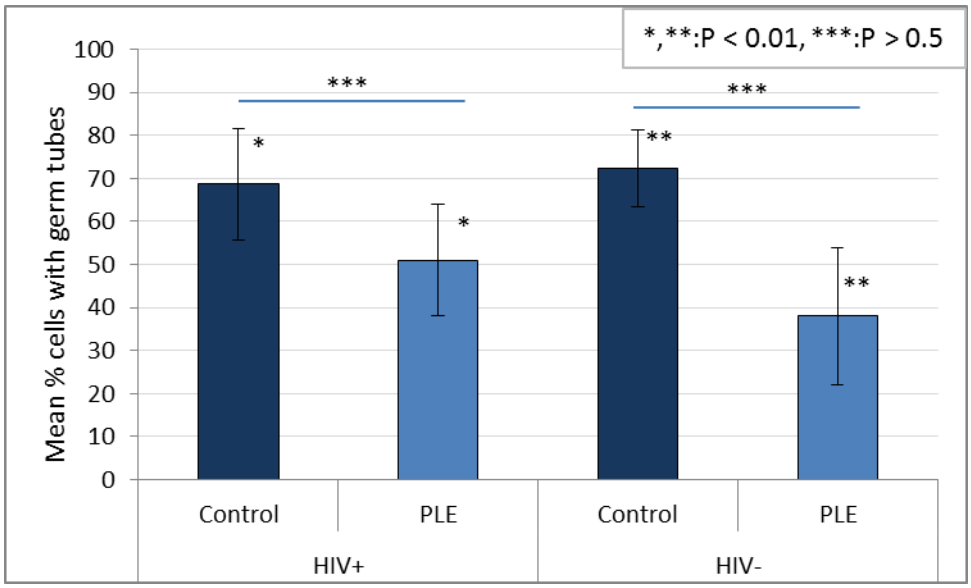

Figure 3.2 Effect of the sub-inhibitory concentration of methanol peel extract (PLE) of *P. granatum* on the germ tube formation by *C. albicans* isolated from the HIV positive and HIV negative patients (summary). Water was used as a negative control. * and **: $p < 0.01$, ***: $p > 0.05$

3.6 Effect of *P. granatum* fruit peel on the production of phospholipase by *C. albicans* isolated from HIV positive and HIV negative patients

Not all the *C. albicans* strains used in the MIC determination, adherence and germ tube formation study produced phospholipase. Therefore, 16 strains isolated from HIV positive patients and 21 strains isolated from HIV negative patients were screened before selecting 10 phospholipase producing strains from each group to study the inhibitory effect of *P. granatum.*

The results of the effect of *P. granatum* fruit peel on the production of phospholipase by *C. albicans* isolated from HIV positive and HIV negative patients are shown in Tables 3.13 and 3.14 respectively, with a summary of results in Table 3.15 and Figure 3.3. In the *C. albicans* isolated from HIV positive patients (Table 3.13), production of phospholipase was increased from 0.76 to 0.73 Pz (4% increase) by the plant extract. This increase was not significant with a p value of > 0.05. Conversely, in the *C. albicans* isolated from HIV negative patients (Table 3.14), the production of phospholipase was slightly reduced from 0.77 to 0.78 Pz (1.3% decrease) by the plant extract. This reduction was also not significant with a p value of > 0.05. In addition, there was no significant difference in the results obtain for the *C. albicans* isolated from both the study groups ($p > 0.05$). Interestingly, *C. albicans* (ATCC 90028) always produced phospholipase and the Pz values were approximately 0.54, while *C. parapsilosis* did not produce phospholipase and the Pz values were 1.

Table 3.13 Effect of the sub-inhibitory concentration of methanol peel extract of *P. granatum* on the production of phospholipase (Pz) by *C. albicans* isolated from the HIV positive patients.

***C. albicans* isolated from HIV positive patients**						
Isolates	**Control**			**Peel extract**		
	Colony diameter (mm)	**Precipitation diameter (mm)**	**Pz**	**Colony diameter (mm)**	**Precipitation diameter (mm)**	**Pz**
2.1	15.00	16.00	0.94	16.00	17.00	0.94
3.1	18.00	22.00	0.82	15.00	25.00	0.60
16.1	14.00	19.00	0.74	12.10	18.40	0.66
24.1	15.00	21.00	0.71	15.00	20.00	0.75
25.1	15.00	17.00	0.88	17.00	19.00	0.89
64.1	14.00	20.90	0.67	13.40	21.20	0.63
77.1	16.00	19.00	0.84	14.50	19.00	0.76
187.1	13.00	24.00	0.54	12.50	25.50	0.49
196.1	11.00	17.00	0.65	12.00	16.00	0.75
C1	13.90	16.50	0.84	13.50	16.20	0.83
Mean			**0.76**			**0.73**

Where Pz = colony diameter divided by the precipitation diameter. Ratio of 1 = no enzyme production whereas a lower ratio indicates a higher production of enzyme.

Table 3.14 Effect of the sub-inhibitory concentration of methanol peel extract of *P. granatum* on the production of phospholipase by *C. albicans* isolated from the HIV negative patients.

***C. albicans* isolated from HIV negative patients**						
Isolates	**Control**			**Peel extract**		
	Colony diameter (mm)	**Precipitation diameter (mm)**	**Pz**	**Colony diameter (mm)**	**Precipitation diameter (mm)**	**Pz**
N3	15.00	19.00	0.79	21.00	26.10	0.80
N8	14.80	16.00	0.93	14.00	17.80	0.78
N16	18.00	21.00	0.86	19.20	20.00	0.96
N40	12.00	26.00	0.48	14.00	27.00	0.52
N43	14.00	18.00	0.78	14.00	17.00	0.82
N44	12.00	20.00	0.60	12.10	22.00	0.55
Y13	11.10	15.50	0.72	11.10	13.00	0.85
Y35	18.00	19.10	0.94	13.00	14.50	0.90
Y46	14.00	15.50	0.90	15.00	15.50	0.97
Y69	11.00	15.00	0.73	12.10	18.90	0.64
Mean			**0.77**			**0.78**

Where Pz = colony diameter divided by the precipitation diameter. Ratio of 1 = no enzyme production whereas a lower ratio indicates a higher production of enzyme.

Table 3.15 Summary of the effect of the sub-inhibitory concentration of methanol peel extract (PLE) of *P. granatum* on the production of phospholipase by *C. albicans* isolated from the HIV positive and HIV negative patients.

Phospholipase (Pz)					
***C. albicans* isolated from HIV positive patients**			***C. albicans* isolated from HIV negative patients**		
Isolates	**Control**	**PLE**	**Isolates**	**Control**	**PLE**
2.1	0.94	0.94	N8	0.79	0.80
64.1	0.82	0.60	N16	0.93	0.78
3.1	0.74	0.66	Y33	0.86	0.96
16.1	0.71	0.75	N35	0.48	0.52
24.1	0.88	0.89	N43	0.78	0.82
25.1	0.67	0.63	Y2	0.60	0.55
26.1	0.84	0.76	Y13	0.72	0.85
77.1	0.54	0.49	Y18	0.94	0.90
187.1	0.65	0.75	Y20	0.90	0.97
C2	0.84	0.83	Y69	0.73	0.64
Mean±SD	**0.76±0.12**	**0.73±0.14**		**0.77±0.15**	**0.78±0.16**

Where Pz = colony diameter divided by the precipitation diameter. Ratio of 1 = no enzyme production whereas a lower ratio indicates a higher production of enzyme.

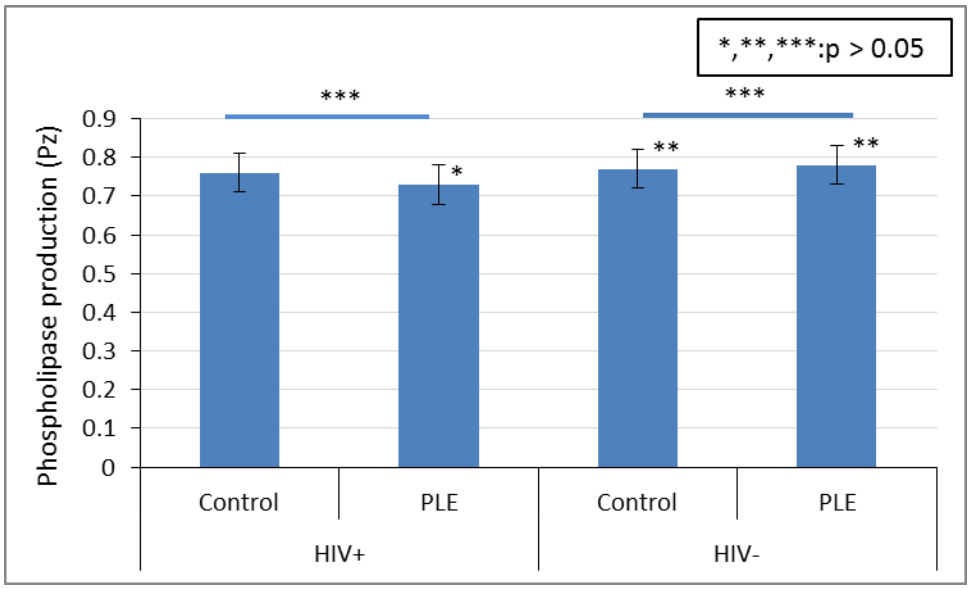

Figure 3.3 Effect of the sub-inhibitory concentration of methanol peel extract (PLE) of

P. granatum on the production of phospholipase by *C. albicans* isolated from the HIV positive and HIV negative patients (summary). Water was used as a negative control. *,** and ***: p > 0.05.

3.7 Effect of *P. granatum* fruit peel on the production of proteinase by *C. albicans* isolated from HIV positive and HIV negative patients

Not all the *C. albicans* strains used in the determination of the MIC, adherence and germ tube formation study produced proteinase. Therefore, 14 strains isolated from HIV positive patients and 20 strains isolated from HIV negative patients were screened before selecting 10 proteinase producing strains from each of the groups to study the inhibitory effect of *P. granatum.*

The results of the effect of *P. granatum* fruit peel on the production of proteinase by *C. albicans* isolated from HIV positive and HIV negative patients are shown in Tables 3.16 and 3.17 respectively, with a summary of results in Table 3.18 and Figure 3.4. In the *C. albicans* isolated from HIV positive patients (Table 3.16), production of proteinase was decreased from 0.93 to 0.95 Pr (2% decrease) by the plant extract. This decrease was not significant with a p value of > 0.05. Similarly, in the *C. albicans* isolated from HIV negative patients (Table 3.17), the production of proteinase was reduced from 0.89 to 0.92 Pr (3.4% reduction) by the plant extract. This reduction was also not significant with a p value of > 0.05. In addition there was no significant difference in the results obtain for the *C. albicans* isolated from both the study groups (p > 0.05). Of importance, *C. albicans* (ATCC 90028) produced proteinase and the Pr values were approximately 0.69, while *C. parapsilosis* produced no proteinase and the values were 1.

Table 3.16 Effect of the sub-inhibitory concentration of methanol peel extract of *P. granatum* on the production of proteinase (Pr) by *C. albicans* isolated from the HIV positive patients.

***C. albicans* isolated from HIV positive patients**						
Isolates	**Control**			**Peel extract**		
	Colony diameter (mm)	**Precipitation diameter (mm)**	**Pr**	**Colony diameter (mm)**	**Precipitation diameter (mm)**	**Pr**
2.1	28.00	28.70	0.98	28.30	28.80	0.98
64.1	27.40	28.20	0.97	25.20	26.10	0.97
3.1	26.10	27.00	0.97	24.50	27.00	0.91
16.1	16.10	18.10	0.89	14.30	15.00	0.95
24.1	26.70	27.10	0.99	25.00	27.50	0.91
25.1	25.00	25.10	1.00	28.40	29.10	0.98
26.1	30.90	32.00	0.97	30.00	30.10	1.00
77.1	15.00	24.10	0.62	16.10	18.10	0.89
187.1	25.50	27.50	0.93	26.30	26.50	0.97
C2	24.00	24.10	1.00	24.10	24.80	0.97
Mean			**0.93**			**0.95**

Where Pr = colony diameter divided by the light colour zone diameter. Ratio of 1 = no enzyme production whereas a lower ratio indicates a higher production of enzyme.

Table 3.17 Effect of the sub-inhibitory concentration of methanol peel extract of *P. granatum* on the production of proteinase (Pr) by *C. albicans* isolated from the HIV negative patients.

***C. albicans* isolated from HIV negative patients**						
Isolates	**Control**			**Peel extract**		
	Colony diameter (mm)	**Precipitation diameter (mm)**	**Pr**	**Colony diameter (mm)**	**Precipitation diameter (mm)**	**Pr**
N8	25.20	26.10	0.97	24.90	26.50	0.94
N16	14.40	15.50	0.93	13.70	14.10	0.97
Y33	17.60	18.60	0.95	26.50	21.90	0.83
N35	21.60	24.50	0.88	16.50	17.40	0.95
N43	24.00	25.10	0.96	24.40	25.30	0.96
Y2	17.00	22.20	0.77	19.20	19.90	0.96
Y13	12.70	16.00	0.79	13.00	14.00	0.93
Y18	27.50	29.00	0.95	26.50	32.40	0.82
Y20	27.10	28.70	0.94	26.60	27.70	0.96
Y69	15.40	18.50	0.83	17.20	19.10	0.90
Mean			**0.89**			**0.92**

Where Pr = colony diameter divided by the light colour zone diameter. Ratio of 1 = no enzyme production whereas a lower ratio indicates a higher production of enzyme.

Table 3.18 Summary of the effect of the sub-inhibitory concentration of methanol peel extract (PLE) of *P. granatum* on the production of proteinase by *C. albicans* isolated from the HIV positive and HIV negative patients.

Proteinase (Pr)					
***C. albicans* isolated from HIV positive patients**			***C. albicans* isolated from HIV negative patients**		
Isolates	**Control**	**PLE**	**Isolates**	**Control**	**PLE**
2.1	0.98	0.98	N8	0.97	0.94
64.1	0.97	0.97	N16	0.93	0.97
3.1	0.97	0.91	Y33	0.95	0.83
16.1	0.89	0.95	N35	0.88	0.95
24.1	0.99	0.91	N43	0.96	0.96
25.1	1.00	0.98	Y2	0.77	0.96
26.1	0.97	1.00	Y13	0.79	0.93
77.1	0.62	0.89	Y18	0.95	0.82
187.1	0.93	0.97	Y20	0.94	0.96
C2	1.00	0.97	Y69	0.83	0.90
Mean±SD	**0.93±0.11**	**0.95±0.04**		**0.89±0.07**	**0.92±0.05**

Where Pr = colony diameter divided by the light colour zone diameter. Ratio of 1 = no enzyme production whereas a lower ratio indicates a higher production of enzyme.

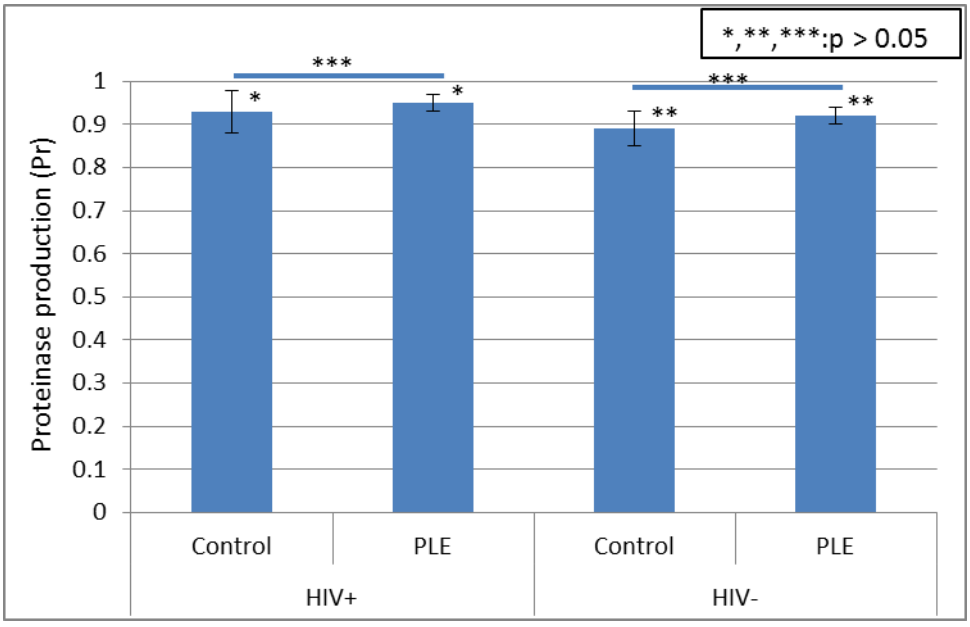

Figure 3.4 Effect of the subinhibitory concentration of methanol peel extract (PLE) of *P. granatum* on the production of proteinase by *C. albicans* isolated from the HIV positive and HIV negative patients (summary). Water was used as a negative control. *,**and ***: p > 0.05

3.8 Effect of *P. granatum* fruit peel on the production of lipase by *C. albicans* isolated from HIV positive and HIV negative patients

Not all the *C. albicans* strains used in the determination of MIC values, adherence and germ tube formation studies produced lipase. Therefore, 19 strains isolated from HIV positive patients and 49 strains isolated from HIV negative patients were screened before selecting 10 lipase producing strains from each group to study the inhibitory effect of *P. granatum*.

The results of the effect of *P. granatum* fruit peel on the production of lipase by *C. albicans* isolated from HIV positive and HIV negative patients are shown in Tables 3.19 and 3.20, with a summary of results in Table 3.21 and Figure 3.5. In the *C. albicans* isolated from HIV positive patients (Table 3.19), production of lipase decreased from 0.67 to 0.73 Pl (9% decrease) by the plant extract. This decrease was not significant with a p value of > 0.05. Similarly, in the *C. albicans* isolated from HIV negative patients (Table 3.20), the production of lipase was reduced from 0.71 to 0.75 Pl (5.6% reduction) by the plant extract. This reduction was also not significant with a p value of > 0.05. In addition, there was no significant difference in the results obtain for the *C. albicans* isolated from both the study groups ($p \geq 0.05$). Importantly, *C. albicans* (ATCC 90028) produced lipase and the Pl values were approximately 0.73, while *C. parapsilosis* did not produced proteinase and the Pl values were 1.

Table 3.19 Effect of the sub-inhibitory concentration of methanol peel extract of *P. granatum* on the production of lipase by *C. albicans* isolated from the HIV positive patients.

***C. albicans* isolated from HIV positive patients**						
Isolates	**Control**			**Plant extract**		
	Colony diameter (mm)	**Precipitation diameter (mm)**	**Pl**	**Colony diameter (mm)**	**Precipitation diameter (mm)**	**Pl**
C1	13.00	22.00	0.59	13.40	20.00	0.67
2.1	11.00	16.50	0.67	11.50	18.90	0.61
3.1	12.60	19.50	0.65	11.50	23.00	0.50
24.1	13.50	20.00	0.68	13.00	16.50	0.79
25.1	12.50	18.50	0.68	13.50	16.00	0.84
26.1	13.20	22.50	0.59	12.00	19.50	0.62
196.1	11.50	19.20	0.59	12.30	16.10	0.76
274	13.20	16.00	0.83	10.50	12.00	0.88
281	12.90	18.00	0.72	11.10	14.00	0.79
329	12.00	16.20	0.74	12.00	14.00	0.86
Mean			**0.67**			**0.73**

Where PI = colony diameter divided by the clear zone diameter.

Ratio of 1 = no enzyme production whereas a lower ratio indicates a higher production of enzyme.

Table 3.20 Effect of the sub-inhibitory concentration of methanol peel extract of *P. granatum* on the production of lipase (Pl) by *C. albicans* isolated from the HIV negative patients.

***C. albicans* isolated from HIV negative patients**						
Isolates	**Control**			**Plant extract**		
	Colony diameter (mm)	**Precipitation diameter (mm)**	**Pl**	**Colony diameter (mm)**	**Precipitation diameter (mm)**	**Pl**
N8	10.00	14.00	0.71	11.00	16.50	0.67
N9	11.60	14.90	0.78	10.50	16.10	0.65
N16	12.50	19.00	0.66	12.00	16.50	0.73
N43	13.00	19.20	0.68	14.40	18.00	0.80
N44	12.00	19.60	0.61	12.50	16.00	0.78
Y18	14.30	17.00	0.84	13.50	15.00	0.90
Y33	12.20	14.00	0.87	11.80	13.50	0.87
Y69	13.00	20.50	0.63	12.00	17.50	0.69
Y77	11.00	19.00	0.58	11.50	19.00	0.61
Y118	10.00	13.10	0.76	10.00	12.10	0.83
Mean			**0.71**			**0.75**

Where Pl = colony diameter divided by the clear zone diameter.

Ratio of 1 = no enzyme production whereas a lower ratio indicates a higher production of enzyme.**Table 3.21** Summary of the effect of a sub inhibitory concentration of methanol peel

extract (PLE) of *P. granatum* on the production of lipase (Pl) by *C. albicans* isolated from the HIV positive and HIV negative patients.

Lipase **(Pl)**					
***C. albicans* isolated from HIV positive patients**			***C. albicans* isolated from HIV negative patients**		
Isolates	**Control**	**PLE**	**Isolates**	**Control**	**PLE**
C1	0.59	0.67	N8	0.71	0.67
2.1	0.67	0.61	N9	0.78	0.65
3.1	0.65	0.50	N16	0.66	0.73
24.1	0.68	0.79	N43	0.68	0.80
25.1	0.68	0.84	N44	0.61	0.78
26.1	0.59	0.62	Y18	0.84	0.90
196.1	0.59	0.76	Y33	0.87	0.87
274	0.83	0.88	Y69	0.63	0.69
281	0.72	0.79	Y77	0.58	0.61
329	0.74	0.86	Y118	0.76	0.83
Mean±SD	**0.67±0.08**	**0.73±0.12**		**0.71±0.1**	**0.75±0.1**

Where PI = colony diameter divided by the clear zone diameter.

Ratio of 1 = no enzyme production whereas a lower ratio indicates a higher production of enzyme.

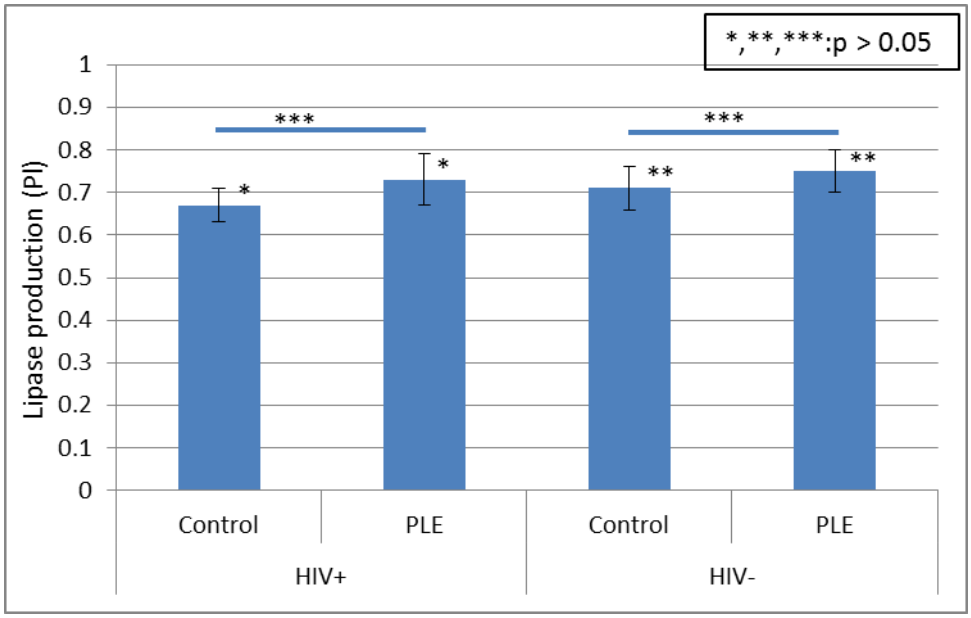

Figure 3.5 Effect of the sub-inhibitory concentration of methanol peel extract (PLE) of *P. granatum* on the production of lipase by *C. albicans* isolated from the HIV positive and HIV negative patients (summary). Water was used as a negative control. *,** and ***: p > 0.05

The results discussed in this section are summarized in Figure 4.1

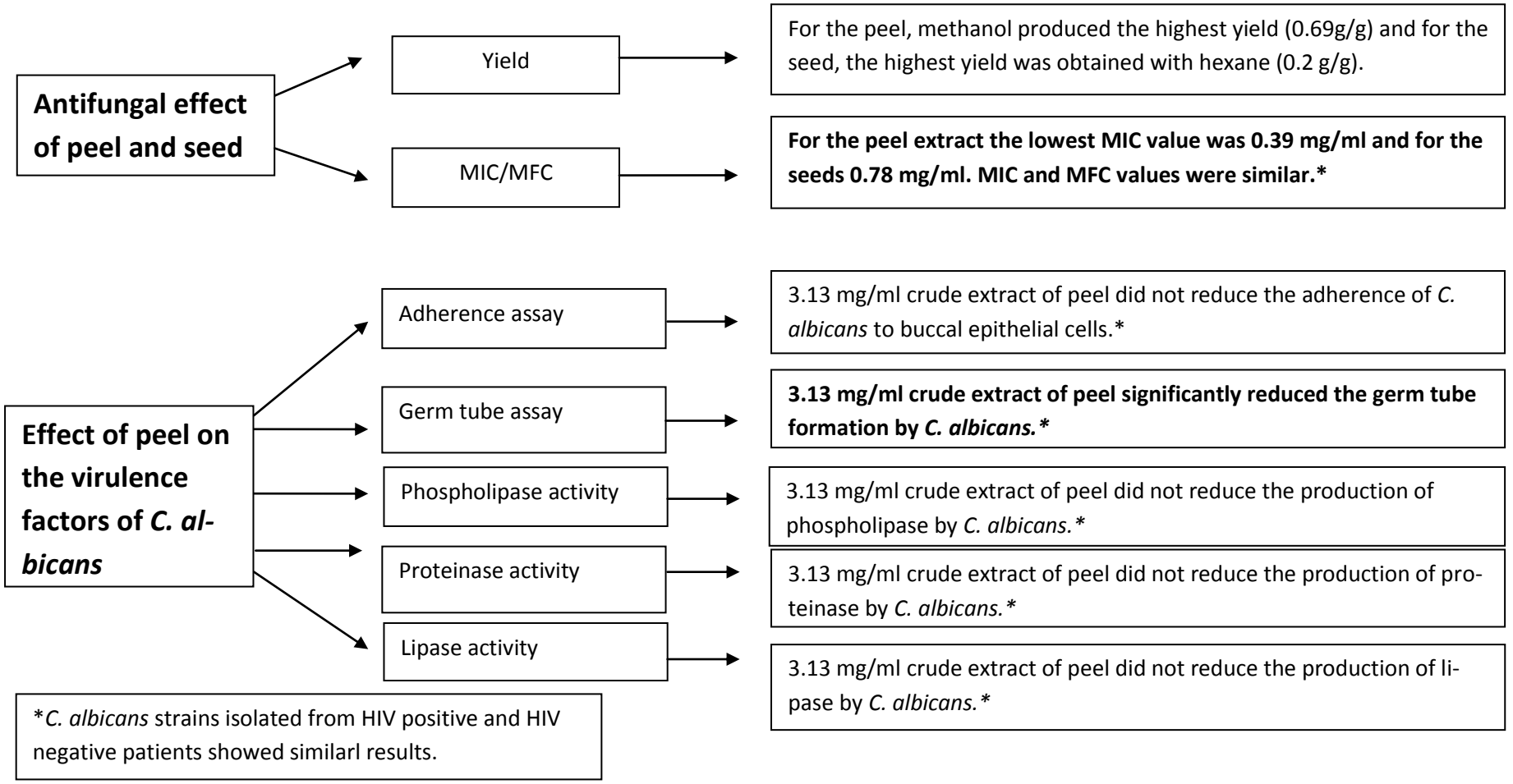

Figure 4.1 Study design and results – *P. granatum* and *C. albicans* isolated from HIV positive and HIV negative patients

CHAPTER 4: DISCUSSION

Oral candidiasis is a fungal infection which is commonly observed in immunocompromised patients especially HIV infected persons, terminally ill patients, people on prolonged antibiotics and with poor oral and denture hygiene. Oral candidiasis is mainly caused by *C. albicans.* Untreated oral candidiasis can cause a tremendous amount of pain, discomfort and eating disability in these patients. In addition, this infection can disseminate and cause oropharyngeal and systemic infections including candidaemia which can be fatal (Akpan and Morgan, 2002).

Compared to antibiotics, few drugs are available for the treatment of fungal infections. The drugs available have poor solubility and stability and they can be toxic because fungal cells are similar (eukaryotic) to the host cells. Furthermore, in many parts of the world *C. albicans* has developed resistance to the commonly used drugs (Ramesh *et al*., 2011, Tripathi *et al*., 2014). With the increasing rate of HIV/AIDS, fungal infections including oral candidiasis is increasing, which has increased the medical management cost of opportunistic infections. This has led to an urgent search for alternative antifungal therapy which can successfully treat oral candidiasis.

A novel therapeutic approach has been described by some investigators. They suggested that instead of developing therapeutic agents that can kill *C. albicans,* drugs should target the virulence factors of *C. albicans* such as adherence ability, hyphae formation and hydrolytic enzyme production (Gauwerky *et al*., 2009). In addition, the oral cavity is a unique site where it is difficult to maintain high concentrations of topical therapeutic compounds due to the constant flow of saliva. Therefore, it is important to study the effect of sub-therapeutic concentrations of topical therapeutic compounds, on the causative agents such as *C. albicans*.

Medicinal plants are an attractive source of therapeutic agents. Many traditionally used medicinal plants have for example an anticandidal effect (Runyoro *et al*., 2006). This study investigated the antifungal effect of *Punica granatum L* commonly known as pomegranate and its effect on the virulence properties of *C. albicans.*

4.1 Antifungal properties of *P. granatum*

4.1.1 Extraction yield

Many solvents such as methanol, ethanol, acetone, ethyl acetate, acetyl acetone and chloroform can be used to prepare extracts of *P. granatum* peel. In this study methanol produced the best yield (0.67 g/g dried peel powder), while water showed the second best results (0.54 g/g). Similar results have been shown by other researchers (Tayel and El-Tras, 2009; Singh *et al*., 2002). In contrast to the peel extract yield results, extracts of seed was found to be similar with different solvents (0.12 - 0.2 g/g seed powder). These results are contradicting the results obtained by Singh *et al*., (2002). They obtained high yields of seed extract using methanol and water. Seeds contain many oils composed of sterols and punicic acid which are conjugated fatty acids (Hora *et al*., 2003) and therefore difficult to extract. Methanol extracts are generally used for its antimicrobial properties (Dahham *et al*., 2010).

4.1.2 Minimum inhibitory concentration study

P. granatum is known to have an antibacterial effect. Much research has been done to determine the antibacterial property of this plant against enteric pathogens such as *Salmonella typhi, Escherichia coli, Bacillus cereus, Staphylococcus aureus* and *Klebsiella pneumonia* (Rani and Khullar, 2004; Dahham *et al*., 2010; Fawole *et al*., 2012). Some research has shown that *P. granatum* has an antibacterial effect against oral pathogens including *Streptococcus mutans*, a caries causing organism and *Porphyromonas gingivalis*, a periodontal pathogen (Abdollahzadeh *et al*., 2011; Rosas-Pinón *et a*l., 2012). It has also been shown that the peel extract can prevent adherence of cariogenic bacteria to the teeth and prevent plaque formation (Vasconcelos *et al*., 2006). Therefore, if these extracts were suggested to be used in the oral cavity, study of antimicrobial activity against other oral pathogens such as *C. albicans* was necessary. The MIC values of the peel extract ranged between 0.39 to 6.25 mg/ml

depending on the type of solvent used for the extraction. These results are much higher than those obtained by Anibal *et al.*, (2013). However, they tested only an ethanolic extract obtaining only a value of 0.125 mg/ml for the MIC. Punicalagin has been isolated from *P. granatum* which has shown stronger anti-*Candidal* activity with a MIC of 3.9-1.9 µg/ml (Endo *et al*., 2010). In addition, they showed some morphological changes in *C. albicans* caused by this extracted compound.

P. granatum seeds are rich in conjugated linolenic acid, linoleic acid, oleic acid, stearic acid, punicic acid and catalpic acid (Viuda-Martos *et al*., 2010). Although the extraction of these compounds including fatty acids from the seeds is difficult, present study showed antifungal activity comparable to the peel extracts. The MIC values of seed extracts also ranged between 0.78 to 6.25 mg/ml, which is similar to the MIC values (> 1000 µg/ml) obtained by Anibal *et al.*, (2013). Seeds generally have a lower polyphenol content compared to the peel and furthermore, the seed only consist of about 3% of the whole fruit weight (Faria and Calhau, 2011) which means that the availability of seeds and the extract amounts would also be less.

C. albicans isolated from HIV positive patients are known to be more virulent and possess greater virulence properties (Sweet *et al*., 1995). This increased virulence suggests that these strains may be resistant to antifungal agents (Zhang *et al.*, 2014). It is possible that their response may be different to plant extracts. However, no difference in the MIC values of isolates from HIV positive and HIV negative patients was found in this study. Similar results have been found by Patel and Coogan, (2008) when they studied *Dodonaea viscosa var. angustifolia*. The results of the present study suggest that this plant extract would be effective for patients with oral candidiasis regardless of their other illnesses including HIV infection.

This study has shown that a high concentration of *P. granatum* peel is required to eliminate *C. albicans*. Nevertheless, high concentrations are difficult to maintain in a body orifice where there are secretions, such as saliva in the oral cavity. It is important to study the effect of the diluted compounds on the virulence properties of *C. albicans*. Therefore, the effect of methanolic peel extract of *P. granatum* on the germ tube formation, adherence ability and the production of phospholipase, proteinase and lipase by *C. albicans* were studied.

A sub-inhibitory concentration of methanol extracts (3.13 mg/ml) was used for the further studies because the MIC values did not differ significantly and the yield was much better than for any other extracts. In addition, methanol extract is known to be the best solvent for the extraction of antioxidants which are abundant in *P. granatum* (Singh *et al*., 2002). Antioxidant properties are also associated with the presence of phenolic compounds, antibacterial - and antifungal activities (Singh *et al*., 2002; Duman *et al*., 2009). In addition, methanol is known to extract many chemicals such as anthocyaninis, terpenoids, saponins, tannins, xanthoxyllines, totarol, quassinoids, lactones, flavones, phenones and polyphenols (Cowan, 1999).

4.2 Effect of *P. granatum* on the adherence ability of *C. albicans*

In the pathogenesis of candidiasis, the initial step is adherence and establishment of *C. albicans* onto the host tissue. Proteins found on the external surface of the cell wall of *C. albicans* called adhesins and lectins, are generally responsible for the adhesion to the host cells (Cannon and Chaffin, 1999; Castillo *et al*., 2008). Furthermore, *C. albicans* can also bind to surfaces such as medical devices and catheters, acrylic dentition and other oral bacteria. In this study the adherence of *C. albicans* to oral epithelial cells and the effect of *P. granatum* were studied. Oral epithelial cells were collected from healthy non-carriers of *Candida*

to reduce the possibility of strain contamination. The cells were washed thoroughly to minimize any bacterial contamination which can interfere with the yeast adherence.

The results showed that although the adherence of *C. albicans* to the oral epithelial cells was reduced (3% in strains from HIV positive and 12.2% in strains from HIV negative patients), it was not significant. Researchers have shown that some plants inhibit adherence of yeast cells to the host cells. For example, Taweechaisupapong *et al.*, (2005) showed that *Streblus asper* leaf extract can inhibit the adherence of *C. albicans* to oral epithelial cells. Similarly, Patel *et al.*, (2009) showed that *Dodonaea viscose var. angustifolia* can reduce the adherence of *C. albicans* and suggested that the extract may have interfered with the synthesis of adhesins or caused disruption or even affected the surface fibrils. Tannins are also known to prevent adherence of *C. albicans* to epithelial cells (Ishida *et al*., 2006). The peel of *P. granatum* has a high polyphenol content including punicalagins, which include the minor tannins called punicalin and gallagic acid, called hydrolysable tannins. These tannins either are not capable of inhibiting the adherence of *C. albicans* or they were not extracted in sufficient quantities with methanol. In addition, in this present study, the *C. albicans* cells were exposed to the peel extract and then these exposed *C. albicans* cells were mixed with the oral epithelial cells. In this procedure the assumption is that the peel extract may alter the physiology of *C. albicans*. However, the same study can be repeated by exposing epithelial cells to the peel extract and then challenge them with unexposed or exposed *C. albicans,* which may give different results. This method should be considered in the future research.

It is also known that chemicals present in the plant extracts may not cause extensive damage to the adhesins but they cause some structural changes to the *C. albicans* cells, which consequently affect other virulence properties. For example, *Cassia spectabilis* extract causes an

alteration in the morphology of *C. albicans* preventing biofilm formation (Sangetha *et al*., 2009). Similarly, the essential oil from *Ocimum gratissium L*. showed to impair bud formation and caused structural cell wall changes in *C. albicans* (Nakamura *et al*., 2004). Although in this present study the ultrastructural changes in *C. albicans* cells due to *P. granatum* exposure was not studied, the effect on virulence factors such as hydrolytic enzyme production was investigated.

It has been found that more *C. albicans* cells isolated from HIV positive patients adhere to oral epithelial cells (Sweet *et al*., 1995; Jain *et al*., 2010). Similarly, in our study, strains from HIV positive patients (Control - 331 HIV positive *C. albicans* vs 242 HIV negative *C. albicans* – Table 3.11) showed increased adherence to epithelial cells, compared to strains of *C. albicans* isolated from HIV negative patients. These results showed an increased ability to cause infection in HIV positive patients. However, the effect of peel extract was similar for both the HIV positive and HIV negative group's isolates. Similar results were observed by Patel *et al*., (2009) when they studied *Dodonaea viscosa var. angustifolia*.

4.3 Effect of *P. granatum* on the germ tube formation by *C. albicans*

Candida albicans that are capable of germ tube formation are known to be more adherent to oral epithelial cells than that do not form germ tubes, which suggests that the germ tube plays an important role in adherence(Kimura and Pearsall, 1980). In addition, the developmentally regulated gene HWP 1 is only expressed by *C. albicans* in a hyphae form producing the HWP 1 antigen. Hyphal wall protein1 operates like an adhesin and has ability to cross-link to the host protein. It was suggested that HWP 1 may be more important in mucosal infection including oral candidiasis rather than in systemic infections such as systemic candidiasis (Sundstrom, 2002).

Although the adherence was not reduced by *P. granatum*, the results showed that germ tube formation was reduced by 26% and 34% in *C. albicans* isolated from HIV positive and HIV negative patients respectively. Oral use of *P. granatum* has been proposed in this study as treatment in cases of mucosal candidiasis. In the oral cavity the germ tube formation would be reduced by the plant extract, which would reduce the HPW 1 antigens that is important in the adherence and development of candidiasis. A recent study has shown that plants such as *Piper betle* and *Brucea javanica* can inhibit the expression of HWP 1 genes and reduce the HPW 1 antigen production respectively (Wan Harum *et al*., 2013), and thus possibly render *C. albicans* avirulent. Limited exposure to some of the commonly used antifungal agents such as nystatin, amphotericin B and ketoconazole can also inhibit germ tube formation in *C. albicans* (Ellepila and Samaranayake, 1998).

In the *C. albicans* cell development and hyphae development, the cell wall is very important. Damage to the cell wall may cause reduction in the germ tube formation. Pomegranate fruit (peel, seed and juice) contains punicalagin (Viladomiu *et al*., 2013). Endo *et al*., (2010) reported that punicalagin increase the thickness the cell wall of *C. albicans,* create vacuoles, perturb cell membranes and alter permeability (Endo *et al*., 2010). This means that the plant extract may cause damage to the cell wall hence preventing it to form germ tubes. Germ tube formation is an important step in the pathogenesis of *C. albicans,*since it allows deeper tissue penetration causing deep tissue and cell damage (invasion). This means that even though the crude plant extract of pomegranate peel was unable to stop the first step of the disease (adherence), it could still prevent the disease process. Similar results have been reported by Bernardes *et al.*, (2011) who showed that *Aloe vera* can reduce growth and germ tube formation of *C. albicans.* Naicker and Patel, (2013) also discovered an inhibitory effect of *Dodonaea viscosa var. angustifolia* against germ tube - and biofilm formation by *C. albicans*.

Although strains of *C. albicans* isolated from HIV positive patients are known to be more virulent, there was no difference in the germ tube formation by the strains isolated from the two groups, HIV positive and HIV negative in this study (69 vs 72 – Table 3.12). These results showed that although germ tube formation is known to be a virulence factor, it is not very discriminatory when it comes to comparing different types of patients because all the strains of *C. albicans* will produce germ tubes. However, the significant reduction in the germ tube formation seen in both the study groups means that pomegranate have therapeutic value for patients infected with *C. albicans,* regardless of their HIV status.

4.4 Effect of *P. granatum* on the phospholipase production by *C. albicans*

Production of phospholipase is considered one of the virulence factors in the pathogenesis of candidiasis. Studies have shown that high levels of phospholipase producing *C. albicans* are more pathogenic (Kothavade and Panthaki, 1998), and showed an increased mortality rate in animals (Ibrahim *et al*., 1995). Most pathogenic *C. albicans* strains have shown higher adherence ability to cells and increased production of phospholipase, compared to non-pathogenic strains (Barrett-Bee *et al*., 1985). Phospholipase cause lysis of the host cells by targeting membrane phospholipids. This host endothelial and epithelial cells injury facilitates the penetration of *C. albicans* into the tissues (Ghannoum, 2000).

Therefore, inhibition of the production of phospholipase with therapeutic agents, may be useful in the treatment of candidiasis. Virulence intensity can be demonstrated in a laboratory by the precipitation of phospholipase on egg yolk media, and can be calculated by a Pz value which is the colony diameter divided by the precipitation diameter.. Using this technique, sub-therapeutic concentrations (0.002% and 0.0012%) of chlorhexidine which is a broad

spectrum topical antimicrobial agent has shown to modulate candida phospholipase activity (Kadir *et al*., 2007). Using a similar technique, the effect of *P. granatum* on the phospholipase production was studied. The results of this study showed that *P. granatum* had no effect on the phospholipase production of *C. albicans* (Table 3.15). Similar results were reported by Patel *et al.*, (2009) when they studied the effect of leaf extracts of *Dodonaea viscosa* var. *angustifolia* on phospholipase activity of *C. albicans*. These results suggest that the plant extracts may not cause sufficient cellular damage at that sub-therapeutic concentrations and may not affect the genes responsible (caPLB1 and caPLB2) for the production of this enzyme. However, *Ocimum sanctum* essential oil has been found to inhibit morphological transitions, production of phospholipase and proteinase, and down regulate the genes HWP1, SAP1 and PLB2, (the genes responsible for the production of hyphae, proteinase and phospholipase) in *C. albicans* respectively (Khan *et al*., 2014a). Importantly, the effect was concentration dependent. Similarly, cinnamic aldehyde derived from cinnamon has also shown antihydrolytic enzyme activity (Shreaz *et al*., 2012). In this present study, the effect of *P. granatum* on the genes responsible for the production of hydrolytic enzymes were not studied but should be considered in the future studies.

It has been stated that HIV positive patients tend to produce more phospholipase (Ribeiro *et al*., 2004), and that *C. albicans* isolated from HIV patients patients who are on antiretroviral therapy produce less hydrolytic enzymes. Similarly, Mane *et al.*, (2012) has also shown that isolates of *C. albicans* from HIV positive patients produce high amounts of hydrolytic enzymes including phospholipase. Contrary to these results we found no difference in the phospholipase production by *C. albicans* isolates from the HIV positive and the HIV negative groups (Table 3.15 – HIV positive control P_z = 0.76 vs HIV negative P_z = 0.77). Similar results were found by Patel *et al.*, (2009) when they studied 20 *C. albicans* isolates from HIV

positive and HIV negative patients. In the present study the effect of *P. granatum* on the phospholipase production by the *C. albicans* isolates from the two study groups was also compared, but showed no difference in phospholipase production in the HIV positive and HIV negative groups. *Dodonaea viscosa* var. a*ngustifolia* is reported to have shown similar results (Patel *et al*., 2009). The effect of *P. granatum* on the actual gene expression of the PLB1 and PLB2 genes may be useful andwill be considered in the future studies.

4.5 Effect of *P. granatum* on the proteinase production by *C. albicans*

Of all the virulence factors of *C. albicans,* Saps have been recognized as the most important virulence factor due to its ability to cause tissue destruction and overcome host response. Secretory aspartyl proteinases are encoded by 10 highly regulated genes (Naglik *et al*., 2003). Production of these hydrolytic enzymes can be detected in a laboratory and therefore, studies have been conducted to investigate the inhibitory activities of therapeutic chemicals on the production of these enzymes. For example, sub-inhibitory concentrations of certain antifungal agents can inhibit production of SAP2 and SAP9 (Copping *et al*., 2005). Antiretroviral drugs, Ritonavir and Saquinavir are also known to inhibit Saps production (Pichová *et al*., 2001). In the present study the effect of *P. granatum* on the production of proteinase was studied. The results showed that *P. granatum* extract had no effect on the proteinase production by *C. albicans*. Similar results were obtained by Patel *et al.*, (2009) when they studied *Dodonaea viscosa* var. *angustifolia.* Contrary to these results Khan *et al.*, (2014b) found that sub-MIC concentrations of oils of *Carum copticum*, and *Thymus vulgaris* can inhibit proteinase production (> 70% reduction) by *C. albicans* and *C. tropicalis*. In addition, they also showed that some oils, including thymol, reduce cell hydrophobicity and hemolytic activity, which contributes to pathogenicity. In these types of studies proteinase were detected collectively,

meaning a mixture of SAP1 to SAP10 were produced. During the infection process, Saps 1-10 genes are expressed and enzymes are produced at different infective stages, as and when required by the organism to cause tissue damage and overcome host responses (Satib *et al*., 2000). Interestingly, enzyme production is infection dependent, for example in oesophageal candidiasis only SAP5 and SAP6 are expressed within 3 days, whereas in the hematogenous infection SAP1, SAP2, SAP3, SAP4, SAP5 and SAP6 genes are expressed (Staib *et al*., 2000). Therefore, some researchers have studied the effect of plant extracts on the actual gene expressions. For example *Ocimum sanctum* essential oil has been found to inhibit proteinase production and the expression of the responsible gene SAP1 (Khan *et al*., 2014a). However, in this particular study by Khan *et al.*, (2014a) the results showed the effect on only one type of SAP rather than collective Saps production.

Although it is known that isolates of *C. albicans* from HIV positive patients produce more proteinase compared to the isolates from HIV negative patients (Ollert *et al*., 1995; Sweet *et al*., 1995), our results showed no difference between these two groups of individuals (HIV positive – control Pr = 0.93 vs HIV negative – control Pr = 0.89. Please see Table 3.18). Nevertheless, the effect of *P. granatum* on the proteinase production by *C. albicans* isolated from both the groups was compared and although slightly reduced, the proteinase production in both these groups was not significantly different. Similar results were obtained by Patel *et al*., (2009) when they studied *Dodonaea viscosa var. angustifolia* on *C. albicans*. No other similar studies have been found in the literature.

4.6 Effect of *P. granatum* on the lipase production by *C. albicans*

Lipases are produced by *C. albicans, C. parapsilosis* and *C. tropicalis* (Bramono *et al*., 2006). Lipases digest lipids and provide nutrients to the organism in a carbohydrate restricted area. In addition, lipases facilitate adhesion between pathogens as well as between pathogen and surfaces. Lipase genes are expressed during *C. albicans* infection and therefore implicated in the pathogenesis of the organism (Stehr *et al*., 2004). Stehr *et al*., (2003) suggested that therapeutic agents can be developed which will inhibit lipase production and thus inhibit the microbial pathogenesis. Culture filtrates of *Staphylococcus epidermidis* are known to inhibit lipase production by *C. albicans* (Bhattacharyya *et al*., 2014). Acetylsalicylic acid commonly known as aspirin is also known to reduce tissue damage by
C. albicans due to the reduction in the lipase production (Trofa *et al*., 2009). However, the present study showed that *P. granatum* caused a non-significant 9% reduction in lipase production in the isolates from HIV positive patients, and a 5.6% reduction in lipase in the isolates from HIV negative patients.

4.7 Use of *P. granatum* and pathogenesis of *C. albicans* in the oral cavity

In the treatment of any infection, the primary objective of a therapeutic agent such as antibiotics, chemotherapeutic agent or a plant derived chemical is to eliminate the causative organism. Therefore, the therapeutic agent has to show some antimicrobial activity. In this study the proposed potential therapeutic agent against mucosal candidiasis was the crude extract of *P. granatum* which showed an antifungal effect with MICs of 0.39 to 6.25 mg/ml. *P. granatum* has shown potential to be used as a therapeutic agent in the treatment or in the prevention of *C. albicans* infection. If this crude extract is developed into a gel form and applied into the oral cavity or the vagina it should eliminate *C. albicans* regardless of the immune

status and other current illnesses the patient might have such as HIV infection. Unfortunately these *P. granatum* extract concentrations will not be maintained due to the body secretions in those orifices which will dilute the antifungal gel and render it sub-therapeutic. Therefore, some *C. albicans* cells will survive, andwill be able to adhere to the oral epithelial cells because *P. granatum* has no effect on this property of *C. albicans*. However, the adherent cells will not be able to produce germ tubes or hyphae, which mean that *C. albicans* cannot penetrate the host tissue and will thus not be able to cause tissue damage and infection. However, once *C. albicans* cells overcome this particular step of the disease process, they will produce hydrolytic enzymes and be able to cause infection.

P. granatum can be developed into toothpaste, mouth rinse or chewing gum and it can be used in the prevention of oral candidiasis which is common in HIV positive patients, cancer patients and diabetic patients. In these patients, regular use of a preventative therapeutic agent such as *P. granatum* will be useful, because it will reduce the number of infectious organisms, in this case *C. albicans,* and it will render the surviving fungal cells weaker and less virulent. Maintained reduction in the number of *C. albicans* cells in the prevention of development of oral candidiasis by *P. granatum* has been suggested (Patel *et al*., 2009). In addition, *P. granatum* is known to kill some other oral pathogens, and therefore, it will improve general oral hygiene as well(Rosas-Pinón *et al*., 2012, Bhadbhade *et al*., 2011). In the oral cavity *C. albicans* is known to be associated with other oral bacteria species (Bertolini *et al*., 2015). Therefore, reduction of oral bacteria together with *C. albicans* will be beneficial. DiSilvestro *et al.*, (2009) did propose the development of a mouth rinse containing *P. granatum* for improved oral health.

Finally, safety of *P. granatum* fruit and seeds has been established in human volunteers and rats respectively. Ingested 800 mg of *P. granatum* derived polyphenol was found not to increase interleukin 6 and considered to be safe (Mertens-Talcott *et al*., 2006). No observable side effects were seen when rats were fed with 50000 parts per million (ppm) pomegranate seed oil (Meerts *et al*., 2009). Furthermore, another study has shown that *P. granatum* has no mutagenic effect (Valadares *et al*., 2010). Although no mutagenic effect and cytotoxicity study was performed in this present study, a topical application rather than systemic therapy is proposed, and therefore, it may be considered safe.

Saliva in the oral cavity contains glycoproteins, lectoferrins, lactoperoxidase, biocarbonates and secretory immunoglobulins. These compounds may interfere with the efficacy of the proposed plant product therapy. Therefore, although *P. granatum* has shown antifungal potential in this study and in the literature, a clinical trial showing the sustained efficacy *in vivo* would be necessary.

CHAPTER 5: CONCLUSIONS, LIMITATIONS TO THE STUDY AND FUTURE RESEACH

5.1 Conclusions

This study showed that crude extracts of *P. granatum* peel and seeds can be prepared using various solvents among which for the peel, methanol produced the best yield followed by water. Hexane produced the best yield for the seed extract. All the test extracts showed antifungal activity against *C. albicans* with the MICs range for the peel being 6.25 to 0.39 mg/ml and for the seeds 6.25 to 0.78 mg/ml. A sub-inhibitory concentration (3.125 mg/ml) of the peel extract was unable to inhibit the *C. albicans* adherence to the oral epithelial cells, however, it significantly reduced the germ tube or hyphae formation, which contributes in the pathogenesis of *C. albicans*. Furthermore, *P. granatum* had no effect on the production of phospholipase, proteinase and lipase enzymes, which cause tissue damage during infection. These results suggest that *P. granatum* reduces the number of *C. albicans* cells and that *P. granatum,* at sub-inhibitory concentration reduce some virulence of this organism. Therefore, *P. granatum* has potential to be developed into an antifungal agent for the prevention and treatment of oral candidiasis.

5.2 Limitations to the study

- Pathogenesis of candidiasis can be best studied in animals. However, studies have used laboratory techniques to detect virulence factors and as such, laboratory based techniques were used to detect adherence ability and the production of hydrolytic enzymes. The proteinase assay needs to be repeated, using a more sensitive technique

such as a liquid medium assay instead of the solid agar medium technique (Khan *et al.*, 2014b).

- The effect of *P. granatum* on the adherence of *C. albicans* to the epithelial cells may have given different results if epithelial cell lines were used. However, due to the unavailability of epithelial cell lines at the time of assay, oral epithelial cells from donors were used. Nevertheless, these cells were collected from non-*Candida* infected healthy individuals and the isolates were thoroughly washed to remove bacteria, debrie and saliva. Indeed, many studies in the literature have used buccal epithelial cells harvested from healthy individuals.

5.3 Future research

- The effect of *P. granatum* on the adherence ability of *C. albicans* can be studied by exposing epithelial cells to the plant extract rather than exposing *C. albicans* to the plant extract.
- The effect of *P. granatum* on the expression of genes responsible for the production of hydrolytic enzymes such as HWP1, SAP1-SAP10, PLB1-PLB2 and LIP1-LIP10 can be studied.
- The active chemical constituents present in the *P. granatum* crude extract can be identified using thin layer chromatography, high performance liquid chromatography, gas-chromatograpgy mass spectrometry and nuclear magnetic resounance
- The effect of *P. granatum* on the adherence ability of *C. albicans* to solid surfaces such as catheters, dentures and other oral prostheses and the prevention of biofilm formation can be studied. In addition, the effect on hemolytic activity, hydrophobicity can also be studied.

- More than one sub-inhibitory concentrations of *P. granatum* can be test for beneficial effects.
- Safe application of the proposed extract is important, therefore, cytotoxicity of the crude extract and the active chemical constituent/s should be established by using epithelial cell lines.
- Laboratory studies which screen medicinal plants and show potential for the test plant to be used as treatment, are generally considered preliminary studies. In clinical applications, plant extracts may behave differently due to the body secretions and other physiological conditions which may alter the plant extract and its effects in any way. Therefore, clinical trials are very important once the therapeutic potential of a plant is established.

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APPENDICES

Appendix 1 Composition and preparation of media

Sabouraud Dextrose Agar

Sabouraud Agar powder (Merk, South Africa) 60g

Distilled water 1L

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

Sabouraud Dextrose Broth

Sabouraud Broth powder (Merck, South Africa) 20g

Distilled water 1L

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

DMSO (Dimethyl Sulfoxide) solution (reconstitution per extract)

Weight of dry plant extract (g)/ 0.050g = Reconstitution DMSO

Iodonitrotetrazolium chloride dye

Iodonitrotetrazolium chloride powder 0.01g

Distilled water 5g

Ingredients were added together and mixed until completely dissolved

(Covered in foil). Solution remained in the foil to avoid light.

Egg Yolk Media plates (for phospholipase)

Sabouraud agar 3g

NaCl 11.7g

$Nacl_2$ 0.11g

Egg Yolk emulsion	20g
Distilled water	184g

Egg yolk emulsion (20 ml) was centrifuged at 5000 rpm for 10 minutes at room temperature. Dissolve SAB agar, Nacl, $CaCl_2$ in water and autoclave at 121 °C for 10 minutes. Mixture was cooled to 45 °C. Egg yolk emulsion was added to the mixture and mixed gently. Plates were then poured to Petri dishes.

Bovine Serum Albumin (BSA) agar plates (for Proteinase)

$MgSO_2.H_2O$	0.04g
K_2PO_4	0.5g
NaCl	1.0g
Yeast Carbon Base	0.2g
Glucose	4.0g
BSA	0.5g
Sterile Columbia agar	140g

Dissolve dry ingredients into 60 ml distilled water, adjust pH to 3.5 and filter sterilize. Add this mixture to 140 ml sterile Columbia medium. Pour plates.

Proteinase staining solution (coomasie blue)

Coomasie blue R250	0.5g
Acetic acid	10ml
Distilled water	45ml

Mix the ingredients.

Destaining solution (for Proteinase)

Ethanol	45ml
Acetic acid	10ml
Distilled water	45ml

Mix the ingredients.

1% Tributyrin plates (for Lipase)

Sabouraud agar	13g
Distilled water	200ml
Tributyrin	0.2µl

Mix ingredients, boil and autoclave at 121 ℃ for 10 minutes. Mixture is then cooled to 45 ℃ and poured into plates.

Appendix 2 Statistical analysis

Adherence:

Adhevence assay stats.

```
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Fundi1.log
  log type:  text
 opened on:  15 Apr 2014, 17:18:02

. use "Fundi1.dta"

.
. *DESCRIPTIVES
.
. table plant, c( mean adherence sd adherence median adherence) format(%9.2f)

----------------------------------------------------------------
    Plant | mean(adhere~e)    sd(adhere~e)   med(adhere~e)
----------+-----------------------------------------------------
        0 |         292.11          261.03          229.00
        1 |         275.73          253.47          228.00
----------------------------------------------------------------

.
. table hiv, c( mean adherence sd adherence median adherence) format(%9.2f)

----------------------------------------------------------------
      HIV | mean(adhere~e)    sd(adhere~e)   med(adhere~e)
----------+-----------------------------------------------------
        0 |         241.42          172.84          168.50
        1 |         326.45          314.60          240.50
----------------------------------------------------------------

.
. table plant hiv, c( mean adherence sd adherence median adherence) format(%9.2f)

----------------------
          |       HIV
    Plant |         0         1
----------+-----------------------
        0 |    252.87    331.48
          |    177.74    322.25
          |    154.86    240.50
          |
        1 |    229.97    321.50
          |    170.85    312.19
          |    179.50    235.00
----------------------

.
. *Questions: 1 Does plant extract have any effect on the adherence? Lump all the results together (compare
> controls to tests)
. ttest adherence, by(plant)

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      60    292.1333    33.69922    261.033    224.7013    359.5653
       1 |      60    275.7333    32.72341   253.4744    210.2539    341.2127
---------+--------------------------------------------------------------------
combined |     120    283.9333    23.39967   256.3385    237.5997     330.267
---------+--------------------------------------------------------------------
    diff |              16.4    46.97296               -76.61925    109.4192
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   0.3491
Ho: diff = 0                     degrees of freedom =      118

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.6362         Pr(|T| > |t|) = 0.7276          Pr(T > t) = 0.3638

. ranksum adherence, by(plant)

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       60        3870        3630
          1 |       60        3390        3630
------------+---------------------------------
   combined |      120        7260        7260

unadjusted variance     36300.00
adjustment for ties        -1.13
                        ----------
adjusted variance       36298.87

Ho: adhere~e(plant==0) = adhere~e(plant==1)
              z =   0.997
    Prob > |z| =   0.3186

.
. *ADHERENCE LOWER WITH PLANT BUT NOT SIGNIFICANT
.
. *Question 2 Is there a difference between the results of HIV positive and HIV negative patients?
.
. bysort hiv:ttest adherence, by(plant)

-------------------------------------------------------------------------------
-> hiv = 0

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      30    252.8667    32.44985   177.7351    186.4993    319.2341
       1 |      30    229.9667      31.046   170.0459    166.4705    293.4629
---------+--------------------------------------------------------------------
combined |      60      241.417    22.31334   172.8388    196.7677    286.0657
---------+--------------------------------------------------------------------
    diff |              22.9    44.80931               -66.99576    112.7958
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   0.5070
Ho: diff = 0                     degrees of freedom =       58

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.6940         Pr(|T| > |t|) = 0.6120          Pr(T > t) = 0.3060

-------------------------------------------------------------------------------
-> hiv = 1
```

```
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      30       331.4    58.83487    322.2518    211.0692    451.7308
       1 |      30       321.5    56.99848    312.1935     204.925     438.075
---------+--------------------------------------------------------------------
combined |      60       326.45    40.61494     314.602    245.1797    407.7203
---------+--------------------------------------------------------------------
    diff |               9.9    81.91684                -154.0744    173.8744
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   0.1209
Ho: diff = 0                                     degrees of freedom =       58

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5479         Pr(|T| > |t|) = 0.9042          Pr(T > t) = 0.4521

. bysort hiv:ranksum adherence, by(plant)

-------------------------------------------------------------------------------
-> hiv = 0

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       30         960         915
          1 |       30         870         915
------------+---------------------------------
   combined |       60        1830        1830

unadjusted variance      4575.00
adjustment for ties        -0.38
                        ---------
adjusted variance        4574.62

Ho: adhere-e(plant==0) = adhere-e(plant==1)
           z =   0.665
    Prob > |z| =   0.5058

-------------------------------------------------------------------------------
-> hiv = 1

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       30         981         915
          1 |       30         849         915
------------+---------------------------------
   combined |       60        1830        1830

unadjusted variance      4575.00
adjustment for ties        -0.25
                        ---------
adjusted variance        4574.75

Ho: adhere-e(plant==0) = adhere-e(plant==1)
           z =   0.976
    Prob > |z| =   0.3292

.
. *HIV DOES NOT HAVE ANY EFFECT
.
.
.
. log close
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Fundil.log
  log type:  text
 closed on:  15 Apr 2014, 17:18:02
-------------------------------------------------------------------------------
```

Germtube Stats.

```
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Fundi2.log
  log type:  text
 opened on:  17 Apr 2014, 15:44:13

. use "Fundi2.dta"

.
. *DESCRIPTIVES
.
. table plant, c( mean germtube sd germtube median germtube) format(%9.2f)

----------------------------------------------------------
   Plant | mean(germtube)  sd(germtube)  med(germtube)
---------+------------------------------------------------
       0 |          71.25         22.62          74.00
       1 |          40.62         30.08          42.00
----------------------------------------------------------

.
. table hiv, c( mean germtube sd germtube median germtube) format(%9.2f)

----------------------------------------------------------
     HIV | mean(germtube)  sd(germtube)  med(germtube)
---------+------------------------------------------------
       0 |          53.85         31.52          60.00
       1 |          58.02         29.77          66.00
----------------------------------------------------------

.
. table plant hiv, c( mean germtube sd germtube median germtube) format(%9.2f)

----------+-----------------
          |       HIV
    Plant |       0       1
----------+-----------------
        0 | 71.20  71.38
          | 18.91  26.14
          | 73.00  75.00
          |
        1 | 36.50  44.73
          | 32.25  27.48
          | 40.50  53.50
----------+-----------------

.
. *Questions: 1 Does plant extract have any effect on germ tube formation? Lump all the results together (co
> mpare controls to tests)
.
. ttest germtube, by(plant)

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      60       71.25    2.91909    22.61815    65.40711    77.09289
       1 |      60    40.61667    3.872625    29.99723    32.86758    48.36577
---------+--------------------------------------------------------------------
combined |     120    55.93333    2.793368    30.59981    50.40219    61.46448
---------+--------------------------------------------------------------------
    diff |            30.63333    4.85011                21.02879    40.23787
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  6.3160
Ho: diff = 0                                     degrees of freedom =     118

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

. ranksum germtube, by(plant)

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       60        4716        3630
          1 |       60        2544        3630
------------+---------------------------------
   combined |      120        7260        7260

unadjusted variance     36300.00
adjustment for ties       -42.23
                        ----------
adjusted variance       36257.77

Ho: germtube(plant==0) = germtube(plant==1)
              z =   5.703
    Prob > |z| =   0.0000

.
. *GERMTUBE LOWER WITH PLANT AND SIGNIFICANT
.
. *Question 2 Is there a difference between the results of HIV positive and HIV negative patients?
.
. bysort hiv:ttest germtube, by(plant)

------------------------------------------------------------------------------
-> hiv = 0

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      30        71.2    3.452602    18.91068    64.13864    78.26136
       1 |      30        36.5    5.888573    32.25304    24.45652    48.54348
---------+--------------------------------------------------------------------
combined |      60       53.85    4.068608     31.5153    45.70873    61.99127
---------+--------------------------------------------------------------------
    diff |            34.7    6.826108                21.03606    48.36394
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  5.0834
Ho: diff = 0                                     degrees of freedom =     58

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

------------------------------------------------------------------------------
```

(2)

```
-> hiv = 1

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      30        71.3    4.772081   26.13776       61.54       81.06
       1 |      30    44.73333    5.017084   27.48408    34.47061    54.99606
---------+--------------------------------------------------------------------
combined |      60    58.01667    3.843881   29.77457    50.32508    65.70825
---------+--------------------------------------------------------------------
    diff |            26.56667    6.924732               12.70531    40.42802
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   3.8365
Ho: diff = 0                     degrees of freedom =       58

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9998         Pr(|T| > |t|) = 0.0003          Pr(T > t) = 0.0002

. bysort hiv:ranksum germtube, by(plant)

-------------------------------------------------------------------------------
-> hiv = 0

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       30        1191         915
          1 |       30         639         915
------------+---------------------------------
   combined |       60        1830        1830

unadjusted variance      4575.00
adjustment for ties       -22.37
                        ----------
adjusted variance        4552.63

Ho: germtube(plant==0) = germtube(plant==1)
             z =   4.091
    Prob > |z| =   0.0000

-------------------------------------------------------------------------------
-> hiv = 1

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       30      1184.5         915
          1 |       30       645.5         915
------------+---------------------------------
   combined |       60        1830        1830

unadjusted variance      4575.00
adjustment for ties        -3.81
                        ----------
adjusted variance        4571.19

Ho: germtube(plant==0) = germtube(plant==1)
             z =   3.986
    Prob > |z| =   0.0001

.
. *SIGNIFICANT REDUCTION WITH PLANT IN HIV+ AND HIV-. PLANT SLIGHTLY MORE EFFECTIVE IN HIV-
.
.
.
. log close
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Fundi2.log
  log type:  text
 closed on:  17 Apr 2014, 15:44:13
-------------------------------------------------------------------------------
```

FUNDI
Enzymes
Stats.

1

```
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Fundi data.log
  log type:  text
 opened on:   9 Apr 2015, 14:43:19

. use "Fundi data.dta"

.
. sktest  pr pz plz

                    Skewness/Kurtosis tests for Normality
                                                      ------- joint ------
    Variable |   Obs   Pr(Skewness)   Pr(Kurtosis)  adj chi2(2)   Prob>chi2
-------------+------------------------------------------------------------
          pr |    40        0.0000         0.0004        23.19      0.0000
          pz |    40        0.2198         0.2730         2.90      0.2344
         plz |    40        0.8991         0.0751         3.42      0.1809

.
. *PR skewed and can only be interpreted with non parametric statistics
.
.
. *1 Questions: 1 Does plant extract have any effect on enzyme production? Lump all
> the results togather (compare controls to tests regardless of HIV status)
. table plant, c(mean pr median pr sd pr)format(%9.2f)

----------------------------------------
    Plant |  mean(pr)   med(pr)    sd(pr)
----------+-----------------------------
        0 |       0.91       0.95       0.10
        1 |       0.94       0.95       0.05
----------------------------------------

. table plant, c(mean pz median pz sd pz)format(%9.2f)

----------------------------------------
    Plant |  mean(pz)   med(pz)    sd(pz)
----------+-----------------------------
        0 |       0.77       0.78       0.13
        1 |       0.75       0.77       0.15
----------------------------------------

. table plant, c(mean plz median plz sd plz)format(%9.2f)

----------------------------------------
    Plant |  mean(plz)   med(plz)    sd(plz)
----------+-----------------------------
        0 |       0.69       0.68       0.09
        1 |       0.74       0.77       0.11
----------------------------------------

.
. ttest pr, by(plant)

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      20       .9145    .0214412    .0958878    .8696231    .9593769
       1 |      20       .9375    .0107819    .0482183    .9149332    .9600668
---------+--------------------------------------------------------------------
combined |      40       .926     .0119872    .0758135    .9017537    .9502464
------------------------------------------------------------------------------
```

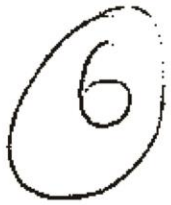
(2)

```
    diff |              -.023    .0239995               -.0715844    .0255844
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -0.9584
Ho: diff = 0                                     degrees of freedom =       38

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1720         Pr(|T| > |t|) = 0.3439          Pr(T > t) = 0.8280

. ranksum pr, by(plant)

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

      plant |      obs    rank sum    expected
------------+---------------------------------
          0 |       20         402         410
          1 |       20         418         410
------------+---------------------------------
   combined |       40         820         820

unadjusted variance     1366.67
adjustment for ties      -15.38
                        --------
adjusted variance       1351.28

Ho: pr(plant==0) = pr(plant==1)
            z =  -0.218
    Prob > |z| =   0.8277

.
. ttest pz, by(plant)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      20        .767    .0300885   .1345597    .7040241    .8299759
       1 |      20       .7545    .0329551   .1473797    .6855242    .8234758
---------+--------------------------------------------------------------------
combined |      40     .76075    .0220471   .1394382    .7161555    .8053445
---------+--------------------------------------------------------------------
    diff |              .0125    .0446246               -.0778378    .1028378
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   0.2801
Ho: diff = 0                                     degrees of freedom =       38

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.6095         Pr(|T| > |t|) = 0.7809          Pr(T > t) = 0.3905

.
. ttest plz, by(plant)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      20        .693    .0196295   .0877856    .6519151    .7340849
       1 |      20       .7445    .0244785   .1094712    .6932659    .7957341
---------+--------------------------------------------------------------------
combined |      40     .71875    .0160256   .1013546    .6863352    .7511648
---------+--------------------------------------------------------------------
    diff |             -.0515     .031377               -.1150193    .0120193
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -1.6413
Ho: diff = 0                                     degrees of freedom =       38
```

```
   Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0545         Pr(|T| > |t|) = 0.1090          Pr(T > t) = 0.9455

. *NO SIGNIFICANT EFFECT OF PLANT ON ENZYME PRODUCTION
.
.
.
. *2 Question: 2 Is there a difference between the results of HIV positive and HIV n
> egative patients? (compare controls and tests separately)
.
. bysort hivpositive:ttest pr, by(plant)

-------------------------------------------------------------------------------
-> hivpositive = 0

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      10        .897    .0236197    .074692    .8435686    .9504314
       1 |      10        .922    .0173718   .0549343    .8827024    .9612976
---------+--------------------------------------------------------------------
combined |      20       .9095    .0145543    .065089    .8790374    .9399626
---------+--------------------------------------------------------------------
    diff |              -.025    .0293201               -.0865992    .0365992
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -0.8527
Ho: diff = 0                     degrees of freedom =       18

   Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.2025         Pr(|T| > |t|) = 0.4051          Pr(T > t) = 0.7975

-------------------------------------------------------------------------------
-> hivpositive = 1

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      10        .932    .0362645   .1146783    .8499641    1.014036
       1 |      10        .953    .0116476    .036833    .9266513    .9793487
---------+--------------------------------------------------------------------
combined |      20       .9425    .0186925   .0835952    .9033763    .9816238
---------+--------------------------------------------------------------------
    diff |              -.021    .0380891               -.1010222    .0590222
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -0.5513
Ho: diff = 0                     degrees of freedom =       18

   Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.2941         Pr(|T| > |t|) = 0.5882          Pr(T > t) = 0.7059

. bysort hivpositive:ranksum pr, by(plant)

-------------------------------------------------------------------------------
-> hivpositive = 0

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

      plant |      obs    rank sum    expected
------------+---------------------------------
```

```
           0 |        10         94.5         105
           1 |        10        115.5         105
-------------+------------------------------------
    combined |        20         210         210

unadjusted variance      175.00
adjustment for ties       -2.37
                         ------
adjusted variance        172.63

Ho: pr(plant==0) = pr(plant==1)
            z =  -0.799
    Prob > |z| =   0.4242

-------------------------------------------------------------------------------
-> hivpositive = 1

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

       plant |       obs    rank sum    expected
-------------+------------------------------------
           0 |        10         111         105
           1 |        10          99         105
-------------+------------------------------------
    combined |        20         210         210

unadjusted variance      175.00
adjustment for ties       -5.92
                         ------
adjusted variance        169.08

Ho: pr(plant==0) = pr(plant==1)
            z =   0.461
    Prob > |z| =   0.6445

.
. bysort hivpositive:ttest pz, by(plant)

-------------------------------------------------------------------------------
-> hivpositive = 0

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      10        .771    .0481998    .1524212    .6619645    .8800356
       1 |      10        .779    .0505404    .1598228    .6646696    .8933304
---------+--------------------------------------------------------------------
combined |      20        .775    .0340008    .1520561    .7038356    .8461644
---------+--------------------------------------------------------------------
    diff |            -.008    .0698395               -.1547273    .1387274
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -0.1145
Ho: diff = 0                     degrees of freedom =       18

   Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
Pr(T < t) = 0.4550         Pr(|T| > |t|) = 0.9101          Pr(T > t) = 0.5450

-------------------------------------------------------------------------------
-> hivpositive = 1

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
```

```
---------+--------------------------------------------------------------------
       0 |      10         .763    .0386738    .1222974    .6755137    .8504863
       1 |      10          .73    .0435635    .1377598    .6314525    .8285475
---------+--------------------------------------------------------------------
combined |      20        .7465    .0286014    .1279093    .6866366    .8063634
---------+--------------------------------------------------------------------
    diff |               .033    .0582533               -.0893856    .1553856
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   0.5665
Ho: diff = 0                                     degrees of freedom =       18

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.7110         Pr(|T| > |t|) = 0.5781          Pr(T > t) = 0.2890

.
. bysort hivpositive:ttest plz, by(plant)

-------------------------------------------------------------------------------
-> hivpositive = 0

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      10         .712    .0310125    .0980703    .6418448    .7821552
       1 |      10         .757    .0301865     .095458    .6887135    .8252865
---------+--------------------------------------------------------------------
combined |      20        .7345    .0216852    .0969794    .6891122    .7798877
---------+--------------------------------------------------------------------
    diff |              -.045    .0432782               -.1359241    .0459241
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -1.0398
Ho: diff = 0                                     degrees of freedom =       18

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1561         Pr(|T| > |t|) = 0.3122          Pr(T > t) = 0.8439

-------------------------------------------------------------------------------
-> hivpositive = 1

Two-sample t test with equal variances

------------------------------------------------------------------------------
   Group |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |      10         .674    .0241845     .076478    .6192909    .7287091
       1 |      10         .732    .0397995    .1258571    .6419673    .8220327
---------+--------------------------------------------------------------------
combined |      20         .703    .0236209    .1056359    .6535609    .7524391
---------+--------------------------------------------------------------------
    diff |              -.058    .0465713               -.1558427    .0398427
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =  -1.2454
Ho: diff = 0                                     degrees of freedom =       18

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1145         Pr(|T| > |t|) = 0.2290          Pr(T > t) = 0.8855

.
. *STILL A BLANK- NO DIFFERENCE
.
.
.
.
.
```



```
. log close
      name:  <unnamed>
       log:  /Users/macbookpro/Documents/Stata/Fundi data.log
  log type:  text
 closed on:   9 Apr 2015, 14:43:19
-------------------------------------------------------------------------------
```

Appendix 3 Ethical clearance certificates

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

Ref: W-CJ-130429-1

29/04/2013

TO WHOM IT MAY CONCERN:

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

Investigator: **Ms T F Mbatha (student no. 794426).**

Project title: **The effect of pomegranate peel extract on the suppression of virulent factors of Candida albicans.**

Reason: This is a wholly laboratory study using *C. albicans* strain isolated in an earlier study approved by the HREC(Medical) M000402. No humans are involved.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2013 -04- 29
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

UNIVERSITY OF THE
WITWATERSRAND
JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172078

Reference: Ms Thokozile Nhlapo
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29 April 2015
Person No: 794426
TAA

Miss TF Mbatha
No 29 Gate Hill
Hillgrove
Newlands West
4037
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Dear Miss Mbatha

Master of Science in Medicine: Change of title of research

I am pleased to inform you that the following change in the title of your Dissertation for the degree of **Master of Science in Medicine** has been approved:

From:	**The effect of Punica granatum L (pomegranate) peel and seed extracts on the virulence factors of Candida albicans**
To:	**Antifungal effect of Punica granatum L (pomegranate) peel and seed extracts and the effect of peel extract on the virulence factors of Candida albicans**

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences