#### **CHAPTER ONE**

## **INTRODUCTION AND OBJECTIVES**

## 1.1 Introduction

*Candida* species are normal oral commensals (Samaranayake, 1990) found in 17-75% of healthy individuals and most debilitated people (Bastiaan and Reade, 1982; Rindum *et al.*, 1994). Commensalism is a symbiotic relationship where the symbiont benefits, but the host neither harmed nor helped (Arendorf and Walker, 1979). Despite representing a very minor component of the normal flora, *Candida* species are responsible for a wide range of systemic and superficial opportunistic infections, which are of particular importance among immunosuppressed and otherwise medically compromised individuals. Changes in the ecological balance within the oral cavity could favour candida to be mutualistic and develops potential pathogenic relationship with the hosts. Besides, its transition to the disease-causing 'parasite' may be associated with the virulence attributes of the microorganism (Samaranayake, 1990).

For years, *Candida albicans* was regarded as the predominant species and most frequent etiologic agent involved in both commensal state and in cases of candidiasis (Back-Brito *et al.*, 2009; Vazquez and Sobel, 2002), based on the high incidences of nosocomial candidemia reported in hospitals. Of late however, the prevalence of *C. albicans* has been outnumbered by the emergence of the non-*Candida albicans Candida* (NCAC) species (Fleming *et al.*, 2002; Kauffman, 2006; Marsh and Martin, 2009) causing infections in immunocompromised individuals (Clark and Hajjeh, 2002; de Pincho Resende *et al.*, 2004; Krcmery and Barnes, 2002; Sánchez-Vargas *et al.*, 2005). Factors such as prescription of broad spectrum antibiotics during therapy of severe underlying disease, diabetics (Soysa *et al.*, 2006, 2008; Tapper-Jones *et al.*, 1981), malnutrition *e.g.* iron deficiency (Cawson, 1963; Joynson *et al.*, 1972), and smoking (Arendorf and Walker, 1980; Kuc *et al.*, 1999; Scully *et al.*, 1994) have been shown to affect the first line of the host's defence which leads to facilitation of candidal colonisation. This may explain the increased resistance of *Candida* species to antimicrobial agents, therefore increases host's susceptibility to candidal infection (Bastert *et al.*, 2001).

Oral candidiasis, also referred to as thrush is a yeast infection of the oral cavity characterised by the creamy white, curd-like patches on the tongue, buccal mucosa, periodontal tissues, and oropharynx. It is generally caused by an overgrowth of the genus *Candida*, predominantly by *C. albicans* (Epstein, 1990; Guida, 1988). The mucosa of the tongue dorsum may represent an attractive colonisation site for *Candida* species. Besides, denture wearing is a causal factor of oral degeneration which acts as a reservoir for candidal colonisation (Critchley and Douglas, 1985). Recently, the incidence of oral candidiasis is on the rise due to the prevalence among individuals wearing denture (He *et al.*, 2006; Nalbant *et al.*, 2008; Pereira-Cenci *et al.*, 2008; Ramage *et al.*, 2006; Scardina *et al.*, 2007). Mizugai *et al.* (2007) also reported that the occurrence of multiple *Candida* species was significantly higher among denture wearers.

Malaysia is blessed with natural products that represent valuable sources of bioactive agents with potent and unique medicinal properties. Many of these natural products are not yet developed as pharmaceutical products (real medicine), but represent as a novel class of dietary supplements or nutraceuticals or exploited as medicaments in traditional medicine. The natural products provide various active compounds that have been shown to have enormous therapeutic potential towards infectious diseases (Galal *et al.*, 1991; Iwu *et al.*, 1999). Previous studies have reported that medicinal plants produce a large number of secondary metabolites with antimicrobial effects on pathogens (Mari *et al.*, 2003; Obagwu and Korsten, 2003). In addition, the increasing prevalence of oral infections and the recent appearance of candidal strains with reduced susceptibility to antibiotics, have led the search for novel compounds from natural resources. Interest in natural antimicrobial product is also driven by the issues concerning the safety aspect of chemical preservatives of current drugs. Contrary to the synthetic drugs, the natural constituents from plants are generally safe for human consumption and are environmentally friendly (Thangavelu *et al.*, 2004).

# 1.2 Objectives of Research

The main objective of this study is to screen for antifungal activities against a variety of *Candida* species, manifested by aqueous extracts from selected local plants.

The specific objectives of this study were to investigate:

- 1. The antifungal properties of crude aqueous (CA) extract of selected plants species against seven common oral *Candida* species.
- 2. The minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC) of the plants extracts that showed antifungal activity against the oral *Candida* species.
- 3. The effects of CA extracts on the growth profile of oral *Candida* species.
- 4. The effects of CA extracts on the cell surface hydrophobicity of *Candida* species.
- 5. The adherence of *Candida* species to acquired pellicle and the anti-adhesion activity of the CA extracts.
- The morphological changes of oral *Candida* species following treatment with the CA extracts.
- 7. The effects of CA extracts towards the expression of secretory aspartyl proteinases (*SAPs*) and hyphal wall protein (*HWP1*) genes, which are responsible for the virulence properties of *Candida* species.

#### **CHAPTER TWO**

#### LITERATURE REVIEW

## 2.1 The Oral Cavity and Its Indigenous Microbial

Human oral cavity is an adequate site for the ecological niches of microbial species, which include bacteria, yeasts, mycoplasma, and protozoa (Samaranayake, 2006). A variety of surfaces *e.g.* mucosa, palate and teeth, produce distinct habitats due to their physical nature and biological properties. There is a dynamic interaction between the oral environments, salivary components, and metabolisms of the resident oral microflora. It is estimated that more than 700 microbial species have been identified from human oral cavity (Aas *et al.*, 2005; Haffajee and Socransky, 2006). A substantial change in a key environmental parameter that affects microbial growth can disrupt the natural balance of the microflora, hence leading to colonisation and infection of the host. Recently, *Candida* infections have received increasing attention, presumably due to the increased prevalence worldwide.

# 2.2 The Genus Candida

## 2.2.1 Biology and Taxonomy

Literature on the genus *Candida* have been well documented in many reviews, covering all aspects of virulence properties and factors that contribute to the infections (Calderone, 2002; Haynes, 2001; Van Wyk and Steenkamp, 2010; Yang, 2003). These yeast-like microorganisms have attracted the interest of investigators for years since it was first discovered in 1844 from the sputum of a tuberculous patient (Bennett, 1844), and *Candida* specieswere later identified as a cause of denture-related infections (Cahn, 1936). In 1980s, a clear surge of interest and associated research of candidal infections

have occurred. This has largely been due to the outbreak of human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) pandemics. The genus *Candida* which consists of 150-200 species is ubiquitous fungi (Odds, 1988), and have now been responsible for a wide range of systemic as well as superficial opportunistic infections (Madigan and Martinko, 2006).

Earlier investigators have assigned this genus *Candida* to the family Deuteromycetes, indicating a lack of sexual reproduction. Several pathogenic and nonpathogenic Candida species however, were reported to have a sexual stage (Calderone, 2002). There are confusions on its taxonomy due the expansion of phenotypic, genotypic and phylogenetic knowledge on the genus. Therefore, in a comprehensive classification of veasts. Kurtzman (1998) placed Candida species order Saccharromycetaceae, under class Hemiascomycetes into which budding yeasts are classified. Although Meyer et al. (1998) have listed a total of 163 Candida species under the genus *Candida*, a significant biodiversity exists among individual species. In addition, applications of molecular sequences in the phylogenetic analysis of various medically important yeasts have also provided information about the heterogeneity of Candida species (Diezmann et al., 2004; Kurtzman and Robnett, 2003). Recent reports have claimed that Candida species are persisting as saprophytes found in soil and aquatic environments as well as colonising in animal reservoirs (Brandão et al., 2010; Hsieh et al., 2010).

#### 2.2.2 General Morphology

All candida cells have unique ability to form pseudohyphae and consequently pseudomycelium on favourable growth conditions except *C. glabrata* which appears only in blastospore form (Richardson and Warnock, 2003). *C. albicans* for instance, is a

pleomorphic fungus that may undergo morphological switching when triggered by environmental conditions, leading to filamentous forms (Sudbery *et al.*, 2004). Basically, the shape of the vegetative cell or the blastospore of *Candida* species is described as a globose, ellipsoidal, and occasionally triangular, or lunate (Kurtzman and Fell, 1998). However, different *Candida* species has various shapes and sizes of blastospores. *Candida* species also produce chlamydospores, thick-walled, spherical, refractory cells (approximately  $8 - 12 \mu m$  in diameter), which are attached to the pseudohypae by elongated, suspensor cells.

# 2.2.3 Structures and Functions of Cell Wall

The cell envelope of microorganisms basically consists of a cell wall and one or two lipid membranes (Beveridge and Graham, 1991). It can differ significantly from one organism to another, and even within a strain it can change as a result of physiological adaptation to the environment (Russell, 1988). For species of the genus *Candida*, cell wall is the sole structure lacking in animal cells, and is situated on the outer surface of the cell. It is essential for its success as a pathogen, which is required for growth (Odds, 1985). The cell wall is also the contact point between the microbe and host surfaces including phagocytic cells. Since the cell wall is not present in mammals, it is a prime target for medical treatments.

The cell wall of *Saccharomyces cerevisiae* has been the most extensively studied, and is likely to be the model for *Candida* species since they have basic similarities (Ballou, 1982; Cabib *et al.*, 1982). The cell wall of yeast may account for up to 30% of the dry weight of the cell (Orlean, 1997) which composed of three basic components representing the major polysaccharides of the cell wall: (i) a highly branched polymers of glucose containing  $\beta$ -1,3 and  $\beta$ -1,6 linkages ( $\beta$ -glucans); (ii)

unbranched polymers of N-acetyl-D-glucosamine (GlcNAc) containing  $\beta$ -1,4 bonds (chitin); and (iii) polymers of mannose (mannan) which is covalently associated with proteins (glyco[manno]proteins) (Fleet, 1991; Valentin *et al.*, 2000).

A high degree of complexity in the protein and mannoprotein components present in the cell wall of *C. albicans* have been reported by several authors (Casanova *et al.*, 1992; Chaffin *et al.*, 1998; Martinez *et al.*, 1998), and were recently reviewed by Chaffin (2008). Mannan constitutes for about 40% of the cell wall biomass. The mannoproteins which appear as a dense network of radially projecting fibrils, designated fimbriae, were synthesized in the cytoplasm and transported through the secretory pathway (Cassone, 1989; Shepherd, 1987). Proteins appear to be either loosely associated with the wall matrix or covalently bound to mannan via phosphatidyl bonds (Casanova and Chaffin, 1991; Elorza *et al.*, 1988). The yeast wall mannoproteins are highly glycosylated polypeptides, thus may be thought of as yeast proteoglycans (van der Vaart *et al.*, 1995). They are densely packed and limit cell wall permeability to solutes (De Nobel *et al.*, 1990; Zlotnik *et al.*, 1984).

The inner part of the cell wall close to plasma membrane is rich in chitin that forms chains of tight antiparallel hydrogen-bonded structures and contributes to insolubility of the fibers. Chitin, a  $\beta$ -(1-4)-linked homopolymer of *N*-acetyl-Dglucosamine (Fig. 2.1), is a simple polysaccharide that is presented in the cell walls of all fungi studied to date (Latge, 2007; Munro and Gow, 2001). Chitin is synthesized by large families of chitin synthase (CHS) enzymes which were assigned into seven classes based on the classification proposed by Niňo-Vega *et al.* (2004) and Roncero (2002). The chitin content of the cell wall increases during morphogenesis and the mycelial cells contain three times more chitin than yeast cells (Shepherd, 1991). Most chitin is concentrated at the septal region, between the mother and the growing daughter bud with a smaller amount evenly distributed in the lateral walls (Cabib *et al.*, 1982; Molano *et al.*, 1980; Roberts *et al.*, 1983). During cytokinesis, a new chitin is deposited in a centripetal fashion in the furrow of the invaginating plasma membrane. A thin disk-like structure so-called primary septum is formed, thus closing the gap between mother and daughter bud. Finally, the two cells are assymmetrically separated so that most of the chitin remains in the mother cells (bud scar) (Horisberger and Vonlanthen, 1977; Molano *et al.*, 1980).

Evidence showed that both chitin and  $\beta$ -glucans polymers are closely integrated (Sietsma and Wessels, 1979; Surarit *et al.*, 1988). The chitin and  $\beta$ -1,3-glucan are synthesized at the plasma membrane and extruded into the periplasm, while  $\beta$ -1,6glucan sythesis occur partially in the endoplasmic reticulum or Golgi complex (Kollár et al., 1995). Although chitin is a minor constituent, but it may suffice to affect the greater amount of glucan (Kollár et al., 1995). Chitin which is randomly located on the wall of mother cell, will be forming part  $\beta$ -1,3-glucan-chitin complex and represent a major constituent of the inner wall (Fig. 2.2). β-1,6-glucan subsequently links the components of the inner and outer walls. While the inner wall layer appears contiguous with the plasmalemma with extensive membrane invaginations involved in the anchoring of the cell wall to the membrane, the outer wall layer appears as a dense network with a fibrillar or flocculent aspect (Cassone, 1989). Covalent linkages are reported to exist as  $\beta$ -1,4linkages between the reducing ends of chitin and the nonreducing end of  $\beta$ -1,3-glucans (Kollár *et al.*, 1995) as well as among glycoproteins;  $\beta$ -1,6-glucans, and  $\beta$ -1,3-glucans (Kapteyn*et al.*, 1996). Most abundant class of cell wall proteins (CWPs) is linked to  $\beta$ -1,6-glucans through a glycophosphatidylinositol (GPI) remnant (GPI-CWP), and the class of Pir (proteins with internal repeats) proteins, is linked directly with  $\beta$ -1,3-glucans with alkali-labile ester linkage (Chaffin, 2008; Ecker *et al.*, 2006; Kapteyn *et al.*, 2000). Hence, they have been generally accepted as polysaccharides that are indispensible for maintaining rigidity and structural integrity of the cells (Gooday, 1995; Kollár *et al.*, 1995).

The cell wall was not only considered as an almost inert structure that protects the protoplast against osmotic pressure, but established as a dynamic and constantly changing structure that contains enzymically active proteins. The cell wall acts as a permeable barrier transporting materials into and out of the cell, maintaining structural integrity (Cabib *et al.*, 1988), and by virtue of its location has a role in physical interaction between the cell and its host. Polypeptides and proteins are intimately bound with cell wall polysaccharides and the fine structures of various wall phosphoglycopeptide oligomers and polymers account for differences in antigenic structures, hydrophobic properties and specific adhesion to host cells and tissues, including the surface of prosthetic devices. It is reported that the development of biofilms is closely associated with the extracellular material that is predominantly composed of cell-wall polysaccharides containing mannose and glucose residues. It does plays an important role in the pathogenicity of microorganisms (Cassone, 1989), and represents a possible target for inhibitors functioning as antifungal agents.



Figure 2.1: Chitin structure – a simple polysaccharide in the cell walls of all fungi.

Note: Chitin is a  $\beta$ -1,4-homopolymer of *N*-acetyl-D-glucosamine that folds in an antiparallel manner forming intra-chain hydrogen bonds. The chains are crossed-linked covalently to  $\beta$ -1,3-glucan (green) to form the inner skeleton of most fungi. (Source: Lenardon *et al.*, 2010).



Figure 2.2: Schematic representation of cell wall components.

Note: The cell wall is external to the cell membrane ( $\bigcirc$ ).  $\beta$ -1,3-glucan network with Pir-CWPs ( $\bigotimes$ ,  $\beta$ -1,3-glucan-chitin complexes represent a major constituent of the inner wall ( $\bigcirc$ ), unattached proteins found in cell wall or in the medium ( $\bigotimes$ ) (Sources: Chaffin, 2008; Klis *et al.*, 2009).

## 2.3 Normal Microbial Flora

Normal microflora is defined as the characteristic mixtures of microorganisms associated with a site in oral cavity (Marsh *et al.*, 2009). Among the members of the normal microflora is yeast, which includes species of the genus *Candida* that live as commensals and coaggregate in harmony with the host (Arendorf and Walker, 1980). The posterior tongue, buccal and other mucosal surfaces of the oral cavity are considered as the primary locations of inhabitance. As commensal microorganism, candida secretes enzymes to break down the carbohydrates via fermentation to yield alcohol and carbon dioxide for their growth and sustenance. Other commensal flora will prevent the colonization of *Candida* species in the oral cavity by inhibition of yeast adhesion, competition for nutrients and production of antifungal agents (MacFarlane and Samaranayake, 1990). *Candida* species simultaneously are 'opportunists' pathogens that are capable of invading the oral mucosa and causing significant damage (Samaranayake, 1990). This usually happens when a variety of predisposing factors implicated in the pathogenesis of oral infection *e.g.* oral candidaiss.

### 2.4 Transition of the Normal Flora to Pathogenic Flora

Oral hygiene is important to assure not only the teeth and gums in a good condition, but also to resist overgrowth of microorganisms from causing oral infections such as cavities, caries and plaque. *Candida* species have complex functions especially when primarily associated with various *Candida*-related infections in human. Species of the genus *Candida* is able to transit from harmless commensal organisms to a pathogen whenever there is a change in the chemistry in the oral cavity. Systemic and local changes within the host may promote proliferation of person's own commensal flora with a possibility of host tissue invasion. The overgrowth of *Candida* species then set off a cycle that leads to the further weakening of the body's defenses, which in turn

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allows *Candida* species to spread even further. In recent years, several species have emerged and justified as potentially pathogenic. At least 17 species of *Candida* have been found to cause diseases in humans (Rinaldi, 1993).

#### 2.5 Types of *Candida* Species

## 2.5.1 Candida albicans

Candida albicans - the currently accepted name was first introduced by Berkhout in 1923 (Kwon-Chung and Bennett, 1992). It has been investigated extensively than the other *Candida* species due to its predominant medical importance, and is generally considered as the most pathogenic member of the genus Candida (Meyer et al., 1998). It once has accounted for most serious nosocomial candidal infections (Trick *et al.*, 2002), and is frequently isolated (in 60 - 80% of the cases) from the oral cavities of both healthy and diseased patients (Rautemaa et al., 2006). It has become a model for fungal cellular development in order to stimulate advances in our understanding of the epidemiology of candidiasis, the pathogenesis of disease as well as the genetics and biochemistry of *Candida* species. There are several known virulent factors that contribute to its pathogenicity, whereby Candidacan grow as yeast cells, pseudohyphae and hyphae, and produces chlamydospores which are round, refractile spore-like structures that are mainly produced at the hyphae ends in vitro under specific environment. Major virulent traits of C. albicans are the ability to switch its morphology, from growth of singular budding cells (blastosphores) to a filamentaous growth form (hyphae) which is called dimorphic transition, crucial for adhesion to host tissues mediated by cell wall components (Calderone and Fonzi, 2001; Cutler, 1991; Odds, 1994). Although the term 'dimorphism' or 'dimorphic fungus' are commonly accepted when referring to C. albicans, the ability to adopt a spectrum of morphologies thus considered this species as a 'polymorphic" or "pleomorphic" organism (Kerridge, 1993). These morphological transitions often represent due to the response of the yeast cells to the changing environmental conditions which may allow the cells to adapt to different biological niches.

#### 2.5.2 Candida dubliniensis

*Candida dubliniensis* was first identified as a new species by Sullivan *et al.* (1995) and its complete genome was recently sequenced (Jackson *et al.*, 2009). *C. dubliniensis* is not a common constituent of the human oral microflora, which approximately 3.5% of healthy individuals carry *C. dubliniensis* in the oral cavity (Pinjon *et al.*, 2005). *C. dubliniensis* however, is now regarded as an emerging pathogenin human oral infections and are most frequently associated with the oral cavities of HIV-infected individuals, particularly those with a history of recurrent oral candidiasis (Coleman *et al.*, 1997; Jabra-Rizk *et al.*, 2000). Moreover, it is now ranked as either the second or third most frequently isolated from HIV/AIDS patients (Badiee *et al.*, 2010). *C. dubliniensis* has also been recovered from vaginal, respiratory, urinary and faecal specimens obtained from both HIV-infected and non HIV-infected patients (Gee *et al.*, 2002; Gutierrez *et al.*, 2002; Jabra-Rizk *et al.*, 2000; Meis *et al.*, 1999; Quindós *et al.*, 2000).

*C. dubliniensis* is phenotypically similar to but genotypically distinct from *C. albicans* (Coleman *et al.*, 1998; Sullivan and Coleman, 1998; Sullivan *et al.*, 1995; Sullivan *et al.*, 2005). *C. dubliniensis* was misidentified as *C. albicans* since isolates of both species are germ-tube-positive and produce chlamydospores (Sullivan and Coleman, 1998; Sullivan *et al.*, 1995). *In vitro* phenotypic studies have shown that *C. dubliniensis* has a few characteristics that distinguish it from *C. albicans* (Hazen *et al.*, 2001). In fact, from electron microscopic studies, both species revealed major

ultrastructural differences (Jabra-Rizk *et al.*, 1999). The higher level of proteases produced by *C. dubliniensis* enables it to adhere more readily to buccal epithelial cells compared to *C. albicans* (McCullough *et al.*, 1995; Sullivan *et al.*, 1999). Gilfillan *et al.* (1998) reported that *C. dubliniensis* is less pathogenic than *C. albicans* based on preliminary phylogenetic studies conducted. Although *C. dubliniensis* has the ability to produce hyphae, it does less efficiently than *C. albicans* under a wide range of hyphae-induction conditions (Stokes *et al.*, 2007). However, there are certain putative virulence factors that have helped this species to sustain its existence and stay competence with the other microorganisms. Hannula *et al.* (2000) have observed that *C. dubliniensis* isolates did. This could be a favourable factor for adaptation of this species to the host environment. In addition, most isolates of *C. dubliniensis* are susceptible to existing antifungal agents but rapidly develops resistance to fluconazole *in vitro* (Kirkpatrick *et al.*, 2000; Moran *et al.*, 1997; Pinjon *et al.*, 2005).

## 2.5.3 Candida glabrata

*Candida glabrata* is phenotypically closer to *Saccharomyces cerevisiae* and considerably the most prevalent amongst the NCAC species (Dujon *et al.*, 2004; Fleck *et al.*, 2007; Redding *et al.*, 2003). While the genus *Candida* was not named until 1913, *C. glabrata* was initially classified in the genus *Torulopsis* due to its lack of pseudohyphae production like other *Candida* species (Kwon-Chung and Bennett, 1992). It is the only *Candida* species that does not form pseudohyphae above 37 °C. The hypha formation is recognized by the increased adherence and tissue invasion (Odds, 1988). *C. glabrata* has been considered as a non-pathogenic saprophyte of the normal flora in healthy individuals (Stenderup and Pederson, 1962) but its implication in human infection has increased. It is often recovered in clinical isolates from AIDS patients

(Miyazaki *et al.*, 1998; Sanglard *et al.*, 1999; Vasquez, 1999) and currently ranks as the second or third most frequently isolated *Candida* species from the reported cases of candidiasis (Fidel *et al.*, 1999; Pfaller and Diekema, 2004; Wingard, 1995). The prevalence of superficial and invasive *C. glabrata* infections has demonstrating concerns since it has often been linked to clinical use of fluconazole of which has apparently appeared to be resistant to moderate levels of fluconazole (Pfaller *et al.*, 1998; Redding *et al.*, 2003; Rex *et al.*, 1995). This explains why most *C. glabrata* were associated with oropharyngeal *Candida* (OPC) infections.

### 2.5.4 Candida krusei

*Candida krusei* species once considered a saprophyte, is recently gaining clinical interest as an emerging pathogen in humans. Further investigations have been carried out over the last two to three decades after it was first suggested by Castellani in early 1900s (Castellani, 1912). C. krusei is different from a majority of other medically important *Candida* species. The cells are elongated and have the appearance of "long grain rice" in its shape (Samaranayake, 1990). In human, this species has been described as a transient organism of the mucosal surface and of minor clinical significance (Samanarayake and Samanarayake, 1994). It is reported that C. krusei mannan is different than those of other Candida species which is being lightly branched and containing (1-2) and (1-6) side chains in the ratio of 3:1 (Kogan et al., 1988). The modified structure of  $\alpha$ -D-mannan of C. krusei has been proposed in which the main chain is lightly branched and consists of 2- and 6-linked units in the ratio 3:1. According to Odds (1988), C. krusei is also believed to stand with free-vitamin media compared to the other medically important Candida species. It was found to be the most distantly related to C. albicans (Barns et al., 1991), and being recognised as a potentially multidrug-resistant (MDR) fungal pathogen. The breakthrough of C. kruseiamong patients have been verified by several authors due to its intrinsic fluconazole resistance and reports of decreased susceptibility to both amphotericin B and flucytosine (McGee and Tereso, 2003; Majoros et al., 2006; Pemán et al., 2006). However, voriconazole has been used successfully to treat C. krusei-infected patients (Ostrosky-Zeichner et al., 2003; Kullberg et al., 2005). Despite the Candida is a genus of asexual yeasts (Odds, 1988), C. krusei is very closely related to a sexual species (Kurtzman et al., 1980). This could explain why C. krusei can be found in two basic morphological forms, as yeast and pseudohyphae. Both are frequently present simultaneously in growing cultures and may not be separated easily. The increasing incidence of C. krusei infections has also been well documented. From a recent study conducted by Pfaller et al. (2008) between January 2001 and December 2005, a total of 10,510 Candida species was isolated in Malaysia alone, out of which 142 were C. *krusei* and represented 1.3%. However, it remains an uncommon clinical isolate due to the very low frequency of isolation in both Asia-Pacific and Latin American regions (Pfaller et al., 2008). In contrast to C. albicans, which is considered to be the most pathogenic and virulent species, C. krusei was classified as an extremely rare nonalbicans Candida species occasionally associated with candidiasis, endocarditis (Rubinstein et al., 1975) and esophagitis (Mathieson and Dutta, 1983).

## 2.5.5 Candida lusitaniae

The first reported cases of human infection caused by *Candida lusitaniae* were in 1979 (Holzschu *et al.*, 1979). *C. lusitaniae* is an infrequent yeast species that is found to be related with human commensal, and less than 1% of samples recovered from hospitalised patients were *C. lusitaniae* (Merz, 1984). *C. lusitaniae* resembles *C. tropicalis* and *C. parapsilosis*, but differs in its ability to ferment cellobiose and assimilate rhamnose (Guarro *et al.*, 1999), and less pathogenic than *C. tropicalis* and *C.*  *parapsilosis*. The most infections are mainly because of immunocompromised hosts with prolonged administration of antibiotics, prolonged hospitalisation, cytotoxic and corticosteriod therapy. *C. lusitaniae* is highly susceptible to azole antifungal (Pfaller *et al.*, 2003) despite having a slightly increased resistance to fluconazole as proven recently in a global antifungal surveillance study (Pfaller *et al.*, 2007). Besides, it is also considered as susceptible *in vitro*, to all the systemic antifungal agents which has characterised by its susceptibility to develop resistance to amphotericin B (AMB) during treatment (Hawkins and Baddour, 2003; Nöel *et al.*, 2003; Papon *et al.*, 2007).

#### 2.5.6 Candida parapsilosis

Candida parapsilosis has now become the second most frequently recovered Candida species from blood culture in Europe, Canada and Latin America, and rank third in the United States (Pfaller et al., 1999, 2000). C. parapsilosis also the most frequent candidal species isolated from oral candidiasis in children and adolescents with cancers (Gravina et al., 2007; Safdar et al., 2001), as well as a frequent cause of endocarditis among parenteral drug abusers (Weems, 1992). The epidemiology of C. parapsilosis in the hospital environment is unique. It has been shown to be the NCAC species most commonly recovered from the hands of health care workers, and this explains why the catheters and other devices are readily contaminated (Hedderwick et al., 2000). The affinity of C. parapsilosis for surfaces of intravascular catheters and prosthetic devices has been recognized (Levin et al., 1998; Moran et al., 2002), and its ability to produce an extracellular polysaccharide in vitro is believed to aid adherence and biofilm formation on artificial surfaces (Levin et al., 1998). Compared to C. albicans, C. parapsilosis exhibits different morphology, with the biofilms being apparently devoid of extracellular matrix and hyphae, and less thickness. Notably, the morphology of C. parapsilosis biofilms is much similar to bacteria (Kuhn et al., 2002; Odds, 1988) and they do grow in pseudohypal or other filamentous forms. *C. parapsilosis* is generally susceptible to all of the major antifungal drugs. Nguyen *et al.* (1996) however, reported that *C. parapsilosis* infection was associated with prior fluconazole therapy and was responsible for a number of cases of breakthrough candidemia in patients receiving fluconazole. In addition, several investigators have also demonstrated that *C. parapsilosis* was tolerant to amphotericin B (Cantón *et al.*, 2003; Seidenfeld *et al.*, 1983).

#### 2.5.7 Candida tropicalis

Candida tropicalis was classified as the second most frequent pathogenic Candida species present in adults and elderly patients, and is a leading cause of candidiasis due to its similar epidemiology to C. albicans (Nucci and Colombo, 2007). Among the non-Candida albicans Candida (NCAC) species, the emergence resistance level of C. tropicalis isolates towards common antibiotics such as fluconazole has increased and the development is considerably important because it was among the common isolated species than C. albicans (Cheng et al., 2004; Yang et al., 2004; Yang et al., 2005). Yang et al. (2003) also reported that C. tropicalis had been more successful than C. albicans or C. glabrata in invading mucosal surfaces or in colonizing catheters. As reported, C. tropicalis makes up to 6% of isolates of yeasts other than C. albicans in HIV-infected individuals (Cartledge et al., 1999). In some perspectives, C. tropicalis could have greater virulence equivalent to the other species. To date, it has been discovered that C. tropicalis possess a unique sexual programme. The ability of C. tropicalis to switch its phenotype in order to regulate the sexual mating, and the extracellular proteolytic activity would have had contributed to its transition from commensal to a pathogen (Porman et al., 2011; Silva et al., 2011).

## 2.6 Emergence of the Non-Candida albicans Candida Species

The non-Candida albicans Candida (NCAC) species are heterogenous organisms that differ from each other, and from C. albicans (Moran et al., 2002). The predominance of NCAC species over C. albicans (Belazi et al., 2004; Davies et al., 2002; Safdar *et al.*, 2001) has become increasingly important especially in high-risk patients with a variety of other members notably C. krusei, C. glabrata, C. parapsilosis, C. dubliniensis and C. tropicalis have been cited as the causative agents of an increasing number of mucosal and systemic infections (Bokor-Brati, 2008; Gilfillan et al., 1998; Hong Nguyen and Peacock, 1996; Krcmery and Barnes, 2002; Madigan and Martinko, 2006). Apart from the latter, C. lusitanie, C. guilliermondii, C. rugosa, C. kefyr, C. stellatoidea, C. norvegensis, and C. famata were among the other NCAC species that caused a small percentage of human fungaemia (Krcmery and Barnes, 2002). There is increasing evidence that more than one Candida species may simultaneously colonise the oral mucosa, tongue and palate, both in healthy and diseased subjects. A number of studies have revealed that the proportion of current infections involving the NCAC species had changed with higher resistance level towards common prescribed antifungal agents (Hong Nguyen and Peacock, 1996). This has become a great concern to society. This is said to be due to the rising number of immunocompromised individuals and a consequence of the heavy usage of antibiotics of such as amphotericin B and fluconazole (Moran et al., 1997, 1998; Moran et al., 2002; Pfaller et al., 2005).

# 2.7 Oral candidiasis

Oral candidiasis, also known as thrush is a common opportunistic infection caused by an overgrowth of the genus *Candida*, predominantly by *C. albicans* (Epstein, 1990; Guida, 1988). It is characterised by creamy white, curd-like patches on the tongue, buccal mucosa, periodontal tissues, and oropharynx. The infection is more

commonly sighted in subjects who are simultaneously suffering from other illness.Neonates and infants are also thought to develop oral candidiasis due to their under developed immune defences.

#### 2.7.1 Classification of Oral Candidiasis

The systematic classification of oral candidiasis was first proposed by Lehner in 1966 (Lehner, 1966). Clinically, oral candidiasis can be classifed into four variants: (i) pseudomembranouscandidiasis, (ii) erythematous (atrophic) candidosis, (iii) chronic hyperplastic, and (iv) angular cheilitis.Other common *Candida*-associated lesions include, (v) *Candida*-associated denture stomatitis, and (vi) median rhomboid glossitis (Lewis and Lamey, 1995; Zegarelli, 1993).

## 2.7.2 Predisposing Factors to Oral Candidiasis

The interaction between *Candida* and the host are extremely complex, involving host factors, systemic factors and iatrogenic factors. The chance of getting oral candidiasis is considerably higher as a result of the local and systemic conditions of the host itself (Samaranayake, 1990; Scully *et al.*, 1994).

## 2.7.2.1 Local Host Factors

For *Candida* species to establish an infective process, the organisms must adhere to a host surface, proliferate and penetrate the first line of the host's defence which is the oral mucosa. Primary function of oral mucosa is to serve as a barrier that protects the underlying tissues. The proteins present in these mucosal cells may act as antifungals and retard candidal invasion (Kashima *et al.*, 1989). Changes in the epithelium of the oral mucosa, atrophy, hyperplasia or dysplasia, may affect the efficiency of the mucosal barrier (Samaranayake, 1990). In addition, a continuous flow rate and the quality of saliva are important in preventing oral colonisation because it removes the loosely attached *Candida* from the oral cavity. Qualitative changes in saliva such as the content of salivary glucose however, may influence the oral carriage of *Candida* species (Samaranayake, 1990). The oral carriage is also significantly higher among patients with burning mouth syndrome and this may be directly or indirectly related to reduced salivary gland function in this group (Lamey and Lamb, 1988). Denture wearing is another factor in the prevalence of *Candida*-associated infections (Bastert *et al.*, 2001; Meurman *et al.*, 1997). When attachment takes place on acrylic denture surfaces, the organisms benefit from the enriched nutrient that exists at the solid-liquid interfaces (Marshall, 1977).

#### 2.7.2.2 Systemic Factors

The increasing numbers of immunocompromised individuals due to the progress made in modern medicine (Richardson, 2005), patients with severe illness, or human immunodeficiency virus (HIV)-infections are the important contributory factors of susceptibility to fungal infections (Nittayananta *et al.*, 2001; Vanden Bossche *et al.*, 1994). The infection was highly (>90%) recovered from HIV/AIDS patients who were immunosuppressed (Back-Brito *et al.*, 2009; Nittayananta *et al.*, 2001). Thus, the existance of harmless oral flora would turn out to be pathogenic and affect the oral health. Moreover, the non-*Candida albicans Candida* strains are now considered as a cause of failure of antifungal therapy as well as increased recurrence in HIV patients (Cartledge *et al.*, 1999). Nutritional factors *i.e.* malnutrition, iron deficiency, high carbohydrate diet, or endocrine disorders *i.e.* diabetes mellitus (Belazi *et al.*, 2005; Willis *et al.*, 1999) and malignant disease may play a major role in enhacing the chronicity of the candidiasis. A high carbohydrate intake for instance, might promote

the growth of *Candida* which utilizes the glucose in saliva despite the presence of many antifungal proteins (Knight and Fletcher, 1971).

## 2.7.2.3 Iatrogenic Factors

The use of broad-spectrum antibacterial antibiotics during hospitalization, and a consequence of extensive corticosteroid therapy (Samaranayake, 1990), cytotoxic therapy and radiotherapy (Peterson and Sonis, 1983), are the iatrogenic factors which induce significant disturbances in the normal microflora. Seelig (1966) demonstrated that the administration of antibiotics is an important predisposing factor in initiating oral candidiasis by suppressing the bacterial flora that used to prevent an overgrowth of yeast cells.



Figure 2.3: Schematic diagram summarizing the possible predisposing factors influenced the interaction between Candida and the host.

## 2.7.3 Denture Wearing Associated with Oral Candidiasis

Despite its low carriage percentage, many possible predisposing factors may contribute to the pathogenesis of oral candidiasis (Fig. 2.3). Numerous reports concerning oral candidiasis suggest that denture wearing has become a significant cause of oral infection (He et al., 2006; Mizugai et al., 2007; Pereira-Cenci et al., 2008; Ramage et al., 2006; Scardina et al., 2007). The incidences of wearing dentures also increases with age and this has promoted candidal colonisation. Moreover, the rise of single and multiple Candida species were distinguished and significantly correlated with denture wearing (Daniluk et al., 2006; Mizugai et al., 2007). This has caused major disturbances to the habitat which perturb the stability of the microflora. Therefore, the non-Candida albicans Candida (NCAC) species have been taken into consideration in recent study in order to preserve oral health effectively. In addition, inadequate cleaning of the oral cavity and denture is a prerequisite for successful colonisation and development of biofilms. The surfaces may serve as a reservoir (Critchley and Douglas, 1985) which allows the attachment of microorganisms including the species of Candida. Researchers had demonstrated that a denture coated with salivary pellicle provides convenient sites for the adherence of microorganisms. In addition, materials with the roughest surface usually exhibit higher yeast counts (Nevzatoðlu et al., 2007; Pereira-Cenci et al., 2007). Chronic atrophic candidiasis, also known as denture stomatitis, is a common form of oral candidiasis associated with the use of dentures. In a study conducted in a hospital population, 50% of denture wearers have been diagnosed with fungal infection consisting of Candida-associated denture stomatitis (Budtz-Jorgensen, 1990). It results in changes of the microbial flora and accumulation of plaque between the palate and the mucosal surface of the denture (Scully et al., 1994). The infection subsequently formed an oral lesion corresponding to the area covered by the denture especially on the upper fitting surface (Hasan *et al.*, 2009; He *et al.*, 2006; Maijala *et al.*, 2007).

## 2.8 Virulence Factors

There are specific factors contributing to the pathogenicity of *Candida* species, including the ability to adhere to epithelial cells (Ellepola *et al.*, 1998) and prosthetic devices, production of hydrolytic enzymes such as extracellular proteinases and phospholipases, including the production of haemolytic factor (Ghannoum, 2000).

## 2.8.1 Adherence Mechanisms

Adherence is a key attribute of virulence among *Candida* species for successful colonisation and infection. Microbial adhesion to mucosal or denture surfaces has been known to be an important stage in infectious disease process, particularly in the oral cavity (Ellepola *et al.*, 1998; Kolenbrander, 1990; Tawara *et al.*, 1996). However, the mechanisms are varied since the *Candida*-host interaction systems are extremely complex and involve a variety of ligand-receptor components (Sturtevant and Calderone, 1997). Generally, there are two mechanisms implicated in facilitating the adherence. First, the adhesion based on the specific-binding which the interaction occurs between the acquired pellicle and the candidal cell surface. The second mechanism which is said to be non-specific, involves physiological forces.

## 2.8.1.1 Specific Adhesion: Acquired Pellicles

Some authors demonstrated that the yeast cell wall is specifically designed with components to bind with a variety of ligands on the host cell surface including protein-protein interactions, protein-carbohydrate interactions and *Candida* mannoprotein

ligands (Calderone and Braun, 1991; Cannon and Chaffin, 1999; Chaffin et al., 1998). Once a denture is placed in oral cavity, the surfaces will be instantaneously coated with a layer of organic molecules in saliva. Hence, promotes the adhesion of the free-floating cells to adhere on the surfaces by providing many specific receptors to the acquired pellicle. The binding forms endowed with hyphae are able to stick and allow easier invasion into the tissues of the host. While it is a natural part of candidal lifestyle, thus one can expect that *Candida* species may have specific affinity and binding to acrylic resin (Candida-associated denture stomatitis) and other plastics (catheter-related candidiasis). Adhesion to biomaterial is not an esoteric interaction, since the binding found may contribute to the development of infection and deterioration of the devices and since adherent organisms may be more resistant to antifungal drugs. The adhesion also depends on the microporosity present on the surface of the denture (Allison and Douglas, 1973; Powers and Sakaguchi, 2006). Such irregularities of surface enable the yeasts to nest, aggregate with bacterial communities and develop into distinct surfaceattached communities called biofilms to avoid dislodgement by cleasing action of mucosal secretions. It has been demonstrated that the adherence and pathogenicity of C. albicans are correlated (McCourtie and Douglas, 1984). There have been a few studies conducted regarding the adhesion of non-albicans Candida species and comparisons were made with C. albicans. In addition, adhering properties such as hydrophobicity, adhesin-like hyphal wall proteins 1 (Hwp1), aspartyl proteinases (Saps) and phosphatases, have been shown to play a crucial role in the ability of the yeast to adhere to human buccal epithelial cells (BECs) and other substrates (Cannon and Chaffin, 1999; Sturtevant and Calderone, 1997).

## 2.8.1.2 Non-Specific Adhesion: Cell Surface Hydrophobicity

Cell surface hydrophobicity (CSH) also plays an important role in the initial candidal adherence to epithelial cells and medical biomaterial (Cannon and Chaffin, 1999; Hazen, 1989; Hazen and Hazen, 1992). This involves physiochemical forces which are the hydrophobicity and electrostatic interactions that bound to promote cell adherence on mucosal tissues or substrates and to form biofilms on the inert surfaces of medical implants (Bos et al., 1999; Busscher et al., 1984; Li et al., 2003). As discussed above, cell wall is a vital component with designed receptors found in extracellular matrix to interact with host proteins under appropriate environments. Hydrophobic proteins embedded in the matrix of the candidal cell wall beneath the fibrillar layer, hence provide the hydrophobic interactions between the two surfaces – the acquired pellicle and the cell wall. Hydrophobic interaction is defined as the tendency of hydrocarbon components to associate with each other in an aqueous environment (Conn and Stumpf, 1976). The fibrils of the outer fibrillar layer of the Candida species appeared to be condensed or dispersed to make a contact between the inner layers of the cell wall and recognition sites on the epithelial membranes (Howlett and Squier, 1980). Such interaction will turn the initial attachment between fungus and the host surface into a strong bond (Hazen, 1989).

Growth temperature has been suggested to have a slight influence in affecting the interactions and coaggregation of microorganisms. Recent comparative studies however have shown that each species possess different cell surface variations in which allowed it to be constantly hydrophobic, regardless of growth temperature. This agrees with previous study which demonstrated the expression of CSH to be different between species (Jabra-Rizk *et al.*, 1999). Hydrophobic components appeared to be more abundant in the extracts from germ tube than from yeast cell. Previous studies also indicated that the hydrophobic proteins are usually smaller than 50 kDa while hydrophilic proteins are predominantly larger than 90 kDa. Based on the freeze-fracture examination, the external cell surface layers had revealed structural differences between hydrophilic and hydrophobic cells. Hydrophilic exhibits a dense layer of fibrils, composed mostly of high-molecular-weight mannoproteins that is absent or scant in hydrophobic cells (Hazen and Hazen, 1992). Hazen and Hazen (1992) also suggested that the amount of hydrophobic proteins is constant while the amount of hydrophilic proteins varies during growth. Being hydrophobic is an advantage for *Candida* species to colonize the pellicle-covered tooth/denture surfaces. Having this property would allow the formation of hydrophobic interactions between the candidal cell surface and the pellicle-covered surface. Samaranayake *et al.* (1995) demonstrated that there was no correlation between hydrophobicity and adherence of two different *Candida* species to denture surfaces. Despite the contradictory findings, more different species should be considered in the present study to understand the variations in adherence capabilities of *Candida* species.

#### 2.8.2 Virulence-Associated Genes in *Candida* Species

Most pathogens, including *Candida* species have developed an effective battery of putative virulence traits to overcome host defences and invade the mucosal tissues in the oral cavity. Despite a panel of virulence attributes are involved in the infective process, no single factor accounts for *Candida* virulence and not all expressed virulence attributes are necessary for a particular stage of infection (Cutler, 1991; Odds, 1994). Although many factors have been suggested to be virulence attributes for *Candida* species, specifically *C. albicans*, extracellular hydrolytic enzymes such as secreted arpartyl proteinases (Saps) and adhesins have widely been studied to understand the inner mechanisms of the cells (Table 2.1).

Virulence attributes	Putative virulence roles	References	
Adhesins (e.g. Hwp1) <sup>a</sup>	Adhesion and colonisation	Calderone and Braun, 1991; Calderone and Gow, 2002; Chaffin <i>et al.</i> , 1998.	
Hydrolytic enzymes ( <i>e.g.</i> Saps) <sup>b</sup>	Invasion, tissue damage, evasion of host responses	Hube, 1996; Hube, 2000; Hube and Naglik, 2001.	

Table 2.1: Virulence attributes of *C. albicans* 

<sup>a</sup> Hwp1, hyphal cell wall protein 1

<sup>b</sup> Saps, secreted aspartyl proteinases 1-10

## 2.8.2.1 Adhesin-like Hyphal Cell Wall Protein 1 (Hwp1)

Hyphal cell wall-specific protein (Hwp1) is among the most well understood and abundantly found *Candida albicans* adhesin (Sundstrom, 2002). It is expressed only during the candidal hyphae form compared to during the yeast forms (Nantel *et al.*, 2002; Sharkey *et al.*, 1999; Staab *et al.*, 1996, 1999; Sundstrom, 2002). A result from recent investigation hassuggested that *HWP1* mRNA may also arise from pseudophyhal growth forms (Snide and Sundstrom, 2006) or possibly from yeast forms, although there is no significant experimental data to support the presence of *HWP1* mRNA in the yeast forms. Hwp1 is a cell surface protein that is covalently linked to the cell wall  $\beta$ -glucan through a remnant of its glycosylphosphatidylinositol (GPI) anchor (Staab *et al.*, 2004) and promotes adhesion of *C. albicans* to epithelial surfaces.

Since adhesion and hyphae formation are well known as the virulence factors and a prerequisite for candidal infection, the presence of adhesin is important for the pathogenesis of candidiasis (Staab *et al.*, 1996, 1999; Sundstrom *et al.*, 2002). This agrees with previous report which concluded that pathogenicity and adherence of C. *albicans* to the host surfaces are positively correlated (Hogan *et al.*, 1996). Furthermore, consistence with the idea that Hwp1 expression is hypha-specific in the context of a biofilm, Hwp1 has a critical role in biofilm formation (Nobile *et al.*, 2006). Taken together, these cohesive relations are important in the regulation of *HWP1* gene expression and Hwp1 secretion. As previously described, the expression is dependent upon Bcr1, a key regulator of biofilm formation (Nobile and Mitchell, 2005; Nobile *et al.*, 2006). The high frequency of *HWP1* expression in oral cavity from carriers strongly implicates the role of Hwp1 and hyphae in establishing and maintaining the presence of *C. albicans* on mucosal surfaces. Hence, Hwp1 and hyphal growth appeared to be important factors in invasive interactions of *C. albicans* to human hosts. Although the regulation of Hwp1 secretion and *HWP1* gene expression have been extensively studied for *C. albicans* (Naglik *et al.*, 2006; Nobile and Mitchell, 2006; Nobile *et al.*, 2006; Sundstrom, 2002), but little is known on whether distinct adhesion of the non-albicans and hyphae formation have the propensity to express distinctive patterns of *HWP1* gene expression, which would secrete different amounts of Hwp1.

### 2.8.2.2 Secreted Aspartyl Proteinases (Saps)

Evidence of proteolytic activity of *Candida* species was first described by Staib (1965), and the secretory aspartyl proteinases (Saps) have been considered as virulence factor (Cutler, 1991; Hoegl *et al.*, 1996; Odds, 1994). *C. albicans* is not the only *Candida* species known to produce extracellular proteinases. Other *Candida* species such as *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis* have also been identified as proteinase secretors (Gilfillan *et al.*, 1998; Monod *et al.*, 1994; Pichova *et al.*, 2001).

At present, *C. albicans* produces at least 10 secreted aspartic proteinases (Saps) encoded by corresponding genes (*SAP1-10*) on five chromosomes (Hube, 1996; Monod

*et al.*, 1994, 1998; Naglik *et al.*, 2003). *In vivo* analysis of samples from patients with oral candidiasis and asymptomatic carriers demonstrated variable patterns of *SAP* gene expression (Naglik *et al.*, 1999; Schaller *et al.*, 1998). Schaller *et al.* (1998) demonstrated that the expression of *SAP* genes is temporally regulated and the expression sequence is *SAP1* and *SAP3* > *SAP6* > *SAP2* and *SAP8* in the *in vitro* model of oral candidiasis by reverse transcriptase polymerase chain reaction. Transcripts for *SAP1* and *SAP3* were predominantly found in oral candidiasis patients but not in asymptomatic carriers (Naglik *et al.*, 1999).

Immunohistochemical studies on *Candida* infected tissues have demonstrated that these proteinases are involved in the invasive pathogenesis of candidiasis (Naglik *et al.*, 2003; Schaller *et al.*, 2000). These enzymes facilitate candidal adhesion to epithelial surfaces by degrading immunoglobulins (Kaminishi *et al.*, 1995) and complement proteins such as keratin (Ray and Payne, 1988) and mucin (de Repentigny *et al.*, 2000). Proposed functions of acid proteinase during infection also include the digestion of host proteins for nutrient supply (Staib *et al.*, 1999), adherence (Ray and Payne, 1988) and degradation of host barriers during invasion (Colina *et al.*, 1996).

Moreover, these *SAP* enzymes are found to be correlated with candidal morphogenesis; *SAP1-3* during the yeast form, and *SAP4-6* during the hyphal form (Schaller *et al.*, 1998).Several mutants lacking *SAP* genes have been shown to have decreased invasion (Hube *et al.*, 1997; Sanglard *et al.*, 1997). Cassone *et al.* (1999) also reported that introduction of proteinase inhibitors such as peptasin-A or HIV proteinase inhibitors either in *in vitro Candida* cultures or *in vivo* murine vaginitis have shown marked reduction of proteolytic activity of *C. albicans*. These indicate that the candidal *SAPs* contribute directly to the virulence of *Candida* species.

# 2.9 Chemical-based Antimicrobial: Clorhexidine gluconate

Mouthrinses have widely been used for medicinal purposes. It is only in recent years that the rationale behind the use of the ingredients has been subject to scientific research and clinical trial. Chlorhexidine gluconate (CHX) is a compound with a very broad antimicrobial spectrum and has the ability to bind to both soft and hard tissues surfaces. This binding property enables CHX to act over a long period after used. Its potency as an antimicrobial compound has been well established (Adams and Addy, 1994; Briner *et al.*, 1986; Grossman *et al.*, 1986; Hope and Wilson, 2004; Jones, 1997). Due to its effectiveness, CHX is normally used as a reference (positive control) in many clinical trials of new mouthrinse formulations.

## 2.10 Plant-based Antimicrobial: An Alternative to Antifungal Agents

Natural products provide various compounds that are useful in biological activities (Galal *et al.*, 1991). Plants remain the most common source of antimicrobial agents and are widely used in traditional medicine by local folklore practitioners. Despite their popularity, a large number of medicinal plants have not been scientifically explored and the knowledgeof the indigenous uses of plants was mostly passed orally from generation to another. In Peninsular Malaysia, 1,200 species of higher plants, and 2,000 species in Sabah and Sarawak are reported to have medicinal values and have been used for generations in various traditional health care systems (Jantan, 1998). Their usage as traditional remedies is popular as they tend to have minimal side-effects upon the host (Babu *et al.*, 2002; Maghrani *et al.*, 2005). World Health Organisation (WHO) has also advocated that traditional medicine as safe remedies for ailments both microbial and non-microbial origin (WHO, 1978). Researchers are increasingly turning their attention to natural products looking for new leads to develop better drugs or an alternative of current antifungal agents against microbial infections (Harvey, 1999;

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Hoffmann *et al.*, 1993). For the past few decades, the bioactivities of the medicinal plants due to their isolated active compounds were recognized. The beneficial medicinal effects of plants typically result from the combinations of secondary products present in the plant. Several local plants were chosen in this study to screen for their antifungal activities against the common oral *Candida* species.

# 2.11. Types of Plant Species

# 2.11.1 Brucea javanica Linn.

# 2.11.1.1 General Information

Brucea javanica L. Merr. (Simaroubaceae) (Fig. 2.4), a synonym of Brucea amarissima (Lour.) Desv., B. sumatrana Roxb., B. sumatrensis Spreng., B. gracilis DC., B. glabra Decene. (Sharma and Agarwal, 1993) is a plant indigenous of China, India, Indonesia, Malaysia, Thailand and Vietnam (Matsuuraet al., 2007). It is locally known as Ya-Tan-Tze (pronouns Yadanzi) (China), Bogo-bogo (Visayan dialect) (Philippines), buah makassar (Indonesia), melada pahit (Malaysia).



Figure 2.4: Brucea javanica Linn.

## 2.11.1.2 The Uses of *Brucea javanica* (L.) Merr.

This medicinal plant used by local practitioner in Southeast Asia to treat dysentery, malaria, cancer (Anderson *et al.*, 1983; Kim *et al.*, 2004), and used as an insecticide (Liu *et al.*, 1990). It is used externally to treat vaginal trichomoniasis and various fungal infections (Tan, 1980). It also demonstrated antipyretic and homeostatic effects (Wagih *et al.*, 2008), and antiplasmodial activities (Murnigsih, 2005; Sriwilaijaroen *et al.*, 2010). Other uses of *B. javanica* are to treat ringworm, whipworm, roundworm and tapeworm, scurf, centipede bites, haemorrhoids, and enlarged spleen (Perry, 1980). The fruit of *B. javanica* contains bruceosides and bruceanols/glycosides called quassinoids which is most effective as an amoebicide (Wright *et al.*, 1993). The seed and seed oil have been used in the treatment of warts and corns. In other countries, the bark or root bark of *B. javanica* is a folk remedy for dysentery and verrucous tumor or cancer (Chang and But, 1987). To the best of our knowledge there has been no reported study on the bark of *B. javanica*.

#### 2.11.1.3 The Chemical Constituents of *Brucea javanica* (L.) Merr.

In a prior phytochemical study, several triterpenoids, two quassinoids (the bitter constituents), and a flavonolignan were identified (Pan *et al.*, 2009). Among these compounds, bruceajavanone A 7-acetate, bruceajavaninone A, bruceajavanone B, bruceajavanone C, bruceantin, bruceine A, and (–)-hydnocarpin, as well as a flavonoid [chrysoeriol] were isolated. Bruceantin and related compounds have shown very promising anti-cancer effects. It was reported in a recent study that these compounds were highly cytotoxic and has anti-cancer properties (Kim *et al.*, 2010). Anderson *et al.* (1983) reported that the compound and its hydroxylated and methoxylated derivatives have demonstrated cytotoxic effect and marked activities against malaria, leukaemia, carcinoma, keratinocytes of guinea-pig ear, and bacteria. Brusatol, another quassinoid
isolated from the seeds of *B. javanica* have also been reported to be effective in the treatment of dysentery (Sato *et al.*, 1980).

#### 2.11.2 Centella asiatica Linn.

#### 2.11.2.1 General Information

*Centella asiatica* L. (Fig. 2.5), a synonym of *Hydrocotyle asiatica* L. and *Trisanthus cochinchinensis* (Lour.) is a weekly aromatic smelling herb of the family Umbelliferae. The herb is also known as *pegaga* (Malaysia), *Indian pennywort* and *Gotu Kola* (Europe and America), *mandookaparni* (India), *pegagan* or *kaki kuda* (Indonesia), *Luei Gong Gen* or *Tung Chain* (China) (Tolkah, 1999). *Centella* comprises some 50 species (James and Dubery 2009), inhabiting tropical and sub-tropical regions throughout Asian countries, including Australia, Madagascar, South Africa, Sri Lanka, China and Japan (Schaneberg *et al.*, 2003). It is a perennial, herbaceous creeper with kidney-shaped leaves and long petiolate. The leaves are entire, crenate orbicular or reniform and sessile flowers in simple umbels. Leaves are 1.5-6.5 cm in diameter, petioles 7.5-15 cm in length, stipules are short forming sheathing base (Zheng and Qin, 2007).



Figure 2.5: Centella asiatica Linn.

#### 2.11.2.2 The Uses of *Centella asiatica* L.

*Centella asiatica* L. have been used widely in folk medicine for many years to treat a wide range of illness (Brinkhaus *et al.*, 2000; James and Dubery, 2009). Its efficacy for the treatment of tuberculosis, syphilis, amoebic dysentery, asthma, bronchitis, allergy, leucorrhea, toxic fever, and skin diseases such as eczema, leprosy and psoriasis is well documented in the literature (Gupta *et al.*, 1999; Kan, 1986). Apart from its use for wound healing (Hong *et al.*, 2005; Kimura *et al.*, 2008; Shetty *et al.*, 2006; Shukla *et al.*, 1999), this plant has been reported to have antioxidant (Pitella *et al.*, 2009; Ullah *et al.*, 2009), anti-ulcer (Abdulla *et al.*, 2010; Cheng *et al.*, 2004), anti-inflammatory (Guo *et al.*, 2004), anti-cancer (Park *et al.*, 2005), antibacterial activity (Zaidan *et al.*, 2005), and anti-rheumatoid arthritic effect (Liu *et al.*, 2008). However, there is no data available on antifungal effect of *C. asiatica* leaf extract.

#### 2.11.2.3 The Chemical Constituents of *Centella asiatica* L.

Several phytochemical constituents are reported in *Centella asiatica* L. such as (1) triterpenes: asiatic acid, asiaticosside, brahmic acid, brahmoside, brahminoside, cenellic acid, centelloside, madecassic acid. madasiatic centic acid. acid. madecassoside, terminolic acid, thankiniside, isothankunisode (Schaneberg et al., 2003; Zheng and Qin, 2007), (2) fatty oil contains glycerides of palmitic, stearic, lignoceric, oleic, linoleic and ascorbic acids, (3) alkaloids: hydrocotylin (Chopra et al., 1956) (4) glycosides: asiaticoside, madecassoide (Schaneberg et al., 2003), centelloside and indcentelloside (Bhattacharya, 1956), and (5) high phenolic contents which contributed by flavonoids such as quercetin, kaempherol, catechin, rutin, apigenin and naringin (Suntornsuk and Anurukvorakun, 2005; Zainol, 2004).

#### 2.11.3 Garcinia mangostana Linn.

#### 2.11.3.1 General Information

Mangosteen (*Garcinia mangostana* L.) (Fig. 2.6) belongs to the family Gulttifeare (Morton, 1987). It is known as *mangosteen* (English), *mangostanier* (French), *mangostan* (Spanish), *manggis* (Malaysian), *manggustan* (Philippine), *mongkhut* (Cambodian), *mangkhut* (Thai) and *can cut* (Vietnamese) (Nakasone and Paull, 1998). The fruit is 4-7 cm across covered by the smooth and hard pericarp about 6-10 mm thick and end with the persistent calyx. The pericarp turns purple at ripening and exudes latex with bitter yellowish and purple-staining juice if damaged (Nakasone and Paull, 1998). Mangosteen has a comparatively small edible partition or aril as the pericarp is thick. The edible aril, which makes up 30% of total fruit weight is of pearly white colour, slightly translucent and consists of 4 or 8 segments and have no limiting membrane.



Figure 2.6: Garcinia mangostana Linn.

#### 2.11.3.2 The Uses of Garcinia mangostana L.

The fruit hull of *Garcinia mangostana* L. has been used as a traditional medicine in Southeast Asia for the treatment of diarrhea, inflammation and ulcers (Farnsworth and Bunyapraphatsara, 1992; Lu *et al.*, 1998; Suksamrarn *et al.*, 2006). The rind of the fruit has also been used as a traditional medicine in Thailand for the treatment of trauma, diarrhea and skin infections (Nakatani *et al.*, 2002). In addition, a decoction of the leaves and bark has been employed as a febrifuge and to treat thrush, diarrhoea, dysentery and urinary disorders (Morton, 1987).

#### 2.11.3.3 The Chemical Constituents of *Garcinia mangostana* L.

Extensive studies of the constituents of Garcinia mangostana L. have lead to the identification of many compounds. Phytochemical studies showed that the fruit hulls contain various secondary metabolites such as tannins, triterpenes, anthocyanins, phenolic compounds, vitamins B1, B2 and C, and other bioactive substances (Farnsworth and Bunyapraphatsara, 1992). Derivatives of xanthones for instance, are biologically active compounds that have received increasing interest in pharmacological studies for a variety of health benefits (Nilar, 2002; Suksamrarn et al., 2002). Four new compounds, including three minor xanthones, garcimangosone A, B and C, benzophenone glucoside, and garcimangosone D were isolated by Huang et al. (2001). Three new xanthones, (1) mangostenol, (2) mangostenone A, (3) and mangostenone B were isolated from the green fruit hulls of G. mangostana, along with the abundant xanthones such as 8-desoxygartanin, trapezifolixanthone, tovophyllin B,  $\alpha$ -,  $\beta$ - and  $\gamma$ mangostins, garcinone B, mangostinone, mangostanol, and flavonoid epicatechin (Chairungsrilerd et al., 1996; Huang et al., 2001; Suksamrarn et al., 2002). These xanthones have demonstrated antibacterial (Iinuma et al., 1996; Moongkarndi et al., 2004), antifungal (Gopalakrishnan et al., 1997), antitumor-promotion (Moongkarndi et al., 2004; Suksamrarn et al., 2002), and cytotoxic characteristics in human leukemia cell lines (Katsumoto et al., 2003). Chen et al. (2007) also demonstrated that  $\alpha$ - and  $\gamma$ mangostins from the fruit hulls of G. mangostana are anti-inflammatory substances which can serve as lead compounds in the development of anti-inflammatory drugs.

#### 2.11.4 Mangifera indica Linn.

#### 2.11.4.1 General Information

*Mangifera indica* L. (mango) (Fig. 2.7) belongs to the family Anacardiaceae, is native from tropical Asia with the highest diversity occurs in Malaysia, particularly in peninsular Malaya, Borneo and Sumatra (Bompard, 2009). It is a large evergreen tree that has been introduced wherever the climate is sufficiently warm and damp, and is now completely naturalized in many parts of tropics and subtropics (Ross, 1999). There are vernacular names which are locally known as *Mangueira* (Brazil), *An Lo Kuo* (China), *Pauh* (Indonesia), *Mangga* (Malaysia and Singapore), *Amba* (Oman) (Kirtikar and Basu, 1993; Ross, 1999).





Figure 2.7: Mangifera indica Linn.

#### 2.11.4.2 The Uses of *Mangifera indica* L.

*Mangifera indica* L. is used medicinally to treat ailments such as asthma, cough, diarrhea, dysentery, pains and malaria (Madunagu *et al.*, 1990). Based on ethnopharmacological knowledge, a standardized aqueous extract of *M. indica* stem bark with antioxidant, anti-inflammatory and immunomodulatory properties has recently been developed in Cuba. Nuñez-Selles (2005) proposed that the extract could

be developed as a nutritional supplement (antioxidant) and an anti-inflammatory, analgesic and immunomodulatory treatment to prevent disease progress or increase the patient's quality of life in gastric and dermatological disorders, AIDS, cancer and asthma. The decoction of stem bark of *M. indica* has been applied for toothache (Ross, 1999). Various pharmacological activities of *Mangifera* have also been reported such as antioxidant (Muruganandan *et al.*, 2002; Leiro *et al.*, 2003; Stoilova *et al.*, 2005), antibacterial (Stoilova *et al.*, 2005), antifungal effects (Stoilova *et al.*, 2005), inhibitory activities on carbohydrate metabolism enzyme and lipolytic activity (Yoshikawa *et al.*, 2001, 2002). In a recent study, the extract of *M. indica* stem bark also exhibited positive effect on the body weight and haemopoietic system of the test rats (Nwinuka *et al.*, 2008).

#### 2.11.4.3 The Chemical Constituents of *Mangifera indica* L.

The phytochemical analysis of aqueous extract of *Mangifera indica* has been reported to contain alkaloids, tanins, phlobatanins, cardiac glycosides, saponin and polyphenol which are of great importance pharmacologically (Madunagu *et al.*, 1990; Ross and Brain, 1977). The chemical constituents of the different organs of *M. indica* are reviewed in Ross (1999) and Scartezzini and Speroni (2000). The bark is reported to contain protocatechic acid, catechin, mangiferin, alanine, glycine,  $\gamma$ -aminobutyric acid, kinic acid, shikimic acid and the tetracyclic triterpenoids cycloart-24-en-3 $\beta$ ,26diol, 3ketodammar-24 (E)-en-20S,26-diol, C-24 epimers of cycloart-25 en 3 $\beta$ ,24, 27-triol and cycloartan-3 $\beta$ ,24,27-triol (Scartezzini and Speroni, 2000). The natural C-glucoside xanthone mangiferin [2-C- $\beta$ -Dgluco-pyranosyl-1,3,6,7-tetrahydroxyxanthone; C<sub>19</sub>H<sub>18</sub>O<sub>11</sub> (Muruganandan *et al.*, 2002) has been reported in various parts of *M. indica*, such as leaves (Desai *et al.*, 1966), fruits (El Ansari *et al.*, 1971), stem bark (Bhatia *et al.*, 1967; El Ansari *et al.*, 1967), and roots (Nigam and Mitra, 1964).

#### 2.11.5 Piper betle Linn.

#### 2.11.5.1 General Information

*Piper betle* L. (Fig. 2.8) is a scientific name of betel vine leaves. It is a tropical creeper plant belonging to the family Piperaceae (Gunther, 1952). It is believed to be indigenous throughout the Indian Malay region (Chopra *et al.*, 1956). About 100 varieties of betel vine are recognized in the world, of which about 40 are found in India and 30 in West Bengal (Guha, 1997). It is also reported that Malaysia is the most probable place of origin of betel vine (Chattaopadhyay and Maity, 1967). However, the plant is much more popular in India due to the significance of the leaves uses in many occasions of human life. In India, betel vine leaves are well known as *Paan* (Guha and Jain, 1997), while in Malaysia, it is called *Sireh*.



Figure 2.8: Piper betle Linn.

#### 2.11.5.2 The Uses of *Piper betle* L.

*Piper betle* L. leaves have been traditionally used as anti-inflammatory, antibacterial and carminative agent. The betel leaves are often chewed by certain ethnics together with areca nut and slaked lime as stimulant and tonic to the chewer (Indu and Ng, 2000; Pickwell *et al.*, 1994). Leaves are also heated and applied to the chest to

relieve cough and asthma. In addition, treatment and prevention of halitosis, headache, joint pain and itches has been implicated with *P. betle* (Chopra *et al.*, 1956; Ong and Nordiana 1999; Ramji *et al.*, 2002). Other biological properties of *P. betle* which have been reported include stimulatory influence on intestinal digestive enzymes activities, antispasmodic, central nervous system depressant, antibacterial, antifungal activities(Fathilah *et al.*, 2009; Himratul-Aznita *et al.*, 2011; Indu and Ng, 2000; Nalina and Rahim, 2006, 2007), and have been associated with the control of caries and periodontal diseases (Fathilah *et al.*, 2000).

#### 2.11.5.3 The Chemical Constituents of *Piper betle* L.

*Piper betle* L. leaves extract contains catechols, terpenes, cardinene, caryophyllene, chavicol, methyl chavicol, chavibetol, estragole, eugenol, methyl eugenol, pinene, limonene, safrole, 1,8-cineole and allylpyrocatechol monoacetate (Atal *et al.*, 1975; Ponglux *et al.*, 1987; Rimando, 1986).Some of these compounds have shown to exhibit antifungal activity towards several strains of microorganisms (Friedman *et al.*, 2002; Kim *et al.*, 1995). Nalina and Rahim (2007) have reported that the main constituents of the crude aqueous *P. betle* extract correspond to hydroxychavicol, fatty acids (stearic and palmitic), hydroxybenzeneacetic acid and hydroxy esters of fatty acids (stearic, palmitic and myristic).

#### 2.11.6 Piper sarmentosum Roxb.

#### 2.11.6.1 General Information

*Piper sarmentosum* Roxb. (Piperaceae) (Fig. 2.9) is an herbaceous plant that is widely cultivated in the tropical regions such as Southeast Asia. Trease and Evan (1983) described that Piperaceae consists of 4 genera and 2000 species. With long creeping stems, this tropical plant is usually found as a weed in villages and places with plenty of

shade (Hsuan, 1990). Leaves alternate, simple, heart shaped and young leaves have a waxy surface. The flower is bar shape that one-celled ovary has a single ovule and develops to a berry. Fruit is small, dry, with several rounded bulges (Hsuan, 1990; Wee, 1992). It is locally known as *daun kadok* (Malaysia), *sireh tanah* (Indonesia), *cha-plu* (Thailand), and *Jia-ju* (China).



Figure 2.9: Piper sarmentosum Roxb.

### 2.11.6.2 The Uses of *Piper sarmentosum* Roxb.

*Piper sarmentosum* Roxb. is popular due to its culinary and medicinal properties. The decoctionis traditionally used to cure many diseases such as pain in bones and applied to the forehead of children suffering from headaches (Saralamp *et al.*, 1996). In Malaysia and the Indonesian Archipalago, the leaves and roots of this plant are used for the treatment of toothache and as an effective remedy for fungoid dermatitis on the feet, coughing asthma and pleurisy (Duke and Ayensu, 1985; Toong and Wong, 1989; Wee, 1992). It also used as muscle pain relieving property (Ridtitid *et al.*, 1998). Previous studies reported that the plant extract possess pharmacological properties like antioxidant (Vimala *et al.*, 2003), anti-tuberculosis and anti-angiogenic (Hussain *et al.*, 2008), anti-cancer (Zainal Ariffin *et al.*, 2009), anti-diabetic and hypoglycaemic effects

(Peungvicha *et al.*, 1998), anti-malarial (Rahman *et al.*, 1999), anti-nociceptive and antiinflammatory (Zakaria *et al.*, 2010).

#### 2.11.6.3 The Chemical Constituents of *Piper sarmentosum* Roxb.

Phytochemically, the plant contains constituents likephenylpropanoids (ascaricin,  $\alpha$ -ascarone) (Masuda *et al.*, 1991), alkaloids (amides) (Stoehr *et al.*, 1999), flavonoids, pyrones, flavonoids, sterols and neolignans (Parmar *et al.*, 1997; Tutiwachwuttikul *et al.*, 2006), tannins, carotenes, xanthophylls and phenols (Chanwitheesuk *et al.*, 2004). Aunpak *et al.* (1997) studied chemical constituents of essential oil distilled from the leaves and fruits of *P. sarmentosum*, and revealed that longifolene,  $\beta$ -caryophyllene, allo-aromadendrene and 9-epi-(E)-caryophyllene were the major constituents of the leaf oil whereas  $\beta$ -caryophyllene,  $\beta$ -asaron, viriflorene, and  $\beta$ -selinene were the major constituents of the fruit oil.

#### 2.11.7 Psidium guajava Linn.

#### 2.11.7.1 General information

*Psidium guajava* L. (Fig. 2.10) commonly known as guava, is a tropical and semitropical plant belonging to the family Myrtaceae. It is a native of South America and has long been naturalized in Southeast Asia (Lozoya *et al.*, 2002). It grows about 6 to 25 feet high with wide-spreading branches and square. The fruit is small, 3 to 6 cm long, pear-shaped and reddish-yellow when ripe. The tree is also known as *guayabo* (Spanish), *guofa* (Yoruba) and *goba* (Nigeria). In Malaysia, it is generally known as *jambu batu* besides other local names given such as *jambu burung*, *jambu pelawas*, *jambu biji*, *jambu berasu*, and *jambu melukut* (Zakaria and Mohd, 1994).



Figure 2.10: Psidium guajava Linn.

#### 2.11.7.2 The Uses of *Psidium guajava* L.

*Psidium guajava* L. is used for prevention and treatment of scurvy in Africa and Asia (CSIR, 1969). The decoction of the plant has long been used in folk medicine to treat fevers, gastroenteritis, vomiting, diarrhoea, dysentery, wounds, ulcers, toothache, sore throats, inflamed gums and as tonic in psychiatry (Iwu, 1993; Morton, 1987; Oliver-Bever, 1986). Tanaka *et al.* (1992) claimed that the bark extract of guava is useful in treating diarrhoea, stomach ache and diabetes. The leaf extract has been reported to have antibacterial, antiplaque, antifungal, anti-proliferative activity, antiseptic, antimalaria, antispasmodic, antiulcerous, anticonvulsant, blood cleansing, digestive stimulant, mestrual stimulant, nervine and vermifuge effects (Abdelrahim *et al.*, 2002; de Oliveira *et al.*, 2003; Fathilah *et al.*, 2005; Goncalves *et al.*, 2005; Lozoya *et al.*, 2002; Manosroi *et al.*, 2006). A strong antimicrobial action of the leaves on Gram-positive and Gram-negative organisms has also been reported (Chulasiri *et al.*, 1986; Oliver-Bever, 1986).

#### 2.11.7.3 The Chemical Constituents of *Psidium guajava* L.

The leaves of Psidium guajava L. are rich in tannins, pentacyclic triterpenoid guajanoic acid, uvaol, oleanolic acid, ursolic acid, maslinic acid, volatile oil, triterpenoids and flavonoids (Begum et al., 2004; Lozoya et al., 1994). Three active flavonoid compounds; (1) quercetin-3-O-alpha-1-arabinopyranoside (guaijaverin), (2) 3-L-4-4-arabinofuranoside (avicularin) and (3) 3-L-4-pyranoside have demonstrated strong antibacterial action (Oliver-Bever, 1986). Limsong et al. (2004) revealed that one of the active compounds namely quercetin-3-O-alpha-1-arabinopyranoside (guaijaverin) possessed high potential antiplaque activity by inhibiting the growth of *Streptococcus* mutans. The growth of Streptococcus aureus was also inhibited by the extract of guava leaf in a study carried out by disc diffusion method (Abdelrahim et al., 2002). According to Henie et al. (2009), the guava methanolic extract (GME) exhibited significant results in disruption of bacterial membrane and the antimicrobial activity may be due to high flavonoids and polyphenols content of GME (Koo and Mohamed, 2001; Meckes et al., 1996). Chen et al. (2011) reported that the leaf of P. guajava is also rich in corosolic acid which has attracted interest due to its activities of antidiabetes and anti-cancer (Sivakumar et al., 2009; Xu et al., 2009). To date, there is little information concerning the effect of P. guajava aqueous extract on the biological properties of the oral Candida species.

# **CHAPTER THREE**

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Plants Samples

- Brucea javanica Linn.
- *Centella asiatica* Linn.
- Garcinia mangostana Linn.
- *Mangifera indica* Linn.
- Piper betle Linn.
- Piper sarmentosum Roxb.
- *Psidium guajava* Linn.

## 3.1.2 Candida Species

The candidal strains were purchased from the American Types Cultures

Collection (ATCC)

- Candida albicans ATCC 14053
- Candida dubliniensis ATCCMYA-2975
- Candida glabrata ATCC 90030
- Candida krusei ATCC 14243
- Candida lusitaniae ATCC 64125
- Candida parapsilosis ATCC 22019
- Candida tropicalis ATCC 13803

# 3.1.3 Culture Media

- Yeast Peptone Dextrose Agar (BD Difco<sup>TM</sup>)
- Yeast Peptone Dextrose Broth (BD Difco<sup>TM</sup>)

# 3.1.4 Commercial Kits

- Easy-RED<sup>TM</sup> BYF Pre-Lysis Buffer (iNtRON Biotechnology)
- GenetBio

# 3.1.5 Consumables

- Agarose Superior Grade Type II (Sisco Research Lab.)
- Antibiotic Assay Disc (Whatman, Maidstone England)
- Aluminium Foil
- C206 Gas Cartridge
- Disposable Pipette Tips
- Falcon Tube 15 mL
- Filter Paper (Whatman No.1)
- Latex Examination Glove (Osaki)
- Microtitre 96-well Plate
- Parafilm M
- Peroxide-Cured Silicone Tubing L/S 16 (MASTERFLEX®)
- Syringe (TERUMO Corp.)

# **3.1.6** Chemical Reagents

- Chlorhexidane-gluconate (ORADEX<sup>TM</sup>)
- Chloroform (ChemAR<sup>TM</sup>)

- Ethanol (MERCK, Darmstadt, Germany)
- Ethidium Bromide (SIGMA-ALDRICH)
- Glutaraldehyde (SIGMA-ALDRICH)
- Glycerol (MERCK, Darmstadt, Germany)
- Hexadecane (SIGMA-ALDRICH, Steinheim, Germany)
- Iso Propyl Alcohol (2-Propanol) (R&M Chemicals, Essex, UK)
- Osmium Tetroxide (MERCK)
- Phosphate Buffered Saline (BIO BASIC INC.)
- Sodium Chloride, NaCl (R&M Chemicals, Essex, UK)

## 3.1.7 Equipments

- Autoclave (HICLA VE HVE-50 Hirayama, Japan)
- Analytical Balance (Mettler AJ100J, USA)
- Analytical Balance (Denver XL-1810, USA)
- Blender
- Centrifuge (Jouan A14, France)
- Centrifuge (Refrigerated) (Jouan GR20 22, France)
- Chiller (4 °C) (Mutiara, Malaysia)
- Freeze drying (EYELA FDU-1200, Tokyo)
- Freezer (-20 °C) (Zanussi, Germany)
- Freezer (-80 °C) (Hetofrig Cl410, Denmark)
- Hot plate / Stirrer (Thermolyne)
- Incubator (Memmert, Germany)
- Incubator (Binder, Germany)
- Ion Sputter Coater (JOEL JFC1100: JOEL, Tokyo, Japan)
- Laminar Flow Unit (ERLA CFM Series, Australia)

- Scanning Electron Microscope (SEM) (Phillips, Japan)
- Spectrophotometer (Shimadzu UV160A, Japan)
- Vortex Mixer (Snijders Scientific, Holland)
- Water Bath (Grants SS-40-A2, Cambridge, England)
- Water Distiller (J Bibby Merit)

#### 3.2 Methods

The study was focused on the screening of seven plants for their antifungal activities against seven of oral candida. Aqueous extracts were used throughout the study. Screening process was carried out based on the Kirby-Bauer test. Based on the results obtained, extracts that showed positive responses were selected for further analysis while those that showed negative reaction was omitted. The approach and methodology of the study is onlined in Figure 3.1.

#### **3.2.1** Collection of Plant Samples

Seven local plants (Section 3.1.1) known to possess antimicrobial properties were chosen as plant materials. The fresh materials which include the leaves and seeds of plants were obtained or purchased from sources in Brickfield, Sekinchan, and Pelabuhan Klang. The species, genus and family of the plants was identified and confirmed by a botanist from the Institute of Biological Sciences, Faculty of Science University of Malaya. The specimens were given voucher number and deposited at the Herbarium of Rimba Ilmu, University of Malaya.

#### **3.2.2 Preparation of Crude Aqueous Extracts**

In this study, an aqueous (water-based) extraction procedure was employed to retrieve the polar components of the crude extract. Fresh plant materials (100 g) were cleaned in running water and oven-dried at 60-65 °C for two days. The dried materials were homogenized in distilled water at a ratio of 1:10 plant material to water. The homogenate was continuously boiled to one-third of the original volume.The homogenate was filtered through filter paper to remove debris before it was further boiled to a final volume of 100 mL. The decoction was then concentrated overnight by



Figure 3.1: An outline of the research procedures

freeze drying. The powder obtained were sealed in sterile Falcon tubes and stored at 4  $^{\circ}$ C. Prior to use, a stock of the extract was prepared in sterile distilled water at a concentration of 200 mg/mL. Following centrifugation for 10 min at 8,000 x g to remove any remaining debris, the stock was then diluted to concentrations required for each experiment. Prior to each experiment, the extract was filtered through a 0.2  $\mu$ m nylon syringe filter to ensure its sterility.

#### 3.2.3 Preparation of Culture Media

Yeast Peptone Dextrose (YPD) agar and broth (Section 3.1.3) were used as growth media for the yeast. The media which comes in powder form was appropriately weighed as directed for the preparation, dissolved in distilled water, boiled and later sterilised at 121 °C for 15 min in an autoclave. The agar plates and broth media were kept refrigerated at 4 °C for later use.

#### **3.2.4** Stock Cultures Preparation and Condition

The lyophilised candidal species in Section 3.1.2 were rehydrated in sterile distilled water and inoculated onto Yeast Peptone Dextrose (YPD) agar media. Following incubation at 37 °C for 24 h, the colonies were sub-cultured on fresh YPD agar slants and stored at 4 °C for further use in the experiment. Stock for long term storage was prepared in 20% v/v glycerol and stored at -70 °C. The purity of the candidal strains was validated with the use of the API Yeast Identification System kit. Throughout the experiment period, the cultures were maintained on YPD agar up to a maximum of two weeks at 4 °C. Regular sub-culturing was carried out every two weeks to maintain viability of the cells. The growth colonies that appeared following the incubation period were used in the preparation of candidal suspension.

#### 3.2.5 Preparation of Standard Candida Cell Suspension

The grown colonies were harvested and dispensed in 5 mL of YPD broth. The turbidity of the suspension was adjusted and standardised spectrophotometrically to an optical density ( $OD_{550nm}$ ) of 0.144 which is equivalent to 10<sup>6</sup> cells/mL or to #0.5 McFarland standard.

#### 3.2.6 Screening of the Plant Extracts for Antifungal Response

Antifungal responses of the plant extracts were screened *in vitro* against seven oral candidal species using Kirby-Bauer susceptibility test. This test was actually for antibiotic testing. Paper discs impregnated with the test plant extracts were laid on top of an inoculated agar plate of the respective candidal suspension. The compound of the extracts diffuses through the agar, setting up a concentration gradient. Inhibition of colony growth which is the measure of antifungal activitywas indicated by a clear growth inhibition zone surrounding the discs. The size of the clear zone is dependent upon the rate of extract diffusion and colony growth. An illustration of the screening procedure carried out on the plant extracts is given in Figure 3.2.

#### **3.2.6.1** The Kirby-Bauer Susceptibility Test

The antifungal activity of the plant extracts was carried out based on the disc diffusion concept of Kirby-Bauer susceptibility test (Bauer *et al.*, 1966). Sterile blank discs of 6 mm diameter were impregnated with a concentration of 100 mg/mL of the respective plant extracts. The discs were air-dried prior to placement on the agar surface which had been seeded with the respective candidal suspension. Throughout this experiment, a blank disc impregnated with sterile distilled water represented as negative control and another disc impregnated with mouthrinse containing 0.12% w/v

chlorhexidine gluconate (CHX) represented as the positive control were included. The volume of the test extracts, positive and negative controls impregnated onto the discs were standardized at 100  $\mu$ L. The same steps were repeated for the other six types of candidal species. All plates were incubated at 37 °C, except for *Candida parapsilosis* which was incubated at 35 °C. The susceptibility of each *Candida* species was determined by the diameter of the inhibited zone surrounding the discs. The experiment was done in triplicate and carried out three times to ensure reproducibility of results.

#### 3.2.7 Screening for Antifungal Activity

Based on the results obtained in Section 3.2.6.1, the plant extracts that exhibited positive antifungal responses were further analysed to validate their antifungal properties. The strength of the antifungal activity of the plant extracts was determined using a broth dilution method (Smith *et al.*, 1985) which is more sensitive. The minimum inhibition concentrations (MICs) (Jorgensen *et al.*, 1999) and minimum fungicidal concentrations (MFCs) (Espinel-Ingroff *et al.*, 2002) were conducted to determine the efficacy of the plant's extract as a potential antifungal agent. An outline of the procedure for the determination of the MIC and MFC of the plant extracts is given in Figure 3.3.

#### **3.2.7.1** Preparation of Candidal Suspension

The preparation of candidal suspension used in this assay was described in Section 3.2.5.



100 µL of each extract was impregnated to paper discs



Figure 3.2: An illustration of the screening method using the Kirby-Bauer susceptibility test.

Note: The paper discs impregnated with the respective extracts were positioned apart to allow for the formation of inhibition zone. The clear zone formed is an indication of a positive inhibitory response of the extracts. A 0.12% w/v CHX-containing mouthrinse was used as a reference (positive control).

#### **3.2.7.2** Preparation of Extract's Stock Solution

To assay the MIC and MFC determinations, the stock solution of respective plants that exhibited positive antifungal responses was prepared in sterile distilled water at concentration of 200 mg/mL. The stock solutions were then centrifuged at 8,000 x g for 10 min. The pelleted debris was discarded and the supernatant was used in this assay.

#### **3.2.7.3** Determination of Minimum Inhibition Concentration (MIC)

The MICs of each plant extract against the seven oral candidal species were determined using the method described by Jorgenson *et al.* (1999). 100  $\mu$ L of YPD broth was dispensed into a microtitre plate labeled as Well 1 (W1) to Well 8 (W8). 100  $\mu$ L of extract stock solution (200 mg/mL) was added into the W1 and two-fold serial dilution was carried out from W1 through W6. Hence, the final concentrations of extract in W1, W2, W3, W4 and W5 were 100, 50, 25, 12.5, 6.25 and 3.13 mg/mL. Throughout the assay, CHX was used in a place of the plant extract as a positive control in W8, while W7 which only contain the mixture of YPD broth and the extract represented the negative control. Except for W7, 20  $\mu$ L of candidal suspension was added to W1 through W8. This step was repeated to the other six *Candida* species. The microtitre plates were incubated overnight at 37 °C except for *C. parapsilosis* which was incubated at 35 °C. Following incubation, growth inhibition in the microtitre wells was observed. The MIC is represented by the concentration in the well that shows absence of growth.

(1) 100 $\mu$ L YPD brothwas dispensed into W1 – W8.								
Well No	W1	W2	W3	W4	W5	W6	W7	W8
VPD broth (uI )		100	100	100	100	100	(-)	(+)
	100	100	100	100	100	100	100	100
<ul> <li>(2) 100 μL of stock extract (200 mg/mL) is dispensed in W1.</li> <li>(2) Two fold sorial microdilution was corriad out from W1. W6</li> </ul>								
(5) I wo-told serial microditation was carried out from w $I - wb$ .								
	- C	2	3	4	5	6	-ve	() () ≠¥e
Final								
concentrations of	100	50	25	12.5	6.25	3.13	0	0
extract (mg/mL)								
(4) 20 $\mu$ L of candidal suspension is dispensed in W1-W8, but W7.								
Total volume (µL)	120	120	120	120	120	120	120	120
(5) The plate is incubated at 37 °C ( <i>C. parapsilosis</i> at 35 °C). The MIC values are determined based on cells turbidity.								
MIC is determined at 25 mg/mL.	-	-	-	+	+	+	+	+
(6) 50 μL of each well was dispensed onto fresh agar plates and incubated at 37 °C ( <i>C. parapsilosis</i> at 35 °C) for 24 to 48 h. The MFC values were determined based on colony growth (Note: CHX +ve, H <sub>2</sub> O –ve).								

Figure 3.3: An illustration of procedure to determine the MIC and MFC of extracts against candidal species.

#### **3.2.7.4** Determination of Minimum Fungicidal Concentration (MFC)

The MFCs of plant extracts were determined according to a standard procedure described by Espinel-Ingroff *et al.* (2002). Briefly, 50  $\mu$ L from wells identified with the MICs of all the respective candida was pipetted out and subcultured onto fresh YPD agar plates. The plates were incubated at 37 °C (*C. parapsilosis* at 35 °C) for 24 to 48 h until visible growth of colonies was observed. The MFC value was the concentration where no growth or fewer than three colonies were obtained or in other words it gives an approximately 99 to 99.5% killing activity (Espinel-Ingroff *et al.*, 2002).

# 3.2.8 The Effect of *Brucea javanica* L. and *Piper betle* L. Extracts on the Growth Profiles of *Candida* Species

#### 3.2.8.1 Experimental Design

Different species of candida may have different ways of adapting to the environment in the oral cavity that is to adhere, grow and colonize. Therefore, an understanding of the growth kinetics of thesecandida is of fundamental importance. In this study, a spectrophotometric assay (Meletiadis *et al.*, 2001) which was based on the continuous monitoring of changes in the optical density of cell growth and enumeration of colony forming unit (CFU) were employed. The growth of different candidal species can be distinguished by measuring the changes of specific-growth rates ( $\mu$ ) and doubling time (g) following equations previously described (Cappucino and Sherman, 2002; Gerhardt, 1981):

(i) Specific growth rate, 
$$\mu = \frac{\text{In} (N_t / N_0)}{t_2 - t_1}$$

(ii) Doubling time,  $\mathbf{g} = \log_{10} (\mathbf{N}_t / \mathbf{N}_o) / \log_{10} 2$ 

where,  $N_t$  represented the number of cells at log phase,  $N_o$  represented the number of cells at zero time,  $t_2$  was the time taken to reach plateau, and  $t_1$  zero time when the cells enter the log phase. Throughout the study, CHX was used in place of the extract as positive control. An illustration of the assay is shown in Figure 3.4.

#### 3.2.8.2 Growth Profiles of Untreated *Candida* Species

To determine the growth curve of candida under the normal condition, 5 mL of candidal suspension was dispensed into three sterile conical flasks, each containing 40 mL of YPD broth. 5 mL of sterile distilled water was added in a place of test extract to give a total volume of 50 mL in each flask. The flasks were placed in a shaking water bath and continuous agitation was carried out at 35-37 °C. One millilitre of cell suspension was taken for turbidity measurement. The cell density was measured periodically at every one hour interval over a period of 18 h using a spectrophotometer read at a wavelength of 550 nm. The OD readings were plotted against time, and the specific growth rate ( $\mu$ ) and doubling time (g) were calculated following equations stated in Section 3.2.8.1. The reproducible growth of the seven *Candida* species was also enumerated by viable cell counts (CFU). For this, the growth suspension from the conical flask was first diluted by serial dilution using a nontoxic diluent (*e.g.* phosphate-buffered saline, pH 7.2-7.4) before plating on YPD agar plates. The candidal cells cultured in normal condition arerepresenting as a standard.

#### **3.2.8.3** Growth Profiles of Extract-treated *Candida* Species

The extracts of *B. javanica* and *P. betle* were prepared into stocks of 10, 30 and 60 mg/mL. Five millilitre of each stock was dispensed into sterile conical flasks containing 40 mL of YPD broth, followed by five millilitre of respective candidal suspension to give a final concentration of 1, 3 and 6 mg/mL. The flasks were placed in

a shaking water bath and continuously agitated at 37 °C (*C. parapsilosis* at 35 °C). In a similar manner to Section 3.2.8.2, the cells growing under the treatment of the extracts was measured periodically at every one hour interval over a period of 18 h. The changes in specific growth rate ( $\mu$ ) and doubling time (g) of growth curves obtained under the treated condition were compared to curves of the normal condition. The effect of the extracts on the growth curves was also determined based on viable cell counts. A flow-chart of the procedure is given in Figure 3.4.



Figure 3.4: An illustration of the growth kinetic assay.

Note: The growth kinetic assay involved the untreated and *B. javanica* and *P. betle* treated candidal cells. CHX-containing mouthrinse was used in place of the extract as reference (positive control).

# 3.2.9 The Effect of *Brucea javanica* L. and *Piper betle* L. Extracts on Cell Surface Hydrophobicity on *Candida* Species

#### 3.2.9.1 Experimental Design

Determination of cell surface hydrophobicity (CSH) was carried out following the protocol of Rodrigues *et al.* (1999). The relative CSH of oral candida tested following treatment with the plant extracts at sub-MICs was investigated. Hexadecane is a hydrocarbon used to represent the hydrophobic nature of the acquired pellicle. The relative cell surface hydrophobicity was expressed as a percentage of adsorption of the candidal cells to hexadecane, as described in the equation:

% change in 
$$A_{550} = (A_t - A_u) \times 100$$
  
 $A_t$ 

where,  $A_t$  is absorbance of the total cell suspension in the absence of hexadecane, and  $A_u$  is absorbance of the total cell in the presence of hexadecane. An outline of the study was shown in Figure 3.5.

#### **3.2.9.2** Preparation of Standard Candidal Cell Suspension

The harvested candidal cells were washed with sterile saline prior to be prepared as a standard cell suspension of  $10^7$  cells/mL with an OD<sub>550nm</sub> of 0.5.



Figure 3.5: An illustration of determination of cell surface hydrophobicity of candidal species.

#### **3.2.9.3** Determination of Cell Surface Hydrophobicity without Treatment

Two millilitre of the respective candidal suspension was dispensed into sterile glass tubes. Another 2 mL of sterile saline was then added to give a final volume of 4 mL. In this assay, the absorbance ( $A_t$ ) of the total cell in the absence of hexadecane was recorded at a wavelength of 550 nm. Following this, 200 µL of hexadecane was pipetted into each of the tubes. The tubes were vigorously agitated (1 min) and left to stand at room temperature (15 min) to allow for separation of hexadecane from the aqueous phase. The lower aqueous phase of the mixture was gently aliquoted out into cuvette and the absorbance ( $A_u$ ) was again read at OD<sub>550nm</sub>. This represented the final absorbance of the respective candida cells after agitation. The percentage of candidal cells adsorbed to hexadecane was determined following the equation stated in Section 3.2.9.1.

# 3.2.9.4 Cell Surface Hydrophobicity of *Candida* Species Following Treatment of the Extracts

Similar procedure as above was repeated but the addition of 2 mL sterile saline was replaced by the extract stocks at appropriate volume to give a final concentration of 1, 3 and 6 mg/mL. The mixture was allowed to stand at room temperature for 2 min prior to the reading of the absorbance. This represented the initial absorbance in the absence of hexadecane ( $A_t$ ). 200 µL of hexadecane was then addedand vigorously agitated. The mixtures were left to stand at room temperature to allow for separation of the aqueous from the non-polar phase. The lower aqueous phase was the gently aliquoted out and put into cuvette and was read a wavelength of 550 nm ( $A_u$ ). The percentage of adsorbed cells to hexadecane was determined using the equation stated in Section 3.2.9.1.

# 3.2.10 The Effects of *Brucea javanica* L. and *Piper betle* L. Extracts on the Adherence of *Candida* Species to Saliva-coated Surface

#### **3.2.10.1** Experimental Design

Adherence of candidal species on the mucosa, teeth and denture surfaces is mediated by specific adhesion mechanism that involves various ligand-receptor reactions of protein-protein and/or protein-carbohydrate between the acquired pellicle and the surface of the candidal cells. In the oral cavity, saliva plays an important role in promoting adherence of these cells to hard surfaces that is providing specific receptors for adhesion to the acquired pellicles.

In this study, an artificial mouth model named the *Nordini Artificial Mouth* (NAM) system (Rahim *et al.*, 2008) was used to mimic condition in the oral environment. Using the NAM, experimental pellicles are developed on glass surfaces to mimic the acquired pellicles on the tooth surface.

In the experiment, experimental pellicles were treated with the extract of *B*. *javanica* and *P*. *betle* for 2 min before being exposed to the respective seven candidal suspensions. The effectiveness of plant extracts as anti-adherence agent was determined based on the reduction in the population of adhering cells comparative to the number that had adhered on the untreated pellicles. Throughout the study, CHX-containing mouthrinse was used as a reference. Besides having a wide spectrum of antibacterial and antifungal activities, CHX has long been considered the gold standard as an antimicrobial in dental and hospital arena (Jones, 1997). A complete experimental design of NAM system is illustrated in Figure 3.6.

#### 3.2.10.2 Collection of Saliva

Ethical approval for the collection of saliva was obtained from Ethical Committee, Faculty of Dentistry under the code of DFOB0702/0002(L) (The Virulence Determination of *Candida* Species in the Oral Cavity). Unstimulated saliva from healthy donor was collected into ice-chilled test tube. A single donor was used throughout the study to minimize variations in the composition of saliva that may arise between individuals. The donor was asked to gargle with distilled water to reduce bacterial contamination before the beginning of collection at 9.00 am to 11.00 am. The saliva was then clarified by centrifugation (17,000 x g) for 30 min and the pelleted debris was discarded while the clarified saliva was sterilized by filtration. Collection from different batches was then pooled and stored at -20 °C until further use.

#### **3.2.10.3** Preparation of Acquired-pellicles

Ten sterile glass beads placed in the NAM chamber were coated with clarified saliva by allowing it to flow into the NAM model for 2 min at a constant rate of 0.3 mL/min. Once formed, sterile distilled water was allowed to run into the system to rinse off excess saliva from the experimental pellicle that has formed on the glass beads. The NAM model was setup and performed following a standard procedure described by Rahim *et al.* (2008).

#### 3.2.10.4 Adherence Affinities of *Candida* Species to Salivary Pellicles

Candidal suspension was pumped into the system and left to circulate overnight (37 °C) at a rate of 0.3 mL/min. Following that, the glass beads were rinsed with sterile distilled water. The population of adherent candidal cells was determined by transferring the beads to a sterile microcentrifuge tube containing one millilitre of

phosphate-buffered saline (PBS). The tubes were sonicated for a few seconds and vortexed for 1 min to dislodge attached cells. The tube containing the dislodged candidalspecies was marked as Tube 1 (T1). The content of T1 was then serially diluted using PBS to a final dilution of 1:10 (6th dilutions) contained in Tube 7 (T7). 100  $\mu$ L of candidal suspensions from each tube was pipetted out and inoculated on three separate YPD agar plates. Following incubation at 37 °C for 24 to 48 h, the plates with dilution that gave a CFU count of between 30 to 300 cells were selected for enumeration. The growth population (CFU/mL) were calculated using the formula:

# Total CFU/mL = Number of formed colonies

#### **Dilution factor x Volume used (mL)**

The results were expressed as the mean of CFU/mL of triplicate determinations.

# 3.2.10.5 Anti-Adherence Activity of *Brucea javanica* L. and *Piper betle* L. Extracts

The freshly prepared experimental pellicles (Section 3.2.10.3) were first treated with the respective extracts by allowing 10 mL of the extracts at equal concentration of 6 mg/mL to flow through the system for 2 min. Following the treatment, candidal suspension was then pumped into the system and left to circulate overnight (37 °C) at a constant flow rate of 0.3 mL/min. Following this, the number of cell adhering to the glass beads were determined following similar method as described in Section 3.2.10.4. The CFU on the extract-coated pellicles was compared to the number that binds to the normal, untreated pellicles.



Figure 3.6: An illustration of the Nordini Artificial Mouth (NAM) model

Note: The model designed in laboratory scale to investigate the adherence affinity of candidal cells to adhere on the salivary pellicles.

# 3.2.11 The Effects of *Brucea javanica* L. and *Piper betle* L. Extracts on the Morphology of *Candida* Species

#### **3.2.11.1** Experimental Design

In this study, freshly prepared candidal suspension was cultured on YPD agar and colonies were allowed to grow for 24 h. Using a sterile blade, the agar was cut approximately 1 x 1 cm<sup>2</sup> and transferred into sterile vials for extracts treatment. The antifungal activity of the respective extracts was further screened for their effect on the morphology of the candida. Under the influence of the extract the candidal cells may have to alter some of its normal physiological functions in an attempt to adapt to the new growth environment. This may interfere with the growth activity of the candidal cells that later may lead to changes in the ultrastructure of the cells. Such adaptation may also affect other biological functions such as reproductivity and adhering property. Ultrastructural study using the scanning electron microscopy (SEM) was performed to investigate changes in the morphology of the candidal cells.

#### 3.2.11.2 Preparation of Samples for Scanning Electron Microscopy

The samples with the 24 h colonies of the respective candidalspecies were cut off the agar and individually placed in a sterile vial containing extract at concentration of 6 mg/mL. The samples were immersed with the extract for 4 h. Prior to the SEM processing, the samples beads were fixed overnight in glutaraldehyde. The samples were then washed with sodium cacodylate buffer (0.1 M at pH 7.4), followed by fixation in 2% w/v osmium tetroxide in the buffer solution overnight. The next day, the samples were gently washed in distilled water twice for 15 min and dehydrated in an ascending series of ethanol concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 95%) for 15 min for each step. Following this, the samples were further dehydrated twice in 100% ethanol for 15 min and subsequently in the ethanol-acetone mixture with a ratio
of ethanol to acetone of 3:1, 1:1 and 1:3 for 15 min each. Then, the samples were treated thrice with pure acetone for 15 min. The samples were then processed for critical point drying for about 2 h. Once the samples were completely dry, they were mounted on aluminium stubs, with copper tape and coated with gold under low-pressure with an ion sputter coater. Three samples from each treatment were visualized with a scanning electron microscope in high-vacuum mode at 10kV, and the images processed. Any considerable changes on the morphology of candidal cells between the untreated and treated were observed.

# 3.2.12 The Effects of *Brucea javanica* L. and *Piper betle* L. Extracts on the Expression of Secretory Aspartyl Proteinases (*SAP1-10*) and Hyphal Wall Protein (*HWP1*) Genes.

#### 3.2.12.1 Experimental Design

The expression of interest genes such as the secreted aspartic proteinase (*SAP*) and hyphal wall protein (*HWP1*) is considered a key attribute of *Candida albicans* that aids in invasive pathogenesis. In this study, the expression of these genes in candidal cells was determined following the treatment with the extracts. The expression of the genes was further analyzed in comparison with the normal cells (in the absence of extract treatment). The *C. albicans ACT1* gene was used as an internal mRNA control for evaluation of the efficacy of the RT-PCR analysis. It has been reported that when actin primer set *ACT1* was reacted with all *Candida* species, the yielded PCR products were of the same size as *C. albicans* (Losberger and Ernst, 1989). Therefore, it served as a positive control to confirm the presence or absence of *Candida* species.







Reverse transcription-polymerase chain reaction (RT-PCR): To synthesis the complementary DNA (cDNA)



Gel analysis

Figure 3.7: An outline of the molecular study.

Note: The expressions of *SAP* (*SAP1-10*) and *HWP1* genes associated with the virulence of *C. albicans* were elucidated in different culture conditions.

#### **3.2.12.2** Total RNA Extraction

*Candida* species were freshly cultured overnight and washed in phosphatebuffered saline (PBS). Total RNA from the cells was extracted using an easy-RED<sup>TM</sup> BYF Total RNA Extraction Kit (Intron Biotechnology Inc.) following the manufacturer's instruction.

Following the washing step, 250  $\mu$ L of pre-lysis buffer was added and resuspended thoroughly. Then, 750  $\mu$ L of easy-RED<sup>TM</sup> solution was added; mix in room temperature for 15 sec by vigorousvortexing, and left to stand at room temperature for 5 min. Following this, 200  $\mu$ L of chloroform was added; mix in room temperature for 15 s by vigorous vortex, and left to stand at room temperature for another 5 min.

Following a centrifugation at 8,000 x g (4  $^{\circ}$ C) for 15 min, the colourless aqueous phase was carefully transferred to a new microcentrifuge tube. An equal volume of isopropanol (2-propanol) was added and mixed well by inverting the tube for 6-7 times and left aside at room temperature for 10 min. The tube was centrifuged at 8,000 x g (4  $^{\circ}$ C) for 10 min, and the supernatant was discarded without disturbing the pellet.

1 mL of 70% ethanol was added and the solution was mix by inverting the tube several times. The mixture was centrifuged at 8,000 x g (4  $^{\circ}$ C) for 5 min. The supernatant was discarded and the remaining RNA pellet was left to dry. The total RNA was dissolved in 50 µL of RNase free water, and stored at -80  $^{\circ}$ C.

#### 3.2.12.3 RNA Quantification

It is vital to get high quality and intact RNA to ensure good results obtained in the experiments. Total RNA integrities of the samples were analyzed using the Agilent 2100 Bioanalyzer, and all samples were considered have high quality RNA by referring to the RIN number (Schroeder *et al.*, 2006).

#### **Specific Primers** 3.2.12.4

The following pairs of specific primers were used to amplify the interest genes to study their expressions.

Table 3.1: Oligonucleotide sequences of SAP1-10 and ACT1 gene.

Gene target <sup>a</sup>	Primer Sequences (5´-3´)	$T_m(^{\circ}C)^{b}$	(bp) <sup>c</sup>
SAP1			
Forward	TCAATCAATTTACTCTTCCATTTCTAACA	56.8	161
Reverse	CCAGTAGCATTAACAGGAGTTTTAATGACA	62.8	
SAP2		<i>(</i> <b>)</b> <i>5</i>	170
Forward		62.5	1/8
Reverse	IGACCATTAGTAACIGGGAAIGCITTAGGA	65.9	
SAP3			
Forward	CCTTCTCTAAAATTATGGATTGGAAC	57.0	231
Reverse	TTGATTTCACCTTGGGGGACCAGTAACATTT	65.6	-01
SAP4			
Forward	CATTCATTCCTTTAATACCGACTATC	56.7	156
Reverse	GGTAACAAACCCTGTAGATCTTTTAAC	60.0	
SAP7			106
Forward	GAAATGCAAAGAGTATTAGAGTTATTAC	55.5	196
Reverse	GAAIGAITIGGITIACAICAICIICAACIG	58.9	
SAP8			
Forward	GCCGTTGGTGCCAAATGGAATAGTTA	65 5	256
Reverse	ATTTGACTTGAGCCAACAGAATGGT	61.2	200
SAP9			
Forward	AACCGACGAGGAAAAAGAAGCACTG	63.8	283
Reverse	GTACCCCCAAATACCAATGGCACTG	67.2	
G4 D 10			
SAP10		(2.0)	150
Forward		63.0 62.5	152
Reverse	IGUCLAAGAGCAICAAICCAIICAI	05.5	
ACT1			
Forward	GGCTGGTAGAGACTTGACCAACCATTTG	67.6	304
Reverse	GGAGTTGAAAGTGGTTTGGTCAATAC	61.4	20.

<sup>a</sup> Gene templates are chosen as prescribed in Tavanti *et al.* (2004). <sup>b</sup> Melting temperatures are analyzed using Oligo Analyzer 1.2.

<sup>c</sup> Product size (bp, base pair).

Genes <sup>a</sup>	Primer Sequences (5´-3´)	$T_m (^{o}C)^{b}$	(bp) <sup>c</sup>	
Forward Reverse	CCATGTGATGATTACCCACA GCTGGAACAGAAGATTCAGG	56.6 57.8	572	

Table 3.2: Oligonucleotide sequences of HWP1 gene.

<sup>a</sup> Gene templates are chosen as prescribed in Naglik *et al.* (2006).

<sup>b</sup> Melting temperatures are analyzed using Oligo Analyzer 1.2.

<sup>c</sup> Product size (bp, base-pair)

#### **3.2.12.5** Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

A single PCR protocol was used with all primer sets (Table 3.1 and 3.2). The total RNA (50 ng/ $\mu$ L) and the specific primers (1  $\mu$ L each) were mixed in 10  $\mu$ L of Prime RT-PCR Premix (2X) (GENET BIO) which contained HS Prime Tag DNA Polymerase, Prime MMLV reverse transcriptase, reaction buffer, 0.1 mM dNTPs mixture, RNase inhibitor, protein stabilizer and enhancers for cDNA synthesis. RNasefree water (or DEPC-treated water) was added up to total reaction volume 20 µL. Reverse transcription was carried out at 42 °C for 30 min, followed by denaturation at 94 °C for 10 min in order to deactivate reverse transcriptase and activate the HS Prime Taq DNA Polymerase. The samples were then subjected to 30 cycles of denaturation (94 °C), annealing (58 °C to 63 °C), and extension (72 °C), each for 30 s. This is followed by a final extension (72 °C) for 5 min. The amplification reaction was carried out using a thermal cycler. A sample (6  $\mu$ L) of each amplified product was separated by electrophoresis in 1.5% w/v agarose gels containing 0.5 µg of ethidium bromide/mL. Tris-borate-EDTA (TBE) was used as the running buffer, and 100 bp DNA ladder (BIO-RAD) was used as a molecular weight marker. The propensity of genes' expressions was visualized by ultraviolet (UV) illumination (Alphalmager 2200, Alpha Innotech).

#### 3.2.13 Statistical Analysis

All results were computed and expressed as mean  $\pm$  standard deviation (SD) from three determinations performed in triplicate (n = 9). Statistical analysis was performed using SPSS software (version 17.0). Analysis of variance (One-Way ANOVA) and post-hoc test Dunnett's T3 were used to compare the significant difference between the groups. A *P*-value of < 0.05 was considered as statistically significant.

#### **CHAPTER FOUR**

#### RESULTS

#### 4.1 Screening of Plant Extracts for Antifungal Activity

#### 4.1.1 The Kirby-Bauer Susceptibility Test

The antifungal activity of aqueous extracts of *Brucea javanica* L., *Centella asiatica* L., *Garcinia mangostana* L., *Mangifera indica* L., *Piper betle* L., *Piper sarmentosum* Roxb. and *Psidium guajava* L. against the seven candidal species – *Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida krusei*, *Candida lusitaniae*, *Candida parapsilosis* and *Candida tropicalis*, were estimated based on the presence or absence of inhibitory zones. The extracts of *C. asiatica*, *G. mangostana*, *M. indica*, *P. sarmentosum* and *P. guajava* showed negative antifungal activity against all candidal species. Positive inhibitory activity was noted in *M. indica* against *C. glabrata* (11.5  $\pm$  0.8 mm), and in *P. guajava* extract against *C. glabrata* (15.3  $\pm$  0.7 mm) and *C. lusitaniae* (19.1  $\pm$  0.6 mm) (Table 4.1).

Out of the seven plant extracts screened for antifungal activities, only the extracts of *B. javanica* and *P. betle* exhibited antifungal responses to all seven *Candida* species. Based on result of the disc diffusion test, all *Candida* species were found to be more susceptible to *P. betle* compared to *B. javanica*. At 100 mg/mL of *P. betle*, the diameters of the growth inhibition zones of the seven candidalspecies were determined within the range of 11.0 mm to 32.1 mm. At similar concentration, the inhibited zones produced by *B. javanica* were in the lower range of 11 mm to 16 mm.

The inhibition zones were observed to be concentration dependent whereby smaller inhibitory zone was recorded at lower range of 50 and 25 mg/mL. Of the seven candidal species, *C. dubliniensis* was the most sensitive to *B. javanica* (22.7  $\pm$  1.0 mm) and *P. betle* (32.1  $\pm$  0.5 mm) extracts. *C. parapsilosis* and *C. tropicalis* were both slightly resistant to *P. betle* (11.0  $\pm$  0.8 mm) and *B. javanica* (11.3  $\pm$  1.0 mm), respectively. CHX exhibited zones of inhibition within the range of 9.0 to 25.0 mm.

Based on the strength and efficacy of all the extracts towards the seven candidal species, only *B. javanica* and *P. betle* extracts were selected as test materials for further tests. The other plant five plants were screened out of the study due to their low or negative antifungal activity.



Figure 4.1: The antifungal responses of *Candida* species using the Kirby-Bauer susceptibility test.

Note: The YPD agar plates which have been seeded with *C. albicans* (A), *C. glabrata* (B), *C. tropicalis* (C), *C. lusitaniae* (D), *C. krusei* (E), and *C. dubliniensis* (F).

- (i) In ABC: Discs a and b were impregnated with 100 μL of 100 mg/mL of *P. betle* and *B. javanica* extracts, respectively.
- (ii) In DEF: Discs were impregnated with 100  $\mu$ L of 100 mg/mL of *B. javanica* extracts.
- (iii) Antifungal response was evaluated by measuring the diameter of inhibition zone of the tested candida (arrow; ↔).

Inhibition zone $(mm \pm 1)$ (Mean $\pm$ SD)							
Plant species <sup>a</sup> (Parts used)	С. а	<i>C. d</i>	С. g	<i>C. k</i>	C. l	С. р	<i>C. t</i>
<i>B. javanica</i> L. (Seeds)	13.8 ± 0.8	22.7 ± 1.0	15.6 ± 1.6	12.0 ± 1.3	15.1 ± 0.8	15.0 ± 0.9	11.3 ± 1.0
<i>C. asiatica</i> L. (Leaves)	NS	NS	NS	NS	NS	NS	NS
G. mangostana L. (Leaves)	NS	NS	NS	NS	NS	NS	NS
<i>M. indica</i> L. Leaves)	NS	NS	11.5 ± 0.8	NS	NS	NS	NS
<i>P. betle</i> L. (Leaves)	29.4 ± 1.1	32.1 ± 0.5	17.9 ± 1.3	21.2 ± 0.6	28.2 ± 1.0	11.0 ± 0.8	28.1 ± 1.0
<i>P. sarmentosum</i> R. (Leaves)	NS	NS	NS	NS	NS	NS	NS
<i>P. guajava</i> L. (Leaves)	NS	NS	15.3 ± 0.7	NS	19.1 ± 0.6	NS	NS
Chlorhexidine	13.0 ± 0.2	11.1 ± 0.3	9.0 ± 0.5	20.2 ± 0.7	12.1 ± 0.4	$\begin{array}{c} 25.0 \pm \\ 0.2 \end{array}$	19.3 ± 0.3
Sterile dH <sub>2</sub> O	NS	NS	NS	NS	NS	NS	NS

Table 4.1: The diameter of growth inhibition zones produced by the seven plant extracts on the seven different *Candida* species.

*C. a: Candida albicans* (ATCC 14053); *C. d: Candida dubliniensis* (ATCC MYA-2975); *C. g: Candida glabrata* (ATCC 90030); *C. k: Candida krusei* (ATCC 14243); *C. l: Candida lusitaniae* (ATCC 64125); *C. p: Candida parapsilosis* (ATCC 22019);

C. t: Candida tropicalis (ATCC 13803). NS: no sensitivity.

<sup>a</sup> Crude aqueous extract was prepared at concentration of 100 mg/mL.

Note: CHX represented as positive and  $dH_2O$  as negative controls in the tests. All values were expressed as mean  $\pm$  SD of three determinations performed in triplicate (n = 9).

#### 4.2 Determination of Antifungal Activity

#### 4.2.1 The MIC and MFC Values of *Brucea javanica* L. and *Piper betle* L.

The MIC and MFC of *B. javanica* and *P. betle* extracts towards all seven *Candida* species showed variations (Table 4.2). The MICs of *B. javanica* were determined within the range of 3.13 to 100 mg/mL, while the MFC values were from 12.5 to 100 mg/mL. For *C. glabrata* and *C. tropicalis*, the MFCs were found to be greater than 100 mg/mL. Among the seven *Candida* species, *C. dubliniensis* was the most susceptible to the *B. javanica* extract.

*P. betle* extract effectively inhibited the growth of all seven *Candida* species at concentration of 12.5 mg/mL. Its MFCs against all species was recorded at 12.5 mg/mL, except for *C. albicans* which was killed at higher MFC value of 25 mg/mL. Based on the results obtained, *Candida* species were comparatively susceptible to *P. betle* extract than to *B. javanica*.

species of candida.		v				
		MIC and MFC	C values (mg/mL) <sup>a</sup>			
	B. jav	vanica L.	P. bet	P. betle L.		
Candida spp. <sup>b</sup>	MIC range (MIC)	MFC range (MFC)	MIC range (MIC)	MFC range (MFC)		

25.0-200

(100)

3.13-100

(12.5)

3.13-100

(50)

25.0-200

(100)

3.13-100

(25)

3.13-100

(25)

25.0-200

(100)

3.13-100

(12.5)

3.13-100

(12.5)

3.13-100

(12.5)

3.13-100

(12.5)

3.13-100

(12.5)

3.13-100

(12.5)

3.13-100

(12.5)

12.5-100

(25)

12.5-100

(12.5)

12.5-100

(12.5)

12.5-100

(12.5)

12.5-100

(12.5)

12.5-100

(12.5)

12.5-100

(12.5)

3.13-200

(50)

3.13-200

(3.13)

3.13-200

(25)

3.13-200

(50)

3.13-200

(25)

3.13-200

(25)

3.13-200

(100)

C. albicans

(ATCC 14053)

C. dubliniensis

C. glabrata

C. krusei

(ATCC 90030)

(ATCC 14243)

C. lusitaniae

(ATCC 64125)

C. parapsilosis

(ATCC 22019)

C. tropicalis

(ATCC 13803)

(ATCC MYA-2975)

Table 4.2: MIC and MFC values of *B. javanica* and *P. betle* extracts towards seven

<sup>a</sup> Antifungal activity was measured by measuring the diameter of inhibition zone (DIZ)
produced by the seven candidal species. The concentration of the extracts was set at 100
and 200 mg/mL.

<sup>b</sup>Values obtain from three sets of determinations each performed in triplicate (n = 9).

# 4.3 The Effect of *Brucea javanica* L. and *Piper betle* L. Extracts on the Growth Profiles of *Candida* Species

#### 4.3.1 Growth Profiles of Candida Species

Figure 4.2 displayed the growth curves of the seven oral candida cultured under the normal, untreated growth condition. It is shown that under ideal growth conditions where all the nutrients are provided, the growth curves of the candidawere all sigmoidal, with clear exhibition of the lag, log and stationary phases. Variations in the onset and duration of the lag and log phases between the various different species were observed. Generally, about 5 h to 7 h were required by the cells to adapt to the normal growth environment before they were ready to proliferate and enter the log phase. *C. tropicalis* showed the highest growth rates  $(0.319 \pm 0.002 h^{-1})$  indicating high reproducibility. The other species were lower in the range of  $0.141 \pm 0.001 h^{-1}$  to  $0.265 \pm 0.005 h^{-1}$ . Of the seven candidal species, the doubling time of *C. dubliniensis*  $(3.330 \pm 0.164 h)$  was slightly more extended than the others which were between  $1.816 \pm 0.052$  to  $2.229 \pm$ 0.037 h. Growth kinetics of the candidal species was evaluated based on the enumeration of the CFU (Fig. 4.3). The profile pattern in Figure 4.3 clearly illustrated that the population of all seven candidal species had increased gradually from approximately  $0.5 \times 10^5$  CFU/mL to  $10.0 \times 10^5$  CFU/mL over an 18 h of incubation.



Figure 4.2: Growth curve of *Candida* species cultured in YPD broth.

Note: Cell growth signified by the sigmoidal curve pattern indicated an orderly increase in the cell mass and size under the normal growth environment. The values plotted were based on the spectrophotometric assay and expressed as mean  $\pm$  SD of three determinations carried out in triplicate (n = 9).





Figure 4.3 (i-vii): A composite figure illustrating the normal growth of *Candida* species cultured in YPD broth.





Figure 4.3 (i-vii): Continued.





Figure 4.3 (i-vii): Continued.



Figure 4.3 (i-vii): Continued.

Note: The cell growth signified by the sigmoidal curve pattern indicating an orderly increase in the cell mass and size. The values plotted were based on the spectrophotometric assay (every one hour interval) and colony-forming unit (CFU) (every two hours interval) enumeration. Data were the mean  $\pm$  SD of three independent experiments performed in triplicate (n = 9).

## 4.3.2 Growth Profiles of *Candida* Species Following Treatment of *Brucea javanica* and *Piper betle* Extracts

The pattern of growth curves of all seven *Candida* species were altered and showed deviations from the normal sigmoidal pattern following treatment with *B*. *javanica* and *P. betle* extracts. The growth curves were observed shifted to the right due to extension of the lag phases (Fig. 4.4 and 4.5). This effect which is an illustration of suppression of cell growth was confirmed by the elevated doubling time (g-values) and reduced specific growth rates ( $\mu$ -values), respectively (Table 4.3 and 4.4). The growth suppression effect of the extracts was found to be concentration dependent and species specific.

Table 4.3 showed the  $\mu$ -values of seven *Candida* species which were significantly deviated by the presence of *P. betle* extract. At 1 mg/mL, the  $\mu$ -values of *Candida* species were observed to reduceby about 15% to 44%. The reduction of *C. parapsilosis* was however found not significant (*P* = 0.537). Drasticreduction of the  $\mu$ -values of all *Candida* species to almost half of the untreated cells was noted at a higher concentration of 3 mg/mL. *C. dubliniensis* was considered as the most susceptible to the extract (98.4%), followed by *C. lusitaniae* (89.8%) and *C. albicans* (89.6%). The  $\mu$ -values reductions of four other species were comparatively lower in the range of 48% to 71%. At 6 mg/mL of *P. betle*, the  $\mu$ -values of all the candida were more than 90% reduced (*P* < 0.05).

*B. javanica* extract also showed similar growth inhibitory effect on the seven *Candida* species (Table 4.4). At 1 mg/mL, the  $\mu$ -values of *C. parapsilosis*, *C. krusei*, *C. dubliniensis* and *C. tropicalis* were significantly reduced by 87.94%, 88.05%, 86.45% and 57.05%, respectively (*P* < 0.05). At higher concentrations of 3 and 6 mg/mL, the  $\mu$ -

values were found further reduced by more than 90% except for *C. albicans* (57.41%) (P < 0.05).

The presences of *P. betle* and *B. javanica* extracts have also changed the gvalues of *Candida* species. At 1 mg/mL of *P. betle* extract, the population of *C. albicans* increases approximately 1.78-fold. The increase in the population of other candidal species was however lower within the range of 0.98- to 1.43-fold. At a concentration of 6 mg/mL, greater suppression of growth was observed whereby the population of *C. albicans* showed an increase of only 0.04-fold, and the others within the range of 0.17to 0.97-fold. Similar pattern of growth suppression was also observed for *B. javanica*. By comparing the g-values, the profile of growth curves for all the seven *Candida* species showed significantdeviation when in the presence of *P. betle* and *B. javanica* extracts (P < 0.05). In *C. krusei* however, the deviation of g-values at 6 mg/mL of *P. betle* extract was not significant (P = 0.513). This had lead to the extension of the lag and log phases. In a similar manner, based on CFU enumeration, the population (CFU/mL) of all the candidal species also showed reduction of an average of 10 x 10<sup>6</sup> to  $6 \times 10^6$  CFU/mL.



Figure 4.4 (A-G): Composite figures illustrating the population of *Candida* species following treatment with *P. betle* extract.



Figure 4.4 (A-G): Continued.



Figure 4.4 (A-G): Continued.

Note: *P. betle* extracts used were at sub-MICs of 1 mg/mL ( $\bigcirc$ ), 3 mg/mL ( $\bigcirc$ ) and 6 mg/mL ( $\bigcirc$ ). Deviation of cell numbers from the normal ( $\bigcirc$ ) is an indication of the effect of the extract on the growth of the candida. CHX was used as a reference ( $\bigcirc$ ). Viable counts of *Candida* species were estimated at 2, 6, 10, 14 and 18 h intervals. Data were mean ± SD of three independent experiments performed in triplicate (n = 9).



Figure 4.5 (A-G): Composite figures illustrating the population of *Candida* species following treatment with *B. javanica* extract.



Figure 4.5 (A-G): Continued.



Figure 4.5 (A-G): Continued.

Note: *B. javanica* extracts used were at sub-MICs of 1 mg/mL ( $\bigcirc$ , 3 mg/mL ( $\bigcirc$ ) and 6 mg/mL ( $\bigcirc$ ). Deviation of cell numbers from the normal ( $\bigcirc$ ) is an indication of the effect of the extract on the growth of the candida. CHX was used as a reference ( $\bigcirc$ ). Viable counts of *Candida* species were estimated at 2, 6, 10, 14 and 18 h intervals. Data were mean ± SD of three independent experiments performed in triplicate (n = 9).

Table 4.3: Changes in the doubling time (g) and specific-growth rates ( $\mu$ ) of the candidal species cultured in the absence (untreated) and presence of *P. betle* extract (1, 3 and 6 mg/mL).

Candida spp.	Doubling time ( & Specific-grow rates (µ)	g) Untreated /th	P. betle (1 mg/mL)	P. betle (3 mg/mL)	P. betle (6 mg/mL)
C. albicans ATCC 14053	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	2.403±0.095 0.263±0.011 -	4.098±0.034 0.152±0.008 1.71-fold 42.21%	2.181±0.044 0.031±0.005 0.91-fold 88.21%	0.094±0.023 0.005±0.005 0.04-fold 98.10%
<i>C. dubliniensis</i> ATCC MYA- 2975	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	3.330±0.164 0.251±0.010 -	3.111±0.038 0.183±0.014 0.93-fold 27.09%	0.586±0.042 0.006±0.004 0.18-fold 97.61%	1.450±0.384 0.004±0.003 0.44-fold 98.41%
<i>C. glabrata</i> ATCC 90030	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	2.229±0.037 0.263±0.004 -	2.864±0.042 0.174±0.008 1.28-fold 33.84%	3.927±0.101 0.099±0.012 1.76-fold 62.36%	1.140±0.093 0.011±0.006 0.51-fold 95.82%
C. krusei ATCC 14243	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	2.542±0.045 0.251±0.006 -	2.478±0.016 0.151±0.006 0.97-fold 39.84%	3.581±0.422 0.077±0.007 1.41-fold 69.32%	2.389±0.290 0.027±0.005 0.94-fold 89.24%
<i>C. lusitaniae</i> ATCC 64125	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	2.694±0.058 0.265±0.005 -	2.944±0.094 0.180±0.009 1.09-fold 32.08%	2.509±0.143 0.030±0.004 0.93-fold 88.68%	1.214±0.076 0.012±0.003 0.45-fold 95.47%
C. parapsilosis ATCC 22019	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	1.816±0.052 0.141±0.001 -	1.581±0.078 0.139±0.002 0.87-fold 1.42%	1.568±0.092 0.074±0.004 0.86-fold 47.52%	0.630±0.107 0.010±0.003 0.35-fold 92.91%
C. tropicalis ATCC 13803	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	2.809±0.017 0.319±0.002 -	4.032±0.020 0.271±0.004 1.44-fold 15.05%	3.419±0.082 0.109±0.004 1.22-fold 65.83%	0.573±0.060 0.008±0.007 0.20-fold 97.49%

Note: Values were obtained from spectrophotometric assay and expressed as mean  $\pm$  SD of three independent experiments performed in triplicate (n = 9).

Table 4.4: Changes in the doubling time (g) and specific-growth rates ( $\mu$ ) of the candidal species cultured in the absence (untreated) and presence of *B. javanica* extract (1, 3 and 6 mg/mL).

Candida spp.	Doubling time ( & Specific-grow rates (µ)	g) Untreated ⁄th	<i>B. javanica</i> (1 mg/mL)	B. javanica (3 mg/mL)	<i>B. javanica</i> (6 mg/mL)
C. albicans ATCC 14053	g(h) $\mu(h^{-1})$ Increase in g Reduction in $\mu$	2.403±0.095 0.263±0.011 -	2.461±0.083 0.257±0.008 1.02-fold 2.28%	2.727±0.101 0.130±0.022 1.13-fold 50.57%	3.021±0.094 0.112±0.006 1.26-fold 57.41%
C. dubliniensis ATCC MYA-	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	3.330±0.164 0.251±0.010	2.526±0.124 0.034±0.012 0.76-fold 86.45%	2.096±0.204 0.014±0.003 0.63-fold 94.42%	0.271±0.016 0.002±0.002 0.08-fold 99.20%
<i>C. glabrata</i> ATCC 90030	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	2.229±0.037 0.263±0.004 -	3.061±0.049 0.249±0.005 1.37-fold 5.32%	2.543±0.041 0.216±0.002 1.14-fold 17.87%	2.843±0.177 0.025±0.002 1.28-fold 90.49%
C. krusei ATCC 14243	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	2.542±0.045 0.251±0.006 -	3.109±0.241 0.030±0.003 1.22-fold 88.05%	1.950±0.096 0.028±0.002 0.77-fold 88.84%	1.024±0.042 0.015±0.002 0.40-fold 94.02%
C. lusitaniae ATCC 64125	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	2.694±0.058 0.265±0.005 -	3.425±0.111 0.238±0.003 1.27-fold 10.19%	2.708±0.046 0.087±0.005 1.01-fold 67.17%	0.867±0.043 0.008±0.002 0.32-fold 96.98%
C. parapsilosis ATCC 22019	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	1.816±0.052 0.141±0.001 -	1.615±0.108 0.017±0.004 0.89-fold 87.94%	1.554±0.098 0.010±0.002 0.86-fold 92.91%	0.614±0.105 0.003±0.001 0.34-fold 97.87%
<i>C. tropicalis</i> ATCC 13803	g (h) μ (h <sup>-1</sup> ) Increase in g Reduction in μ	2.809±0.017 0.319±0.002 -	3.967±0.089 0.137±0.003 1.41-fold 57.05%	3.466±0.108 0.026±0.005 1.23-fold 91.85%	0.568±0.055 0.005±0.003 0.20-fold 98.43%

Note: Values were obtained from spectrophotometric assay and expressed as mean  $\pm$  SD of three independent experiments performed in triplicate (n = 9).

# 4.4 The Effect of *Brucea javanica* L. and *Piper betle* L. Extracts on the Cell Surface Hydrophobicity of *Candida* Species

#### 4.4.1 Cell Surface Hydrophobicity of Selected Oral Candida Species

The cell surface hydrophobicity (CSH) of the *Candida* species was represented in Fig. 4.6. CSH of each candidal species was expressed as the percentage of the total cells to adsorb to hexadecane. Comparatively, *C. krusei* was found to be the most hydrophobic ( $30.23 \pm 4.63\%$ ) followed by *C. dubliniensis* ( $26.19 \pm 4.44\%$ ) and *C. tropicalis* ( $19.70 \pm 4.11\%$ ). These percentages were significantly higher than the four other candidal species (P < 0.05). The hydrophobic binding affinity of the others; *C. albicans*, *C. glabrata*, *C. lusitaniae* and *C. parapsilosis* were comparatively lower in the range of 7.95% to 10.01% (Fig. 4.6). Subsequent post hoc test (Dunnett T3) however showed that the mean percentage adsorption to hexadecane between *C. krusei* and *C. dubliniensis* was not significant (P = 0.706).



<sup>a</sup> Bars denoted by asterik in each sub-figure differ significantly at P < 0.05.

Figure 4.6: The cell surface hydrophobicity of *Candida* species measured by their binding affinities to hexadecane.

Note: Each bar represents the mean  $\pm$  SD of three independent experiments performed in triplicate (n = 9).

- (\*) Not significant (P > 0.05)
- (\*\*) Significant compared to (\*)
- (\*\*\*) Significant compared to (\*) and (\*\*)

## 4.4.2 Cell Surface Hydrophobicity of Selected Oral *Candida* Species Following Treatment of *B. javanica* and *P. betle* Extracts

Figure 4.7 illustrated the significant effect of both extracts on the CSH of the seven *Candida* species (P < 0.05). An exposure to 1 mg/mL of *B. javanica* extract had drastically reduced the CSH of most candidal species by more than 50% of the untreated condition (82.08%, *C. albicans*; 81.52%, *C. krusei*; 74.60%, *C. lusitaniae*; 72.24%, *C. parapsilosis*; 60.57%, *C. tropicalis*; 58.64%, *C. dubliniensis*; 53.31%, *C. glabrata*). The reductions were however less drastic when higher concentrations of the extract (3 and 6 mg/mL) were used. Comparative to *P. betle* exposure to 6 mg/mL of *B. javanica*, showed significant reduction of CSH by more than 90% (P < 0.05).

Similar patterns of reduction in CSH were observed when the cells were treated with *P. betle*. At 1 mg/mL, *P. betle* extract was able to reduce the CSH of *C. dubliniensis* (78.27%), *C. glabrata* (71.36%), *C. lusitaniae* (67.54%), *C. parapsilosis* (48.11%), *C. albicans* (38.56%), *C. krusei* (31.57%) and *C. tropicalis* (29.69%). Greater reduction effect was demonstrated at higher concentration of 3 and 6 mg/mL towards all candidal species, an indication that the effect was concentration dependent. Comparing to 1 mg/mL, the *P. betle* extract of 6 mg/mL could drastically reduced the CSH of *C. dubliniensis* (95.13%), followed by *C. parapsilosis* (94.20%), *C. glabrata* (91.34%), *C. lusitaniae* (84.53%) and *C. albicans* (80.35%). *C. krusei* and *C. tropicalis* however, were remained the least affected with reduction of 67.69% and 55.42%, respectively.

Most interestingly, the degree of the effect of *B. javanica* was relatively higher compared to the effect of *P. betle* (Fig. 4.7). *C. krusei* and *C. tropicalis* for instance, have shown higher hydrophobic binding affinity in the presence of 1 mg/mL *P. betle* extract, yet were strongly influenced by the same concentration of *B. javanica*. In this

study, the independent t-test was also performed to compare the mean percentage of adsorption of each candidal species to hexadecane between the treatments of *B. javanica* and *P. betle*. Based on independent t-test, the means percentage adsorption of five candidal species (*C. albicans, C. dubliniensis, C. krusei, C. parapsilosis* and *C. tropicalis*) to hexadecane were significantly different (P < 0.05) between the treatments of *B. javanica* and *P. betle*, with *B. javanica* outstrips the *P. betle* extract. The means percentage adsorption of *C. glabrata* and *C. lusitaniae* however were not significantly different (P > 0.05) with P = 0.150 and P = 0.105, respectively (Table 4.5). Comparing these two extracts at 6 mg/mL, the results showed that *B. javanica* extract exhibited an average of 10% more effective than *P. betle* in reducing the CSH of *Candida* species tested. Likewise responses to *B. javanica*, both *C. dubliniensis* and *C. glabrata* were more susceptible to the *P. betle* extract with greater reduction of the CSH at 1 mg/mL.





Figure 4.7: Comparison of the effect of *P*. *betle* ( ) and *B. javanica* ( ) extracts on the CSH of oral candidal species.

Note: The percentages were mean  $\pm$  SD of three independent experiments performed in triplicate (n = 9) (*P* < 0.05). CHX ( $\blacksquare$ ) as a positive control.

Table 4.5: The means percentage of adsorption of seven *Candida* species to hexadecane following treatment with *B. javanica* and *P. betle* extracts at different concentrations of 1, 3 and 6 mg/mL.

Variables	B. javanica	P. betle	Mean diff.	<i>t</i> -statistic	<i>P</i> -
	(n=27)	(n=27)	(95% CI)	(df)	value
	Mean (SD)	Mean (SD)			
C albicans	1.110	3.174	-2.065	-5.420 <sup>a</sup>	0.001
C. aibicans	(1.094)	(1.650)	(-2.832,-1.297)	(45.163)	0.001
C dubliniansis	6.044	3.485	2.559	2.952 <sup>a</sup>	0.005
C. uubiiniensis	(3.891)	(2.269)	(0.809, 4.308)	(41.845)	0.005
C alabrata	2.851	2.198	0.653	1.461	0.150
C. giubraia	(1.867)	(1.381)	(-0.244, 1.550)	(52)	0.150
C krusoi	2.882	14.834	-11.952	-11.121 <sup>a</sup>	0.001
C. Krusei	(2.506)	(4.990)	(-14.127,-9.777)	(38.329)	0.001
C lusitaniaa	1.402	2.026	-0.624	-1.651	0 105
C. iusiiuniae	(1.516)	(1.252)	(-1.384, 0.135)	(52)	0.105
C naransilosis	0.803	2.457	-1.654	-3.061	0.003
C. parapsilosis	(2.090)	(1.876)	(-2.739,-0.570)	(52)	0.005
C tropicalis	5.437	10.945	-5.508	-7.151	0.001
C. Hopicaus	(2.844)	(2.817)	(-7.054,-3.963)	(52)	0.001

<sup>a</sup> Equal variance not assumed (Levene's test P-value < 0.05)

Note: Values were expressed as mean  $\pm$  SD of three independent experiments performed in triplicate (n = 9).

# 4.5 The Effect of *Brucea javanica* L. and *Piper betle* L. Extracts on the Adherence Affinity of *Candida* Species to Acquired Pellicles

#### 4.5.1 The Adherence Affinity of *Candida* Species

Figure 4.8 showed the adherence affinity of the *Candida* species towards salivacoated glass surface. The saliva coating or pellicle consists of adsorbed proteins and other macromolecules from the saliva which provide specific recognition of salivary receptors for the candidal cells to adhere. By using the NAM model as described in Materials and Methods (Section 3.2.10), the population of candida cells (CFU/mL) able to adhere to the experimental salivary pellicle was to have variation. Different species showed different adhering capacity with *C. parapsilosis* exhibiting the highest capacity at  $18.72 \pm 0.71 \times 10^5$  CFU/mL, followed by *C. lusitaniae* (14.39 ± 2.38 x 10<sup>5</sup> CFU/mL), *C. tropicalis* (8.58 ± 0.53 x 10<sup>5</sup> CFU/mL), *C. albicans* (6.22 ± 0.87 x 10<sup>5</sup> CFU/mL) and *C. glabrata* (7.17 ± 1.87 x 10<sup>5</sup> CFU/mL). *C. dubliniensis* and *C. krusei* possessed the least adhering ability at 2.76 ± 0.10 x 10<sup>5</sup> CFU/mL and 2.13 ± 0.38 x 10<sup>5</sup> CFU/mL, respectively.


Figure 4.8: The adherence of candida to saliva-coated glass surface.

Note: Results were expressed as the mean  $\pm$  SD of three independent experiments performed in triplicate (n = 9) (P < 0.05).

#### 4.5.2 Anti-adherence Activity of Brucea javanica and Piper betle Extracts

Figure 4.9 comparatively showed the maximum adhering capacity of the candida on salivary pellicle (untreated) and the reduced population able to adhere following treatment with the plant extracts. This reduction illustrated the anti-adherence effect of *B. javanica* and *P. betle* on the specific adhesion of the candida.

The anti-adherence effect of *P. betle* and *B. javanica* extracts was presented in Figure 4.10. The anti-adherence effect of both extracts varied between the *Candida* species. Exposing the candidal cells to *P. betle*-treated pellicle drastically reduced three out of seven *Candida* species by more than 50% (*C. tropicalis* by 86.10%, *C. albicans* 61.41% and *C. krusei* 56.34%). *C. lusitaniae*, *C. parapsilosis* and *C. dubliniensis* were reduced by 47.60%, 46.53% and 21.38%, respectively. The reduction of *C. glabrata* however was found to be the least at 12.41%.

The aqueous extract of *B. javanica* also exhibited similar effect by reducing five *Candida* species tested at more than 50% (*C. tropicalis* 89.86%, *C. lusitaniae* 88.95%, *C. albicans* 79.74%, *C. glabrata* 76.85% and *C. krusei* 67.61%). The reduction in adhesion of *C. parapsilosis* and *C. dubliniensis* were comparatively lower at 48.99%, and 27.90%, respectively.

Based on the results, we may suggest that the anti-adherence effect of the aqueous extract of *B. javanica* towards the *Candida* species was found to be slightly higher compared to that of the aqueous extract of *P. betle*. Compared to the anti-adherence effects shown by the extracts, the CHX-containing mouthrinse remains as an effective antifungal agent by reducing the *Candida* species by more than 90%.





Figure 4.9: The adhering capacity of oral candida to salivary-pellicle (untreated) and extract-treated pellicle.

Note: Results were expressed as mean  $\pm$  SD of triplicate in three determinations (n = 9). (\*) indicates the mean was not significantly different (*P* > 0.05) from the untreated.



Figure 4.10: The anti-adherence effect of *P. betle* and *B. javanica* extracts on the binding capacity of oral *Candida* species to the acquired pellicles.

Note: The anti-adherence effect was measured by the difference in % of cells adhering to the pellicles. The percentage plotted was expressed as the mean  $\pm$  SD of three independent experiments performed in triplicate (n = 9). CHX (0.12% w/v) represented as a positive control, significantly reduced the adhesion of candida to the pellicles by more than 90% (*P* < 0.01).

# 4.6 The Effect of *Brucea javanica* L. and *Piper betle* L. Extracts on the Morphology of *Candida* Species

#### 4.6.1 Scanning Electron Microscope Examination

Figure 4.11 – 4.17 represented the SEM micrographs of seven *Candida* species – *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis* and *C. tropicalis*, respectively. In the absence of the extracts, the untreated candidal cells were all intact and had attained optimum cell sizes within the range of  $3.13 \times 2.33 \mu m$  to  $6.65 \times 1.95 \mu m$  (Table 4.6). These non-treated colonies were smooth-surfaced, rounded and some were elongated in their well-developed structure. Most of these cells were also shown to be in the active dividing stateand existed either as individual or/and in chain. As with all species examined, interconnecting processes, buds and bud scars were seen.

Some physical changes and morphological alteration on the candida cells were observed following treatment with *B. javanica* and *P. betle* extracts (Table 4.7). It was found that the cells sizes were slightly smaller and elongated compared to the untreated cells. Amongst the treated candidal cells, deposition of heavy mesh-like extracellular matrix around the cells was observed, which resulted in a fluffy appearance with aggregated cells forming large clumps. Figure 4.13 and Figure 4.14 clearly demonstrated that the candidal cells treated with the extracts underwent considerable damage as some of them were shriken due to the decomposition of cell wall.

Table 4.6: Deviations in the sizes of candidal cells following treatment of *P. betle* and *B. javanica* extracts.

	Normal		P. betle		B. javanica	
Canalaa species	Length (µm)	Width (µm)	Length (µm)	Width (µm)	Length (µm)	Width (µm)
C. albicans	$3.93\pm0.30$	$3.07\pm0.15$	$3.20\pm0.51$	$2.73\pm0.19$	$3.60\pm0.33$	$3.03\pm0.51$
C. dubliniensis	$4.11 \pm 0.48$	$2.40\pm0.15$	$3.57\pm0.47$	$2.43\pm0.13$	$3.50\pm0.18$	$2.26\pm0.33$
C. glabrata	3.13 ± 0.39	$2.33\pm0.39$	$2.43\pm0.23$	$1.87\pm0.45$	$2.73\pm0.24$	$2.17\pm0.27$
C. krusei	$6.65\pm0.52$	$1.95\pm0.16$	$6.00 \pm 0.71$	$1.85 \pm 0.48$	$5.77\pm0.53$	$2.10\pm0.58$
C. lusitaniae	$3.80 \pm 0.44$	$2.56\pm0.25$	$3.16\pm0.24$	$2.40\pm0.21$	$3.32\pm0.28$	$2.36\pm0.24$
C. parapsilosis	$4.56\pm0.34$	$2.36\pm0.28$	$4.04\pm0.63$	$2.32\pm0.25$	$3.36\pm0.43$	$2.28\pm0.25$
C. tropicalis	$3.80\pm0.51$	$2.80\pm0.18$	$3.77\pm0.59$	$2.90\pm0.33$	$3.73\pm0.39$	$2.83\pm0.39$

Note: Values are expressed as mean  $\pm$  SD of nine determinations (n = 9).

Table 4.7: The changes in the ultrastructural morphological features of seven *Candida* species cells observed following treatment of *P. betle* and *B. javanica* extracts (6 mg/mL).

<i>Candida</i> species	Morphological features of cells grown under normal	Morphological features of cells under treatment of plant extract			
	physiological condition	P. betle	B. javanica		
C. albicans	<ul> <li>Figure 4.11a:</li> <li>Oval-shaped cells are seen on the surface of YPD media.</li> <li>Individual cells, rounded and had a smooth surface.</li> <li>Cells had attained optimum size with an average of 3.93 ± 0.30 µm x 3.07 ± 0.15 µm.</li> </ul>	<ul> <li>Figure 4.11b:</li> <li>&gt; Oval-shaped cells are seen on the surface of YPD media.</li> <li>&gt; Individual cells and the deposition of extracellular matrix (→) on the surface are seen.</li> <li>&gt; Cells sizes were relatively smaller with an average of 3.20 ± 0.51 µm x 2.73 ± 0.19 µm.</li> </ul>	<ul> <li>Figure 4.11c:</li> <li>Oval-shaped cells are seen on the surface of YPD media.</li> <li>Individual and the deposition of extracellular matrix (-&gt;) on the surface are seen.</li> <li>Cells had attained optimum size, but slightly smallerin sizes with an average of 3.60 ±</li> </ul>		
	<ul> <li>Interconnecting processes, the buds and bud scars (-&gt;) are seen.</li> </ul>	<ul> <li>No buds or scars are seen.</li> </ul>	<ul> <li>0.33 µm x 3.03 ±</li> <li>0.51 µm.</li> <li>&gt; Some buds and the scars (→) still can be seen.</li> </ul>		

Table 4.7: Continued.

<i>Candida</i> species	Morphological features of cells grown under normal	Morphological features of cells under treatment of plant extract		
	physiological condition	P. betle	B. javanica	
C. dubliniensis	<ul> <li>Figure 4.12a:</li> <li>Conical-shaped, elongated cells are seen on the surface of YPD media.</li> <li>Cells are seen as individual cells and in chain. The surface of cells appeared smooth.</li> <li>Cells had attained optimum size with an average of 4.11 ± 0.48 μm x 2.40 ± 0.15 μm.</li> </ul>	<ul> <li>Figure 4.12b:</li> <li>Conical-shaped, elongated cells are seen on the surface of YPD media.</li> <li>Cells are seen as individual and in chain. The deposition of extracellular matrix (→) on the surface isseen.</li> <li>Cells sizes were relatively smaller with an average of 3.57 ± 0.47 µm x 2.43 ± 0.13 µm.</li> </ul>	<ul> <li>Figure 4.12c:</li> <li>Conical-shaped, elongated cellsare seen on the surface of YPD media.</li> <li>Cells are seen as individual. The depositionof materials (→) on the surfaceis seen.</li> <li>Cells had attained optimum size, relatively smaller with an average of 3.50 ± 0.18 µm x 2.26 ± 0.33 µm.</li> </ul>	
	Interconnecting processes and the buds (→) are seen.	➤ The formation of buds (→) still can be seen.	➤ The buds (→) are seen.	

Table 4.7: Continued.

Candida species	Morphological features of cells grown under normal	Morphological features of cells under treatment of plant extract		
	physiological condition	P. betle	B. javanica	
C. glabrata	Figure 4.13a:	Figure 4.13b:	Figure 4.13c:	
	Conical-shaped,	➤ Conical-shaped,	<ul> <li>Conical-shaped,</li> </ul>	
	elongated cells are	elongated cells are	elongated cells	
	seen on the surface	seen on the surface	are seen on the	
	of YPD media.	of YPD media.	surface of YPD	
			media.	
	Individual cells	➤ Individual cells.	Individual cells.	
	and the surface of	The deposition of	The deposition of	
	cells appeared	extracellular	extracellular	
	smooth.	matrix $(\rightarrow)$ and	matrix $(\rightarrow)$ and	
		decomposition of	decomposition of	
		the cell walls	the cell walls	
		occurred (O). The	occurred (🔵).	
		surfaces appeared	The surfaces	
		rough.	appeared rough.	
	Cells had attained	Cells didn't	Cells didn't	
	optimum size with	attained optimum	attained optimum	
	an average of 3.13	size than the	size than the	
	$\pm$ 0.39 $\mu m$ x 2.33 $\pm$	untreated,	untreated,	
	0.39 µm.	relatively smaller	relatively smaller	
		with an average of	with an average	
		$2.43\pm0.23~\mu m~x$	of $2.73 \pm 0.24$	
		$1.87\pm0.45~\mu m.$	$\mu m \; x \; 2.17 \pm 0.27$	
			μm.	
	> The buds ( $\rightarrow$ ) are	➢ Some buds (→)	➢ Some buds (→)	
	seen.	still can be seen.	still can be seen.	

Candida species	Morphological features of cells grown under normal	Morphological features of cells under treatment of plant extract		
	physiological condition	P. betle	B. javanica	
C. krusei	<ul> <li>Figure 4.14a:</li> <li>Rod-shaped, elongated cells are seen on the surface of YPD media.</li> </ul>	<ul> <li>Figure 4.14b:</li> <li>Rod-shaped, elongated cells are seen on the surface of YPD media.</li> </ul>	<ul> <li>Figure 4.14c:</li> <li>Rod-shaped, elongated cells are seen on the surface of YPD media.</li> </ul>	
	Individual cells, the surface of cells appeared slightly rough and had a randomly positioned punctates (	Individual cells. The cell structures were mostly changed, shrinked and flaccid (○).	Individual cells. The cell structures were mostly changed, shrinked and flaccid (○).	
	<ul> <li>Cells are relatively bigger than the other untreated <i>Candida</i> species whichhad attained optimum size with an average of 6.65 ± 0.52 μm x 1.95 ± 0.16 μm.</li> <li>The buds (→) are seen.</li> </ul>	<ul> <li>Cells didn't attained optimum size due to its shape, relatively smaller with an average of 6.00 ± 0.71 µm x 1.85 ± 0.48 µm.</li> <li>No buds are seen.</li> </ul>	<ul> <li>&gt; Cells didn't attained optimum size due to its shape, relatively smaller with an average of 5.77 ± 0.53 µm x 2.10 ± 0.58 µm.</li> <li>&gt; Some buds and buds cars (→) also can be seen.</li> </ul>	

<i>Candida</i> species	Morphological features of cells grown under normal	Morphological features of cells under treatment of plant extract			
	physiological condition	P. betle	B. javanica		
C. lusitaniae	<ul> <li>Figure 4.15a:</li> <li>Oval-shaped cells are seen on the</li> </ul>	<ul> <li>Figure 4.15b:</li> <li>Oval-shaped cells are seen on the</li> </ul>	<ul><li>Figure 4.15c:</li><li>➢ Oval-shaped cells are seen on the</li></ul>		
	surface of YPD media.	surface of YPD media.	surface of YPD media.		
	<ul> <li>&gt; Individually arranged, rounded and had a randomly positioned punctates (→) on the surface.</li> <li>&gt; Cells had attained optimum size with an average of 3.80 ± 0.44 µm x 2.56 ± 0.25 µm.</li> <li>&gt; The buds (→) are seen.</li> </ul>	<ul> <li>&gt; Individually arranged, rounded and the punctates on the surface seem to have disappeared or damaged.</li> <li>&gt; Cells had attained optimum size and still intact but were relatively smaller with an average of 3.16 ± 0.24 µm x 2.40 ± 0.21 µm.</li> <li>&gt; The buds (→) still can be seen.</li> </ul>	<ul> <li>➢ Individually arranged, rounded and the punctates on the surface seemto have disappeared or damaged.</li> <li>➢ Cells had attained optimum size and still intact but were relatively smaller with an average of 3.32 ± 0.28 µm x 2.36 ± 0.24 µm.</li> <li>➢ The buds (→) still can be seen.</li> </ul>		

Candida species	Morphological features of cells grown under normal	Morphological features of cells under treatment of plant extract			
	physiological condition	P. betle	B. javanica		
C. parapsilosis	<ul> <li>Figure 4.16a:</li> <li>Conical-shaped, elongated cellsare seen on the surface of YPD media.</li> <li>Cells are seen as individual, rounded and had a smooth surface.</li> <li>Cells had attained optimum size with</li> </ul>	<ul> <li>Figure 4.16b:</li> <li>Conical-shaped, elongated cells are seen on the surface of YPD media.</li> <li>Individual cells and the deposition of extracellular matrix (→) on the surface are seen.</li> <li>Cells had attained optimum size but</li> </ul>	<ul> <li>Figure 4.16c:</li> <li>Conical-shaped, elongated cells are seen on the surface of YPD media.</li> <li>Individual cells and the deposition of extracellular matrix (-&gt;) on the surface are seen.</li> <li>Cells had attained optimum</li> </ul>		
	<ul> <li>an average of 4.56 ± 0.34 μm x 2.36 ± 0.28 μm.</li> <li>&gt; The buds (→) are seen.</li> </ul>	<ul> <li>were relatively smaller with an average of 4.04 ± 0.63 µm x 2.32 ± 0.25 µm.</li> <li>&gt; The scars of the buds (→) still can be seen.</li> </ul>	size but were relatively smaller with an average of 3.36 ± 0.43 µm x 2.28 ± 0.25 µm. ➤ No buds are seen.		

<i>Candida</i> species	Morphological features of cells grown under normal	Morphological features of cells under treatment of plant extract			
	physiological condition	P. betle	B. javanica		
C. tropicalis	<ul> <li>Figure 4.17a:</li> <li>Oval-shaped cells are seen on the surface of YPD media.</li> <li>Individually arranged, rounded and had a smooth surface.</li> </ul>	<ul> <li>Figure 4.17b:</li> <li>Oval-shaped cells are seen on the surface of YPD media.</li> <li>Individually arranged, rounded and had a smooth surface. Cell structures remain intact.</li> </ul>	<ul> <li>Figure 4.17c:</li> <li>Oval-shaped cells are seen on the surface of YPD media.</li> <li>Individually arranged, rounded and had a smooth surface. The deposition of extracellular matrix (</li></ul>		
	<ul> <li>Cells had attained optimum size with an average of 3.80 ± 0.51 µm x 2.80 ± 0.18 µm.</li> <li>Interconnecting processes and the buds (→) are seen.</li> </ul>	<ul> <li>Cells had attained optimum size with an average of 3.77 ± 0.59 µm x 2.90 ± 0.33 µm.</li> <li>Interconnecting processes and the buds (→) are seen.</li> </ul>	<ul> <li>Cells had attained optimum size with an average of 3.73 ± 0.39 µm x 2.83 ± 0.39 µm.</li> <li>Interconnecting processes and the buds (→) are seen.</li> </ul>		



Figure 4.11: SEM micrographs of *C. albicans*.

Note: (A) The non-treated cells. (B) Cells under treatment of *P. betle*. (C) Cells under treatment of *B. javanica* (5000x). Buds / bud scars ( $\rightarrow$ ), deposition of extracellular matrix ( $\rightarrow$ ).



Figure 4.12: SEM micrographs of C. dubliniensis.

Note: (A) The non-treated cells. (B) Cells under treatment of *P. betle*. (C) Cells under treatment of *B. javanica* (5000x). Buds / bud scars ( $\rightarrow$ ), deposition of extracellular matrix ( $\rightarrow$ ).



Figure 4.13: SEM micrographs of C. glabrata.

Note: (A) The non-treated cells. (B) Cells under treatment of *P. betle*. (C) Cells under treatment of *B. javanica* (5000x). Buds / buds cars ( $\rightarrow$ ), deposition of extracellular matrix ( $\rightarrow$ ), changes / decomposition of the cell wall ( $\bigcirc$ ).



Figure 4.14: SEM micrographs of C. krusei.

Note: (A) The non-treated cells. (B) Cells under treatment of *P. betle*. (C) Cells under treatment of *B. javanica* (5000x). Buds / buds scars ( $\rightarrow$ ), deposition of extracellular matrix ( $\rightarrow$ ), decomposition of the cell wall (O). Punctuates on the cell surface ( $\rightarrow$ ).



Figure 4.15: SEM micrographs of C. lusitaniae.

Note: (A) The non-treated cells. (B) Cells under treatment of *P. betle*. (C) Cells under treatment of *B. javanica* (5000x). Buds / bud scars ( $\rightarrow$ , the cells showing randomly positioned punctates on the cell surface ( $\rightarrow$ ).



Figure 4.16: SEM micrographs of C. parapsilosis.

Note: (A) The non-treated cells. (B) Cells under treatment of *P. betle*. (C) Cells under treatment of *B. javanica* (5000x). Buds / bud scars ( $\rightarrow$ ), deposition of extracellular matrix ( $\rightarrow$ ).



Figure 4.16: SEM micrographs of *C. tropicalis*.

Note: (A) The non-treated cells. (B) Cells under treatment of *P. betle*. (C) Cells under treatment of *B. javanica* (5000x). Buds / bud scars ( $\rightarrow$ ), deposition of extracellular matrix ( $\rightarrow$ ).

# 4.7 The Effect of *Brucea javanica* L. and *Piper betle* L. Extracts on the Expressions of Secretory Aspartyl Proteinases (*SAP1-10*) and Hyphal Wall Protein (*HWP1*) Genes

The secreted aspartyl proteainases (Saps) and hyphall wall adhesins (Hwp1) have long been recognised as virulence traits in the pathogenicity of *Candida* species, most notably in *C. albicans*. Saps encoded by 10 *SAP* genes all have been well characterised in *C. albicans*, besides Hwp1 that associated in the cell adhesion. Therefore, an extended study on the antifungal effect of *B. javanica* and *P. betle* extracts was performed to investigate the expression patterns of the interest genes *e.g. SAP1-10* and *HWP1* after 24 h of growth in response to the extracts at respective concentrations of 1, 3 and 6 mg/mL. The changes in gene expression between test samples were justified. Total RNA extracted from *C. albicans* cells was quantified and the concentration used for RT-PCR was standardised at 50  $\mu$ g/ $\mu$ L.

#### 4.7.1 Differential Expression of Secretory Aspartyl Proteinases (SAP1-10)

As shown in Figure 4.18, the multigene family of *SAP* (*SAP1-10*) were highly expressed in *C. albicans* cells grown in the normal, untreated YPD medium. The expression of each individual gene was thought to reflect the secretion of aspartic proteinases (Saps) *in vivo* to survive in the oral mucosa. It is demonstrated that the candidal cells tend to secrete the proteinases under an ideal condition, where the nutrients required for the growth are sufficient. As specific primers (Table 3.1) used in this study, the sizes of amplified products of *SAP* are expected to be in the range of between 152 to 283 bp.

*SAP4, SAP5* and *SAP6* are nearly identical in the sequences of both translated and untranslated regions of their respective mRNAs. Therefore, a single set of PCR primers which produces a 150 bp product derived from any members of the three; *SAP4, SAP5* and *SAP6* gene subfamily, was chosen. In this study, the sequence was repeated twice in tandem, resulting another additional PCR product of 181 bp. A primer set to detect *Candida* actin gene (*ACT1*) served as a positive control. Using RT-PCR, the actin primer was consistantly positive, indicating the presence of *Candida* mRNAs in the samples tested.

SAP gene expression is extremely sensitive and responsive to the changes in the environmental conditions. The results revealed that *SAP1-10* expressions were faint in comparison to the normal cells, which indicates each gene was down-regulated when the candida cells grown in the extract-treated medium. Marked differences in the expression of *SAP1-10* were observed when high concentration of the extracts (6 mg/mL) was added. No distinct band for the certain *SAP* genes but a smear pattern was observed. One possible explaination might be the involvement of repeatative sequences dispersed in the genome. *SAP1-10* genes were differentially expressed under the influence of *B. javanica* and *P. betle* extracts. The extracts added in the growth medium probably have created environmental stress for the cells, which subsequently affects several functional pathways.

#### 4.7.2 Differential Expression of Hyphal Wall Protein (*HWP1*)

As shown in Figure 4.19, *HWP1* was expressed in *C. albicans* cultured in normal, untreated condition and the size of RT-PCR product is 572 bp. However, the cells treated for 24 h with *B. javanica* and *P. betle* extracts displayed reduced levels of *HWP1* expression in a dose-dependent manner compared to the untreated cells. Similar

to the family of *SAP* above, down-regulation of *HWP1* has also been observed for the candidal cells grown in the extract-treated medium. The addition of extracts could possibly suppressed the biological system of the cells and delayed the transcription of the gene.



Figure 4.18: Differential expression of *SAP1-10* genes in *C. albicans* cultured in different conditions.

Note: (A) Grown in the normal, untreated condition. (B) Under treatment of *P. betle* extract at concentrations of 1, 3 and 6 mg/mL. (C) Under treatment of *B. javanica* extract at concentrations of 1, 3 and 6 mg/mL. Reverse transcription of mRNA followed by gene specific PCR was used to demonstrate *SAP1-10*. M: molecular weight marker (100 bp), 1-10: *SAP1-10* genes, A: *ACT1* gene (positive control).

[bp]	М	12	34	M 5	678	8 M A
3000						
1500						=
1000						
900	_					=
800						
700						
500						
400						-
300						
200						
100						

Figure 4.19: Differential expression of *HWP1* gene in *C. albicans* cultured in different conditions.

Note: Lane 1 and 5 : Cells grown in the normal, untreated condition.

Lane 2	: Under treatment of 1 mg/mL P. be	etle extract
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Lane 3 : Under treatment of 3 mg/mL *P. betle* extract

Lane 4 : Under treatment of 6 mg/mL *P. betle* extract

Lane 6 : Under treatment of 1 mg/mL *B. javanica* extract

- Lane 7 : Under treatment of 3 mg/mL *B. javanica* extract
- Lane 8 : Under treatment of 6 mg/mL *B. javanica* extract

M: molecular weight marker (100 bp), A: ACT1gene (positive control).

#### **CHAPTER FIVE**

#### DISCUSSION

## 5.1 The Susceptibility of Oral Candida to *Piper betle* and *Brucea javanica* Extracts

*Candida* species possess unique ability to adapt to adverse environmental conditions in order to sustain their cellular system despite fluctuations in the external surroundings. Being a unicellular organism, they require specific internal conditions for optimal growth and function. Although *C. albicans* is the most pathogenic organism in this genus, the non-*Candida albicans Candida* (NCAC) species have recently emerged as significant pathogens of clinical importance (Krcmery and Barnes, 2002; Nguyen *et al.*, 1996). The proportion of fungal infections in the oral cavity has also changed due to the increased resistance to current chemical or synthetic antifungal agents. Therefore, researchers began to embark on serious efforts to discover potential compounds from natural products that can be used as antibacterial and antifungal agents (Arif *et al.*, 2009; Mahesh and Satish, 2008).

In this study, seven local plants were first screened for their antifungal activity against seven *Candida* species common to the oral cavity. From the Kirby-Bauer sensitivity test, all seven oral candidal species were found susceptible to only two plants that include the extracts of *P. betle* and *B. javanica*. Thus, the other five plant extracts were omitted from subsequent analysis. Based on the diameter of inhibition zone (DIZ) values, the extracts of *P. betle* and *B. javanica* were found to exhibit varying degree of antifungal activity towards different species of oral candida. Compared to *B. javanica*, *P. betle* extract exhibited much stronger antifungal activity (Table 4.1). In previous

studies, *P. betle* extract has been reported to possess antibacterial properties towards *Streptococcus mutans* (Nalina and Rahim 2006, 2007), as well as *Streptococcus mitis, Streptococcus sanguis* and *Actinomyces viscosus* (Fathilah, 2005) which are the early colonizers of dental plaque. Despite the many biological activities of *B. javanica* and *P. betle*, to our knowledge, there is little information with regards to their antifungal activity against the oral candidal species. In the present study, the extent of antifungal activity of these extracts was further studied on the seven species of oral candida that may be involved in their virulence. The determination of growth inhibitory responses of *Candida* species, the adherence mechanisms, the ultrastructural changes of the cell surfaces, and the differential expression of the interest genes of the candidal species in the normal and stressed environment were analysed to help better understand the sensitivity and resistance of *Candida* species in adapting to the oral environment. This information will contribute to the development of effective preventive measures against candidal infection in the oral cavity in the future.

Based on the MIC and MFC determinations, the antifungal activity of *P. betle* was greater compared to that of *B. javanica* extract. Although these two values provide same information on the concentration of the extracts that will exert antifungal activity, it must be acknowledged that these data do not allow for the prediction of activity or specificity of the extracts *in vivo*. This is because in this study, the MIC and MFC of the extracts were determined on individual species unlike the oral cavity where the other microorganism exist. The extracts were also prepared as crude, which may contain impurities that could have masked their potent antifungal potential against the candida. This may explain the high MIC and MFC of the plant extracts compared to the CHX-containing mouthrinse (positive control). Nevertheless, *P. betle* and *B. javanica* extracts showed satisfactory antifungal activity towards all seven *Candida* species tested.

Realising that the susceptibility of *Candida* species may not be uniform, three different sub-MICs concentrations (sub-MICs) (1, 3 and 6 mg/mL) were used throughout the study.

# 5.2 Growth Inhibitory Responses of *Candida* Species to *Piper betle* and *Brucea javanica* Extracts

The growth inhibitory response of *Candida* species to *P. betle* and *B. javanica* was investigated using a microdilution broth kinetic system which was based on continuous monitoring of changes in the optical density of cell growth over time (Meletiadis *et al.*, 2001). To standardise the inoculum, the suspension of microbes was kept constant at  $10^6$  cells/mL (OD<sub>550</sub> of 0.144). In addition, the temperature of growth incubation was kept constant at 37 °C, except for *C. parapsilosis* which was set at 35 °C. In normal condition, the duration of the lag phase (the period until the first significant change in OD) of candidal species was found to have range from 5 to 7 h indicating that different species in the genus *Candida* have different reproducibility when adapting to different growth environment. Among the seven species tested, *C. krusei* and *C. tropicalis* exhibited the shortest lag phase of 5 h, whereas *C. parapsilosis* showed the longest at 7 h (Fig. 4.2). The results observed were in agreement with Pang *et al.* (2010), who reported that the growth rates of *C. krusei*, *C. albicans* and *C. glabrata* are relatively higher compared to *C. parapsilosis*.

It was found that *P. betle* and *B. javanica* extracts exhibited varying degree of growth inhibitory effect on *Candida* species. Disruption of the normal physiological growth process of the *Candida* species was indicated by the deviation of the growth curves from the normal pattern. Upon the addition of the extracts into the growth environment, the log phase of *Candida* species were very much reduced and shifted to

the right. The candidal cells may be under the fungistatic influence following the addition of the extracts. The biophysical changes of the growth environment upon the introduction of the extracts require the candida to adapt appropriately to survive. When curves of growth in the presence of increasing concentrations of *P. betle* and *B. javanica* extracts were studied, both parameters; the doubling time (g) and specific-growth rates ( $\mu$ ) of seven candidal species were affected compared with those of the extract-free (untreated) growth media. It was shown that the higher the concentration, the longer the lag phase of the growth curves (Fig. 4.4 and 4.5). This effect was previously observed for *Candida* species when growth curves based on capacitance were obtained (Chang *et al.*, 2000). The candidal cells were not able to grow as normal since they were not able to attain the predetermined size and volume required to enable them to divide successfully. The presence of extract has decreased the germination and elongation rates of spores and germinated spores, respectively. Therefore, the critical turbidity was delayed, resulting in longer lag phases of *Candida* species were also reduced.

At 3 mg/mL of *P. betle* extract, the reduction in the specific growth rates ( $\mu$ ) of *C. parapsilosis* and *C. tropicalis* were 48.23% and 66.77%, respectively. In the treatment with *B. javanica*, *C. dubliniensis* and *C. tropicalis* showed the highest reduction of  $\mu$ -values with 87.04% and 57.28%, respectively. At higher concentration (6 mg/mL), both extracts exhibited significant reduction towards the growth (*P* < 0.05). *C. krusei* however showed insignificant reduction of  $\mu$ -values following treatment with *P. betle* (*P* = 0.153). The doubling time (g) of most *Candida* species were also reduced by 90%, specifically at concentration of 6 mg/mL.

Based on the  $\mu$ - and g-values obtained, it is suggested that the *P. betle* and *B. javanica* extracts have a fungistatic effect on the oral *Candida* species. The extended lag phases strongly suggest that the extracts have successfully suppressed the cells to become dormant and unable to proliferate actively. This also indicates that candida grown in the presence of the extract could experience environmental stress, which may influence their normal biological functions (Gerhardt *et al.*, 1981) and the ability to use nutrients efficiently, thereby has slowed down its growth. At this point, the cells may deactivate its metabolism while waiting for the environment to revert back to the normal condition. The need to synthesis the essential enzymes by the suppressed cells in preparing for growth may possibly explains the extended lag phase of the *Candida* species (Pirt, 1975). Fathilah *et al.* (2009) reported that the early settlers of dental plaque have also experienced the bacteriostatic effect when treated with the *P. betle* extract.

Among the seven oral *Candida* species, *C. krusei* was observed to be highly resistant towards *P. betle* extract. Despite the cells experiencing an extension of the lag phase, proliferation of the cells was resumed after 12 h of incubation. The resistance may possibly be due to the distinct structure of its cell wall which constitute of a specific mannan not found in other *Candida* species (Kogan *et al.*, 1988). Moreover, *C. krusei* is recognized as a potentially multidrug-resistant (MDR) fungal pathogen which is able to withstand a vitamin-free media, or the shortage of nutrients compared to the other *Candida* species (Odds, 1988). In addition, the ability of *C. krusei* to switch into certain form could help the cells to tolerate with the stressed surroundings. In this study, *C. dubliniensis* was found to be most susceptible species towards both the *P. betle* and *B. javanica* extracts.

# 5.3 Adherence Capacity of Oral *Candida* Species: The Cell Surface Hydrophobicity and Specific Adhesion Mechanism of Candidal Cells under Influence of *Brucea javanica* and *Piper betle* Extracts

Adherence to surfaces may occur through hydrophobic interaction, only if the associating sites have sufficiently high densities of non-polar areas. It is reported that the presence of cell surface proteins in the cell membrane moiety contribute to the hydrophobicity of the candidal cells (Rosenberg et al., 1983). The surface proteins comprised of hydrophobic domain that consists of non-polar amino acids which may represent as a measurable physiochemical variable to estimate the overall adhesion potential of candida to surfaces. The involvement of cell surface hydrophobicity (CSH) in the adhesion of other microorganisms was also reported in previous studies (Courtney et al., 1990; Busscher et al., 1992; McNab et al., 1995; Davies et al., 1996; Van der Mei and Busscher, 1996; Vázquez-Juárez et al., 1997). Hexadecane, a hydrocarbon is used to mimic the hydrophobic surfaces of the teeth or dentures in the oral cavity. The hydrophobic surface of the cell wall may be driven by the forces to adhere on the surfaces. This effect is fundamentally based on the tendency of water molecules (polar) to exclude non-polar molecules, leading to segregation of polar and non-polar substances (Chandler, 2005). Deviation to the candidal hydrophobic affinity may cease the adherence mechanism of the cells.

The CSH of seven oral *Candida* species was significantly reduced (P < 0.05) following treatments with the *B. javanica* and *P. betle* extracts. It was shown that the extracts exhibited varying degree of inhibitory effects on the binding affinities of candidal cells to hexadecane. *B. javanica* extract has remarkably reduced the CSH of *C. albicans*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis* and *C. tropicalis*. In contrast, *C. dubliniensis* and *C. glabrata* were found to be more susceptible to *P. betle* (Fig. 4.7).

Initially, C. krusei was highly hydrophobic among the other candidal species due to its high percentage adsorption to hydrocarbon. This was in an agreement with previous studies which have reported that C. krusei was highly hydrophobic compared to C. albicans, C. tropicalis and C. glabrata (Klotz et al., 1985; Minagi et al., 1985; Samaranayake et al., 1995). Silva et al. (2010) reported that C. glabrata, C. parapsilosis and C. tropicalis were all hydrophilic. In this study, C. tropicalis seem to be hydrophobic than C. glabrata and C. parapsilosis. Meanwhile, C. dubliniensis is more hydrophobic than C. albicans despite being phylogenetically closer to the latter. Findings reflect that each candidal species has distinct capacity of surface proteins encompassing the hydrophobic that confer the CSH status. The cell membrane can differ significantly from one organism to another, and even within a strain it can change as a result of physiological adaptation to the environment (Russell, 1988). Other possible reasons could be attributed by the surface physiochemical characteristics of the candidal species that were species-specific and the binding capacities are probably determined by differences in the chemical composition in the cell membrane (Christophe et al., 1997). The CSH values however, were diminished uniformly with increased concentrations of the extracts, indicating the effect is concentration dependent.

A study on the adherence of seven candidal species specifically on the salivary pellicle was carried out by using an artificial mouth model system. Saliva was earlier deposited as pellicle onto glass beads which mimic the environment in the oral cavity (Gocke *et al.*, 2002). The presence of salivary pellicle may provide receptor sites that assist the candidal to adhere. Adhesin proteins on the candidal cell walls may act as ligands to mediate attachment of the organisms to specific receptors on the salivary pellicles covering the surfaces in the oral cavity. Therefore, the specific interaction was clearly displayed by the varying adhering ability of each of the candidal species to the saliva-coated glass surfaces (Fig. 4.10), which is similar to other reported findings (Moura *et al.*, 2006; Pereira-Cenci *et al.*, 2007).

It is important to note that C. parapsilosis showed the highest ability of adherence to salivary pellicle than the others. The mechanism of each candidal species is considerably complex but evidence on the capabilities of the non-Candida albicans Candida (NCAC) species to adhere have been shown C. parapsilosis exhibited a higher degree of intra-species heterogeneity compared with the other species. The intra-species variation in the adherence of C. parapsilosis to different biomaterials has also been reported previously (Panagoda et al., 2001; Trofa et al., 2008). Such characteristics reflect the pathogenic potential of C. parapsilosis isolated from oral candidiasis. C. dubliniensis and C. krusei showed the lowest number of adhered cells with 2.76 x  $10^5$ and 2.13 x 10<sup>5</sup> CFU/mL, respectively. Elguezabal et al. (2004) reported that the adherence of C. dubliniensis to oral surfaces has been shown to decrease despite the presence of saliva. In addition, C. dubliniensis has been found to produce hyphal at lower rate compared to other Candida species (Gilfillan et al., 1998). As the hyphae are related to adherence, this could explain the lowest number of cells being adhered on the pellicle due to the low number of hyphae present. C. krusei cells were found to be less adherent on the salivary pellicle through specific bindings and it is already known that C. krusei is less invasive on the superficial epithelium (Samaranayake et al., 1994). This may contribute to the less virulence attributes of C. krusei than C. albicans.

The extracts of *P. betle* and *B. javanica* have shown anti-adherence effects against the specific adhesion of the *Candida* species to the acquired pellicle. A brief treatment (2-3 min exposure) of the experimental pellicle with the extracts (6 mg/mL)

was able to reduce significantly the adhering capacity of five out of seven *Candida* species tested to the pellicle (P < 0.05). Concentration of 6 mg/mL was applied due to its remarkable reduction against the non-specific adhesion in an earlier study. The anti-adherence activity of the extracts was shown to be species specific. It was shown that the population of *C. dubliniensis* was not significantly reduced on the treated pellicles with the *B. javanica* extract and the reduction of *C. glabrata* was not significant on the *P. betle*-treated pellicles. Therefore, we suggest that the receptors in the experimental pellicle may have been modified by the extracts. The existence of some active compounds in the extracts could have altered the receptors of the pellicle resulting in failure of recognition of by the candidal cells.

The physiochemical forces of the hydrophobic interaction between the candidal cells and the experimental pellicle may be implicated in assisting the adherence processes. In this study, no correlations of CSH in any of the species studied were detected for binding to the pellicle. Although *C. krusei* was found to be most hydrophobic *Candida* species, the result merely did not reflect the number of cells that adhered to the experimental pellicle. In addition, the population of *C. parapsilosis* (CFU/mL) that adhered on the pellicle was significantly higher despite of having lower surface hydrophobicity. This indicates the CSH may slightly facilitate the adherence mechanism but is not a major contributing role in facilitating the adherence. The results obtained can be supported by the findings of a previous study which reported that saliva does not contain unique macromolecules to serve as receptors for the hydrophobic microorganisms to adhere (Gibbons and Etherden, 1983). Some investigators have suggested that CSH is involved in adherence to epithelial cells and is associated with the pathogenic potential (Hazen *et al.*, 1991). In contrast, other studies have shown that CSH has little effect on adherence (Hazen, 1983). Ellepola and Samaranayake (1998)

reported that the antifungal agents such as amphotericin B, nystatin, fluconazole, ketoconazole and 5-flurocytosine have decreased the adherence to buccal epithelial cells, but do not affect the CSH of the yeasts. Further studies will be required to elucidate the role of CSH in the specific binding mechanism.

## 5.4 Ultrastructure of *Candida* Species Following Treatment with the *Piper betle* and *Brucea javanica* Extracts

The antifungal activity of P. betle and B. javanica extracts was further studied for their effect on the morphological appearance of the candidal cells by using a scanning electron microscopy (SEM). When analyzing the morphological characters, it can be concluded that the normal, untreated candidal cells had attained optimum sizes in their well developed-structures. C. albicans, C. dubliniensis, C. glabrata, C. parapsilosis and C. tropicalis cells were rounded and had a smooth surface. Based on the cellular phenotype, the cells may be considered as white colonies (Anderson and Soll, 1987). In contrast, C. krusei and C. lusitaniae had a rough surface. It is much easier to recognise C. krusei since it has different structure with rod-shaped cells and the size is bigger than the other *Candida* species. This could influence the metabolic features of C. krusei which exhibits different behaviour patterns of growth rates and resistance toward host defences as reported by Samaranayake and Samaranayake (1994). The pimple-like and punctates observed on the cell surface of C. krusei and C. lusitaniae indicated that the cells had the possibility of having undergone phenotype switching following exposure of the extracts to ensure its survivability in the stressed environment (Anderson and Soll, 1987; Soll, 1992). The presence of punctates on C. krusei surface and its ability in phenotype switching was also reported by Hafiz et al. (2012).

Following treatment with the extracts, most candidal cells managed to retain their structures and the distribution of buds still can be seen. As the sub-MIC of 6 mg/mL was used in this assay, the extracts probably have suppressed the metabolic activity of candida cells and slowed down the growth due to its fungistatic activity. The fungistatic activity of the extracts towards candidal cells has been shown in the results and discussed earlier whereby the growth profiles of candida cells were all shifted to the right. Therefore, the destructive effect of the extracts towards candida colonies would be minimal and the formation of budding cells might be delayed. However, the cells sizes were relatively smaller and the depositions of extracellular matrix proteins were observed on the surfaces of candida following treatment of the extracts. The extracts may cause environmental stress for candida colonies, which would restrict the ions and nutrients uptake mechanisms that normally take place on the cell surfaces. One explaination of the apparent differences on the cell surfaces is that certain cell wall constituents which are less firmly bound to the rigid glucan-chitin network may be lost during the extracts treatments. These constituents may represent a relatively small proportion of cell wall materials. The active compounds present in the extracts might have binded to the cell surface and penetrate the target sites, possibly the phospholipid bilayer of the cytoplasmic membrane. Several authors have suggested that the loss of a few wall components and damage to the cytoplasmic membrane could lead to the loss of structural integrity and the ability of the membrane to act as a permeability barrier (de Billerbeck et al., 2001). C. glabrata and C. krusei (Fig. 4.13 and 4.14) clearly demonstrated that the cells undergone decomposition of the cell walls whereby some of them were shrinking and apparently have lost cell densities. In addition, the punctates that randomly positioned on the surface of untreated C. krusei and C. lusitaniae have disappeared following treatment of the extracts, thus confirms the direct effect of the extracts on the cell walls. The distortion of the cell physical structure would cause
expansion and destabilization of the membrane fluidity, which in turn would increase passive permeability (Ultee *et al.*, 2002). The extensive loss of cell contents, the exit of critical molecules and ions or the initiation of autolytic processes may lead to cell death. From the SEM examination, it seems that the extracts of *P. betle* and *B. javanica* caused slight changes on the surfaces of candida. This is in agreement with a previous study which reported that many antifungal drugs and plant extracts can affect the morphology and ultrastructure of yeasts (Nakamura *et al.*, 2004).

# 5.5 Differential Expression of *SAP1-10* and *HWP1* Genes Following Treatment with the *Piper betle* and *Brucea javanica* Extracts

Interactions between pathogens and the host are typically mediated by molecules that are either secreted or displayed on the cell wall. Extracellular hydrolytic enzymes such as secreted aspartyl proteinases (Saps) seem to play an important role in the invasion and pathogenesis of oral candidiasis. Every member of *SAP (SAP1-10)* genes is responsible in secretion of Saps, a hydrolytic enzyme that contribute to the virulence properties of *Candida* species (Naglik *et al.*, 2003). The Saps are able to degrade many human proteins found at lesion sites, such as albumin, hemoglobin, keratin, collagen, mucin and secretory immunoglobulin A (sIgA) (Colina *et al.*, 1996; Hube, 1996). Degradation of these molecules may allow the candidal cells to restore their inhabitant in the oral cavity. The cell wall proteins (CWPs) also play important roles in the fitness and virulence of *Candida* species. Among the CWPs, hyphal cell wall protein 1 (Hwp1) is a transglutaminase substrate that functions as adhesins to promote adhesion to epithelial surfaces (Staab *et al.*, 1996, 1999, 2004; Sundstrom *et al.*, 2002).

The expressions of *SAP* and *HWP1* genes have been extensively studied in different experimental conditions, reflecting the growing realisation of how important

their role is in the various stages of oral infection (Koga *et al.*, 2006; Naglik *et al.*, 2006; Nobile *et al.*, 2008; Schaller *et al.*, 1998, 2000; Tavanti *et al.*, 2004). Although the transcriptional networks that orchestrate gene expression are complex, an assessment has been carried out to gain insight into the development of antifungal agents based on the plant crude extracts. Therefore, the efficacy of *P. betle* and *B. javanica* extracts on the temporal expression of the *SAP* and *HWP1* genes were investigated. Of the seven *Candida* species, *C. albicans* is widely recognised as the most pathogenic species and predominantly isolated from oral candidiasis patients. Although the other *Candida* species; *C. dubliniensis, C. parapsilosis* and *C. tropicalis* are known to possess *SAP* genes (Monod *et al.*, 1994), *C. albicans* is by far the best characterised proteinases where at least 10 members of a *SAP* gene family have been sequenced. Therefore, *C. albicans* was considered as a good exemplary to understand the regulation of the genes following treatment with the extracts.

This study demonstrated that the level of *SAP* (*SAP1-10*) and *HWP1* mRNA expression was positively regulated in the normal growth condition. In such condition, the nutrients required for the growth are sufficient and the cells are freely consuming it to accelerate their metabolic pathways. At this point, the regulatory elements of the interest genes in the normal cells are well functioning. The pathway of proteinase synthesis starts with the transcription of *SAP1-10* mRNA in the nucleus, and translated into the preproenzyme on the rough endoplasmic reticulum. Once the N-terminal signal peptide is removed by a signal peptidase, the proenzyme was further processed by a Kex2-like proteinase (Newport and Agabian, 1997). Transcription factors that are present at significant level in cells under basal condition are activated by signalling the cascades to activate transcription of responsive target genes. The expression of the interest genes was thought to reflect the production of proteinases (Sap1-10) and

adhesins (Hwp1) *in vitro*. Naglik *et al.* (1999) reported that the expression of *SAP1-7* genes *in vivo* has provides an evidence of their role in human oral candidiasis. Meanwhile, regulation of *HWP1* mRNA suggests that the transcriptional level may arise from hyphal and pseudohyphal forms in both commensalism and candidiasis (Snide and Sundstrom, 2006).

Majority of the genes are expressed according to the proteins they encode which are responsible in contributing to the pathogenesis and oral infection of *Candida* species. It has been reported that the expression of *SAP1* and *SAP3* were due to phenotypic switching of candidal cells (Morrow *et al.*, 1994; White *et al.*, 1995). In circumstances where the candidal cells can get enough nutrients required for their growth, the switching phases are essential for the cells to attain maturity. In previous study, *SAP1* and *SAP3* were only detected in patients with oral candidiasis, but not in *Candida* carrier (Naglik *et al.*, 1999). In contrast, *SAP2* was frequently expressed in nearly all *C. albicans* strains during log-phase growth, and *SAP3* has been reported to express when *SAP2* is expressed in some strains (Smolenski *et al.*, 1997). Nevertheless, regulation of *SAP1*, *SAP2* and *SAP3* transcripts are expected as the three shared up to 67% identical (Fig. 4.18).

Experiments on *C. albicans* have revealed that the expression of *SAP4-6* transcripts indicated that *C. albicans* has undergone a transition from yeast to hyphae (White *et al.*, 1995). *C. albicans* is a polymorphic pathogen, which can exist in yeast or a hyphal state. The ability of *C. albicans* to transform into hyphae was considered a pathogenic determinant in tissue invasion, where the hyphae may promote the adherence and penetration of *C. albicans* to host tissues. Relationship between the proteinases and hyphae formation strongly indicates the Sap4-6 has equally contributed

to *C. albicans* pathogenicity. The production of Sap4-6 appears to protect *C. albicans* from phagocytic killing by murince macrophages (Borg-von Zepelin *et al.*, 1998), hence leading to immune evasion. While *SAP1-3* were clustered in a group by sequence homology, *SAP4, SAP5* and *SAP6* are up to 89% identical in the sequences of both translated and untranslated regions of their respective mRNAs. Interestingly, the primer designed to detect *SAP4-6* mRNA managed to amplify two RT-PCR products of 156 bp and 181 bp. Although *SAP5* and *SAP6* are reported to be able to amplify the PCR products of 156, 181 and 206 bp (Monod *et al.*, 1994), no band indicates the fragment of 206 bp was observed. It was noted that this proteinase subfamily is unique, and warrants further investigation on the speciality of *SAP4-6* genes. Meanwhile, *SAP7* appears to be divergent amongst the *SAP* family with only 20 to 27% identical. *SAP7* expression was also associated with candidal infection as the transcripts were detected in 60% of oral candidiasis patients (Naglik *et al.*, 1999).

Based on the dendrogram, *SAP8* is slightly closer to *SAP1* to *SAP6* but is a member of the *SAP* family that has had minimal study. Nevertheless, reports suggested that Sap8 does have a role in oral infection isolates (Naglik *et al.*, 2003). It is demonstrated that *SAP8* was highly regulated in deeper sites of infection where the oxygen may be depleted (Schaller *et al.*, 2000). Unlike Sap1 to Sap8, Sap9 and Sap10 both have C-terminal consensus sequences typical for GPI proteins that allow the incorporation of the proteinases into the cell wall via GPI anchor (Albrecht *et al.*, 2006). There is also an evident showing Sap9 and Sap10 are not secreted from the cell, instead are regulatory proteinases that play a role in maintaining cell surface integrity (Naglik *et al.*, 2003).

Apparently, the presence of P. betle and B. javanica extracts has created an inadequate condition for the cells. Most genes probably contain response elements that confer responsiveness to physiologic signals. It is demonstrated that all respective genes were down-regulated when the candidal cells were cultured 24 h in the presence of extracts. The extracts not only disrupt the cell walls and altered the membrane-bound enzymes, but the environmental stresses triggers a direct inhibitory effect on the intrinsic pathways of the cells and distort the responsible transcription factors. Downregulation of the SAP1-10 and HWP1 genes in response to the environmental stress could be a reflection of the suppression to the synthesis of essential enzymes (Saps and Hwp1). One possible reason is that, the regulatory elements were disturbed and therefore the transcription of respective genes was deactivated. General reduction rather than a specific effect on the transcription of specific genes observed in the study may be a result of the slowing growth rate of the candida when treated with the extracts. Therefore, transcription study is necessary to confirm the specific effect of the extracts on the genes. The lack of these enzymes will impair the virulence properties of the candidal cells. In addition, the features and functions of the Saps and Hwp1 proteins which are covalently linked to the skeletal cell polysaccharides (Staab et al., 2004) and contribute to the cell wall integrity (Albrecht et al., 2006) may be interfered in the addition of the extracts. This explains the loss of the structural integrity of the cells and causing alteration to the cell membrane permeable barrier, which in turn inhibits the active transport. The impaired biological system in the cells will delay the growth.

Most importantly, this is the first study that shows the differential expression of the responsible genes associated with the virulence and adherence properties in *C*. *albicans* being down-regulated in the presence of plant extracts. The results may provide evidence of the efficacy of the *P*. *betle* and *B. javanica* extracts in suppressing

the genes. The suppression mechanism induced by the extracts towards these two important genes might reflect a similar effect on the other putative genes and the growth regulatory genes as well. However, further study is required to extend our understanding over the mechanism.

Based on the results gathered, the extracts of *P. betle* and *B. javanica* exhibited antifungal effects against the seven *Candida* species. The *P. betle* leaf extract had different constituents in accordance with the study of Atal *et al.* (1975) and Rimando (1986). Carvacrol, eugenol and chavibetol, were among the bioactive components isolated from the *P. betle* extract (Dorman and Deans, 2000). Mechanism of action of monoterpenes (*e.g.* 1,8-cineole, pinene and limonene), sesquiterpene (*e.g.* caryophyllene and cadinene), phenylpropanes (*e.g.* chavibetol, eugenol, methyl eugenol, chavicol, methyl chavicol) and phenol (*e.g.* carvacrol) (Pauli, 2001) in *P. betle* may be involved in the degradation of certain metabolic pathways. Hydroxychavicol is also one of the major constituents of *P. betle* which has been reported for antibacterial activity (Sharma *et al.*, 2009). Several triterpenoids, two quassinoids (the bitter constituents), and a flavonolignan have been identified in *B. javanica* (Pan *et al.*, 2009). Despite the fact that the mode of action of *B. javanica* compounds on the *Candida* species is relatively unknown, its principles should be similar to the other organic compounds such as terpenes and phenols, which affect the initial interaction with the cell membrane.

Faleiro *et al.* (2003) have shown that the antimicrobial action is determined by more than one compound. The major compound in the plant extracts is responsible not only for the antimicrobial activity, but also the synergistic effect that may take place. These constituents can either affect the physiological functions of candida or cause structural changes of hyphae and spores (Zambonelli *et al.*, 2004). It also appears that

different species of candida react differently to these compounds. The activity is probably due to the lipophilic character of these compounds which was reported to exhibit toxic effects on microorganisms (Uribe, 1985). As a result, the changes in the structure and integrity of the cell membranes are considerably affected to a major extent and thus leading to disruption of the cytoplasmic membrane and coagulation of cell content. However, the microbial strains were reported to be able to tolerate the threat in the environment by modifying their membranes (Levy, 1992). The changes in the functional properties of the surface proteins could have been induced as a result of its responses to the stressed environment. Subsequently, the interactions with the hydrophobic parts of the call were altered. This may explain the reduction of hydrophobicities of the candidal species tested in the addition of extracts. The receptors that played critical roles in assisting the adherence may also be inhibited by constituents of the extracts. As a result, the candidal population adhered on the experimental pellicle was greatly reduced due to the blockade.

#### **CHAPTER SIX**

#### SUMMARY AND CONCLUSIONS

The main conclusions of this dissertation are:

- I. The aqueous extracts of *B. javanica* and *P. betle* possessed significant *in vitro* antifungal properties against seven oral *Candida* species *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis* and *C. tropicalis*, and the effect is concentration dependent. These susceptibilities showed variations of MIC and MFC within the range of 3.13 to 100 mg/mL.
- II. The growth of seven *Candida* species was inhibited by *B. javanica* and *P. betle* extracts. It is suggested that the extracts has created environmental stress and suppressed the cell growth, rendering the candidal cells unable to perform the normal biological activities. The specific-growth rates (μ) and doubling time (g) of all seven *Candida* species were deviated from the normal profile, indicating the extracts demonstrated a fungistatic activity. The population (CFU/mL) of *Candida* species was also reduced thus confirms the suppression of growth activity by *B. javanica* and *P. betle* extracts.
- III. The extracts of *B. javanica* and *P. betle* have significantly reduced the cell surface hydrophobicity of *Candida* species which in turn may have caused adhesion of the candidal cells via hydrophobic interaction to decrease. Treatments of salivary pellicle with *B. javanica* and *P. betle* have interfered with the ability of the candidal cells to form specific binding on the experimental pellicles. As a result, the number of the cells anchored on the salivary receptors had been reduced, suggesting the extracts may have modified the salivary receptors.

- IV. Morphological changes of the candidal cells were also observed by SEM, indicating the extracts of *B. javanica* and *P. betle* have a direct effect on the cell walls.
- V. Differential expressions of *SAP1-10* and *HWP1* genes were noted following treatment of the candida with *B. javanica* and *P. betle* extracts. This suggests that the extracts had succeeded penetrating and disrupting the cell wall to interfere with the metabolic activity of the cells. Such event may have affected the transcription of the genes.

## Suppression and Inhibitory Effects on Candidal Growth

Deviation in the specific growth rates  $(\mu)$ and doubling time (g) suggests that the extracts possessed **fungistatic activity** towards the *Candida* sp. It is observed that the population (CFU/mL) of these candida was **reduced** when the concentration of extracts was increased.

## Reduced Cell Surface Hydrophobicity

The cell surface hydrophobicity of the *Candida* sp. was **significantly reduced** following treatment with the extracts. The extracts mayhave **disturbed cell adhesion via hydrophobic interaction**, thus leading to **reduction of cell adhesion**.

## Anti-adherence on Salivary Pellicle

The extracts could possibly **modify the characteristics of the salivary pellicle**, thus rendering them to be inactive in recognizing the complementary adhesins on the surfaces of the candidal cells. This explains the **reduction of the candidal population** from adhering to

## Morphological Changes

Morphological changes and deposition of extracellular materials on the cells were observed by SEM. The reduction in cell size and damage to cell structure may indicate the direct effect of the extracts on the cell walls.

## Suppression of Virulence-Associated Genes

SAP1-10 and HWP1 genes were down-regulated following treatment with the extracts. The extracts may have disrupted the cell wall, penetrate the cytoplamic membrane and coagulate the intracellular contents. Gene transcription will be affected and lack of these enzymes could impair the virulence attributes of candidal cells.

Several Antifungal Activities of *B. javanica* and *P. betle* Promising Their Potential as Antifungal Agent V

Figure 6.1: Summary of the effect of crude extract of B. javanica and P. betle on oral candida

#### **CHAPTER SEVEN**

### SIGNIFICANCE OF THE STUDY AND FUTURE RECOMMENDATIONS

#### 7.1 Significance of the Study in Clinical Application

Human oral cavity is a dynamic environment for microbiome including *Candida* species. Healthy people might not have problems with the oral candida as the immune system is functioning to maintain the oral health. However, this problem may have an effect on aging people as some of the bodily functions have lost the efficiency and causing oral degeneration. However, denture wearing has been known to be a causal factor of oral infection *e.g.* denture stomatitis, is a common form of oral candidiasis.

First attachment of *Candida* species to the surfaces of mucosa and/or prosthetic devices will lead to the development ofbiofilms. The overgrowth of *Candida* species and its co-aggregation with the microbiome in the oral cavity are rapidly built when the host is under immunocompromised state. Although there is availability of antifungal agents to be used to treat the infection, recent reports on the increasing resistance of *Candida* species to amphotericin B and azoles group have open doors to develop alternatives of the antifungal agents. Plants have highly recommended for their medicinal values.

Microorganisms growing in a biofilm are highly resistant to antimicrobial agents. The colonisation and overgrowth of *Candida* species may initiate an acute disseminated infection in the oral cavity. In the present study, the antifungal effects of *B. javanica* and *P. betle* on the virulence attributes of seven oral *Candida* species including the growth, the adherence mechanisms, alterations of the cell wall surface and

the suppression activity on the genesregulation, all have demonstrated that the extracts possessed constituents that could inhibits or at least diminish the cell colonisation.

In a nut shell, findings have shown that *B. javanica* and *P. betle* are the promising candidates to be developed as alternatives to current drugs/antifungal agents. The present study would be fundamental scientific bases of defining the strategies to inhibit or at least diminish the colonisation of *Candida* cells in the oral cavity. Findings would also have given additional informations to the other researchers to extend the investigation in any field of research and benefiting the society.

#### 7.2 **Recommendations for Future Research**

This study demonstrates that the crude aqueous extracts of *B. javanica* (seeds) and *P. betle* (leaves) possess antifungal effects towards oral *Candida* species. Seven different *Candida* species were susceptible to the extracts, suggesting that these extracts had a potential to be developed as antifungal or anti-candida agents. Further investigation is needed to identify and isolate the active constituents of the crude extracts. The characterisation of the chemical constituents in plants is important as the milestone of this study is to produce oral health products.

An extended analysis of the antifungal activity of the compounds will be carried out, focusing on the intrinsic response pathways of the candidal cells. In addition, as human oral cavity comprises many commensal microorganisms, the interactions between the mixed *Candida* species and *Candida*-bacteria in response to the compounds will also be recommended. This will broaden our understanding on the mechanisms of the antifungal agent in *B. javanica* and *P. betle* specifically on the oral *Candida* species. There are many active constituents derived in plants that can be used as an addative agent to develop the antifungal related products. The products would be more cost effective, less side-effects to human and can be used to treat fungal infection in groups that have high susceptibility to infection such as denture wearers, immunocompromised individuals and babies with undeveloped immune system.