IN VITRO EVALUATION OF ANTIFUNGAL, ANTIOXIDANT AND CYTOTOXICITY ACTIVITY OF *Couroupita guianensis* WITH IDENTIFICATION OF ANTIFUNGAL ACTIVE FRACTION

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by

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% (V/V)	Percentage volume by volume
AST	Antimicrobial Susceptibility Test
ATCC	American Type Culture Collection
BHT	Butylated Hydroxyl Toluene
BuOH	Butanol
BST	Brine Shrimp lethality Test
^{o}C	Degree Celsius
CA	*Candida albicans*
CO2	Carbon dioxide
CFU	Colony Forming Unit
cm	Centimeter
CWP	Cell wall protein
DMSO	Dimethyl sulfoxide
DMEM	Advanced Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DPPH	– 1,1-diphenyl-2-picrylhydrazyl
EtOAc	Ethyl acetate
EUCAST	European Committee for Antimicrobial Susceptibility Testing
ET	Electron transfer
FBS	Foetal Bovine Serum
g	Gram
GPI	glycosyl-phosphatidyl-inositol
HIV	Human Immunodeficiency Virus
H_2O_2	Hydrogen peroxide
IC_{50}	50% Inhibitory Concentration
LC50	50 % Lethal concentration
M	Molar
MeOH	Methanol
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
µg	Microgram
µL	Microliter
milligram/milliliter	mg/ml
µM	Micro molar
MRSA	Methicillin-resistant *Staphylococcus*

	aureus
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCCLS	National Committe for Clinical Laboratory Science
NYS	Nystatin
OD	Optical Density
PBS	Phosphate buffered saline
rpm	Rotation per minute
ROS	Reactive Oxygen Species
S.E	Standard Error
SDA	Sabouraud Dextrose Agar
SEM	Scanning electron microscopy
STD	Sexually transmitted disease
UV	Ultraviolet
WHO	World Health Organization

KAJIAN *IN VITRO* ANTIKULAT, ANTIOXIDANT DAN AKTIVITI SITOTOKSITI DARI *Couroupita guianensis* DENGAN PENGENALAN FRAKSI AKTIF ANTIKULAT

ABSTRAK

Kajian ini telah dijalankan untuk menilai antikulat, antioksidan dan sitotoksiti aktiviti ekstrak bunga dari *C. guianensis* . Ekstrak ini dipilih daripada 125 ekstrak tumbuhan perubatan tempatan dengan kaedah cakera resapan terhadap *Streptococcus aureus* , *E. coli* dan *C. albicans*. kajian ini didapati bahawa *C. guianensis* ekstrak bunga mempunyai spektrum aktiviti antimikrob yang luas. Cara pertumbuhan *C. albicans* dan peranannya dalam jangkitan manusia semakin menarik perhatian kerana rintangannya terhadap agen antikulat yang digunakan secara komersial. Oleh itu, kajian ini telah diteruskan untuk menentukan aktiviti anti-*Candida* oleh ekstrak bunga *C. guianensis.* Ujian aktiviti anti-*Candida* telah dijalankan dengan menggunakan kaedah cakera resapan, kaedah pencairan kaldu dan profil membunuh mengikut masa. Kesan *C. guianensis* ekstrak bunga kepada struktur morfologi dan *C. albicans* telah diperiksa dengan mengimbas mikroskop elektron (SEM). Aktiviti anti-*Candida* menunjukkan bahawa zon min perencatan adalah 18.2 ± 0.3 mm. MIC dan nilai MFC untuk *C. albicans* didapati masing-masing 12.5 mg/mL dan 25 mg/mL masing-masing . Keputusan kajian profil membunuh mengikut masa menunjukkan bahawa perencatan pertumbuhan *C. albicans* bergantung kepada kepekatan *C. guianensis* ekstrak bunga. Pengimejan SEM mendedahkan perubahan secara beransur-ansur dari masa ke masa pada mikrostruktur luar *C. albicans*. A kemusnahan lengkap *C. albicans* sel-sel telah

diperhatikan selepas 36 jam terdedah kepada ekstrak bunga *C. guianensis.* Pengimejan SEM mendedahkan perubahan secara beransur-ansur dari masa ke masa pada mikrostruktur luar *C. albicans*. A kemusnahan lengkap *C. albicans* sel-sel telah diperhatikan selepas 36 jam terdedah kepada ekstrak bunga *C. guianensis*. Ekstrak bunga *C. guianensis* disaringkan untuk mengetahui komponen fitokimia dengan menggunakan kaedah piawai dan menunjukkan kehadiran bahan fitokimia seperti alkaloid, flavonoid, tanin, steroid, glikosida dan saponin. Jumlah kandungan fenolik dalam ekstrak bunga *C. guianensis* telah ditentukan dan didapati sebanyak 32.20 ± 0.22 mg GAE/100 gram ekstrak. Kajian aktiviti antioksidan secara *in-vitro* telah di nilai dengan menggunakan kaedah asai hapus-sisa radikal bebas 2, 2-difenil-1-pikrilhidrazil (DPPH) dan asai pemusnahan sisa radikal bebas hydrogen peroksida. Nilai IC_{50} ekstrak bunga *C. guianensis* ekstrak bunga untuk menghapus radikal bebas DPPH dan hidrogen peroksida masing-masing ialah 93.2 μg/mL ± 0.011 dan 46.48 ± 0.13 μg/mL. Kesan sitotoksik daripada ekstrak bunga *C. guianensis* dinilai menggunakan ujian keracunan anak udang dan asai MTT. Sebatian dalam ekstrak bunga *C. guianensis* mepamerkan hasil bukan toksik dengan nilai LC_{50} = 1210.65 μg/mL dan IC_{50} = 513.22 μg/mL. Kajian ketoksikan pada DNA dari ekstrak bunga *C. guianensis* bunga dinilai oleh gel elektroforesis (comet assay). Hasil kajian menunjukkan bahawa rawatan dengan ekstrak bunga *C. guianensis* tidak menyebabkan kerosakan DNA yang jelas dalam sel vero. Ekstrak bunga *C. guianensis* telah difraksinasi berturut-turut dengan heksan, etil asetat, butanol dan air untuk aktiviti anti-*Candida* dengan menggunakan kaedah cakera resapan. Fraksi etil asetat dianggap sebagai fraksi aktif berdasarkan nlai zon perencatan yang tinggi (18.9 mm). Fraksi aktif etil asetat yang mempamerkan aktiviti antikulat dianalisis dengan menggunakan kaedah kromatografi gas-jisim spektrometri (GC-MS).

Analisis GC-MS menunjukkan kehadiran asid Docosanoic, asid n-heksadekanoat, asid Eikosanoat, benzamid, 2, 3, 4, 5-tetrafluoro-n(-3-methylthio-1, 2, 4-triazole-5-YL), asid 9, 12-Octadekanoat (Z, Z), asid Octadekanoat, asid Pentadekanoat, 14-metil, Squalene dan asid tetradekanoat di dalam fraksi aktif etil asetat yang mungkin bertanggungjawab tehadap aktiviti antimikrob dan antikulat yang diperhatikan dalam kajian ini. Hasil kajian ini mencadangkan bahawa ekstrak bunga *C. guianensis* mempunyai aktiviti anti-*candida* dan antioksidan yang baik. Kajian lanjut diperlukan untuk mengenal pasti bahan fitokimia yang bertanggungjawab untuk aktiviti biologi dipamerkan dalam kajian ini**.**

IN VITRO EVALUATION OF ANTIFUNGAL, ANTIOXIDANT AND CYTOTOXICITY ACTIVITY OF *Couroupita guianensis* WITH THE IDENTIFICATION OF ANTIFUNGAL ACTIVE FRACTION

ABSTRACT

The current study was undertaken to evaluate the antifungal, antioxidant and cytotoxicity activities of *Couroupita guianensis* flower extract. The *Couroupita guianensis* flower extract was selected from 125 local medicinal plant by disc diffusion method against *Streptococcus aureus*, *E. coli* and *C. albicans*. It was found that the *C. guianensis* flower extract possessed a broad spectrum of antimicrobial properties. With respect to the emergence of susceptibility of *C. albicans* to antifungal agent, this study was conducted to determine the anti-*Candida* activity of *C. guianensis* flower extract. Anti-*Candida* test was carried out using disc diffusion assay, broth dilution method, and time killing profile. The effect of *C. guianensis* flower extracts upon the morphological and structure of *C. albicans* were examined by scanning electron microscope (SEM). Anti-*Candida* activity indicated that the mean zone of inhibition was 18.2 ± 0.3 mm. The MIC and MFC value for *C. albicans* found to be 12.5 mg/mL and 25 mg/mL respectively. Time kill experiments indicated an intense concentration-dependent fungicidal effect against *C. albicans*. Imaging of SEM revealed a gradual alteration over time on the outer microstructure of *C. albicans*. A complete destruction of *C. albicans* cells were observed after 36 hours of exposure to flower extracts *C. guianensis*. Qualitative phytochemical screening *C. guianensis* flower extract showed the presence of alkaloids, flavonoids, tannins, steroids, glycosides and saponin. The Total phenolic content of the C.

guianensis flower extract was determined and exhibited moderate value with 32.2 ± 0.22 mg of GAE/100 gram of extract. The free radical scavenging activity of *C. guianensis* flower extract was assessed by 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging assay and hydrogen peroxide radical scavenging activity. The IC_{50} values for *C. guianensis* flower extract for DPPH and hydrogen peroxide free radicals scavenging activities was 93.2 µg/mL ± 0.011 and 46.48 ± 0.13 µg/mL respectively. The cytotoxic effect of the *C. guianensis* flower extract was evaluated using brine shrimp assay and MTT assay. The compounds in *C. guianensis* flower extract exhibited non-toxic results with LC_{50}= 1210.65 µg/mL and IC_{50}= 513.22 µg/mL. Genotoxicity study on *C. guianensis* flower extract was assessed by the single cell gel electrophoresis (comet assay). Genotoxicity study results showed that treatment with *C. guianensis* flower extract did not cause obvious DNA damage in *vero* cells. The flower exract of *C. guianensis* was serially extracted with solvents of varying polarities, with hexane, ethyl acetate, butanol and water and tested for anti-candida activity by disk diffusion method. Ethyl acetate fraction was regarded as active fraction on the basis of highest zone of inhibition (18.99 mm). The active ethyl acetate fraction were analysed with Gas Chromatography-Mass Spectrometry (GC-MS). The GC-MS analysis showed the presence of docosanoic acid, eicosanoic acid, n-hexadecanoic acid, benzamide, 2, 3, 4, 5-tetrafluoro-N(-3-methylthio-1, 2, 4-triazol-5-yl), 9, 12-octodecadienoic acid (Z, Z), octadecadienoic acid, pentadecanoin acid, 14-methyl-, squalene and tetradecanoic acid in the ethyl acetate fraction which may responsible for observed antimicrobial and antifungal activities in this study. The results of this study suggested that *C. guianensis* flower extract possess good antifungal and antioxidant activity. Further study is required to identify the chemical compounds responsible for their biological activity.

CHAPTER 1.0: INTRODUCTION

1.1 Overview

The new-fangled drug resistance to human pathogenic fungus is repetitively being reported from all over the world (Piddock & Wise, 1989). Nonetheless, this situation is a threat in developing as well as developed countries (Pagano *et al*., 2006). Resistance to antifungal drug has taken to undesirable implication for mortality, morbidity and health care in the community. In Malaysia, HIV/AIDS cases have been reported since 1986 by the Ministry of Health (Naning & Kamarulzaman, 2014). Since then, the number of patients with HIV positive has been increasing. In such condition, where patient's immune system is compromised, infections that are opportunistic such as cryptococcosis, penicilliosise and candidiasis are common (Shai *et al*., 2008). Indirectly, fungal infection holds a critical problem to health and major root for mortality worldwide. Despite the increase in the spectrum of antifungal agents, the antifungal regimen has fallen far behind bacterial chemotherapy (Duraipandiyan & Ignacimuthuthe, 2011). For instance, Amphotericin B is a macrocyclic type compound and is used as "gold standard" for being less toxic. It was made available in the early 60's and prescribed until now. Griseofulvin, terbinafine and itraconazole are considered as drug of choice for fungal infections. Unfortunately, these drugs have been withdrawn from market as it has been superseded by new antifungal drugs (Abad *et al*., 2007).

Regardless of dedication to the development of new therapeutic strategies, there are only a limited number of available drugs to fight against fungal infections. Indeed, only four molecular classes that target three distinct fungal

metabolic pathways are currently used in clinical practice to treat essentially fungal infections: fluoropyrimidine analogs, polyenes, azoles, and echinocandins. Several other classes, such as morpholines and allylamines are only used as topical agents due to poor efficiency or several adverse effects when administered systemically. Thus the choice of suitable antifungal agents remains relatively limited due to the emergence of resistant fungal species and infection breakthrough (Pasqualotto & Denning, 2008).

Ignorance of the antifungal drug's drawback added more to the existing complexity. Health care providers have often overlooked this problem; being if one antifungal did not treat the infection, another was usually available. Coupling to this problem, the undesirable side effects of long term treatment including hypersensitivity, allergic reaction and immune-suppression pose a real challenge to their clinical use. This fact is supplemented with slow mycological diagnosis, variable drug bioavailability in immunocomprimised patients and lack of either oral or intravenous preparation. Since then, doctors and nurses are facing with treat infection where antibiotic options are very finite (Ponnusamy *et al*., 2010).

The increase in the prevalence of multiple drug resistance has slow down the development of new synthetic antifungal drugs. A new synthetic antifungal drug may have a limited time in which fungal may develop resistance. This situation has forced scientist to search for novel compound with different mode of action (Monroe & Polk, 2000). Therefore, a search for novel antifungal drugs selectively acting on new targets with fewer or no side effects is extremely necessary. Against this backdrop, researchers are forced to identify and explore a non-chemical, non-classical approach which is plant-based therapeutic agents that are cheaper, safer and effective antifungal drugs (Suleiman *et al*., 2010).

1.2 Plant as antifungal agent

Medicinal plants rich in natural sources have been used to treat mankind for various diseases since antiquity. The utilization of crude extracts of plant parts and phytochemicals for treating diseases is as old as the human species. Popular observations on the use and efficacy of the medicinal plants significantly contribute to the discovery of their therapeutic properties, so that they are frequently prescribed, even if their chemical constituents are not always completely known. Moreover, phytotherapies assures a shorter and cheaper manufacturing as medicinal plant does not demand for a strict quality control concerning safety and effectiveness compared to the synthetic drugs (Maregesi *et al*., 2008).

Medicinal plants appeared to be an excellent system of antimicrobial therapeutic agent compared to western medicine because of their limitless availability and affordability. Plants are capable of synthesizing secondary chemical substances which play a pivotal role in their ecophysiology, counting a safeguarding role against herbivores, pathogen attack and play an attractant role against beneficial organisms such as symbionts (Briskin, 2000). Thus, potential secondary-derived compounds may therefore have beneficial goods in combating microbial infections in animals and humans (Suleima *et al*., 2010).

Generally, plants produce secondary metabolite which exhibits antibacterial, antifungal and insecticidal with minimal environmental impact and not toxic to human cells in contrast to the synthetic antifungal agent. Thus, the health risk by using medicinal plant is minimized (Pathwardhan, 2004). Plant system is less complicated than other bio-sources like animals, so the purification and extraction techniques for the antifungal agent are less complicated. Moreover, plants are

considered as an excellent system because of their ability to regulate human protein expression for palliation of a disease. Besides, identification of phyto-compounds in plants can significantly contribute to the development of plant crops by altering crop genetics with aim to enhance durable resistance mechanisms in crop plants against pathogens (Verma & Dubey, 1999).

Medicinal plant either as pure compounds or extracts, present boundless prospect for new therapeutic agent by reason of the unmatched availability of chemical diversity in plant group (Cos *et al*., 2006). Their distinctive chemical structures differ from those synthetic antimicrobial drugs and hence their modes of action are most likely to vary. This expands the interest in phytochemical constituents of medicinal plant with its pharmacological activity (Roy *et al*., 2009). For these reasons, medicinal plant that is traditionally used by local population in Malaysia has been chosen as source of medicinal plant for antifungal agent development in this study.

1.3 Problem statement

Malaysian medicinal plants have played a significant role in traditional medicine among local people and also have been consuming medicinal plant as the dietary intake since ancient times. Due to emergence of antifungal drug resistance, lack of curative effect, high cost and toxicity, a new prototype antifungal agent is needed to address this situation (Nostro *et al*., 2000). This urged the evaluation of medicinal plants as a source of potential antifungal agent based on their usage as therapeutic agents. It is postulated that a naturally produced agent, *Couroupita guianensis* flower extract could fill this need as this flower was reported to be rich in phytocompounds.

1.4 Objectives

The current study was conducted with the following objectives:

1. To screen for antimicrobial activity of the 125 of local medicinal plants.
2. To evaluate the anticandidal activity of *C. guianensis* flower extract against *C. albicans.*
3. To evaluate the antioxidant activity of *C. guianensis* flower extract.
4. To determine the *in-vitro* cytotoxicity and genotoxicity of *C. guianensis* flower extract.
5. To identify the active anticandidal fraction from *C. guianensis* flower extract.

CHAPTER 2: LITERATURE REVIEW

2.1 Medicinal plants

2.1.1 Introduction

Traditional medicine refers to indigenous or folk medicine incorporating the use of plant and their components to treat diagnose and prevent illness or maintain well-being. Traditional medicine combines health practices, knowledge, beliefs and integrates plant and mineral based medicines to treat, diagnose and prevent illnesses or maintain well being (Lulekal *et al*., 2008). Plants are considered as medicinal if they are collected only for their therapeutic use. Examples of plants derived medicines which serve as new source for drugs and this includes theophylline from Chinese tea (bronchodilators), morphine from poppies (pain inhibitors) and vincristine from periwinkle (antineoplastic) (Licciardi & Underwood, 2011).

Medicinal plants contain components of therapeutic value have continued to play a dominant role in the maintenance of human health for centuries (Nair *et al*., 2005). Plant extract or their active compounds are used in traditional therapies of 80% of the world's population. The diverse range of active compound or bioactive molecules makes them a rich source of different types of medicines. Today, a great number of different medicinal plant products are available on market including pharmaceuticals and cosmetics. Example is the latest discovery of a new cancer-killing compound, taxol which is originate from the Pacific Yew tree (Nahrstedt *et al*., 2000). There has been revival of interest in herbal medicines compared to

modern conventional pharmaceuticals partly due to usage of herbal medicines lower the incidence of adverse reactions and reduced cost with strong biological activity. In addition, the limited ability of synthetic pharmaceutical products to control major disease has increased the awareness among many scientists and encouraged them to screen plants to study the biological activity of their constituents in therapeutic aspects (Sies *et al*., 1996).

2.1.2 Phytochemistry of medicinal plants

The beneficial of medicinal plant result from the secondary metabolites present in the plant. The medicinal actions of a plant are specific to particular species or group as the presence of secondary metabolites in a particular plant are distinct consistent with their taxonomical classification (Wink, 1999). Secondary metabolites have been defined historically as a chemical without any pivotal role contradicting to primary metabolites (carbohydrates, lipids, protein and nucleic acid) which are common to all plants and involved in primary metabolic process of building and maintaining plant cell (Kaufman *et al*., 1999).

Recent research has also revealed that secondary metabolites have protective roles in relation to pathogen attack, inter-plant competition, abiotic stress linked with changes in temperature, water status, light level, UV exposure and mineral nutrients. Potential role of secondary metabolites at cellular level were also reported plant as growth regulators, modulators of gene expression, and in signal transduction (Kaufman *et al*., 1999). Secondary products have a variety of ecological functions in plants such as protects plants from being infected by microbial pathogens and as agents of plant-plant competition and plant-microbe symbioses which are likely to have bearing on potential medicinal effects for humans. For an example, secondary

products involved in plant defence through cytotoxicity towards microbial pathogens could serve as antimicrobial agent in humans, if not too toxic. Similarly, secondary metabolites involved in defence against herbivores through could act as antidepressants, sedatives, muscles relaxants, or anaesthetics in humans (Wink & Schimmer, 1999). In this regard, the secondary plant product may exert their action in human as endogenous metabolites, hormones or signal transduction molecules to combat diseases in human (Kaufman *et al*., 1999).

2.1.3 Major phytochemical groups of antimicrobial activity

Phytochemical constituents are compounds that present in the plant which lead to the pharmacological properties of the plant. These compounds are recognised as secondary metabolites and can be classified based on composition (presence or absence of nitrogen, chemical structure (contain ring or sugar), the pathway which they are synthesized or their solvent solubility (Agrawal & talele, 2011). A basic classification of secondary metabolites includes terpenoids, phenolic and nitrogen containing compound, nitrogen and sulphur containing compound. Chemical analysis showed most plant is presented with flavonoids and phenolic together with their derivatives (Rafael *et al*., 2008). Table 2.1 shows the most common secondary metabolites in plants.

Antimicrobial phytochemicals can be categorized into several groups. Simple phenols and phenolic acids simplest are bioactive phytochemicals consist of a single substituted phenolic ring. Phenolic compounds holds a C_3 side chain at a lower level of oxidation and containing no oxygen often cited as antimicrobialCinnamic and caffeic acids are most common examples of a widespread group of phenylpropane derived compounds considered bacteriostatic against both fungi and bacteria

(Pengelly, 1996). Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are responsible for the browning reaction in cut or injured fruits and vegetables. Quinones are excellent source of stable free radicals and had potential antimicrobial effects (Kazmi *et al*, 1994).

Flavones, flavonoids, and flavonols are derivates phenolic structures containing carbonyl group. They are reported to be synthesized in response to microbial infection by plants. Also said, they have been establish *in-vitro* to be effective antimicrobial compound against a wide range of microorganisms (Tsuchiya, 1996). Tannins are categorized into two groups, hydrolyzable and condensed tannins and are substance capable of tanning leather or precipitating gelatin, also known as astringency properties. Their mode of antimicrobial action is by inactivation of microbial adhesins, enzymes and cell envelope transport proteins (Jones et al., 1994). Alkaloids are heterocyclic nitrogen compounds. The mechanism of action of highly aromatic alkaloids is their ability to intercalate with DNA. Reports mention that they are potentially effective against trypanosomes and plasmodia (Phillipson *et al*., 1987).

Table 2.1: Major groups of secondary metabolites in plant

Class	Example Compounds	Example Sources
Nitrogen containing Alkaloids	Nicotine, cocaine, theobromine	Tabacco plant, cocao
Nitrogen and sulphur containing Glucosinolates	Sinigrin	Cabbage, relatives
Terpenoids		
Monoterpenes	Menthol linalool	Mint and relatives, many plants
Diterpenes	Gossypol	Cotton
Triterpenes, cardiac,glycosides	Digitogenin	Gigitalis (foxglove)
Tetraterpenoids	Carotene	Carrots, sweet potato, mangoes
Terpene polymers	Rubber	Hevea (rubber) trees,dandelion
Sterols	Spinasterol	
Phenolics		
Phenolic acids	Caffeic, chlorogenic	In all plants
Coumarins	Umbelliferone	Carrots, parsnip
Flavonoids	Anthocyanin, catechin	All plants
Tannin	Gallotannin, condensed tannin	Oak, hemlock trees

(Adapted: Cowan, 1997)

2.2 Biodiversity prospect of medicinal plant in Malaysia

2.2.1 Country profile

Malaysia consists of Peninsular Malaysia and east Malaysia, the states of Sabah and Sarawak separated by South China Sea into two similarly sized region with total landmass of 329, 847 sq km. It consists of thirteen states and three federal territories. Malaysia is expected to become 43rd most populous country in the world by 2015 with a population of over 30 million (U.S and world population clocks, 2014). 91.8% of total population are Malaysian citizen, with the Malay counting 63.1% being the predominant ethnic group in Peninsular Malaysia while the Ibans constituted 30.3% of the total citizens in Sarawak while Kadazan/Dusun made up 24.5% in Sabah. Malaysia is multi-racial, multi-cultural and multi-religious country with Malay, Chinese and Indian ethnic groups (John and Brownsey, 1988).

2.2.2 Use of medicinal plant in Malaysia

Malaysia has a unique locality which is diverse in climate, geology and geography. Though Malaysia is relatively a small country, it is characterized by a great variation in wild plants. Malaysia has around 3200 wild plant species, of which 100 species are endemic, belonging to about 700 genera. From these plants, more than 1200 species are categorized as medicinal plant**s** which is broadly scattered all over the country and enormously used in traditional medicine. In addition to this unique botanical heritage, traditional healing is integral to each ethnical group (Ibrahim, 2004).

In Malaysia, traditional health practices involving the use of remedies derived from plants are common and widespread such that 60-80% of the population belief and culture relies solely or partially on such practices to treat a variety of mild

diseases. Apart from their cultural significance, these medicines are generally more accessible and affordable. These medicines plants are used in Malaysia for different biological and pharmaceutical activities including antifungal, antibacterial and insecticidal properties (DeSmet, 1997). A study conducted in Malaysian population analysing the utilization between traditional and modern medicine revealed that 41% of participant using traditional medicines was much higher compared to previous years (Jamia, 2006) An increasing utilization by every year, point towards the awareness of the people on the safety and efficiency of plant medicine utilization containing natural compounds for ailment (Zabidah *et al*., 2005). This reliance on plants as source of medicines warrants scientific validation of their safety, efficiency, quality and the appropriate dosage of the plant material used (Masika & Afolayan, 2002).

2.2.3 Disease transition in Malaysia

Occurrence of disease in Malaysia is a fragment of a complex interaction between humans and their social and physical environment. From the time when independence, the Malaysian healthcare system has provided great and valuable service to Malaysia via a broad network of both public and private facilities. Malaysia as a developing country is experiencing a rapid socio-economic growth in a much shorter time, gaining to an extent the experience of the developed world. Accessible through 349 hospitals (140 government & 209 private), 7700 clinics with doctors (public & 6675 private) and 2009 community clinics run by paramedics (klinink Desa), the Malaysian healthcare has improved over the years with doctors to patient ratio from 1:1437 to 1:758. This transition proposes that as people revolutionise with age, the balance between death and disease also changes. The expectancy in gradual changes of infectious disease to more chronic is predicted.

Since the state of nation's health has economic value, the sickness due to infectious diseases is a calculable economic loss to the community (Ledergerber, 1990).

With the introduction of socio-economic development, the common cause of mortality has shift from poverty and underdevelopment related communicable microbial disease such as malaria, tuberculosis, gastroenteritis to modernisation related diseases such as hepatitis, gonorrhoea and syphilis. These diseases that were relatively unrecognised in the past are increasingly being identified. These incidences of disease are most commonly transmitted sexually. Currently, sexually transmitted disease (STI) is the leading or top infectious killer in Malaysia. Untreated STI may lead to severe physical and psychosocial morbidity and mortality. More importantly, such infections have been shown to enhance the transmission and/ or acquisition of HIV. With the recent increase of STI(s), this may lead to an increase in the number of patient infected with Human immunodeficiency virus/Acquired immunodeficiency syndrome (HIV/AIDS) (Fleming & Wasserheit (1999).

2.3 Opportunistic fungal infection associated with HIV/AIDS in Malaysia

Acquired immunodeficiency syndrome or more generally known as AIDS is a critical public health concern. Scientific resources supports that HIV is the causative agent of AIDS (Alizon *et al*., 2010). HIV is viruses that alter the host immune system, making them more vulnerable to secondary infections and disease. HIV is found in the infected human body fluid including semen, vaginal fluids, and blood and breast milk. The virus is passed either via sexual intercourse, injection drug use, occupational exposure and blood transfusion (Khobotlo, 2009). Symptoms may be developed shortly after being infected, being nausea, vomiting, reduced appetite, weight loss, headache, diarrhoea, constipation, anxiety and depression. Despite the

ready access to antiviral therapy and conventional treatments for secondary or opportunistic infections, The demand for alternative HIV medicine for addressing drug resistance, to prevent or relieve treatment side effect, some of which are not easily treatable with conventional medicine. Adding to complementary therapies assets are immunity booster, stress reliever or to improve general health or wellbeing (Woolridge *et al*., 2005).

Malaysia has become home to one of the fastest growing AIDS epidemics in the East Asia and Pacific region. Malaysia HIV/AIDS cases have been detected since 1986. From 1986 till 2012, 101,672 men, women and children have been confirmed with HIV and 16,360 deaths have been reported. Males make up the majority of HIV cases (90%), but the percentage of women reported with HIV increased greatly in the last decade, from 4% of new cases in 1995 to 12% in 2005 and 18% in 2011 (Masur, 2014).

The causative agent or the virus of AIDS infects the cells of the immune system. HIV can infect multiple cells in the patient, but its main target is the CD4 lymphocytes, also known as T-cell or CD4 cell. CD4 cells are a type of white blood cell (WBC) which is part of infection fighting immune system (Catalfamo *et al*., 2008). Once infected with HIV, the virus instigates the attack and destruction of the CD4 cells of infected person affecting the CD4 count in the body. As the infection progress, the number of CD4 cells decline with the strength of immune system. At advanced stage of HIV infection, people are vulnerable to secondary infections and malignancies that are generally termed as opportunistic infections ().

People with healthy immune system can be exposed to bacteria, fungus or bacteria, would not produce any significant disease. Contrasting to people living with

HIV/AIDS can face serious health threats when there are exposed to opportunistic infections as their immune system have been suppressed and cannot fight disease. These infections are called “opportunistic” as they benefits of only from weakened immune system and resulting in distressing illness (Benson *et al*., 2005).

Fungal infection accounts for a large number of AIDS-index diagnosis and complicate the course of most patients with HIV/AIDS disease compared to other microbial pathogen in Malaysia. Table 2.3 list the most common occurrence of microbes in HIV patient among Malaysian. Fungi are said to be the common and difficult to avoid because they are natural part of environment or more commonly known as normal flora. They are also found outdoors such as in soil, on plants, trees and other vegetation. Besides, they also present on the surface of human skin (Romani, 2008).

Opportunistic fungal infections continue to be associated with high mortality and morbidity. Comparing to most bacterial infections, diagnosis of fungal infection is not an easy task. Fungi are a slow growing and difficult to identify. Unlike for bacterial infection, the serum concentrations of most antifungal agents are not routinely examined. Due to the poor knowledge, the relationship between plasma concentration, susceptibility and clinical effectiveness is still unidentified. Adding to the high prevalence of fungal infection in HIV/AIDS is the range and diversity of fungi that causes the disease to broadened (Pappas, 2006).The emergence of drug resistance towards the existing antifungal agent correspondingly become the cause of death among HIV/AIDS patients (Pappas *et al*., 2009).

The most common fungal infection seen in association with HIV infection is candidiasis and represent a major threat to HIV/AIDS infected people (Morgan,

2005). Candidiasis is a disease caused by genus *Candida*, predominately by *Candida albicans. C. albicans* produce wide array of disease ranging from superficial, mucocutaneous to invasive infection. They can be found in the mouth, skin, scalp, vagina, fingers, nails (superficial), bronchi, lung or gastrointestinal tract (mucocutaneous) and may progress to systemic circulation where they are found in blood, consequences can be deadly (Badiee, 2011).

Infection of dermal candidiasis can involve almost any skin on the body. Rashes from candidiasis can appear either as white or red skin. The common site for candidiasis is areas of skin that are moist o sweaty. Therefore the common sites affected are in fold of skins, groin, and armpits and around the breast area (Pappas *et al*., 2009). Oropharyngeal candidiasis is asymptomatic. Patient's maybe be presented with either soreness or burning sensation in the mouth and throat (Johnson, 2000). Patients with genital, vaginal candidiasis may experience an itchy rash on the affected area, white, discharge, and soreness (Umeha & Umeakanne, 2010). Figure 2.1 shows most common infected area of candidiasis.

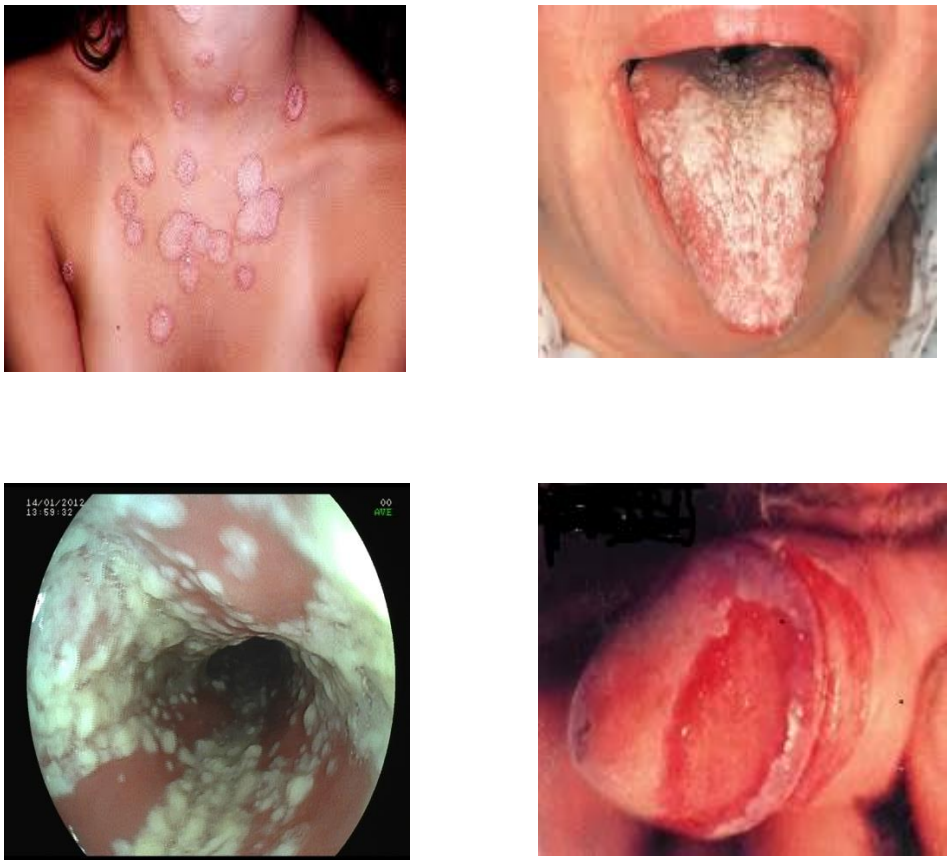


Figure 2.1: Clinical manifestation of candidiasis; upper left: dermal candidiasis, upper right: oral candidiasis, lower left: oropharyngeal candidiasis, lower right: genital candidiasis. (Adapted: Pfaller, 2006)

Table 2.2: Microbial profile of HIV positive in Malaysia

Pathogens	No of cases (%)
Bacterial respiratory pathogens	
Mycobacterium tuberculosis	23
Klebsiella pneumonia	6
***Pseudomonas* spp**	4
Staphylococcus aureus	3
Escherichia coli	2
Fungal pathogens	
Candida albicans	27
Candida tropicalis	5
Candida Krusie	1
Cryptococcus neoformans	2
Intestinal parasites	
Cryotosporidium	20
Isospora beli	3
Giardia lamblia	2
Entamoeba histolytica	1
Lodoamoeba butschii	1

(Adapted: Koh, 2014)

2.4 Genus candida

Genus *Candida* consists of 150-200 species. *C. albicans* is one of the most common species in medical practice (Pfaller & Diekema, 2002). In general, these organisms are isolated from the oral cavity, epidermis, genital and gastrointestinal tract in healthy humans as microflora. The immune system keep a balance from other microorganism from enviroment or the microflora itself from becoming pathogenic in healthy people. However, a weak immune system as in HIV/AIDS makes it easier for microorganism such as *C. albicans* to grow and cause infection. A serious outbreak occur when CD4 count are decline up to 100 and below. Normal range of CD4 count in healthy humans is above 500 cells/mm^3. At this point, candidiasis can persist and difficult to be treated (Rautemaa *et al*., 2006). Although there are over 200 species of Candida, only few species have been associated with human disease as listed in table 2.3.

Table 2.3: The medically significant *Candida* species

Pathogenic species
Candida albicans (50-60 %)
Candida parapsilosis (10-20 %)
Candida glabrata (15-20 %)
Candida tropicalis (6-12 %)
Candida rusei (1-3 %)
Candida kkefyr(≤ 5%)
Candida lusitaniae (≤ 5%)

(Adapted: Pfaller & Diekema, 2002)

2.4.1 Polymorphic species

C. albicans is a polymorphic fungus, ranging from unicellular budding yeast to true hyphae with parallel side wall. The cell wall structure ultimately responsible for given morphology. They grow either as yeast with budding ovoid-shaped or pseudohypae with elongated cell with constriction at the septa. *C. albicans* reproduces by budding to form unicellular yeast cell (blastospores). The hypae (mycelial form) are the germ tubes that result from a filamentous growth (Sudbery *et al*., 2004). Pseudohypae is formed when the blastopores elongate without detachment. Under non-optimal condition, *C. albicans* can also form chlamydospores, which are round, refractile spores with thick cell wall as shown in Figure 2.2 (Lo *et al*., 1997).

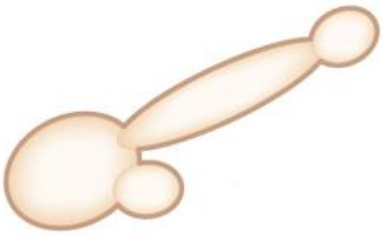
	Yeast cell with blastospores
	Pseudohypae
	True hypae
	Chlamydospores

Figure 2.2: The various cell types of *C. albicans*, each with different roles in growth and disease in the human host (Adapted Neil, 2013).

The ability to switch morphologically represents a response to undesirable changes in the environmental condition and adaptation to different biological niche. A range of environmental cues affect *C. albicans* morphology including response to pH, physiological temperature, CO_2 and starvation. The production of unicellular yeast form is triggered by low temperature, low pH (acidic condition) and high glucose concentration (Whiteway & Bachewich, 2006). At high pH (>7) and temperature, low concentration of glucose (starvation), hypal growth is induced. Yeast cell are have important roles in establishing and disseminating *Candida* infections, whereas hypae are responsible for tissue penetration and invasion (Neil, 2013). Yeast cell have different properties than hypae as the biological attributes, ultrastructure and the composition of the cell wall contrast each other. According to Yang (2003), mutant cells, lacking genes coding for the ability to switch cell type are less virulent. The ability to switch between various morphological forms is an important feature of C. *albicans* to infect and survive in the human host (Kurtz *et al*., 1990).

2.4.2 Cell wall composition and plasma membrane of *C. albicans*

The cell wall of *C. albicans* is composed of 80-90% carbohydrates and 10% proteins. The polysaccharides of the cell wall represented by three basic constituents including mannans, β-glucons and chitins. In addition the cell walls contain minor amount of lipids (1-7%). The structural component of the cell wall represented by β-glucons and chitins (microfibrillar polymers). They serve as a solid skeleton that function as strong physical protection to the cell. Mannan (mannose polymer) represents 40% of the total cell wall polysaccharide and the main component of matrix cell wall (Cassone, 1989). The cell wall composition between the yeast and hyphae vary according to the *C. albicans* growth phase (Klis *et al*., 2001). The hyphal cell holds

more chitin than yeast cell. Immature hyphal cell more contain β-glucons than that found in mature hyphal. Chittin and β-glucons are mainly present in inner cell wall and mannons are present in the outer layer (Sullivan *et al*., 1983).

The selectively permeable plasma membrane is required by *C. albicans* for survival. It contains glycolipids, sphingolipids, sterols and free fatty acid. The lipid bilayer of plasma membrane comprised with ergosterol instead of cholesterol. Both yeast and hyphae contain ergosterol. This sterol provides a useful target in the development of antifungal agent. As for the lipid content of the plasma membrane, the composition can vary according to the changes of duration of biofilm, pH, oxygen pressure, host substrate and presence of antifungal agent (Baek *et al*., 2006).