ANTIMICROBIAL, ANTIOXIDANT AND TOXICITY STUDIES OF *Cassia* SPECIES AND THE POTENTIAL AS ANTICANDIDAL AGENT

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UNIVERSITI SAINS MALAYSIA

2009

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by

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Thesis submitted in fulfillment of the
requirements for the degree of
Master of Science

June 2009

ACKNOWLEDGEMENTS

I believe God provides an umbrella when it rains and sends a rainbow after every storm. I am very blessed to get the opportunity to work with wonderful people who have been my umbrella and rainbow in the times of adversity.

First and foremost, I extend my sincere gratitude to my main supervisor, Assoc. Prof. Dr. Zuraini Zakaria for the encouragement, advice and support all through this research. Next, a reliable person that I could always count on in times of stumbling block, my co-supervisor Dr. Sasidharan Sreenivasan. I wish to express my highest appreciation to him for guiding, helping and sharing his expertise. A note of thanks also goes to Mr. Aman, and Kak Ary from the School of Distance Education, Mr. Muthu, Cik Faizah, Mr. Letcu, Kak Fida, Puan Siti, En. Mutalib, Kak Nurul, En. Sulaiman and Ms. Shanthini from the School of Biological Sciences and finally, Dr. Surash Ramanathan from the School of Pharmacy. I thank you all for the chemicals, facilities, help, support and assistance given to me all along. It has been pleasure knowing and working with all of you.

Next, my highest gratitude goes to my lovely family. I could not have come this far without all of you. Thanks mom, dad, sis and bros for the love and encouragement you all gave me. I cant ask for more. An extra special note of of love and thanks to Guna who have been my strength, support and a helping hand throughout my research. I feel deeply humbled and blessed to have such amazing people around me. Finally, my appreciation goes to Universiti Sains Malaysia for offering me the USM Fellowship during my studies here in completing my MSc studies. The recognition goes to the Dean of School of Distance Education, Associate

Prof. Omar Majid and the Deputy Dean of Graduate Studies and Research of School of Distance Education, Prof. Dr. Hanafi Attan for approving the extension of USM fellowship each semester.

My sincere appreciation to all of you, best wishes and may God bless you all. Thank You!!

SANGETHA NEHRU

School of Distance Education

April 2009

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of varians
Amp B	Ampotericin B
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CFU	Colony forming units
CV	Coefficient Variation
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
FLZ	Fluconazole
GAE	Gallic Acid Equivalents
GC-MS	Gas Chromatography-Mass Spectrometry
GHS-px	Glutathione peroxidase
HIV	Human Immunodeficiency Virus
IC_{50}	Inhibitory Concentration at 50%
LC_{50}	Lethality Concentration at 50%
LDL	Low-Density Lipoprotein
LD_{50}	Lethality Dosage at 50%
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibition Concentration
PL	Phospholipases
Rf	Retention factor
RSA	Radical-Scavenging Activity
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species

ROS	Reactive Oxygen Species
SAP	Secreted Aspartyl Proteolytic
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
SEM	Scanning Electron Microscope
SOD	Superoxide dismutase
TEM	Transmission Electron Microsope
TLC	Thin Layer Chromatography
v/v	Volume per volume
w/w	Weight per weight
XO	Xanthine Oxidase
XOI	Xanthine Oxidase Inhibitory

KAJIAN ANTIMIKROB, ANTIOKSIDAN DAN KETOKSIKAN SPESIES *Cassia* SERTA POTENSI SEBAGAI AGEN ANTIKANDIDA

ABSTRAK

Dalam penyelidikan ini tiga *Cassia* spesies dikaji untuk aktiviti antimikrob, antioksidan dan ujian ketoksikan. Ekstrak metanol daun, bunga, batang dan lengai *Cassia spectabilis*, *Cassia surattensis* dan *Cassia fistula* telah disaring untuk aktiviti antimikrob melalui kaedah resapan cakera terhadap bakteria Gram positif dan Gram negatif, serta kulat. Keputusan menunjukkan bahawa ekstrak daun *C. spectabilis* mempunyai spektrum aktiviti antimikrob yang luas oleh itu ekstrak ini dipilih untuk menentukan Kepekatan Perencatan Minimum (MIC) dan kajian lanjutan secara mendalam untuk aktiviti antikandida. Nilai MIC terhadap bakteria Gram-positif dan Gram-negatif adalah dalam julat 0.195 hingga 50 mg/mL. Bagi aktiviti antikandida, ekstrak daun menunjukkan aktiviti yang baik terhadap *Candida albicans* dengan nilai MIC 6.25 mg/mL. Kesan ekstrak daun terhadap profil pertumbuhan *C. albicans* menunjukkan ekstrak telah mengubah pertumbuhan normal yis tersebut maka mengesahkan aktiviti antikandida terhadap *C. albicans*. Sel *C. albicans* yang dirawat dengan ekstrak daun menunjukkan pengurangan pembentukan biofilem disebabkan oleh kesan tindakan ekstrak. Pengimejan SEM dan TEM untuk menentukan perubahan drastik pada mikrostruktur dalaman dan luaran sel *C. albicans* menunjukkan perubahan abnormal yang ketara. Perubahan morfologi dan pemecahan sel yis secara meyeluruh dapat diperhatikan selepas 36 jam pendedahan terhadap ekstrak daun. Penyaringan fitokimia ekstrak daun *C. spectabilis* melalui kaedah analisis GC-MS menunjukkan kehadiran asid asetik dan digliserol yang

mana telah dilaporkan sebagai agen antimikrob. Aktiviti antioksidan melalui ujian DPPH terhadap ekstrak bunga *C. surattensis* menunjukkan aktiviti yang menyerlah dengan nilai RSA 93.54% dan nilai IC_{50} 423.3 µg/mL. Tambahan pula, ekstrak bunga tersebut mempunyai kandungan fenol yang tinggi, 657.24 mg GAE/g ekstrak jika dibandingkan dengan bahagian tumbuhan lain. *C. surattensis* telah dikaji secara mendalam untuk aktiviti perencatan xanthine oksidase dan didapati ekstrak bunga *C. surattensis* menunjukkan aktiviti perencatan yang tinggi terhadap xanthine oxidase dengan nilai perencatan sebanyak 76.8% dan nilai IC_{50} sebanyak 11.3 µg/mL. Ujian ketoksikan anak udang brin dengan ekstrak daun *C. spectabilis* tidak menunjukkan kesan toksik yang signifikan dengan nilai LC_{50} 2.20 mg/mL. Tambahan pula, kajian ketoksikan akut oral *in vivo* terhadap tikus juga menunjukkan tidak toksik dengan nilai LD_{50} lebih dari 2000 mg/kg berat badan. Tiada sebarang kesan atau simptom toksik pada mencit yang dirawat berbanding dengan tikus kawalan. Pemerhatian histopatologi menyokong keputusan ini, dimana tiada perubahan besar diperhatikan pada ginjal, hati, paru-paru dan limpa. Secara keseluruhan keputusan yang didapati mencadangkan ekstrak daun *C. spectabilis* sebagai pilihan terbaik untuk menghasilkan agen antikandida yang baru dari pada sumber alam. Di samping itu, ekstrak bunga *C. surattensis* kemungkinan besar boleh menyumbang sebagai sumber antioksidan semulajadi pada masa hadapan.

ANTIMICROBIAL, ANTIOXIDANT AND TOXICITY STUDIES OF *Cassia* SPECIES AND THE POTENTIAL AS ANTICANDIDAL AGENT

ABSTRACT

In this study three *Cassia* species were subjected to antimicrobial, antioxidant and toxicity studies. Methanol extracts of the leaf, flower, stem and pod of *Cassia spectabilis*, *Cassia surattensis* and *Cassia fistula* were screened for antimicrobial activity by disc diffusion assay against Gram-positive and Gram-negative bacteria, and fungi. It was found that the leaf extract of *C. spectabilis* possessed a broad-spectrum of antimicrobial properties, hence this extract was evaluated to determine the Minimum Inhibitory Concentration (MIC) and further detailed to anticandidal activity study. The MIC values against the Gram-positive and Gram-negative bacteria ranged from 0.195 to 50 mg/mL for the extract. In the anticandidal activity study, the leaf extract showed a favorable activity against *Candida albicans* with MIC value of 6.25 mg/mL. The effect of the leaf extract on growth profile of *C. albicans* was examined and results revealed that the extract altered the normal growth of the yeast thus confirming the anticandidal effect on *C. albicans*. The treated cells of *C. albicans* by the leaf extract revealed that the biofilm formations have decreased. Imaging using SEM and TEM to determine the major alteration on the microstructure of the outer and inner cells of *C. albicans* showed distinct main abnormalities. Alterations in morphology and complete collapse of yeast cells were observed after 36 hours of exposure to the leaf extract. Phytochemical screening of the leaf extract of *C. spectabilis* by GC-MS analysis, showed presence of acetic acid and diglyserol which were reported as antimicrobial

agents. Antioxidant activity of *C. surattensis* flower extract via DPPH assay revealed a remarkable scavenging activity with RSA value of 93.54% and IC_{50} value of 423.3 µg/mL. In addition, the flower extract also possessed a higher phenolic content 657.24 mg GAE/g extract compared with other parts of the plant. *C. surattensis* extracts were further evaluated for the xanthine oxidase inhibitory assay. Results showed that the flower extract of *C. surattenisis* exhibited a high inhibitory activity of xanthine oxidase with value of 76.8% and IC_{50} value of 11.3 µg/mL. The brine shrimp toxicity assay of *C. spectabilis* leaf extract showed no significant toxicity with LC_{50} value of 2.20 mg/mL. Furthermore, the *in vivo* oral acute toxicity study in mice also revealed that the leaf extract showed no toxicity with LD_{50} value greater than 2000 mg/kg body weight. There were no toxic signs or symptoms on the treated mice compared with control. Histopathological examinations supported this finding where there were no major alterations observed in the kidney, liver, lungs and spleen. In conclusion, the obtained results suggested that the *C. spectabilis* leaf extract as an excellent candidate to develop a new anticandidal agent from nature. Additionally, *C. surattensis* flower may highly provide as a natural source of antioxidant agent in future.

CHAPTER ONE

INTRODUCTION

In the pre-antibiotic era, a major determinant of human morbidity and mortality are infectious diseases. Today multidrug resistant organisms are costly to treat and the treatments which are done most of the times are prone to failure. These pathogens are resistant to multiple antimicrobial classes which cover most or sometimes all the antimicrobials in clinical usage (Deshpande *et al*., 2004; D'Agata, 2004). The conventional view of antibiotic resistant is one where microorganisms exhibit significantly reduced susceptibility to antimicrobials in laboratory tests by mechanisms such as altered drug uptake, altered drug target and drug inactivation. Unfortunately, a different scenario typically prevails in the clinic where treatment fails in spite of antibiotic sensitivity in laboratory tests. In other words, clinical failure is often due not to infections with microorganisms harboring mechanisms resulting in high level antibiotic resistance, but rather to organisms that are phenotypically resistant *in vivo*.

An example of phenotypically resistant *in vivo* is the biofilm growth which almost always leads to a significant decrease in susceptibility to antimicrobial agents compared with cultures grown in suspension. To be precise, when biofilm microorganisms are grown in conventional laboratory suspension culture they become susceptible to antimicrobials (Poole *et al*., 2005). A number of elements in the process of biofilm formation have been studied as targets for novel drug delivery technologies. These include, surface modification of devices to reduce microorganisms attachment and biofilm development as well as incorporation of antimicrobials agent to prevent colonisation (Smith, 2005). One of an example of biofilm associated microorganism is the *C. albicans*. It causes infection in its biofilm

mode of growth and has gain attention with an increasing recognition of resistant to phenotypic adaptation within the biofilm (Jain *et al*., 2007).

During the past several years, there have been increasing incidences of fungal infections by *C. albicans* due to a growth in immunocompromised population such as organ transplant recipients, cancer, and HIV or AIDS patients. This fact coupled with the resistance to antibiotics and with the toxicity during prolonged treatment with several antifungal drugs (Giordani *et al*., 2001; Fostel and Lartey, 2000) have been the reasons for an extended search for newer drugs to treat opportunistic fungal infections. Studies of AIDS all over the world show that 58 to 81% of all patients contract a fungal infection and also at some time during the primordial stage or after developing AIDS. In addition, 10 to 20% have died as a direct consequence of fungal infections (Motsei *et al*., 2003).

In HIV patients, the presence of oral candidiasis is the earliest opportunist infection (Fan-Havard *et al*., 1991). Clinically, the fungal infection is identified as creamy-white, curd-like patches on the tongue or other oral mucosal surfaces which are removed by scrapings. If left untreated, this leads to difficulty in chewing and swallowing and is sometimes associated with severe diarrhoea (Dube and Mutloane, 2001). Hence, those who suffer from oral *Candida* often lose a lot of weight because of a sore throat, which prevents them from eating (Sanne, 2001). *C. albicans* is the most frequent etiological agent associated with this oral infection (Patel and Coogan, 2008). Other related diseases caused by *C. albicans* are genital candidasis (Sobel, 2005), inflammatory lesions in muscular and soft tissues (Ruiz-Cabello *et al*., 1999), and lung infection (Goldenberg and Price, 2008).

In the past the wide range of antimicrobial agents from lower organisms and synthetic drugs sufficed in the treatment or control of infectious diseases. However, the microbial drug resistance and the increase of opportunistic infections especially with AIDS patients and individuals on immunosuppressive chemotherapy currently change the whole scenario. Many antifungals are of limited use due to toxicity, while other infectious diseases have not yet found a cure. These problems pose a need for searching more new substances from other sources especially plants (Cowan, 1999). Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug discovery because of the unmatched availability of chemical diversity (Cos *et al*., 2006). According to the World Health Organization (WHO), about three quarter of the world population depends on traditional remedies for their health care (Gilani and Rahman, 2005).

The international market of herbal for herbal medicine is more than $ 60 billion a year and growing at the rate of 7 % annually. It is believed that the world demand for herbal medicine is likely to rise to $ 3 trillion by the year 2050 from the existing level. However, the past few years have seen a major increase in their use in the developed world. In Germany and France, many herbs and herbal extract are used as drugs prescription and their sales in the countries of European Union were around $ 6 billion in 1991 and may be over $ 20 billion now. In USA herbal drugs are currently sold in health food store with a turnover of about $ 4 billion in 1996 (Rawls, 1996). In India the herbal drug market is about $ 1 billion and the export of plants based crude drug is around $ 80 million. Herbal medicine also find market as nutraceuticals or ‘health food’ where sales have reached about $ 60 billion globally and $ 20 billion in the USA in 2004, experiencing all average annual growth rate of

approximately 4% since 2000 (Saivaino, 2006). All this statistical data are evidence for the bright future of natural product based antimicrobial development program.

Since plants produce a variety of compounds with antimicrobial properties, it is expected that screening programs for some under-represented targets such as antifungal activity, may yield candidate compounds for developing new antimicrobial drugs (Ahmad and Beg, 2001). In addition, it is expected that plant compounds showing target sites other than those currently used by antibiotics, will be active against drug-resistant microbial pathogens such as *C. albicans* (Duarte *et al*., 2005). Yet, the information available on plants, particularly medicinal plants, and active against this yeast species has until recently, not resulted in effective formulations for humans or animal use. According to literature, the investigation of natural products active against *Candida* species increased significantly in the last 10 years, with investigations of approximately 258 plant species from 94 families (Duarte and Figueira, 2005).

Another major hitch to humans is free radicals. Free radicals are molecules produced when our body breaks down food, or by environmental exposures like tobacco smoke and radiation. However, oxygen-centered free radicals and other reactive oxygen species (ROS) which are continuously produced *in vivo*, result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1985). Antioxidant compounds reduce the action of ROS in tissue damage (Lillian *et al.,* 2007). The search of new products with antioxidative properties from natural resources such as medicinal plants, is a very active domain of research (Cengiz *et al.*, 2008). Although humans and other organisms possess antioxidant defence enzymes, such as superoxide dismutase and

catalase, or compounds such as ascorbic acid, tocopherols and glutathione and repair systems to protect them against oxidative damage, these systems are insufficient in totally preventing the damage (Simic, 1988; Mau *et al*., 2002). However, antioxidant supplements or foods containing antioxidants, may help the human body reduce the oxidative damage (Yanga *et al*., 2002).

Malaysia is known as one of the world's 12 mega-biodiversity hotspots in the world, where it's a home to an estimated 9% of the world known species (Saidin, 1993). These statistics provide us unlimited opportunities of our country's natural resources which could be explored and investigated to develop natural product based antiinfectious and antioxidant agent. In Malaysia, various medicinal plants are used to treat diverse diseases. From approximately 250,000 higher plant species that exist on our planet, only 15% have been studied phytochemically and 6% have been screened for diverse biological activities. The genus *Cassia* has been used as a potential medicinal plant since long ago (Chopra *et al*., 1956; Ayo *et al*., 2007). Thus in this study, the extracts of leaf, flower, stem and pod of *C. spectabilis, C. surattensis* and *C. fistula* was evaluated in several distinct studies.

The current study was undertaken with the following objectives:

1) To obtain the optimum extract of *Cassia* leaf, flower, pod and stem for antimicrobial, antioxidant and toxicity testings.
2) To study the *in vitro* and *in vivo* antimicrobial activity of *Cassia* species.
3) To find the phytochemicals in the selected *Cassia* species from the antimicrobial studies.
4) To study the antioxidant activity of the *Cassia* species.
5) To investigate the toxicity of the selected *Cassia* species.

CHAPTER TWO

LITERATURE REVIEW

2.1 Antimicrobial

2.1.1 Antimicrobial agents

Infectious diseases by microorganism actions daily caused about 50 thousand of premature deaths (Carey, 2004; Esterhuizen *et al*., 2006). This signifies that the control of microorganism is crucial in prevention and curing of diseases caused by their action. An antimicrobial agent is a substance that kills or inhibits the growth or prevents damage due to the action of infectious microorganisms. Antimicrobial agents comprise of antibacterial, antifungal, antiprotozoal, antihelminthic and antiviral agents (Baron *et al*., 1994). Antibacterial agents are also closely associated with antibiotics. Antibiotics are substances produced by bacteria and fungi that inhibit the growth and kill other bacteria (Purohit *et al*., 2003). Substances that kill or inhibit the growth of fungi are known as antifungal agent (McDonnell, 2007).

Since the discovery of the antimicrobial activity of penicillin by Alexander Fleming, the field of antimicrobial agent or drug discovery has been largely dominated by whole-cell screening assay. The capability to inhibit the growth of actively multiplying bacteria is preferred in selecting the best antimicrobial compounds, although the mechanism of action of such compounds is not always understandable. However, this approach was successful in the early days of antibiotic development and still holds a good prospective for the screening of large synthetic chemical libraries with novel chemistries or naturally occurring antimicrobials, including peptides (Bax *et al*., 1998; Overbye and Barrett, 2005). Now, research of antimicrobial drug discovery has integrated a complementary

strategy where the potential novel targets, which are important for the survival or growth of bacteria, are well identified. A better understanding of the metabolism and the sequencing of the genes, which involved in the process, makes the identification of novel targets possible (Brazas and Hancock, 2005).

Although the foundation for a whole new era in antimicrobial agent or drug discovery are related to bacterial genomics and its related technologies, there are still no new antimicrobial agents in late clinical development that have originated solely from genomics-based approaches (Coates *et al.*, 2002). A few antimicrobial agents have recently entered into early clinical development by the attempt of several pharmaceutical companies using such strategies in antimicrobial drug discovery. However, this attempt has also resulted without a major return on the investment made (Bush *et al*., 2004). This failure in an area of research with so much prospective, leads one to wonder that there might be other antibacterial targets and modes of action left to be discovered (Coates *et al.*, 2002).

2.1.2 Characteristics of antimicrobial agent's activities *in vitro*

2.1.2.1 Antimicrobial activity is measurable

An antimicrobial activity can be measured by using two growth-based methods; through determination of the inhibitory and the killing capabilities. When measuring the inhibitory activity, the results are referred as Minimum Inhibitory Concentration (MIC) which is a common method used in *in vitro* antimicrobial susceptibility testing. The MIC is defined as the lowest concentration of an antimicrobial agent that inhibits visible growth of microorganism (Andrews, 2001). The results generated from this method are interpreted as semi-quantitative or qualitative. By using the disc diffusion method, susceptibility testing could also be

carried out and the activity is measured relative to the size of the zone of inhibition around the disc embedded with an agent. *In vitro* susceptibility testing is normally performed either in clinical or veterinary laboratories. Interpretations of these *in vitro* results are based upon standards within the United States which recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2003).

At the concentration equal or higher than MIC, some antimicrobials are lethal to bacteria while others have primarily bacteriostatic activity. The term bacteriostatic or bactericidal, describes an agent's antibacterial mode of action. The bactericidal effect of a particular drug can be determined *in vivo*, but it is not always tested due to technical and interpretive difficulties (Lorian, 1996; Lopes and Moreno, 1991). Antimicrobial killing effect can be measured by exposing microbes to a given concentration of drug and the reduction of the population is measured over time. This method is also known as 'killing-curve' where the killing of an *in vitro* response relationship is observed. The bactericidal effect can be also measured in the presence of serum, where it shows the *in vivo* antimicrobial activity due to the impact of the serum components (MacGowan *et al*., 1997).

2.1.2.2 Antimicrobial activity is specific

Antimicrobial agents act on microbes by targeting one or more specific components of the microbe cells that are essential for their physiological and replication functions. Many antimicrobial are categorised based on their mechanisms of action. Microorganisms that are inhibited by antimicrobial agents are target-specific and it dependent upon the drug's mode of action. Nevertheless, effect exerted by antimicrobial drugs can be either a direct event subsequent to the

inhibition of the same cellular targets or cascade of reaction resulting from a particular drug interaction (Yan and Gilbert, 2004).

2.1.3 Evaluation of antimicrobial activity

By observing the growth response of various microorganisms towards natural extracts and pure compounds, the antimicrobial activity could be observed. A number of methods for detecting activity are available, but since these methods are not equally sensitive or not based upon the same principle, the results will be profoundly influenced by the methods. The antibacterial and antifungal test methods are classified into two main groups; agar-diffusion and dilution method.

2.1.3.1 Agar-diffusion method

In the agar-diffusion technique, a reservoir containing the test compound at a known concentration is brought into contact with an inoculated medium and the diameter of the clear zone around the reservoir is measured at the end of the incubation period. The inoculated system is kept at lower temperature for several hours before incubation to enhance compound diffusion over microbial growth. Many types of reservoirs can be used, such as filter paper discs or stainless steel cylinders placed on the surface and holes punched in the medium. The hole-punch method is only suitable for aqueous extracts, because of the interference by particulate matter is much less than with other types of reservoirs. However little sample requirements and the possibility to test up to six extracts per plate against a single microorganism are its specific advantages (Hadacek and Greger, 2000). For testing non-polar samples or samples that do not easily diffuse into agar this hole-punch method is not appropriate. Generally, the relative antimicrobial potency of different samples may not always be compared. This is due to the differences in

physical properties, such as solubility, volatility and diffusion characteristics in agar (Cos *et al*., 2006).

2.1.3.2 Dilution method

In the dilution method, the test compounds are mixed with a suitable medium that has previously been inoculated with the test organism. This method can be carried out in liquid as well as in solid media. The growth of the microorganism can be measured in a number of ways such as the agar dilution method and liquid or broth dilution methods where turbidity and redox-indicators are most frequently used in these last two methods. Turbidity can be estimated visually or obtained more accurately by measuring the optical density at 405 nm. However, test samples that are not fully soluble may interfere with turbidity readings, emphasizing the need for a negative control or sterility control. As an example an extract dissolved in blank medium without microorganisms is referred and compared. As for the liquid dilution method it also allows determination whether a compound or extract has a cidal or static action at a particular concentration (Cos *et al*., 2006).

2.2 Antioxidants

Antioxidants are elements of a collection of processes that hinder *in vivo* free radical oxidation. The term antioxidation includes all of the processes that slow down or stop free radical oxidation. Antioxidation processes include, scavenging radicals to prevent their propagation and enzymatic hydrolysis of ester bonds to remove peroxidized fatty acids from lipids, also facilitates processes such as sequestration of transition metal ions, and enzyme-catalyzed reduction of peroxides.

The scavenging radicals that prevent propagation define how an antioxidant works. As for the other three processes, it does not stop the reactions of radicals. As an alternative, they prevent the accumulation of molecules that can enhance free radical reactions (Thomas, 2000).

Antioxidants minimize radical-caused damages by reducing the energy of the free radical. The reduced energy prevents the free radical from forming, or interrupting the oxidation chain reaction itself. To control the number of free radicals, the body produces enzymatic scavengers called endogenous antioxidants, which include superoxide dismutase (SOD), glutathione peroxidase (GHS-px), and catalase. However, even with maximum production of endogenous antioxidants, the body's defenses can sometimes be overwhelmed. That is when exogenous antioxidants, such as particular vitamins, minerals and herbs may be used to aid the body (Greenly, 2004).

2.2.1 Enzymatic and non-enzymatic antioxidants

Antioxidants that react directly with radicals or other reactive species to prevent cellular compounds from becoming oxidized can be divided into enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants react with reactive species and then efficiently recycled after the process. Examples of important antioxidant enzymes are superoxide dismutases, catalase and glutathione peroxidases (Diplock *et al*., 1998). As for the non-enzymatic antioxidants it can be divided into hydrophilic and hydrophobic antioxidants. Hydrophobic antioxidants include α-tocopherol (vitamin E), carotenoids, and ubiquinol are mostly detected in lipoproteins and membranes. Hydrophilic scavengers include glutathione (GSH), ascorbate and uric acid which are predominantly found in cytosolic, mitochondrial and nuclear aqueous compartments (Chaudiere and Ferrari-Iliou, 1999).

2.2.2 Free radicals

Free radicals are defined as reactive molecules due to the presence of one or more unpaired electron(s). These free radicals are generated in the human body either as an essential mediator in vital processes including neurotransmission and inflammatory reactions, or as a byproduct that does not have a role in the actual process. Oxygen is essential to life but at the same time, it is a dangerous culprit. Oxygen molecules become very unstable and highly reactive when one or more electrons are stripped of from their outer orbits and left with unpaired electrons. In this oxidation process, the stripped oxygen molecules that are referred as free radicals, aggressively attack other stable molecules and snatch away their electrons. After having their electrons stolen, the formerly stable molecules then become free radicals themselves. In the consequent chain reaction, the cascading numbers of free radicals may overwhelm the body's defenses and impose lethal damage. Free radicals have been implicated, in more than 60 diseases including coronary and blood vessel diseases, cancers, cataracts, gastrointestinal diseases, and the aging process itself (Greenly, 2004). Thus the body's exposure to free radicals should be reduced, consequently reducing the risk or likelihood of many health problems.

2.2.2.1 Free radical sources and types

Free radicals are usually produced by the body itself as byproducts of metabolism. Heavy exercise can produce higher quantities of free radicals. Not only because that, many free radicals continually enter the body from exogenous sources such as air pollution. The examples are ozone, nitrous oxide, cigarette smoke through active or passive, drugs, pesticides, contaminated or rancid foods, unsaturated fats and exposure to ionizing radiation example ultraviolet light, X-rays and cosmic. There are four main types of destructive oxygen species. The hydroxyl

radicals (OH^-), are the most highly reactive radicals, followed by superoxide radicals ($O_2\cdot$), oxygen that has gained one unpaired electron. Next is the oxygen singlet ($^1O_2$) which is a non-radical reactive oxygen species and finally hydrogen peroxide (H_2O_2), a non-radical reactive oxygen species (Greenly, 2004).

2.2.2.2 Damaging effects of free radicals

If the body is overwhelmed by excess free radicals, damages can occur to cell nuclei, cellular DNA, and the DNA repair process. This may cause uncontrolled cell growth that leads to malignant lesions and tumors. Excess free radicals also contribute to coronary atherosclerosis where low-density lipoprotein (LDL), the so-called "bad cholesterol", forms cholesteric plaques that accumulate when oxidized by free radicals. Macrophages attempting to eliminate the damaged LDL particles cannot drive out the cholesterol thus swell up to become foam cells. The building up of foam cells thicken the arterial walls consequently narrows the openings of the artery where it restricts the blood flow (Greenly, 2004).

Oxidizing radicals also target cell membranes rich in polyunsaturated fatty acids (Cheesman and Slater, 1993). Specific enzymatic oxidation of polyunsaturated fatty acids leads to the formation of extremely potent and biologically important compounds such as prostaglandins and leukotrienes. In contrast, nonspecific oxidation of polyunsaturated fatty acids can lead to lipid peroxidation via a radical mediated pathway (Al-Omar *et al*., 2004)

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) react with practically all bio molecules, including DNA, RNA, proteins, carbohydrates and lipids, thus damaging the attacked molecule (Diplock *et al.*, 1998). Oxidative damage caused by ROS and RNS will lead to DNA lesions (Spencer *et al*., 2000; Waris and Ahsan, 2006), function loss of enzymes (Sastre *et al*., 2000), increased

cell permeability (Yorimitsu *et al*., 2004; Kim *et al*., 2006), disturbed signaling over the cell and eventually even necrotic cell death or apoptosis (Shen and Liu, 2006). Consequently, damage induced by reactive species is often suggested to play a role in the patho-physiology of various diseases, including diabetes (Mehta *et al*., 2006) and cancer (Valko *et al*., 2006). Lung diseases such as chronic obstructive pulmonary disease (Boots *et al*., 2003), the interstitial lung diseases sarcoidosis (MacNee, 2001; Kanoh *et al*., 2005) and idiopathic pulmonary fibrosis are also the effects caused by free radicals (Rahman *et al*., 1999). Reactive oxygen species generated by mitochondria or from other sites within or outside the cell, cause damage to mitochondrial components and initiate degradative processes. Such toxic reactions contribute significantly to the aging process and form the central dogma of "The Free Radical Theory of Aging" (Enrique and Kelvin, 2000).

2.3 Toxicological study

Toxicology derived from the Greek words of *toxicos* and *logos*. It means science that studies the adverse affects of chemical and biological substances for example heavy metals like cadmium, copper and lead. The study is performed to evaluate the effect of plant extracts on human, animal, or microbe. Toxicology is an inter-disciplinary science that integrates the principles and methods of many fields such as chemistry, biology, pharmacology, molecular biology, physiology and medicine (Sasidharan *et al*., 2008b).

Toxicological study is essential in modern drug development to identify and isolate new compounds from natural resources such as plants. Validation and selection of primary toxicological screening methods are essential to guarantee the

selection of extracts or molecules with relevant pharmacological action and worth following-up. Primary toxicological screening methods are generally designed for rapid screening of large numbers of extracts with a potential biological activity. This method should be simple, precise and easy to be implemented. The results should be obtained quickly and the method use is preferably low in cost.

2.3.1 Toxicity test

Toxicity test procedures are composed of the following components; a biological system, an endpoint which refers to the processes responses or effects assessed and an endpoint measurement that refers to the techniques used to assess endpoints. It is also a data analysis method and a way of expressing the result, a prediction model for converting the test result into a prediction of toxicity *in vivo*, and a means of expressing toxic hazard where it is a quantitative prediction of the adverse effects of a chemical under defined conditions (Balls and Fentem, 1999).

2.3.1.1 Brine shrimp lethality test

The brine shrimp *Artemia salina* L. (Artemiidae) is an invertebrate component of the fauna of saline aquatic and marine ecosystems. It plays an important role in the energy flow of food chain (Sanchez-Fortun *et al*., 1995). *Artemia salina* are used in laboratory bioassays in order to determine toxicity by the estimation of the medium lethal concentration (LC_{50} values) (Lewan *et al*., 1992), which have been reported for a series of toxins and plant extracts (Meyer *et al*., 1982). This method determines the LC_{50} value of the active compounds and extracts in saline medium in µg/mL (Massele and Nshimo, 1995). It has been implemented in research on medicinal plants carried out in different countries in order to evaluate toxicity, gastro-protective action, and other biological actions. In some cases, it has

also been related to pharmacological studies carried out for different chemical compounds (Mathews, 1995) where it acts as a screening method mainly for plant origin products (Parra *et al*., 2001).

2.3.1.2 Oral acute toxicity test

Acute toxicity standard tests are simple and a routine method where it can be conducted easily in laboratory basis. However, this test is strictly run in accordance to simple requirements, observing recommended condition and using indicated organisms. Acute toxicity test is aimed to determine the concentration of plant extracts which produces harmful effects on a group of test organisms such as mice in a short-term exposure under controlled condition. The most common acute toxicity test applied to animals is the acute lethality test (Sasidharan *et al*., 2008b). Lack of movements is normally recognized as criteria of death (Rand and Petrocelli, 1985).

Historically, the first toxicity test performed was the acute toxicity study (Casarett and Doull, 1999). The main objective of the study is to discover a single dose causing major unfavorable effects or life threatening toxicity. This often involves an estimation of the minimum dose causing lethality (Robinson *et al*., 2008). Studies are usually carried out on rodents where it consists of a single dose up to a limit of 2000 mg/kg (OECD, 2001). In development of drug in pharmaceutical line, this is the only type of study where lethality or life-threatening toxicity is an endpoint as documented in current regulatory guidelines (CDER, 1996; ICH-Japan, 1999).

2.4 *Candida*

Candida is ubiquitous and more than 200 species have been studied and described (Armstrong, 1995). Most *Candida* species are part of our microbiological flora and only 10% are responsible for infections towards human (Jarvis, 1995). The *Candida* genus characteristic is white asporogenous yeasts that are capable of forming pseudohyphae. Within this genus, species are characterized primarily based on colonial morphology, carbon utilization and fermentation. The most important and virulent which is frequently isolated species is the *C. albicans* (McCullough *et al*., 1996).

2.4.1 Microbiological characteristics

The colonies of *Candida* species, macroscopically are cream coloured to yellowish. Their texture may be pasty, smooth, glistening or dry, wrinkled and dull depending on the species. In the microscopic features, important species related variations are observed. All species produce blastoconidia which may be round or elongated. Most produces pseudohyphae that are long, branched or curved. In addition, true hyphae and chlamydospores are produced by some *Candida* strains which can be observed in Plate 2.1. The photograph shows the yeast and septate hyphae of *C. albicans* by Gram stain done on vaginal smear to observe the epithelial cell. Although all the members are from the same genus, the various species present a degree of unique behavior. This is with respect to their colony texture, microscopic morphology on cornmeal Tween 80 agar at 25°C (Dalmau method), and fermentation or assimilation profiles in biochemical tests that help to differentiate *Candida* from other yeasts (Freydiere *et al*., 2001).

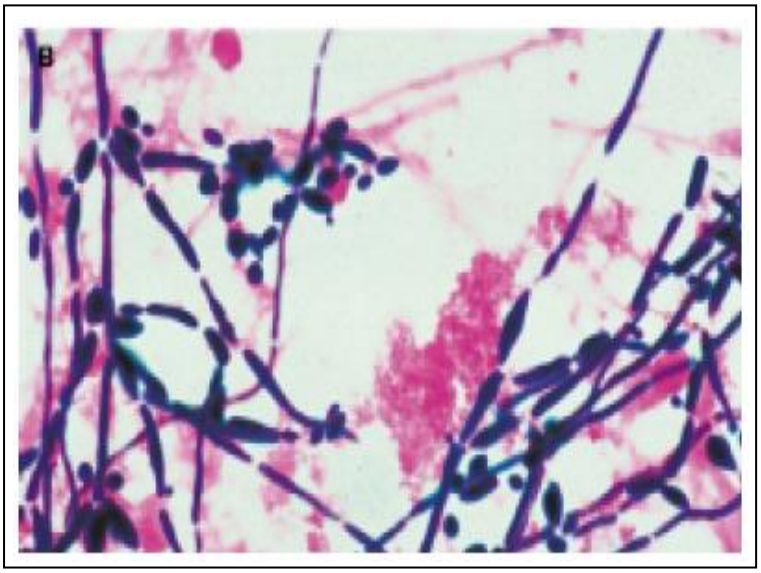

Plate 2.1: Microscopic features of *Candida* species
(Adapted: Figure B, pg 686, Eggimann *et al*., 2003)

2.4.2 Mechanisms of resistance

There are several resistance mechanisms that have been seen in *Candida* species. Resistance often arises from different synergistic combinations of a limited number of molecular mechanisms. These include changes in the cell wall or plasma membrane leading to an impaired uptake of antifungals, efflux pumps that take antifungals outside the cell, overexpression of the antifungal targets and mutation of the antifungal target that decrease their binding ability (Bossche *et al.*, 1998). Other resistant mechanisms are activation of alternate pathways that increases the metabolism of the antifungal, sequestration of the antifungal in organelle-like vacuoles and chromosomal changes to increase the number of copies of the required gene (Sanglard *et al*., 1997). The susceptibility of *Candida* species to antifungal agents is not uniform (Eggimann *et al*., 2003).

2.4.3 Virulence factors

Candida species have the ability to produce virulence factors that enhance their capacity to colonies mucosal or synthetic surfaces (Brassart *et al.*, 1991) and to invade host tissues by disrupting the host cell membranes. Proteinases and species-specific phospolipases account for most secretory proteins acting as virulence factors in host cell and animal models of candidiasis (Ghannoum, 2002). The secreted aspartyl proteinases (SAP) and phospholipases (PL) are two rather large families of *C. albicans* enzymes, some of which have been associated with virulence (Calderone and Fonzi, 2001). The ability of *Candida* species to switch between different phenotypic forms in response to environmental conditions has been studied. Increased secretion of proteolytic enzymes and hyphae formation have been associated with the switching phenomena. *C. albicans* isolates from active infection have been reported to show a higher prevalence of phenotypic switching than those associated with commensalism (Eggiman *et al.*, 2003). Moreover, some characteristics of azole resistance may be related to phenotyping switching (Sanglard and Odds, 2002). Freshly isolated strains from vaginitis or systemically infected patients have higher frequencies of switching (Eggiman *et al*., 2003).

2.4.4 *Candida albicans*

C. albicans is dimorphic yeast that is commonly isolated as a commensal microorganism from the human body. It is a very important and prevalent human fungal pathogen which is superficial as well as potentially life-threatening systemic mycoses. In tissues and in histological samples, the presence of hyphae, pseudohyphae and blastoconidia are known as pathogenic factors of *C. albicans*. Another important pathogenic factor is the production of germinative tubes (Drago *et al*., 2000).

C. albicans is a diploid microorganism (N=8) and genetic evidence suggests a primarily colonel mode of reproduction (Lott and Evat, 2001). However, there has been recent evidence for the manifestation of sexual cycle in *C. albicans* (Hull *et al*., 2000). *C. albicans* is part of the normal microbial flora in human beings and domestic animals. It is associated with the mucous surfaces of the oral cavity, gastrointestinal tract and vagina. However, immune dysfunction can allow *C. albicans* to switch from a commensal to a pathogenic organism that is capable of infecting a variety of tissues and causing a possibly fatal systemic disease when there is immune dysfunction (Traynor and Huffnagle, 2001).

Miller and Johnson (2002) challenge the concept that *C. albicans* mating is an artificial when they discovered a high efficiency mating in *C. albicans* in a laboratory engineered event. The super-mating opaque discovered cells indicates that *C. albicans* has maintained a specialized cell state for the purpose of sexual reproduction. The sexual cycle is an important part of the life cycle for most eukaryotes. One of the advantages of sexual reproduction is that it allows recombination between different genetic backgrounds to facilitate the spread of beneficial mutations into the population (Burt, 2000). Then again, recombination might help to eliminate deleterious mutations efficiently from the population (Zeyl and Bell, 1997). Either way, recombination is important enough to drive many organisms to invest energy in the processes of mating and meiosis. Studies of the population structures of *C. albicans* indicate that, while most genes are inherited clonally, some low-level recombination may be occurring (Anderson *et al*., 2001). This yeast possesses a large spectrum of hydrolytic enzymes with relatively broad substrate specificities including proteases, phospholipases and lipases, which might

be the reason for the outstanding position of this human pathogen (Stehr *et al*., 2003).

2.4.4.1 Formation of biofilm by *Candida albicans*

In natural environment, microorganisms exist predominantly in biofilms which is the surface-attached communities of organisms. Although numerous definitions of a biofilm are introduced, the definition proposed by Donlan and Costerton (2002) is 'mature biofilm is a community of microorganisms irreversibly attached to a surface, containing exopolymeric matrix and exhibiting distinctive phenotypic properties'. *C. albicans* causes infection in its biofilm mode of growth and has taken centre stages with the increasing recognition of its role in human infections due to the development of resistant or phenotic adaptation within the biofilm (Jain *et al*., 2007). *C. albicans* biofilms formed under static conditions where it contains small amounts of exopolymeric material (Hawser *et al*., 1998).

The overall organisation of *C. albicans* biofilm is generally similar to a bacterial biofilm but the details of *C. albicans* biofilm structure are highly dependent upon the conditions under which the biofilm formed (Chandra *et al*., 2001a). This plasticity in structure suggests that biofilms formed in the human host may also vary depending upon the nature of the implanted device and its location (Kumamoto, 2002). The most important feature of biofilm growth is the high resistance to antimicrobial agents exhibited by organisms in a biofilm. The action of an antifungal is limited by their penetration and chemical reaction into biofilm matrix (Vishnu *et al*., 2008). The increasing resistant of *C. albicans* towards these antifungal compounds and the reduced number of available drugs led to the search of new therapeutic alternatives.

2.4.5 Diseases caused by *Candida*

The *C. albicans* yeast is the most common cause of human fungal infections leading to candidiasis (Calderone and Fonzi, 2001; Calderone, 2002). The origin of invasive candidiasis is known as endogenous where from commensal yeasts of the gut or the skin, distributes influenced by a number of factors. Candidiasis is mainly observed in wards of at-risk patients. Nevertheless, colonization and subsequent infection can also be acquired during hospitalisation (Vazquez *et al*., 1998). This is clear by the occurrence of outbreaks, suggesting a common source of contamination, associated with either hand transmission or contaminated materials or infusions (Shin *et al*., 2000; Kuhn *et al*., 2004).

It has been reported by Fidel (2002) that immune protection against candidiasis could be site specific emphasizing the complex nature of the disease. During the development of disease, the microorganism invades tissues by yeast-hyphal transition. Direct infection of yeast cells by mucosal cells has also been observed (Calderone and Fonzi, 2001; Leigh *et al*., 2001). The oral mucosa is a highly specialized stratified epithelia that protects the body from physical and chemical damage, infection, dehydration and heat loss through interactions with the mesenchymal tissues (Nomanbhoy *et al*., 2002; Rouabhia *et al*., 2002; French and Pollitt, 2004). However, the mechanism by which *Candida* an innocuous oral commensal, may occasionally becomes pathogenic and induces oral lesions despite an intact immune surveillance system, remains unclear (Nomanbhoy *et al*., 2002; Rouabhia *et al*., 2002).

Figure 2.1 shows the early events in the pathogenesis of candidiasis on a mucosal surface. The yeast cell of *C. albicans* is either at the budding or germinating

stage. On the mucosal surface the germination of yeast cells and penetration of the mucosa is shown. The persorption of yeast cells also results in the uptake of budding cells into the submucosa. On the far right, phagocytosis of yeast by a mucosal cell is pictured. These actions are facilitated by adhesins such as 'Als1p, Als5p, Hwp1p and Int1p' and 'Saps and Plb1p' enzymes (Calderon and Fonzi, 2001). Persorption and induced phagocytosis have been described by others (Enache *et al.*, 1996).

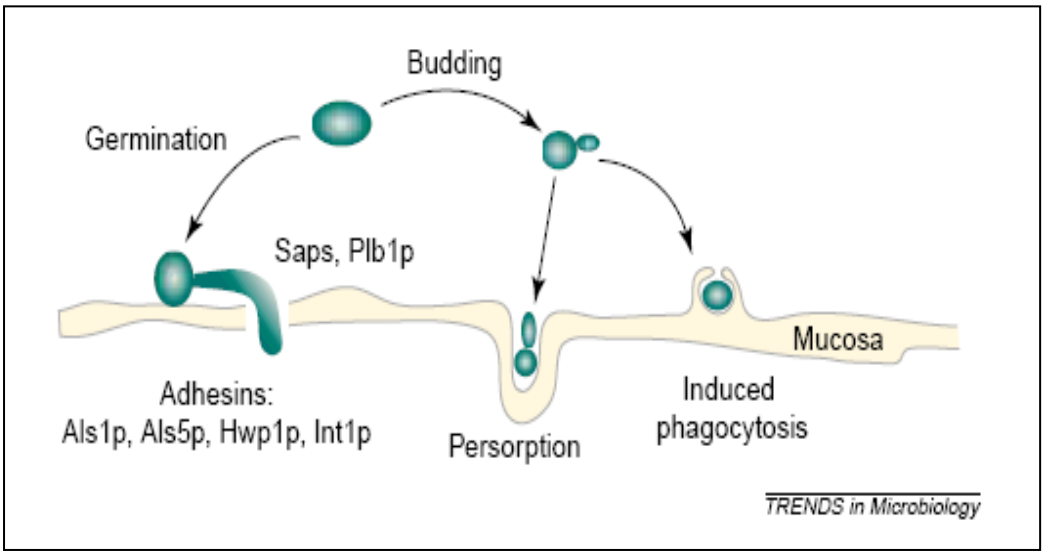

Figure 2.1: Early events in the pathogenesis of candidiasis portrayed on mucosal surface (Adapted: Figure 1, pg 328, Calderon and Fonzi, 2001)

2.4.5.1 Genital candidiasis

In the USA, vaginal infection caused by a *Candida* is the second most common disease, after the bacterial vaginosis infection (Sobel, 2005). During the childbearing years, 75% of women experience at least one episode of vulvovaginal candidiasis, and 40-50% of these women experience a second attack. From this a small subpopulation of women suffers repeated recurrent episodes of *Candida vaginitis. Candida* gains access to the vaginal lumen and secretions predominantly from the adjacent perianal area, and then adheres to vaginal epithelial cells. The

numbers of *C. albicans* that adhere to the vaginal epithelial cells are significantly greater than *Candida tropicalis, Candida krusei* and *Candida glabrata*. As for mens, two forms of balanoposthitis or balanitis are associated with the *Candida* species where both are obtained sexually. A true superficial but invasive infection occurs particularly in uncircumcised males and those with diabetes. It is characterized by intense pruritus, discomfort, erythema and swelling that are localized primarily to the glans, but may extend to involve the penile shaft and scrotum (Sobel, 2005).

2.4.5.2 Inflammatory lesions in muscular and soft tissues

Once *C. albicans* gains access to the bloodstream, it can cause lesions in several organs and tissues, including muscle. There are cases where myocarditis and skeletal muscle infections been reported in patients with disseminated candidiasis (Odds, 1988). Furthermore, muscle infections induced by *C. albicans* have been described in experimental animal models for characterizing immune response to fungal infections (Ruiz-Cabello *et al.*, 1999).

2.4.5.3 Fungal infection in leukemia patient

The incidence of fungal infection in acute leukemia is increasing (Jarvis, 1995). The reasons for this trend are, the increasing intensity of chemotherapy due to new aggressive cytotoxic protocols causing profound and long lasting neutropenia, mucositis (Morrison, 1994). Bone marrow transplantation with multifactorial immunosuppression, prolonged survival in previously rapidly fatal neoplasias, broad spectrum antimicrobial therapy or prophylaxis, prolonged stays in hospitals and extensive use of vascular catheters are the other factors (Jarvis, 1995).

2.4.5.4 Lung infection

In hospitalised patients, *Candida* species are also frequent colonizers of the respiratory tract. True *Candida* pneumonia is extremely rare and only ever occurs in