IN VITRO AND *IN VIVO* STUDIES OF *Cassia surattensis* FLOWER AGAINST *Aspergillus niger*

SUMATHY VELLO

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IN VITRO AND *IN VIVO* STUDIES OF *Cassia surattensis* FLOWER AGAINST *Aspergillus niger*

by

SUMATHY VELLO

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LIST OF ABBREVIATIONS

ABA	Acute Bronchopulmonary Aspergillosis
AM	Alveolar macrophage
Amp B	Amphotericin B
ANOVA	Analysis of Variance
CFU	Colony Forming Unit
CT	Computed tomography
CY	Cyclophosphamide
ELISA	Enzyme Linked Immunosorbent Assay
GI	Gastrointestinal
GM	Galactomannan
GMI	Galactomannan Index
GMS	Gomori's Methenamine Silver
H&E	Hematoxylin and Eosin
HIV	Human Immunodeficiency Virus
i.p	Intraperitoneal
i.v	Intravenous
IA	Invasive Aspergillosis
IC_{50}	Inhibitory concentration at 50%
IPA	Invasive Pulmonary Aspergillosis
LC_{50}	Lethality concentration at 50%
MIC	Minimum Inhibitory Concentration
NA	Nutrient Agar
NB	Nutrient Broth

OD	Optical density
OECD	Organisation for Economic Co-operation and Development
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
SD	Standard deviation
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
SEM	Scanning Electron Microscope
v/v	Volume per volume
w/w	Weight per weight

KAJIAN *IN VITRO* DAN *IN VIVO* BUNGA *Cassia surattensis* TERHADAP *Aspergillus niger*

ABSTRAK

Penyakit berjangkit adalah salah satu masalah kesihatan yang paling lazim di negara membangun dengan peningkatan kerintangan antibiotik. Aspergilosis menjadi punca kematian yang tinggi dan kadar morbiditi di kalangan pesakit berimuniti rendah. Maka, terdapat keperluan segera untuk terapi antikulat baru bagi mengawal kadar kematian populasi. Pemilihan tumbuhan ubatan telah menjadi kecendurungan semasa di kalangan penyelidik sains kerana tumbuhan kaya dengan bahan bioaktif. Bunga *C. surattensis* dikaji untuk mengenalpasti organ ini sebagai suatu agen antikulat yang berpotensi. Saringan mikroorganisma patogen terpilih yang merangkumi Gram positif bakteria, *Bacillus subtilis, Bacillus thuringiensis*, *Micrococcus* sp dan *Staphylococcus aureus*, Gram negatif bakteria, *Escherichia coli, Proteus mirabilis, Salmonella* sp dan kulat, *Candida albicans*, *Aspergillus niger* dan *Rhizopus* sp Gram negatif dan kulat terhadap ekstrak bunga mendapati bunga ini memiliki sifat antimikrob. Zon perencatan untuk mikroorganisma daripada kaedah resapan cakera adalah dalam julat 13 ± 0.04 mm hingga 18 ± 0.00 mm. Mikroorganisma yang lemah terhadap ekstrak bunga seterusnya dinilai melalui Kepekatan Perencatan Minimum (MIC) untuk menentukan aktiviti ekstrak bunga terhadap mikroorganisma dalam cara yang bergantung pada dos. Nilai MIC bagi kedua-dua bakteria Gram positif dan Gram negatif adalah antara 3.125 mg/mL hingga 25.00 mg/mL. Bagi kulat, nilai MIC adalah antara 3.125 mg/mL hingga 6.25 mg/mL. Ini diperhatikan melalui pemeriksaan ultrastruktur *A. niger* yang

menunjukkan hifa yang terlipat, runtuh, terhimpit dan pecah. Konidiofor yang mengecut merupakan perubahan struktur utama yang dikesan pada konidia. Kehadiran agen antikulat dalam ekstrak bunga menyebabkan kerosakan kepada hifa dan konidiofor *A. niger*. Saringan kualitatif fitokimia ekstrak metanol bunga mendapati bahan karbohidrat, tanin, saponin, steroid, fenol dan flavonoid hadir sebagai sebatian aktif. Kajian ketoksikan menggunakan kaedah anak udang air marin dijalankan untuk menguji ketoksikan ekstrak bunga. Keputusan menunjukkan ketoksikan adalah negatif dengan nilai LC_{50} 3.32 mg/mL bagi ekstrak bunga dan LC_{50} 0.27 mg/mL bagi kalium dikromat yang digunakan sebagai kawalan positif. Kajian ketoksikan akut oral dijalankan untuk menyokong keputusan ujian anak udang. Tikus yang menerima dos tunggal 5000 mg/mL ekstrak bunga tidak menunjukkan sebarang perubahan rekabentuk pada paru-paru, jantung, hati, limfa dan buah pinggang dalam pemeriksaan histopatologi dan mencadangkan ekstrak bunga sebagai tidak toksik. Aktiviti antikulat bunga *C. surattensis* terhadap *A. niger* diuji lebih lanjut dengan model sistemik aspergilosis. Tikus dijangkiti dengan konidia dikaji melalui penentuan beban keadaan spora, paras galaktomanan dan kajian histopatologi. Jumlah koloni dalam organ dan Indeks Galaktomanan (GMI) meningkat secara berterusan dalam kawalan negatif sepanjang kajian. Tikus dirawat dengan ekstrak bunga menunjukkan corak penurunan indeks GMI dan beban spora selepas hari ke tujuh walaupun saiz penurunan adalah lebih kecil berbanding tikus kawalan positif yang menerima Amfoterisin B. Keputusan ini disokong oleh kajian histologi yang mana bahagian tikus yang dirawat dengan ekstrak bunga memaparkan pemulihan daripada kecederaan tisu pada semua organ yang diperiksa berbanding kerosakan teruk yang diperhatikan pada kawalan negatif di akhir kajian. Pada hari terakhir jangkitan berkurangan dalam hati serta tiada pada paru-paru dan buah

pinggang dalam kawalan positif. Kesimpulannya, bunga *C. surattensis* mempunyai potensi untuk dimajukan sebagai agent antikulat dalam bidang industri farmaseutikal.

IN VITRO AND _IN VIVO_ STUDIES OF _Cassia surattensis_ FLOWER AGAINST _Aspergillus niger_

ABSTRACT

Infectious disease is one of the commonest health problems in developing countries with the rise of antibiotic resistance. Invasive aspergillosis is causing high mortality and morbidity rate among immunosuppressed patients. Therefore, there is an urgent need for novel antifungal therapy to control the fatality rate in the population. Opting on medicinal plants has become the current trend amongst scientific investigators as plants are rich with biological activities. *Cassia surattensis* flower was studied to identify this organ as a potential antifungal agent. Screening of selected pathogenic microorganisms which were inclusive of Gram positive bacteria, *Bacillus subtilis*, *Bacillus thuringiensis*, *Micrococcus* sp and *Staphylococcus aureus*, Gram negative bacteria, *Escherichia coli*, *Proteus mirabilis* and *Salmonella* sp and fungi *Candida albicans*, *Aspergillus niger* and *Rhizopus* sp against the flower extract revealed this flower to possess antimicrobial properties. The zone of inhibition for microorganisms from disc diffusion assay ranged from 13 ± 0.04 mm to 18 ± 0.00 mm. Microorganisms that were weak against the flower extract were further evaluated with Minimum Inhibitory Concentration (MIC) assay to determine the flower extract activity against the microorganisms in a dose dependent manner. MIC values for both Gram positive and Gram negative bacteria ranged from 3.125 mg/mL to 25.00 mg/mL. For fungi, the MIC value ranged from 3.125 mg/mL to 6.25 mg/mL. The presence of antifungal agent in the flower extract caused damage to the hyphae and conidiophores of *A. niger*. This was observed via ultrastructural

examinations on *A. niger* which demonstrated folded, collapsed, squashed and broken hyphae. Shrunken conidiospores were the major structural alteration detected on the conidia. Qualitative phytochemical screening of the methanolic flower extract found that carbohydrates, tannins, saponins, steroids, phenols and flavonoids were present as active compounds. Toxicity study via brine shrimp assay was performed to ensure the safety of the flower extract. Results turned out to be negative for toxicity with LC_{50} 3.32 mg/mL for flower extract and LC_{50} 0.27 mg/mL for potassium dichromate used as positive control. *In vivo* oral acute toxicity study was performed to support the nontoxic data. Mice administered with single dose of 5000 mg/mL of the flower extract did not show any architectural alterations on the lung, heart, liver, spleen and kidney sections in the histopathology examinations and thus strongly suggested the flower extract as toxic free. Antifungal activity of *C. surattensis* flower against *A. niger* was further studied with systemic aspergillosis model. Mice succumbed with conidia was studied via organ fungal burden determination, galactomannan levels and histopathology analysis. Colony counts in the organs and Galactomannan Index (GMI) increased steadily in the negative control as the study prolonged. Mice treated with flower extract showed a reduction pattern in fungal burden and GMI after Day 7 onwards although the reduction size was smaller compared to the positive control mice which received Amphotericin B. This was supported with histological analysis whereby sections from the flower extract treated mice featured recovery of the injured tissue for all the examined organs compared to severe damage observed in the negative control by end of the study. By Day 28 conidia infection reduced in liver and was cleared from lung and kidney in positive control. As a conclusion, *C. surattensis* flower could be a

promising candidate in the pharmaceutical industry for new antifungal drug discovery using plant as the main ingredient.

CHAPTER ONE

GENERAL INTRODUCTION

Million years back, oceans conquered the earth but evolution has totally formed an unexpected scenario today. Besides *Homo sapiens*, other living and non-living organisms have crammed the world today. History stated the existence of all living creatures on this planet at present primarily diverged from the earliest organism recognized as bacteria. At present, different types of diseases have born together with the growing world. Apparently, diseases can be caused by internal or external factors. Internal factors refer to diseases caused due to autoimmune condition. Autoimmune diseases result from the attack of immune system in human body against its own tissues and cells in the body. There are nearly 70 different autoimmune disorders. Cancer, diabetes mellitus and cardiovascular diseases are some of the classified autoimmune diseases (Lleo *et al*., 2010). Meanwhile, infectious organisms are the dominant external factors leading to infectious diseases. The pathogenic microbial agents include bacteria, fungi, viruses, parasites, protozoa and prions (Roche and Guegan, 2011).

Although the age of disease is as old as human species its implications on man's shelf life are stable. Infectious diseases were treated with the discovery of antibiotic substance the penicillin by Alaxender Fleming in the earlier days, thus keeping the diseases in control and significantly increased the survival rate. Unfortunately, with the parallel usage of antibiotics the microorganism resistance towards antimicrobial agents has emerged tremendously (Seyyednejad and Motamedi, 2010). Variation and diversity of genetic elements in the microorganisms

over the years could best explain the reason for resistance against available commercial drugs over the years.

Usually, outbreaks of infectious diseases such as Severe Acute Respiratory Syndrome (SARS), influenza flu, hand, foot and mouth disease, hepatitis and human immunodeficiency virus (HIV) are the commonly known by public (Zhang *et al*., 2011). These epidemic diseases causes sudden panic in the society as people become aware of the consequences from the outbreak. However, there are very few or limited people who recognize diseases such as aspergillosis, candidiasis, histoplasmosis, cryptococcosis or salmonellosis although these are also classified as infectious diseases (Saag *et al*., 2000; Rex *et al*., 2000). Mycological infections have emerged as an important cause of morbidity and death amongst critically ill patients (Meersseman *et al*., 2004). Fungal infection is a result of fungal invasion into human body at a distinct location and leads to deteriotion of human health. There are different types of fungal infections such as superficial mycoses, cutaneous mycoses, subcutaneous mycoses, systemic mycoses, opportunistic mycoses and non-opportunistic mycoses (Hean *et al*., 2011). Infections caused by opportunistic fungi are classified as new spectrum of fungal pathogens as they were earlier recognized as plant pathogens and now causing disease in human beings (Singh, 2001).

Aspergillus sp is the main agent causing aspergillosis, a type of opportunistic fungal infection (Alexander, 2002). *Aspergillus niger* is identified as one of the primary causative agents to Invasive Aspergillosis (IA) from the genus *Aspergillus* (Taylor *et al*., 2009). Although recognized as notorious pathogenic fungi, *A. niger* is classified as an important class of organism in the biotechnology industry. This

filamentous fungus is used to produce a variety of products and chemicals which represent billions of dollars in annual sales (Kim *et al*., 2007). The escalating incidence of coupled to the staggering overall case fatality ratio in excess of 50% indicating that our therapeutic outcomes are suboptimal (Lin *et al*., 2001). Varieties of antifungal drugs are available but limit to resistance or causing toxicity and side effects. Available treatments fail to complement with IA, thus urging scientists to opt for new sources for drug inventory.

Aspergillosis involves both infection and growth of fungus as well as allergic responses (Hean *et al*., 2011). Aspergillosis develops as a result of inhalation of *Aspergillus* spores. *Aspergillus* sp is an opportunistic pathogen which affects cavities that have formed in lungs from preexisting lung diseases. Ideally, aspergilli form tangled mass of fungus fibers. The fungal mass enlarges gradually and destroys the lung tissue in the process, but usually does not spread to other areas unless under certain circumstances (Bansod and Rai, 2008). IA dominates in patients with neutrophil abnormalities or in immunocompromised patients. For example, patients who have or are receiving intensive treatments for haematological malignancies such as remission-induction chemotherapy and haematopietic stem cell transplantation, with advanced AIDS or chronic granulomatous disease have greater risk for this opportunistic disease (Neofytos *et al*., 2009).

Living in a prebiotic era does not stop us from turning back to traditional prescriptions to treat any types of illness when the role of modern medicines is becoming mild. The use of plants is most accepted compared to those derived from animal source for some religious reasons in some communities. Today,

'Ethnopharmacology' has become an open access platform for bioscientific and clinical research on medicinal and food plants used throughout the world. This multidisciplinary approach is an essential basis for exploitation of the plant resources as tomorrow's medicine. Until today some of the neglected diseases such as diarrhea and tuberculosis are treated with herbal medicines (Heinrich, 2010).

The term phytomedicine was born after the 'marriage' of phytochemical and medicine. Phytomedicines have become the major component of traditional system of healing in almost all developing countries and used as drugs for millennia (Briskin, 2000). Most of current research on phytochemical investigations is devoted to higher plants which are associated with ethnobotanical information. With respect to the current tendency for discovering a new antimicrobial agent from natural resources, the native plants from any region can become a good choice for research and for mass production of natural antibiotics. Surely this is the right time to address the fast-approaching population crunch with the currently available advanced technology.

Malaysia is well known for an astonishing biodiversity amongst other Asian countries. It has a tropical climate with fertile lands that provide a healthy growth of flora. Plants are considered as an important asset to mankind not only for therapeutic reasons but for providing shelter and protection in the earlier days. Natural resources from tropical rainforests have literally become the life-support system to man since ancient times (Gupta *et al*., 2005; Ahsan *et al*., 2009). In the last few decades plants have been the important essence for chemists and biotechnologists. Most of the prescribed drugs around the world are derived directly from plants. Hence, the latter

are called medicinal plants as the naturally developed secondary metabolites are used to design drugs. There is very limited literature on the potential bioactivity of *Cassia surattensis* plant especially on the flowers. Thus, this plant was chosen to evaluate the potential activity of the flowers in warding off infectious diseases caused by pathogenic microorganisms.

1.1 OBJECTIVE

This study was carried out to achieve the following aims:

(a) To evaluate the antimicrobial activity of *Cassia surattensis* flower against pathogenic microoorganism

(b) To study the effects of *C. surattensis* flower extract on the morphology of *Aspergillus niger*

(c) To screen the phytochemical constituents in the methanol extract of *Cassia surattensis* flower extract

(d) To study the toxicity effect of C*assia surattensis* flower extract

(e) To evaluate *in vivo* antifungal activity of *Cassia surattensis* flower extract against *Aspergillus niger*

CHAPTER TWO

LITERATURE REVIEW

2.1 History on use of Plants as Medicine

Recognition on potential health promoting ingredient in plants was traced in the earliest recorded history. The power of plants was explored by the Indians, Chinese, Egyptians, Greeks, Romans and Syrians about 5000 years back from the evidence of ancient records. About 500 plants with medicinal use are mentioned in ancient text and used in indigenous medical system. Up to date, various indigenous systems such as Ayurveda, Siddha and Allopathy use different kinds of plant species to treat many types of diseases (Samy and Gopalakrishnakone, 2007). Over the centuries, plants have become a source of food and medicine for man. The use of herbal remedies has become deeply rooted in the folkoric culture. These beliefs and attitudes have accounted for a brighter modern medical treatment these days. Hipocrates once said 'thy food shall be thy remedy' and today it has become a fact.

2.1.1 Natural resource: Plants as target

Natural resources particularly plants have always persisted as the first target for many purposes especially in drug discovery when the synthetics fail to function effectively. Flora has strong connections with man and it is a gift from God that perishes the globe. The extensive use of herbal remedies and medicinal plants are clearly described in holy texts such as the Bible and Vedas. Thus, various plants are used for therapeutic effect for years in daily living to treat diseases all over the world (Nair *et al*., 2005). Current researches on natural molecules and products primarily focus on plants since they can be sourced more easily and selected on basis of their ethno-medicinal use (Verpoorte *et al*., 2005). Traditional knowledge

provides a better and enhances the probability success in pharmaceutical industry since individual mass screening of plants is vastly expensive, inefficient and time consuming. Vascular plants such as angiosperms, gymnosperms, conifers, ferns and clubmosses are the common identified classes of medicinal plants.

In the current scenario, almost throughout the globe medicinal plants are playing the superior role in health care due to the vast biodiversity and side effects caused by synthetic drugs. Out of the total 422,000 flowering plants reported from the world, more than 50,000 are used for medicinal purposes (Poonam and Singh, 2009). The revival interest in herbal remedies especially using plants is due to the presence of active constituents in this resource. Plant develops secondary compounds consisting of biochemical compounds with active molecular structures which benefit in the findings of lead compounds for synthetic field. Moreover, most of the modern drugs molecular structures were designed using the chemical structures of the active principles as reference.

Plants always receive more attention due to the lower incidence of adverse reactions to plant based products compared to synthetics. This encourages the nations to consider plant as a new source for medicines as an alternative to modern medicines. Thus, the academia researches are diverting their work towards natural resources particularly plants to discover new drugs in order to control the toxicity and mortality caused by current medicines. Edible medicinal plants receive more attention as they are rich with variety of minerals, vitamins and practiced very often in daily living (Ramos *et al.*, 1995). Apart from using medicinal plants in primary

health care system they were also the alternate sources of income for the unprivileged communities (Bussmann *et al*., 2007).

2.1.2 Phytomedicines from plants

Naturally, the use of medicinal plants would have never existed throughout ages if not for the observed pharmacological actions on subjected diseases. While plants are widely practiced as a therapeutic source for years, single compound therapy has dominated in the pharmaceutical industries. The evolving medicines from these compounds are recognized as phytomedicines in health care system (Ekstein and Schachter, 2010). Natural products are promising candidates for drug discovery and will still continue to play an important role in future drug development (Newman and Cragg, 2007). About 25% of drugs in modern pharmacopoeia were derived from plants whilst many others were synthetics mimicked from plants isolated compounds (Rao *et al*., 2004). Triptolide, celastrol, artemisinin and capsaicin are some of the promising phytomedicines in the pharmaceutical industry (Heinrich, 2008). Besides, many others have been marketed and actively used as shown in Table 2.1.

Table 2:1 Plant derived phytomedicines

Plant	Drug	Biomedicine use	Reference
Adhatoda vasica	vasicin	antiplasmodic, cough suppresion	Gurib-Fakim, 2006
Allanblackia monticola	lupeol	arthritis, diabetic	Siddique and Saleem, 2011
Cannabis sativa	nabilone, dranabinol	sclerosis, epilepsy, glaucoma	Amar, 2006
Cathranthus rosesus	vinblastine, vincristine	leukimia, lymphomas	Aslam *et al*., 2009
Condrodendron tomentosum	D-tubocurarine	muscular relaxation	Gurib-Fakim, 2006
Curcuma longa	curcumin	hepatoprotective	Srivastava *et al*., 2011
Gingko biloba	ginkgolides	dementia, cerebral deficiencies	Beek and Montoro, 2009
Harpagophytum procumbens	harpagoside	rheumatism, kidney inflammation	Stewart and Cole, 2005
Mentha piperita	menthol	analgesic, digestive disorder	Galeotti *et al*., 2002
Panax ginseng	ginseng	anemia, hypertension	Wang and Ma, 2006; Choi,2008
Prunus agricana	sitosterol	prostate hyperplasia	Stewart, 2003
Taxus baccata	taxol	ovarian cancer,	Malik *et al*., 2011

2.1.3 Drug discovery: Current scenario

Pharmaceutical industries were amongst the rich sectors making pennies ever since the world moved on with globalization. The credit for the incredible growth in this industry is primarily due to the discovery of blockbluster drugs inventory with advent of modern technology assistance (Patwardhan and Vaidya, 2010). Generally, the process of drug discovery is time consuming and arduous as it begins from the stage of drug design, identifying potential clinical candidates for the drug and finally introducing into the market. Sometimes it spans the course of more than a decade and the expenses could exceed to 800 million USD in United States of America (Schmid and Smith, 2006). Approval from the Food and Drug Administrations (FDA) remains as regulatory before a modern drug receives the commercial licensing. However, at present the trend suggests a decline in the market for the new drugs and it is no longer the economic growth asset. As stated by Hughes (2008) the number of new drugs approval declines to 17 in the 21st century compared to 53 in the year 1996. Hence, the industry is facing tremendous crisis that can progressively worsen if the current situation is not well managed.

Over this background, the pharmaceutical industry is giving attention to experiential wisdom and holistic approach to offer new potential drugs that are safer and more effective. Natural products are believed to perform various functions and most possess interesting and useful biological activities (Philip *et al*., 2009). Thus, ethnopharmacology, traditional and complementary medicines were focused as new strategic options (Patwardhan and Mashalkar, 2009). In fact, over 50% of all the modern clinical drugs are derived from natural product origin that plays role in drug development programs in pharmaceutical industry (Nair *et al*., 2005). Since

traditional medicines are notified for the affordable health remedies for therapeutic reasons, thus the current interests on medicinal plants are growing worldwide.

2.2 *Cassia* spp

Cassia is a family of Caesalpiniaceae or Leguminosae in the subfamily Caesalpinioideae comprising about 600 species (Panda *et al*., 2010) among which are from delicate, annual herbs to highly attractive tall flowering trees present mostly in the tropics and subtropics (Tripathi and Goswami, 2011). *Cassia* spp tolerates a wide range of climates and temperatures, though it prefers warm condition. Within its bounds *Cassia* spp may be seen with great diversity in habit, ranging from tall trees to delicate, prostrate and annual herbs (Irwin and Turner, 1960). *Cassia* spp are also used as favourite food plants by the caterpillars.

2.2.1 *Cassia surattensis*

Cassia surattensis is an impressive small flowering tree and commonly planted as a street and landscape tree whereby it makes a nice dooryard or entrance gate welcoming plant. *C. surattensis* is native in Australia and to most of Asian countries like India, Thailand, Vietnam, Indonesia, Malaysia, Laos, Ceylon, Polynesia and the Philippine islands (El-Sawi and Sleem, 2010). In Malaysia this plant is easily seen along the roadside and being recognized as 'bushy cassia' (Chew *et al*., 2009). Locally, this flowering plant is known as 'Kembang Kuning' or 'Kassia Gelenggang'. The beautiful bright yellow flower blooms in cycles year-round. In Thailand this plant is known as Kalamona Tree or 'magic' tree because the Thai's believe that the plant offers security and fulfill wishes, goals and hopes.

2.2.2 Taxanomy of *Cassia surattensis*

Scientific classification of *Cassia surattensis* is as follows:

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Fabales

Famili : Caesalpiniaceae alt Leguminoseae

Subfamily : Caesalpinioideae

Tribe : Cassieae

Subtribe : Cassinae

Genus : Cassia

Species : *C. surattensis*

Synonmous name: *Cassia suffruticosa, Cassia glauca., Senna surattensis, Cassia fastgiata*

Local Common names: Kembang Kuning, Busshy Cassia, Scrambled egg bush**,** Glaucous Cassia, Golden Senna

Other Common names: Singapore Shower, Sunshine Tree, Sulphur-flowered senna (Anonmyous, 2011)

2.2.2.1 Plant morphology and characteristics

External features are normally important to identify and differentiate plant species from its genus. *Cassia surattensis* (Plate 2.4) is one of the few *Cassias* likely to grow in a shrub form up to 7 m tall with many branches. The dark green leaves are bipinnate, each bearing 14-18 oblong leaflets measuring 25 mm long and 12 mm broad (Chew *et al*., 2009). Its clusters of yellow blooms are present with 10 stamens 1-1 ½ inches wide in semi-erect panicles. *C. surattensis* bears fruits called pods which are normally flat, papery and measures to about 7 inches long. This plant is drought tolerant once established and is a rapidly easy growing shrub.

Plate 2.1 *Cassia surattensis*

2.2.2.2 Traditional uses

In Chinese tradition, the leaves are boiled and the infusion is consumed as a medicine to treat constipation, cough and sore throat (Chew *et al*., 2009). *C. surattensis* leaves are practiced in both internal and external cooling medicine in Balinese community (Sangetha *et al*., 2008). In folk medicine, *C. surattensis* bark and leaves are used for treatment of fungal skin diseases, diabetes and gonorrhea (Gritsanapan and Nualkaew, 2001; Petchi *et al*., 2012). Roots of this plant are blended, filtered and consumed orally as juice to heal snake bites in Purandhar region of Maharashtra, India.

2.2.2.3 Pharmacological activities

Plants are classified as medicinal plants when they are detected with biological activities. *Cassia surattensis* is generally recognized as a medicinal, ornamental and economically important plant (Vajpayee *et al*., 2000) as other *Cassias*. Leaves of this plant were also found to be antihyperlipidemic when tested against hyperlipidemic rats (El-Sawi and Sleem, 2009). The bright golden yellow flowers are also found to be a good antioxidant (Chew *et al*., 2011).

Various pharmacological properties such as antioxidant, anti-inflammatory, antidepressant, muscle relaxant and many others are possible with the presence of different types of phosphodiesterase inhibitors (PDEIs). PDEIs are seen as potential agents for therapeutic reasons which will be helpful for diseases like asthma, depression, schizophrenia and congestive heart failure. Studies found *C. surattensis* as one of the potential herbal phosphodiesterase inhibitors (Rahimi *et al*., 2010).

2.2.2.4 Phytochemical studies

Phytochemicals are chemical compounds developed naturally in plants allowing the latter to possess medicinal properties. Up to date, there is limited literature on the phytochemistry of *C. surattensis* plant.

Flavonoids a large group of polyphenolic compounds was detected in the leaves of *C. surattensis* plant (Rahimi *et al*., 2010). Phytochemical analysis on the flavonoids revealed the presence of quercetin, rutin and quercetin-3-O-glucoside 7-O-rahmnoside. These compounds are reported to inhibit xanthine oxidase, protein kinase C and phosphodiestearases. Quercetin help to inhibit lipid peroxidation by blocking xanthine oxidase enzyme, chelating iron, scavenging hydroxyl, peroxy and superoxide radicals which reveals its antioxidant property. This compound helps to inhibit structural damage to protein (Salvi *et al*., 2001) and protects the oxidative products generated by the respiratory burst in phagocytes (Zielinska *et al*., 2000).

Another study revealed the presence of unsaturated fatty acids, sterols, phytols and squalene in *C. surattensis* leaves which are responsible for improvement on hyperlipidemic. Phytol, the common terpenoid esterified to chlorophyll to confer lipid solubility is valuable in controlling lipid abnormalities in diseases like obesity and diabetes (El-sawi and Sleem, 2009; Schluter *et al*., 2002). Squalene, an isoprenoid intermediate of cholesterol biosynthesis obtained from diet was found to reduce serum triglyceride and cholesterol levels in humans (Simonen et al., 2007). Anthraquinone also a compound that was detected in the leaves is generally used as laxatives mainly from their glycosidic derivatives (Gritsanapan and Nualkaew, 2001).

2.3 Antimicrobial nature

Microorganisms are indispensable components in our ecosystem. Their contribution on this globe is greater than human. These organisms benefit the society in many aspects such as in food industries, health care and agriculture. The role of microorganisms in the environment becomes transparent as they are always pointed for the reasons of many types of diseases. Even though they are always recognized as the smallest living organisms, their capabilities are unchallengeable to those of humans. Infections by these nano creatures can lead to severe disease conditions especially in immunosuppresed patients. In addition, infectious diseases caused by microorganisms remain as a major threat to public health particularly in developing countries due to lack of medical facilities plus emergence of drug resistance (Okeke *et al*., 2005).

At the current state, the commercially available antibiotics are losing their potentials and capabilities to control the colonization of microbes in human host. Efforts to identify and develop new antibiotics are actively practiced by researchers in every part of the world in order to reduce the incidence of opportunistic diseases. Developing a novel drug involves many procedures and regulations. Initially, the potential drug source is targeted and antimicrobial assays are performed to screen the activity against microorganisms. The drug is than tested on animal models followed by clinical trials before it is being commercialized.

2.3.1 Antibacterial and antifungal assays

The validation of antimicrobial activity exhibited by crude extracts from promising antimicrobial source can be evaluated based on the growth response from the tested microorganism. In the test, microorganism has direct contact with the sample and percentage inhibition reflects the potency of the sample as an antibiotic. There are many assays that can be used in an antimicrobial study but the common classical methods are still being applied up to date as they are sensitive to the tested materials. The common *in vitro* antibacterial and antifungal test methods implemented in almost all antimicrobial studies are the diffusion and dilution methods.

2.3.1.1 Agar diffusion method

This test involves the use of solid medium; agar and filter paper disc. The streaked inoculum becomes contact with the sample and forms a clear zone called zone of inhibition (inhibition diameter) as a result of exhibiting antimicrobial activity. A larger zone indicates a greater chance of the sample as an antibiotic agent. In this technique, the specific advantages are that only a small sample is required and can be tested up to six extracts per plate against a single culture (Hadacek and Greger, 2000). In agar diffusion test, the sample basically diffuses into the agar and inhibits the growth of the tested microorganism when it penetrates to obtain food from the medium for survival. Thus, this method is not suitable for testing non-polar samples as the latter cannot diffuse into the agar. Sometimes, the inoculated plates are kept at a lower temperature for several hours before incubation to favour the compound diffusion over microbial growth, thereby increasing the diameter of clear zone (Cos *et al*., 2006).

2.3.1.2 Broth dilution method

Broth dilution uses liquid medium to measure the growth of microorganisms inoculated into dilution series of antimicrobial agents. It is a commonly used method to determine the minimum inhibitory concentration (MIC) of antimicrobial agents that will kill (bactericidal or fungicidal) or inhibit the growth (bacteriostatic or fungistatic) of bacteria or fungi. The presence of turbidity or sediment indicates growth of the microorganism (Wiegand *et al*., 2008). MIC value is determined as the lowest concentration of the antimicrobial agent that prevents visible growth of a microorganism under defined conditions (Gabrielson *et al*., 2002). MIC determination can be used for monitoring the development of antibiotic drug resistance (Kahlmeter *et al*., 2003). There are two variations of broth dilution method which are macrodilution and microdilution. If the final volume of the test is 2 mL or more it is termed macrodilution. Microdilution is performed in microtiter plates using volume of ≤ 500 μL per well (Wiegand *et al*., 2008).

2.3.1.3 Inhibition radial growth assay

Filamentous fungi do not grow as single cells, thus a better challenging standardization method is required than unicellular organism like bacteria and yeast. Besides agar disc diffusion and broth dilution methods for determining antifungal activity, the inhibition radial growth assay can be another useful tool to measure the sensitivity of filamentous fungi to antifungal compounds (Slawecki *et al*., 2002). The assay uses agar medium where the growth on the media is superficial. Mycelial plugs excised from the edges of actively growing colonies are inoculated on the Petri plates with antifungal compound (Engelmeir and Hadacek, 2006). Growth on the solid media has one advantage over the liquid medium. In liquid medium, the

submerged hyphal growth takes place under low oxygen concentrations but fungi growing on agar surfaces are less subjected to this constrain (Hadacek and Greger, 2000). The inhibition effect is determined by measuring the colony diameters in Petri plates.

Radial growth assay was found to be economical, easy to perform and require minimal laboratory equipment. Thus, this technique can be used for a large scale sample screening for antifungal agents. Due to its ease of operation, it can provide fast and effective screening method for the discovery of new types of antifungal agents in future (Liu *et al*., 2002). However, the drawback of this technique is addition of antifungal compounds in the agar medium whereby if the media is hot the compounds can be denatured resulting in poor outcome.

2.3.2 Specific conditions for antibacterial and antifungal screenings

2.3.2.1 Panel for test organisms

To ensure a good outcome in antimicrobial activity screening with new sample, variety of strains should be subjected to this test. The choice of test organisms should consist of Gram positive bacteria, Gram negative bacteria and fungi. Cultures like *Candida albicans*, *Aspergillus niger*, *Rhizopus stolonifer* and *Fusarium solani* are common fungal strains used in antimicrobial study.

2.3.2.2 Growth medium

Appropriate media is important for microorganism growth in antimicrobial study. Mueller-Hinton Agar (MHA) or Mueller-Hinton Broth (MHB) and Nutrient

Agar (NA) or Nutrient Broth (NB) are the common growth media for bacteria, while Sabouraud Dextrose Agar (SDA) or Sabouraud Dextrose Broth (SDB) and Potato Dextrose Agar (PDA) or Potato Dextrose Broth (PDB) are for fungi. The enrichment component in each of this medium differs according to the preference of microbes. The slight differences in the composition of the growth medium can influence the antimicrobial activity of a compound (Butaye *et al*., 2000). The choice of medium used has to meet the requirements of National Committee for Clinical Laboratory Standards (NCCLS) which is recommended as the reference medium for agar and broth dilution assays (Anonmyous, 2000).

2.3.2.3 Inoculum

Standardization of the inoculum size is important to prevent influence on the potency antimicrobial activity of the sample. In disc diffusion test, only a single colony is used to streak the plate to avoid overgrowth of the culture on the plate. The inoculum size used in dilution methods are 10^5 CFU/mL for bacteria and 10^3 to 10^4 CFU/mL for yeast and fungi (Hadacek and Greger, 2000). Generally, too low inoculums size will lead to many false- positive results and too high inoculums size increase the chances of false-negative (Anonmyous, 2003).

2.4 Toxicity

Drug resistances or side effects like toxicity are the common hitch of many medicines. New drug discovery brings excitement especially if it is affordable by everyone. Keeping the healing properties in mind safety measures to ensure the drug is non-toxic remains a major precaution. In laboratory conditions, toxicity can be evaluated in terms of *in vitro* and *in vivo*. Generally, for *in vitro* study, brine shrimp

lethality test remains as the simplest and economical probe. Further toxicity study is conducted with animal models for *in vivo* study. Toxicity studies allow us to understand the symptom, mechanism, treatment and poisoning effect of the material. Toxicology data can also provide a better chance for a new drug formulation in the pharmaceutical industry (Sasidharan *et al*., 2010).

2.4.1 Brine shrimp assay

Brine shrimp or *Artemia salina* is an invertebrate found in sea-water and other saline ecosystems (Bussmann *et al*., 2011). This method was developed by Meyer in 1982 to monitor the biological response of *A. salina* against a test sample. *Artemia* is frequently used agent in laboratory assays to determine the toxicity values by estimating LC_{50} (median lethal concentration) (Coe *et al*., 2010). The lethality can be determined based on the mortality of the brine shrimps. Toxicology is pharmacology at higher doses, thus findings of lower or non-toxic activity might be very useful in the pharmacology and physiological systems (Montanker *et al*., 2002).

2.4.2 Oral acute toxicity

Acute toxicity refers to the adverse effects that occur on first exposure to a single dose of substance. At *in vivo* level animal models like mice and rats are common used in experiments to evaluate the toxicity level. Acute systemic toxicity is assessed by administration of a single dose of compound orally, dermally or by inhalation. The aim of the study is for pharmaceutical reasons, as results from the animal test are used for deriving potential new medicines. In this context, the toxicity data provides information whether the drug outweigh any health risks like

adverse side effects and to establish safe drug dosage. This study is normally carried out according to the Organization of Economic Cooperation and Development (OECD) 420 (2001).

2.5 Tools for Fungal Diseases Diagnosis

Poor treatment facilities and delayed diagnosis are some of the reasons for the rise of fungal infectious diseases observed in developing countries. An accurate diagnosis of aspergillosis is important as early diagnosis has been associated with improved survival rate among patients. Currently, with advance technology there are many alternative tools for pathologist in an effort to decrease the statistics of fungal infections. Leaving the computed tomography (CT) scanning there are other effective models for diagnosing invasive aspergillosis (Ellis, 2002).

2.5.1 Histochemistry

This technique has the potential to depict the colonized fungal species on screened tissues. Visualization of the hyphae and spores of the fungi helps to determine the degree of infections and the invaded area in the particular tissue.

2.5.1.1 Fungal stains

Use of specific fungal stains can be considered as a possible diagnostic for fungal infections like aspergillosis (Denning *et al*., 2003). Besides, haematoxylin and eosin special fungal stains such as Gomori's Methenamine Silver (GMS) can be applied on the histological sections whereby GMS helps to stain the fungal elements. Therefore, GMS stain acts as a sensitive stain to detect any small fungal fragments in the infected host tissue (Hope *et al*., 2005).

2.5.2 Serological technique

2.5.2.1 Galactomannan assay

Galactomannan determination is becoming another important early diagnostic means. This test is performed using the commercial assays for galactomannan detection which is known as Platelia Enzyme linked immunosorbent assay (ELISA) (BioRad, Marnes-La-Coquette, France) (Ascioglu *et al*., 2002). Galactomannan is a component in the fungal cell wall of most *Aspergillus* and *Penicillium* species. Generally, serum samples and bronchoalveolar lavage fluid (BAL) are subjected to galactomannan assay. Nevertheless, galactomannan was also found to be present in other body fluids including cerebral spinal fluid (CSF), peritoneal fluid, pericardial fluid and urine (Klont *et al*., 2004). This technique uses the antibody-antigen binding principle whereby the monoclonal antibody from rat EB-A2 binds with the β (1-5)-linked galactofuranoside side chain residue of galactomannan molecule. The detection uses sandwich ELISA concept. Optical density (OD) obtained from this assay confirms the diagnosis of aspergillosis and the degree of infection (Maertens *et al*., 2001).

2.5.2.2 Other techniques

Polymerase chain reaction (PCR) using nucleic acids can also be used for diagnosis of aspergillosis. However, lack of standardization on the technical issues remains as a barrier to use this application as a diagnostic model (Bretagne, 2003). *Aspergillus* species are widely used in food industries due to the production of secondary metabolites. Hence, metabolites were found to be another diagnostic tool. Unfortunately, the complexity of measurements that requires gas liquid

chromatography and mass spectroscopy becomes a major concern and the technique is less preferred (Lewis *et al.*, 2005).

2.6 Fungi

Fungi fall in the group of microorganisms and are represented by a large number of species. Their ubiquitous distribution in the biosphere, longevity and ability to survive in a wide range of environmental conditions has made fungi the most successful group of organisms. Fungi are heterotrophic organisms whereby they are dependent on dead or living organisms for their growth. This organism comprises approximately 25% of the global biomass (Eduard, 2009). Since ancient days, fungi are present as both saprobes and parasites. Fungi are recognized as decomposer agents as thay are able to degrade complex organic materials to a simpler compound in the environment. By this way, critical constituents such as nitrogen, carbon and phosphorus are released to the atmosphere for the survival of organisms.

Fungi, especially those which are filamentous are used in biotechnological processes due to their metabolic versatility and capability for secreting enzymes, proteins and other important industrial metabolites (Papagianni, 2004). Thus, they remain as an important research tool for biotechnologists and microbiologists in biological processes. On the contrary, fungi have many negative aspects in that they cause major diseases in plants and animals. The ability of these fungi to release chemical substances like mycotoxins in foods and invading human cells causing infectious diseases have inspired fear and superstition in man. However, not all fungi are harmful to man as some are considered as safe.

2.6.1 Species characteristics

Like bacteria, fungi have their own subgroup of organisms namely mushrooms, moulds and yeasts. Classification of fungi depends upon morphological characteristics, reproduction structures and growth conditions. Each species bears different characteristics and is grouped in different genera. Genomics approach is used to identify and classify some fungi when the traditional identification approach not sufficient (Eduard, 2009).

2.6.2 Biological and physical properties

Fungi are eukaryotic organisms and monopoly on other dead or living material for survival. Fungi are dependent on oxygen, water and organic materials for growth whereby oxygen and organic materials are readily available in the environment but access to water sometimes remains the limiting factor. However, water content of about 12-15% in materials is usually sufficient to sustain fungal growth as some fungi are capable of growing in lower water content when the relative humidity in the air is above 85% (Green *et al*., 2005). The growth of fungi is also dependable upon other conditions like temperature. Most fungi are classified as mesophillic because they grow at 15-30°C. In contrast, *Cladosporium herbarum* is able to grow below 5°C and is known as psychrotolerant species. *Aspergillus fumigatus* and *Aspergillus niger* have an optimum growth close to human body temperature which allows these species to invade the human cells and cause opportunistic diseases (Eduard, 2009).