# ANTICANDIDAL, ANTIOXIDANT, TOXICITY STUDIES AND ISOLATION OF COMPOUNDS FROM *Cassia fistula* SEEDS

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This thesis is the end of my long journey in obtaining my MSc degree in Universiti Sains Malaysia. I have not travelled in a vacuum in this journey. In the name of God foremost, I would like to express my heartfelt thanks to Almighty for giving me the most gracious, the most merciful strength and the perseverance to pursue and complete my Master of Science (MSc). It would not have been possible to complete this study without His grace. Moreover, there are some people who have made this journey easier with words of encouragement and intellectually satisfying by offering different places to look to expand my theories and ideas.

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# Dedication

I would like to dedicate this thesis and work to my beloved parents Mr. Subramanion Murugan and Mrs. Kamala Santhanam, my sisters Kasthuri, Samenthy and my nieces and nephews. Thank you for your love, care and moral support.

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| | |
|---|---|
| ABS | Absorbance |
| AIDS | Acquired Immune Deficiency Syndrome |
| ALP | Alkaline phosphate |
| ALT | Alanine aminotransferase |
| Amp B | Ampotericin B |
| ANOVA | Analysis of variance |
| AST | Aspartate aminotransferase |
| BHT | Butylated hydroxytoluene |
| *C. albicans* | *Candida albicans* |
| *C. fistula* | *Cassia fistula* |
| CFU | Colony forming units |
| DMSO | Dimethyl sulfoxide |
| DPPH | 2, 2-diphenyl-1-picrylhydrazyl |
| GAE | Gallic acid equivalents |
| HIV | Human Immunodeficiency Virus |
| HPTLC | High Performance Thin Layer Chromatography |
| $IC_{50}$ | Inhibitory Concentration at 50% |
| $LC_{50}$ | Lethal Concentration at 50% |
| LC-MS | Liquid Chromatography-Mass Spectrometry |
| $LD_{50}$ | Lethality Dosage at 50% |
| MIC | Minimum Inhibitory Concentration |
| OD | Optical Density |
| $R_f$ | Retention Factor |
| ROS | Reactive oxygen species |
| SD | Standard Deviation |
| SEM | Scanning Electron Microscope |
| TBIL | Total Bilirubin |
| TEM | Transmission Electron Microscope |
| TLC | Thin Layer Chromatography |
| v/v | Volume per volume |
| w/v | Weight per volume |
| XO | Xanthine oxidase |

**ANTIKANDIDA, ANTIOKSIDAN, KETOKSIKAN DAN PEMENCILAN SEBATIAN DARIPADA BIJI *Cassia fistula***

## ABSTRAK

Dalam kajian ini, ekstrak metanol biji *Cassia fistula* telah dicerakinkan untuk aktiviti antimikrob dan antioksidan, dipencilkan, ditulenkan untuk sebatian bioaktif serta ditaksir kesan toksiknya. Ekstrak biji telah diuji untuk aktiviti antimikrob terhadap bacteria Gram-positif dan negatif serta jenis kulat yang mempunyai kepentingan perubatan. Aktiviti antimikrob telah ditentukan melalui kaedah peresapan disk dan pencairan kaldu. Ekstrak didapati berkesan terhadap mikroorganisma ujian dan nilai kepekatan perencatan minimum (MIC) didapti berada dalam julat 1.563-50.00 mg/mL. Ekstrak biji menunjukkan aktiviti yang amat baik terhadap kulat patogen *C. albicans* dengan nilai MIC 6.25 mg/mL, dan keputusan ini disahkan melalui kajian asai bunuh mengikut masa. Kesan ekstrak biji *C. fistula* terhadap *C. albicans* telah diuji melalui perubahan dalam profil pertumbuhannya, dan ini mengesahkan aktiviti antikandida terhadap *C. albicans*. Metamorfosis morfologi *C. albicans* kawalan serta yang dirawat dengan ekstrak yang dicerap melalui SEM dan TEM menunjukkan perubahan yang ketara terhadap dinding luar sel dan kandungan sitoplasma *C. albicans* serta kemusnahan sel yis tersebut yang didedahkan pada ekstrak biji dengan kepekatan 6.25 mg/mL selama 36 jam. Analisis penyaringan fitokimia ekstrak biji *C. fistula* menunjukkan kehadiran lima metabolit sekunder utama termasuk antrakuinon, terpenoid, flavonoid, saponin, tanin serta ketidakhadiran alkaloid dan gula penurunan. Maka, penulenan dan identifikasi lanjutan sebatian bioaktif telah dilakukan dengan menggunakan pelbagai teknik kromatografi. Dua jalur utama telah dipisahkan dengan nilai $R_f$ 0.39 dan 0.69 melalui kaedah kromatografi. Seterusnya,

pecahan aktif yang telah dipencilkan tadi dianalisis dengan mengunakan kaedah HPLC dan LC/MS, dan keputusan menghasilkan puncak jelas yang menunjukkan kehadiran sebatian bioaktif utama dengan aktiviti antikandida. Analisis MS terhadap komponen ini telah seterusnya disahkan sebagai roseanon. Aktiviti antioksidan ekstrak biji *C. fistula* telah diuji dengan menggunakan kaedah DPPH dan keputusan menunjukkan aktiviti penjerapan dengan nilai 25.2% dan nilai $IC_{50}$ 11.07 mg/mL. Kandungan jumlah fenol dan flavonoid didapati sangat tinggi di dalam ekstrak biji iaitu sebanyak 474.25 mg bersamaan dengan asid galik per gram berat sampel kering dan 70.86 mg bersamaan katekin per gram berat sampel kering, masing-masing. Ekstrak biji seterusnya diuji untuk asai perencatan xantin oksidase dan ekstrak didapati mempamerkan aktiviti perencatan dengan nilai sebanyak 64.56% dan nilai $IC_{50}$ 49.5 µg/mL. Kajian awal ketoksikan ekstrak biji *C. fistula* telah dilakukan dengan menggunakan esei kematian anak udang brin dan kaedah ketoksikan oral akut. Keputusan menunjukkan ekstrak biji *C. fistula* tidak toksik terhadap *Artemia salina* dengan nilai $LC_{50}$ 2.11 mg/mL. Keputusan ini telah disahkan melalui kajian ketoksikan oral akut dan pemberian ekstrak pada dos yang tinggi iaitu sebanyak 5,000 mg/kg berat badan melalui oral yang tidak menunjukkan sebarang kematian atau bukti kesan sampingan. Ini membuktikan bahawa ekstrak biji *C. fistula* yang diuji adalah tidak toksik. Analisis histopatologi organ penting buah pinggang, hati, paru-paru, limpa dan jantung telah mengesahkan keputusan tersebut. Sebagai kesimpulan keputusan kedua-dua ujian toksisiti ini menunjukkan ekstrak *C. fistula* tidak toksik dan adalah selamat untuk digunakan bagi tujuan komersil khasnya dalam memajukan agen antikandida.

**ANTICANDIDAL, ANTIOXIDANT, TOXICITY STUDIES AND ISOLATION OF COMPOUNDS FROM *Cassia fistula* SEEDS**


## ABSTRACT

In the present study, methanol seeds extract of *Cassia fistula* was assayed for antimicrobial and antioxidant activities, isolated and purified for bioactive compounds as well as evaluated for the toxicity effect. The seeds extract was tested for potential antimicrobial activity against medically important Gram-positive and Gram-negative bacteria and fungal strains. The antimicrobial activity was determined by the disc diffusion and the broth dilution methods. The extract was effective on tested microorganism and the minimum inhibitory concentration (MIC) values were found in the range of 1.563-50.00 mg/mL. The seeds extract showed a favourable activity against pathogenic fungi *C. albicans* with MIC value 6.25 mg/mL, and this was confirmed by time kill assay study. The effect of *C. fistula* seeds extract on *C. albican* was examined by the alteration in normal growth profile curve and this further confirms the anticandidal activity on *C. albicans*. The SEM and TEM observations were carried out to distinguish the metamorphosis in the morphology of control and seeds extract treated *C. albicans.* The results revealed adverse effects on the outer cell wall and cytoplasmic content of the *C. albicans* and complete collapsed of the yeast cell exposed to seeds extract at concentration 6.25 mg/mL for 36 hrs. The phytochemical screening analysis of *C. fistula* seeds extract revealed the presence of five major secondary metabolites including anthraquinone, terpenoids, flavonoids, saponins, tannins and the absence of alkaloids and reducing sugars. Hence, further purification and identification of bioactive compounds were done by various chromatography techniques. Two separated bands were separated

from the developed chromatogram with $R_f$ value 0.39 and 0.69. The isolated active fractions were tested via HPLC and LC/MS, and the results revealed distinct peaks which indicated the presence of major bioactive compound with anticandidal activity. The MS analysis on this component was subsequently confirmed as roseanone. The antioxidant activity of *C. fistula* seeds extract was evaluated by DPPH assay and the result revealed the scavenging activity with value 25.2% and $IC_{50}$ value 11.07 mg/mL. Total phenolic and flavonoid contents were found to be highest in seeds extract with 474.25 mg of gallic acid per gram dry sample and 70.86 mg of catechin equivalent per gram of sample respectively. The seeds extract was further evaluated for the xanthine oxidase inhibitory assay and the seeds extract exhibited inhibitory activity with value 64.56% and $IC_{50}$ value of 49.5 µg/mL. Preliminary toxicity properties of *C. fistula* seeds extract was investigated by brine shrimp lethality assay and acute oral toxicity study. The result indicated the *C. fistula* seeds extract was not toxic against *Artemia salina* with $LC_{50}$ value 2.11 mg/mL. Further confirmation of this finding obtained from acute oral toxicity study and oral administration of seeds extract at the highest dose of 5,000 mg/kg, resulted in no mortalities or evidences of adverse effects, implying that *C. fistula* seeds extract was nontoxic. Histopathology analysis of the vital organs of kidney, liver, lung, spleen and heart confirmed the evidence. In conclusion, the results of both toxicity tests confirmed that *C. fistula* seeds extract was nontoxic and hence safe for commercial utilization especially for the development of anticandidal agent.

# CHAPTER 1.0: INTRODUCTION

In pre-globalization era, infectious diseases caused by microorganisms are still a major threat to public health due to drastic alarming increase in rate of antibiotics resistance. Many people around the world are suffering from infectious diseases such as AIDS, malaria and tuberculosis. The impact is particularly large in developing countries, mainly due to the relative unavailability of medicines and the emergence of widespread drug resistance. The antibiotic resistance is a dangerous trait which enables microbes to survive and continue to grow instead of being inhibit or destroy, and this raise the risk of medical miracles at all times. Moreover, the ability of microorganisms to inactivate current drugs makes the situation even more complicated.

During the past several years, infectious diseases caused by pathogenic microorganisms associated with adherence of biofilms has determined as the major trigger of human morbidity and mortality in non-industrialized countries (Ramage *et al.*, 2001). In addition, huge modifications in genetic variant of target pathogens are subsequently developing resistance to commercially available antibiotics (Hawkey, 1998). Therefore, increasing in resistance to antibiotics greatly demonstrates the potential for the rapid spread of resistant microbial strains and which could stimulate permissive life-threatening infections. One example of biofilm associated microorganism is the *Candida albicans*. It is a well known opportunistic fungal pathogen capable of triggering infections by its biofilm mode of growth particularly in immunocompromised patients (Jain *et al*., 2007) such as cancer, AIDS or HIV. *C. albicans* is able to colonize on mucosal surface and epithelium by its biofilms and have important clinical repercussions due to their increased resistance to antifungal agents which result in treatment failure.

Therefore, research on new drugs development must continue and all possible strategies should be explored to combat antibiotic-resistant fungal pathogens which are mutant to commercially available antifungal agents (Clark and Walker, 1999).

Hence, natural products are one of the best alternatives to be explored to combat antibiotic resistant *Candidal* pathogens. In general, natural products have played a pivotal role in antibiotic drug discovery with most antimicrobial drugs being derived from a natural product or natural product lead. However, the rapid onset of resistance to most antimicrobial drugs diminishes their effectiveness considerably and necessitates a constant supply of new antibiotics as alternative treatment for infectious diseases. The biologically active compounds isolated from plants that contribute to medicinal field are largely known as plant-derived drugs. Furthermore, the activities of compounds found in natural products are thousand times much different compared to the available synthetic products. This may due to the variability in chemical structures of secondary metabolites produced by natural products with increasing potential in new mechanisms to defense the pathogenic microorganisms (Cowan, 1999). Traditionally, the terrestrial plants were the major source which possesses abundant pharmaceutical agents and their potential uses have been documented until today. Recently, many new metabolites have been identified from plant-based natural products and many of which possess highly interesting pharmaceutical properties. Apart from that, natural products are often very different in their chemical structures compared to those found from synthetic compounds. Moreover, most of the synthetic compounds are derivatives of these natural compounds. Therefore, natural products based pharmaceuticals in many cases provide completely new pharmacological mode of actions. Thus, this has attracted the interest of numerous natural

product researchers to further extend their finding towards natural products (Cowan, 1999).

In the past few decades, diseases caused by free radicals have been increased astronomically due to less consumption of high antioxidant food supplements. Natural products also can play an important role to overcome diseases caused by free radicals. Moreover, the food product from natural resources contains high antioxidant properties such as green tea, vegetables and fruits. Since antioxidant compounds possess the power to reduce the reaction of ROS in tissue damage, therefore such situation diverts researches towards finding new products with antioxidative properties from natural resources such as medicinal plants, which are very active domains in today's research world. Although humans and other organisms possess antioxidant defense enzymes to protect against oxidative damage but they are totally insufficient in preventing the overall damage (Mau *et al.,* 2002). Antioxidant supplements or foods containing antioxidants may help the human body to reduce the oxidative damage (Yanga *et al.,* 2002) since these sources are rich with various types of bioactive compounds able to donate electrons to terminate the chain reactions. Hence, in this study the antioxidant activity of *C. fistula* seeds extract was explored.

The exploration of local medicinal plants in this study also brings economic values to the countries. Today herbal markets in the world mainly United States, Germany, France, India and Japan have become huge potential markets with a great room for scientific research and technology. According to statistics being reported, Europe has a huge herbal market with about $ 70 billion a year and is growing at the rate of 6% annually. Germany and France, both the largest European market, use herbs as

pharmaceutical prescriptions and their sales channels were around $ 7.1 billion in 2003 and may be over $ 25 billion. Apart from that, the UK which ranged as the fourth largest European market showed a slight rise from $ 0.5 billion in 2003 to $ 0.7 billion in 2007. Herbs and botanical sales of US were $ 4.4 billion in 2005, with an estimated growth rate of nearly 4% and the sales grew up to $ 4.6 billion in 2006 and currently estimates to about $ 4.8 billion (Verma and Singh, 2008). In India the market for ayurvedic medicines is estimated to be expanding at 20% annually and the sales of medicinal plants have grown by nearly 25% over the past 10 years. According to the statistic analysis reported by the Export Import (EXIM) Bank, the growth rate of international market for medicinal plants related market were 7% for per annum in India in 2004. In addition, the shares in the world herbal market for both China and India were $ 6 and $ 1 billion respectively and it is rapidly growing yearly form 2003 to 2006 (Verma and Singh, 2008). World Health Organization (WHO) believes that the international market for herbal products estimated to be $ 62 billion is poised to grow up to $ 5 trillion by the year 2050 (Rath, 2005). All the statistical data above are evidence for the bright future of natural product-based antimicrobial development program. If *C. fistula* seeds extract was chosen by any pharmaceutical companies will bring economics benefits to the local famers.

Hence, the current research was undertaken to explore the potential pharmaceutical activities of *C. fistula* seeds extract as an anticandidal and antioxidant agents.

## 1.1 Objectives

The current study was undertaken with the following objectives:

- To investigate the rationale of the usage of *C. fistula* as a curative in traditional medicine by studying the antimicrobial and antioxidant activities.
- To study *in vitro* and *in vivo* toxicity of *C. fistula* seeds extract.
- To isolate and identify the active fractions or bioactive compound(s) with anticandidal activity from *C. fistula* seeds extract by using column chromatography, preparative TLC, bioautography, HPLC and LCMS techniques.

# CHAPTER 2.0: LITERATURE REVIEW

## 2.1 Antimicrobial agents

Infectious diseases are still the second leading cause of death worldwide and the number of emerging or re-emerging bacterial, fungal and viral pathogens are continue to increase. This signifies the need of substances to control the microorganisms in prevention and curing the diseases caused by their actions. An antimicrobial agent is one of the substances that kills or inhibits the growth and prevents damage due to the action of infectious microorganisms. Microbiologists have distinguished two groups of antimicrobial agents used to treat infectious diseases (Chattopadhyay *et al*., 2010). Firstly the antibiotics, which are natural substances produced by certain group of microorganisms with potential to inhibit and kill growth of other microorganisms and secondly, chemotherapeutics agents which are chemically synthesized. In medical and pharmaceutical worlds, all antimicrobial agents used in the treatment of diseases are referred as antibiotics which interpret the word literally. Therefore, today the term antibiotic is largely used to refer to any drug that attempts to prevent or cure infectious diseases. Meanwhile, substances that kill or inhibit growth of fungi are known as antifungal agents (McDonnell, 2007).

The discovery of a powerful bactericidal substance, the penicillin by Alexander Fleming which possesses a broad antimicrobial activity begins the golden period of pre-antibiotics era and until now the drug discovery has been largely dominated by the whole-cell screening assay. The capability of the substances to inhibit the growth of the multiplying bacteria is preferred best as antimicrobial agents, although their reaction mechanism behind the action is not always understandable. However, this approach was

successful in the early days of large synthetic chemical libraries with novel chemistries or naturally occurring antimicrobials including peptides (Bax *et al.,* 1998; Overbye and Barrett, 2005). In the past two decades, there has been unprecedented activity in the discovery and development of new antimicrobial agents and such situation resulted in unrelated research and discovery efforts in many different chemical areas. In addition, the technical advances in penicillin chemistry have helped in subsequent rapid development of cephalosporins and other beta-lactams leading to development of structure activity relationship (SAR) which directs the chemical modification of antibiotics to improve bacterial activity. This approach also triggers the discovery of new antifungal agents active against all pathogenic fungus that had acquired resistance to commercially available agents (Gootz, 1990).

### 2.1.1 Resistant microorganisms towards available antimicrobial agents

Antimicrobial agent medications are used to kill pathogenic microorganisms, which can cause severe infections and diseases. The agents have made major contribution to human health and many diseases leading to death can now be treated effectively with antibiotics. However, some microorganisms have become resistant to commonly used antimicrobial agents. The antibiotic resistant strains are not controlled or killed by antibiotics and thereby are able to survive and multiply even in the presence of an antibiotic. Most of infection-caused strains become resistant to one or two antibiotics and in certain cases some become resistant to more antibiotics which are known as multi-resistant organisms (MROs). The molecular mechanisms by which bacteria become resistant to antimicrobial agents are diverse and complex. Overall, the mechanisms of resistant can be categorized according to their principle mechanisms of action known as

genetic and biological alterations (Brazas *et al*., 2007). The genetic mechanisms mostly involve in acquisition of new genetic material by antimicrobial susceptible microorganism from resistant strains which occurs through conjugation, transformation or transduction with transposons, often facilitate the incorporation of the multiple resistance genes into the host genome or plasmids (Foley and Lynne, 2008). The biological mechanism or also known as natural selection generally involves mutation. When bacterial strains grow rapidly and mutate frequently at a rate of 1 in every 100,000 to in every million, such situation triggers biochemical alteration and this often leads to DNA base pair mutation which finally enhances changes in protein shape, function or both. The potential of mutation increases the chances for development of antibiotics resistant strains **(**Hutter *et al.,* 2004).

### 2.1.2 Types of antimicrobial agents and the resistant mechanisms

Antimicrobial chemotherapy has played an important role to overcome severe infectious diseases caused by microbial pathogens. Antifungal and antibacterial agents can be classified based on the chemical structures and its corresponding therapeutic potential mechanisms. In addition, there are three new azole drugs that have been developed, and used in both systemic and superficial fungal infections. The voriconazole, ravuconazole and posaconazole are triazoles, with broad-spectrum activity. Voriconazole has a high bioavailability, and has been used with success in immunocompromised patients with invasive fungal infections. Ravuconazole has shown efficacy in candidiasis in immunocompromised patients, and onychomycosis in healthy patients. Meanwhile, the preliminary *in vivo* studies with posaconazole indicated potential use in a variety of invasive fungal infections including oropharyngeal candidiasis. Echinocandins and

pneumocandins are a new class of antifungals, which act as fungal cell wall beta-(1, 3)-D-glucan synthase enzyme complex inhibitors (Gupte *et al*., 2002). Other antibacterial agents are β-lactams (penicillins and cephalosporins), macrolides (erythromycin and azithromycin), tetracyclines (tetracycline), aminoglycoside (streptomycin) and chloramphenicols (Gootz, 1990).

Infection caused by microbial strain which is resistant to available anti-infectious agents, is particularly a serious problem in patients at risk for infections. Most resistances currently seen are the result of plasmid transfer rather than mutational events. However, extensive use of antimicrobial agents in hospitals has caused the selection of organisms resistant to many agents by virtue of chromosomally mediated mechanisms (Harold, 1984). Many researchers have documented the outline of resistance mechanisms by which bacteria or fungi evade the antimicrobial agents. In general, these mechanisms can be divided into six major categories (Figure 2.1): 1. Modification of a target enzyme where all antimicrobial agents have specific functions towards their target microorganisms to which they interact. In many cases, if the microorganism can modify a target enzyme, then that enzyme may become insensitive to the former inhibitor but still functions and the organisms will not be inhibited (Harold, 1984). 2. Reduction in physiologic importance of target. The mechanism of resistance is due to reduction in the physiological importance of target. In certain streptococci and some gram-negative species, the role of peptidoglycan in maintaining bacterial cell viability is significantly reduced (Shockman *et al.,* 1981). 3. Duplication of target enzyme. Some bacterial strain tends to duplicate targets and this is seen with trimethoprim in which an altered dihydrofolate reductase decreases the affinity for trimethoprim to be synthesized.

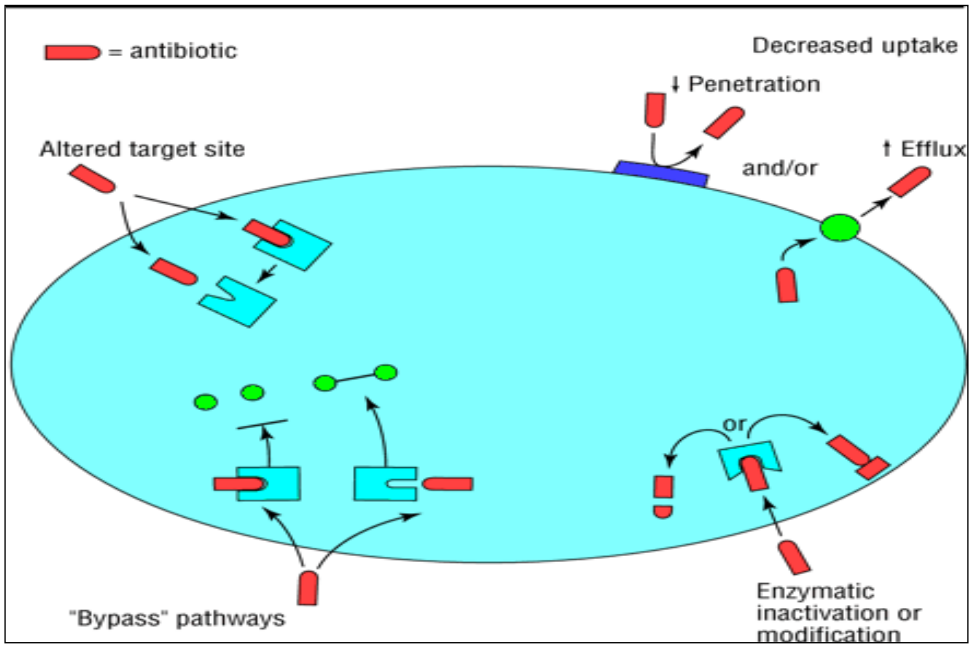


**Figure 2.1:** Major biochemical mechanisms of antibiotics resistance

Source: http://chem3513-2007.pbworks.com/w/page/15648409/Antibiotic-Resistance

Altered penicillin-binding proteins have also been found in beta-lactam-resistant *Streptococcus pneumoniae*, *Neustria gonorrhoeae*, and *Staphylococcus aureus* (Karchmer *et al.*, 1983). 4. Prevention of access to the target. Such situation triggers to prevent the access of the antimicrobial agent to its receptor and this leads to alteration of porin channels, as occurs with beta-lactam antibiotics and aminoglycosides, or loss of a transport system which is an extremely effective form of bacteria resistant to fosfomycin and fosmidomycin. Resistance to tetracycline is primarily the result of exclusion of the drug by insertion of proteins in the bacterial membrane (Chopra and Howe, 1978). 5. Depression of metabolic activity that normally converts an inert agent into an active agent. Some bacteria may depress their usual metabolic pathways to avoid activating an agent, as occurs with metronidazole which must be converted to an active form in the

bacteria (Georgopapadaku and Liu, 1980). 6. Synthesis of enzymes that inactivate antimicrobial agents or modify the agent to alter entry or binding receptor. Finally, bacteria can synthesize enzymes that inactivate the agent by destroying the substance, such as the beta-lactamases (DelBene *et al.,* 1979) or can modify the antibacterial agents. This modified drug either enters the bacterial cell poorly or, when it enters the cell, fails to bind adequately to its target (Bryan, 1982).

### 2.1.3 Synergetic and antagonistic actions of antimicrobial agents

Synergism refers to the combined action of two drugs to produce a marked increase in the bactericidal rate within first 24 hours of exposure as compared with the rate of either drug alone. Such phenomenon expects greater number of bacteria being killed compared to simple summation of single drug effect. Antagonism refers to a marked decrease in the early bactericidal rate as compared with that of the more active single drug even though the combination after prolonged incubation may kill more organisms than either drug alone. These definitions have been chosen because they seem to correlate well with results in the treatment of experimental infections in mice and with certain clinical evidence (Gunnison *et al*., 1955).

## 2.2 Plants as potential natural source

Mankind has been using plants as therapeutic agents for thousands of years and continues to rely on them for primary health care. Plants do not only provide food and shelter but serve humanity to cure different ailments and serve as an excellent source for bioactive compounds which contain potent antimicrobial activities (Khan *et al.,* 2010). Plants that act as herbal medicines are also defined as traditional or natural medicines, which exist in one way or another in different cultures or civilizations such as Indian,

Egyptians, Western, Chinese, Japan and Greco-Arab or Unani. Medicinal plants have always been the source of medicine since they possess pharmacological activities and potential therapeutic uses. Rural areas of many developing countries claim that medicinal plant products are cheaper, effective and impart least effects as compared synthetic products not affordable to those with low-income (Ahsan *et al*., 2009). Medicinal plants research have been supported world widely, the primary goal behind such situation is the recognition of active principle medicinal plants and the pharmacological investigation of the extracts which enhance their safety, effectiveness and constant activities. It is estimated that at the beginning of 21st century, 11% of 252 drugs considered as basic essentials by World Health Organization (WHO) are exclusively of flowering plant origin (Rates, 2001).

Interest in medicinal plants as re-emerging diseases has been fuelled by the rising costs of prescription drugs in maintaining personal health and the bioprospecting of new plant-derived drugs. Furthermore, indiscriminate use of antimicrobial drugs, have developed microorganisms resistance to many antibiotics. This creates several clinical problems in the treatment of infectious diseases and stimulates interests in isolation of plant derived-drugs in a number of countries. Substances with diverse pharmacological properties that can either inhibit the growth of pathogens or kill them with least toxic to host cells are considered as candidates for developing new antimicrobial agents (Ahmad and Beg, 2001). Based on current research and financial investments, medicinal plants seem continue to play an important role as treatment in various ailments (Hoareau and DaSilva, 1999). Since ancient time, plants have served as natural source in treatment of various kinds of diseases; therefore, scientists have been widely using these renewable

resources to produce a new generation of therapeutic solutions with the help of biotechnology.

The search for new anti-infective agents has been taken over by many research groups in the field of ethnopharmacology. Ethnopharmacology provides as an opportunity for both multidisciplinary and interdisciplinary scientific collaboration between the investigations of botany, pharmacology and toxicology, chemistry, anthropology and sociology. Now research on natural molecules and products primarily focuses on medicinal plants since they can be sourced more easily and be selected on the basis of their ethnomedicinal use (Verpoorte *et al.*, 2005). The discovery of plant-derived drugs allows rational planning of new drugs with new biological activity not related to the known compounds (Rates, 2001; Arya *et al.,* 2002). It is expected that plant compounds with diverse chemical structure have great pharmacological effects against drug-resistant microbial pathogens compared to currently used antibiotics (Duarte *et al.,* 2005).

### 2.2.1 Plant as antimicrobial source

Plants have been used as therapeutic agents for thousands of years and continue to rely on them for primary health care needs. “An apple a day keeps the doctor way” according to the traditional American rhyme, and since ancient time plant source has been widely known to have high potential as healing powers (Cowan, 1999). Further acquaintance with different ethnic groups has gain knowledge of ethnopharmacology use of certain compounds from various plant parts including stem, root, leaf, flowers and fruits, based upon the understanding of animal/insect- plant interrelation (Doughari *et al*., 2011).

According to Hippocrates, the Father of modern medicine who claimed the gold coin "Food as medicine and medicine as food" mentioned about 300 to 400 medicinal plants with pharmaceutical properties in late fifty century B.C (Schultes, 1978). Apart from that, it is estimated that there are 250,000 to 500,000 species of plants on Earth (Borris, 1996) and relatively small percentage of these are used as foods by both humans and animal species. In the West, the Renaissance years saw a revival of ancient medicine, which was built largely on plant medicinal. Native American use of plant medicinal has been reviewed extensively in a series of articles by Moerman (1996). He reported that while 1,625 species of plants have been used by various Native American groups as food 2,564 have found use as drugs. Furthermore, Asian cultures were also busy compiling their own pharmacopoeia using medicinal plants. Today many people are interested in having more autonomy over their medical care. In addition, multitude plant compounds are readily available over the counter from herbal suppliers and also natural-food stores for self-medication to cure almost half of infectious diseases around the world. The use of plant extracts, as alternative forms of medical treatments and is enjoying its great popularity since late 1990s (Klink, 1997). Thus, medicinal plants make many researchers to be alert towards their traditional uses through the verification of pharmacological effects which can be natural composite source activity as new anti-infectious agents.

### 2.2.2 Plant-derived antimicrobial agents

Plant-derived antimicrobial agents were discovered from different sources of plant parts and plant origins for many years, and the plant metabolites generally were considered as a source of antinutritional factor. There are a number of medicinal plants with their pharmacological and biological properties such as the essential oils of

cinnamon (*Cinnamomum cassia*), were found to possess *in vitro* antimicrobial properties and shown to inhibit the growth of *Bacillus cereus* (Kalemba and Kunicka, 2003; Valero and Salmeron, 2003). Alcoholic extracts of cinnamon were found most effective against *Helicobacter pylori*, in reducing its growth (Tabak *et al.*, 1996). The ethanol or water extract of cinnamon bark inhibited the activity of bacterial endotoxin. The bark of *Cinnamomum zeylanicum* was found effective against fluconazole resistant *Candida* species, which is an emerging problem. Whereby, *Curcuma longa (Turmeric)* are considered to have natural medicinal properties, including antibacterial, anti-inflammatory, antineoplastic, and analgesic activities because they contains a number of moniterpenoids, sesquiterpenoids, and curcuminoids (Tang and Eisenbrand, 1992; Fang *et al*., 2003). It is also reported to have insecticidal activity (Chander *et al*., 1991a, b). In addition, wound healing and detoxifying properties of curcumin have also received considerable attention (Joe *et al.*, 2004). Curcumin, an active antioxidant from *C. longa* did not produce any effect on aflatoxin production by *Aspergillus parasiticus* (Soni *et al.*, 1992). Meanwhile, the *Piper nigrum (Black pepper)* is used to treat asthma, chronic indigestion, colon toxins, obesity, sinus congestion, fever, intermittent fever, cold extremities, colic, gastric ailments and diarrhea. It has been shown to have antimicrobial activity (Perez and Anesini, 1994; Dorman and Deans, 2000). The both aqueous and ethanol extracts of black pepper screened for antibacterial activity against a penicillin G resistant strain of *Staphylococcus aureus*, showed antibacterial activity (Perez and Anesini, 1994). The *Thymus vulgaris (Thyme)* components are becoming increasingly popular as a naturally occurring antimicrobial and also as an antioxidant agent (Dursun *et al.*, 2003). Thyme showed broad antibacterial activity by inhibiting the growth of both

gram-positive and gram-negative bacteria. However, gram positive bacteria *Clostridium botulinum* and *C. perfringens* appeared to be more sensitive than the gram-negative organisms (Nevas *et al.,* 2004). The alcohol and ethanol extracts of thyme, thyme essential oil, thymol and carvacrol were found to have strong inhibition activity against *Bacillus subtilis*, *Shigella sonnei*, *E. coli* (Fan and Chen, 2001). Aqueous extracts of thyme significantlt inhibited the growth of *H. pylori*, reducing its growth (Tabak *et al.,* 1996). Thymol showed antagonistic effect against *S. sonnei* in anaerobic conditions *in vitro* (Juven *et al.,* 1994).

**2.3 Quantitative evaluation of antimicrobial activity**

Antimicrobial agents are categories based on their mechanisms of action, chemical structure or spectrum of activity. Microorganisms that are inhibited by antimicrobial agents are target-specific and dependent upon the drug's mode of action. Antimicrobial activity of natural extract and pure compounds can be observed based on the growth response of particular microorganisms placed in contact with that active compound. There are a number of methods available for detecting activity however, since these methods are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method (Cos *et al*., 2006). In general, the antifungal and antibacterial test methods are classified into three main groups as follows.

**2.3.1 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 a lower temperature for several hours before incubation to

favours compound diffusion over microbial growth, thereby enhancing the inhibition zone. Many types of reservoir can be used, such as filter paper discs, stainless steel cylinders placed on the surface and holes punched in the medium. The hole-punch method is the only suitable diffusion technique for aqueous extracts, because interference by particulate matter is much less than with other types of reservoirs. Meanwhile, small sample requirements and the possibility to test up to six extracts per plate against a single microorganism are specific advantages (Hadacek and Greger, 2000). In general, 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 characteristic in agar (Cos *et al*., 2006).

### 2.3.2 Dilution method

In the dilution method, 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 test microorganism can be measured in number of ways such as agar dilution method and liquid or broth dilution method based on the Minimal Inhibitory Concentration (MIC), defined as the lowest concentration of antimicrobial agent which inhibits any visible microbial growth by plating-out samples after incubation. The turbidity and redox-indicators are most frequently used in liquid or broth dilution methods. Turbidity can be estimated visually or obtained more accurately by measuring the optical density at 405nm. However, test samples that are not fully soluble may interfere with turbidity readings, emphasizing the need for a negative or sterility control (Cos *et al*., 2006).

### 2.3.3 Bioautographic method

Bioautography is a useful technique to determine bioactive compound with antimicrobial activity from plant extract. TLC bioautographic methods combine chromatographic separation and *in situ* activity determination facilitating the localization and target-directed isolation of active constituents in a mixture (Sasidharan *et al*., 2011). Traditionally, bioautographic technique uses the growth inhibition of microorganisms to detect antimicrobial components of extracts chromatographed on a TLC layer. This methodology has been considered as the most efficacious assay for the detection of anti microbial compounds (Shahverdi *et al*., 2007). Bioautographic localizes the antimicrobial activity on a chromatogram using three approaches: 1) Direct bioautography, where the microorganism grows directly on the thin layer chromatographic plate, 2) Contact bioautography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, and 3) Agar-overlay bioautography, where a seeded agar medium is applied directly onto the TLC plate (Hamburger and Cordell, 1987; Rahalison *et al*., 1991). The inhibition zones produced on TLC plates by one of the above bioautographic techniques will be used to visualize the position of the bioactive compound with antimicrobial activity in the TLC fingerprint with reference to $R_f$ values (Homans and Fuchs, 1970). Despite high sensitivity, its applicability is limited to microorganisms that easily grow on TLC plates. Since biouatography allows localizing antimicrobial activities of an extract on the chromatogram, thus it supports a quick search for new active antimicrobial compounds through bioassay guided isolation (Cos *et al*., 2006). The agar overlay method is advantageous in that, it uses very little amount of sample compared to the normal disc diffusion method (Sasidharan *et al*., 2011).

## 2.4 Antioxidants

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, free radicals and other reactive oxygen species (ROS) are continuously produced *in vivo* by the body itself as byproducts of metabolic processes resulting in dangerous culprit of cell death and tissue damages. The excessive free radicals production and lipid peroxidation are actively involved in the pathogenesis of a wide number of diseases including atherosclerosis (Parthasarathy *et al*., 1998), cardiac and cerebral ischemia, neurodegenerative disorders, carcinogenesis and the aging process (Hu *et al.,* 2000). Oxygen free radicals can initiate lipid peroxidation which stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basements and other membranes, and also crucial in the long-term complication of diabetes (Saha *et al.*, 2004; Polterait, 1997).

Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress (Ozsoy *et al.*, 2008a, b). Antioxidants may also be defined as radical scavengers which protect human body against free radicals that slow down or stop free radical oxidation. Antioxidants minimize radical-caused damages by reducing the energy of free radicals. The reduced energy prevents free radicals from forming, or interrupting the oxidation chain reaction itself. Furthermore to control the number of free radicals, the body produces enzymatic scavengers called endogenous antioxidants such as superoxide dismutase (SOD). Even with maximum production of endogenous antioxidants, the body's defenses sometimes can be overwhelmed; therefore in such a case, exogenous antioxidants particularly vitamins, minerals and herbs may be used to aid the body (Greenly, 2004).

Plant-derived substances such as vitamins, flavonoids, catechins and anthocyanins are secondary metabolites widely distributed with antioxidant and antiradical properties, and becoming increasing suggested dietary factors (Ferguson, 2001). Medicinal plants and herbs are potent biochemical with phytomedicine properties and have obtained a wondrous assortment of industrial chemicals. This has attracted worldwide trend towards the use of natural phytochemicals present in plant sources such as beans, berry crops, tea, oilseeds and vegetables. Several herbs and spices have been reported for antioxidant activity including sage, chili pepper, white pepper ginger, nutmeg, turmeric and several Chinese medicinal plants extracts (Ara and Nur, 2009).

**2.5 Toxicological tests**

Paracelsus, the Father of toxicology said, "All substances are poison and nothing is without poison, only the dose of that substances makes it poison". Ancient Tamil literatures cite "The over dose of a substance makes the poison". Toxicology derived from the Greek words of '*toxicos'* and '*logos',* means science that studies the adverse effects of chemicals and biological substances for example heavy metals like cadmium, chromium, copper and mercury. Toxicology study is to evaluate the plant extract effects on human, animal or microbes. Apart from that, toxicology study 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).

Toxicology study plays a crucial role in identification and isolation of new compounds from natural resources. In addition, the validation and selection of primary toxicological screening methods are essential to guarantee a sound selection of extracts or

molecules with relevant pharmacological action and worthy of following-up. In practical, it is an important method to declare the safety of a developed drug at lower dosages to compare the safety of higher dosages. Primary toxicological screening methods are generally designed for rapid screening of large numbers of extracts with biologically active compounds. These methods should be simple, precise and easy to implement and produce results quickly and preferably at low costs. The primary toxicity assay included the Brine shrimp lethality test and Oral acute toxicity test.

### 2.5.1 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 the food chain (Sanchez-Fortun *et al.,* 1995). *Artemia salina* is used in laboratory bioassay in order to determine toxicity through the estimation of 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 assay is considered a useful tool for preliminary assessment of toxicity since brine shrimp is highly sensitive to a variety of chemical substances (Solý's *et al.*, 1993).

This method determines $LC_{50}$ value of the active compounds and extract in saline medium in µg/mL (Massele and Nshimo, 1995). It has been used 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 been related to pharmacological studies carried out for different chemical compounds (Mathews, 1995; Fumaral and Garchitorena, 1996) where, it acts as a screening method mainly for products of plant origin (Parra *et al.,* 2001).

### 2.5.2 Oral acute toxicity test

Although acute toxicity test is considered simple, standard routine method, it has to be performed strictly in accordance with standard requirements, observing recommended conditions and using indicated organisms. Acute toxicity test is aimed to determine the concentration of plant extract that produces a harmful effect on a group of test organisms such as mice during a short-term exposure under controlled conditions. The most common acute toxicity test applied to animals is the acute lethality test (Sasidharan *et al.,* 2008a). Lack of movement is normally recognized as a criterion of death (Rand and Petrocelli, 1985). Mortality is usually expressed as the median lethal concentration ($LC_{50}$) whereby the estimated concentration of plant extract kills or immobilizes 50% of test organisms throughout the predetermined duration of time.

## 2.6 *Candida*

*Candida* species are ubiquitous fungi representing the most fungal pathogens that affect humans. The genus *Candida* includes around 200 species and the species are characterized primarily on morphology of the colony and carbon utilization. *Candida* is white asporogenous yeast capable of forming pseudohyphae. It is a yeast-like fungus which normally is present in bowel and feeds on sugars, simple carbohydrates and fermented products like alcohol and cheese. Some species are part of our microbiological flora and only 10% are known to be responsible for infections in people (Jarvis, 1995). There are seven *Candida* species of major medical importance; it most important and virulent which is frequently isolated is *Candida albicans* (McCullough *et al.,* 1996).

### 2.6.1 Micromorphological characteristics of *Candida* spp.

The colonies of *Candida* spp. microscopically are cream coloured to yellowish. The texture may be pasty, smooth, glistening or dry, wrinkled and dull depending on species. Microscopic features show important species related variations. All species produce blastoconidia, which may be rounded or elongated. Most produce pseudohyphae that are long, branched or curved. In addition, true hyphae and chlamydospores are produced by some *Candida* strains which observed in (Figure 2.2). Although members of the same genus, the various species present a degree of unique behavior with respect to their colony texture, microscopic morphology on cornmeal Tween 80 agar at 25$^{o}$C (Dalmau method), and fermentation or assimilation profiles in biochemical tests that help to differentiate *Candida* from other yeasts (Freydiere *et al*., 2001).

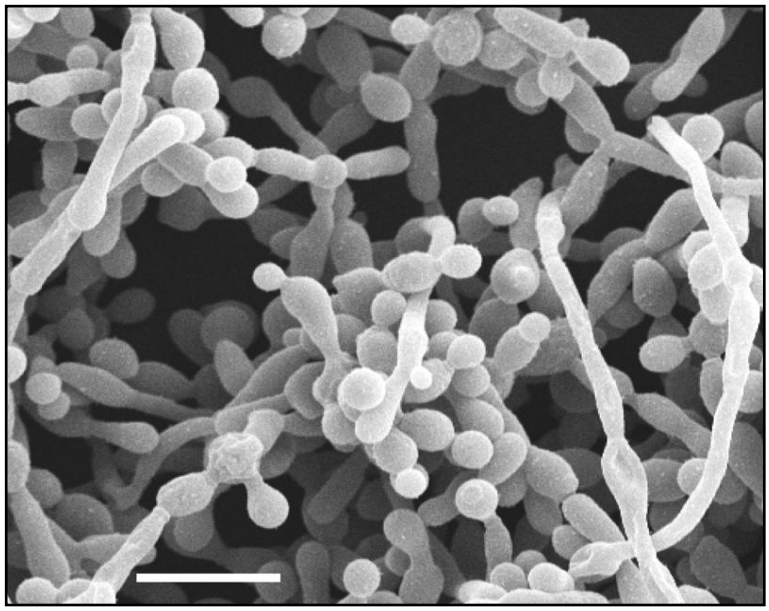

**Figure 2.2:** Scanning electron microscopy image of a mature *Candida* spp. Biofilms are composed of yeast, hyphal and pseudohyphal elements. (Source: Lopes-Ribot *et al*., 2004)

### 2.6.2 Mechanisms of resistance of *Candida* spp.

There is several resistance mechanisms of *Candida* species been extensively documented by review journals (Sanglard and Odds, 2002). In brief, resistance often arises from different synergetic combinations of a limited number of molecular mechanisms. These include the changes in 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 mutations of the antifungal target that decrease its binding ability. Apart from that, the activation of alternate pathways that increase the metabolism of the antifungal and also the sequestration of the antifungal in Organelle-like vacuoles which change the structural content of cell wall are associated with the ability of some *Candida* strains to resist polyenes (Sanglard *et al.*, 1997; Vanden Bossche *et al.*, 1998; Marr *et al.*, 2001).

### 2.6.3 Pathophysiology and virulence factors of *Candida* spp.

*Candida* spp. is part of the normal endogenous flora which is temporary or permanent carriage in the gastrointestinal tract. The mucocutaneous surface colonization is rare under normal condition (Jarvis, 1995) and it will be prerequisite for the development of candidiasis (Pittet *et al.*, 1994; Wright and Wenzel, 1997). Secondary changes in the ecology of the endogenous flora will trigger the overgrowth of *Candida* spp. on mucosal and skin surfaces (Samonis *et al.*, 1993). Furthermore, *Candida* spp. can also translocate across the gut barrier, while its integrity lost (Kennedy and Volz, 1985; Gianotti *et al.*, 1993). The continuous exposure to risk factors is responsible for further invasion with possible secondary haematogenous dissemination (Figure 2.3) (Blumberg