EVALUATION OF THE ANTIFUNGAL PROPERTIES OF TWELVE MEDICINAL PLANTS AND ISOLATION OF ACTIVE COMPOUNDS FROM *Nepenthes gracilis*

By

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ABSTRACT

EVALUATION OF THE ANTIFUNGAL PROPERTIES OF TWELVE MEDICINAL PLANTS AND ISOLATION OF ACTIVE COMPOUNDS FROM *Nepenthes gracilis*

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The increase of opportunistic fungal infections and the development of side effects by synthetic drugs have seriously impacted chemotherapeutic agents available today, making the search for new antifungal agents from natural sources such as medicinal plants becomes necessary. Fresh plant materials were sequentially extracted using solvents of increasing polarity namely hexane, chloroform, ethyl acetate, ethanol, methanol and water. The fungistatic and fungicidal activities of 72 extracts from 12 medicinal plants (*Acmella ciliata*, *Arundina graminifolia*, *Cocos nucifera*, *Diodia sarmentosa*, *Diplazium esculentum*, *Hydrocotyle sibthorpioides*, *Muehlenbeckia platyclada*, *Nepenthes gracilis*, *Parkia speciosa*, *Passiflora edulis*, *Sechium edule* and *Solanum muricatum*) were evaluated in triplicate against six fungal species (*Candida albicans*, *Candida parapsilosis*, *Issatchenkia orientalis*, *Cryptococcus neoformans*, *Aspergillus brasiliensis* and *Trichophyton mentagrophytes*) using a colorimetric broth microdilution method. All the plant extracts showed antifungal activity with 69.21% and 41.20% of the bioassays demonstrating fungistatic and fungicidal activities, respectively. The hexane extract of the leaves of *N. gracilis* showed the lowest minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of 0.02 mg/mL

against *C. albicans*, *I. orientalis* and *T. mentagrophytes*. Bioactivity-guided fractionation and isolation using PLC, HPLC and HPTLC techniques led to the isolation of compound B_1, which has a melting point of 78-79°C. Structural elucidation using FT-IR, GC-MS, LC-MS and NMR techniques (^{1}H, ^{13}C NMR, HMQC and HMBC) established that compound B_1 is plumbagin (5-hydroxy-2-methyl-naphthalene-1,4-dione). Plumbagin exhibited potent antifungal activity, with the MIC values ranging from 2 to 31 μg/mL against all the fungi. Cytotoxic testing using rhesus monkey kidney epithelial cell (LLC-MK2) revealed that the 50% cytotoxicity concentration (CC_{50}) of plumbagin was 0.60 μg/mL. The selectivity indices of plumbagin against all the fungi were less than 1.0, indicating plumbagin is more toxic to mammalian cell than fungal cells. The antifungal properties of *N. gracilis* against human fungal pathogens are reported, to the best of my knowledge, for the first time. The results from this study show that some extracts of these medicinal plants have significant antifungal activity. This corroborates their use in traditional medicine.

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Last but not least, I would like to thank myself for everything.

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SUBMISSION OF DISSERTATION

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I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIST OF ABBREVIATIONS

AR	Analytical Reagent
ATCC	American Type Culture Collection
˚C	Degree Celsius
cfu	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylene diamine tetraacetic acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FSI	Fungal Susceptibility Index
FT-IR	Fourier Transform-Infrared Spectroscopy
GC-MS	Gas Chromatography- Mass Spectrometry
h	Hour
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
Hz	Hertz
i.d.	Internal diameter
INT	*p*-iodonitrotetrazolium violet

KBr	Potassium bromide
L	Liter
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LLC-MK2	Rhesus Monkey Kidney Epithelial Cells
mbar	Millibar
mg	Milligram
µg	Microgram
µL	Microliter
mL	Milliliter
MIC	Minimum Inhibitory Concentration
MFC	Minimum Fungicidal Concentration
min	Minute
NMR	Nuclear Magnetic Resonance
nm	nanometer
NR	Neutral Red
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PTLC	Preparative Thin Layer Chromatography
R_f	Retention factor
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
s	Second
TLC	Thin Layer Chromatography
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Long before human discovered the existence of microorganisms, the possibility that plants have curative abilities which contained antimicrobial properties was well accepted. Human first used plants as food, due to this ingestion; the link to the therapeutic powers of the plants was discovered. The techniques for transforming the healing properties of plants into forms beneficial to human were passed down through oral history (Wee, 2005). Ancient remedies may have associated this knowledge with witchcraft, mysticism, astrology, or religion; but eventually those effective treatments were documented, leading to the early herbals (Dewick, 2002). Archives of early civilizations such as the Assyrian, Egyptian, Chinese and Greek mentioned the use of herbs and herbal drugs to alleviate diseases (Waksmundzka-Hajnos et al., 2008).

In the early 1900s, the golden age of phytotherapy, medicinal plants were the main source use to maintain health. Fluid extracts administered through tinctures, teas and decoctions were used (McChesney et al., 2007). Kipling (1910) wrote: "Anything green that grew out of the mould was an excellent herb to our fathers of old." Man believed that there existed a cure in the plants for every ill; such extensive dependence of human beings on natural products

has invoked great interest in the scientific world to undertake exploration of nature as a source of novel lead for drug development (Brahmachari, 2009).

Modern pharmacognosy concentrates on the science of biogenic or naturally derived pharmaceuticals and poisons from various sources such as plants, bacteria, fungi and marine organisms (Phillipson, 2007). The goal of pharmacognosy is comprehensive studies of natural resources by using physical, chemical, biochemical and biological methods to search for potential drugs or drug substances of natural origin for the discovery of new drugs (American Society of Pharmacognosy, 2004). The plant kingdom is an infinite source of novel chemotypes and pharmacophores. This has continued to attract the interest of modern pharmacognosy in the development of new drugs (Aḥmad et al., 2006).

Fungal infections are the fourth most common cause of life-threatening infections, in part as the result of alterations in immune status associated with Acquired Immune Deficiency Syndrome (AIDS) epidemic, cancer chemotherapy and, organ and bone marrow transplantation (Pfaller and Diekema, 2007). The most common fungal infection agents are those ubiquitous colonizers such as *Candida* spp., *Cryptococcus* and *Aspergillus* spp. with the overall mortality rate of 25-50% for invasive diseases (Perfect and Casadevall, 2002; Heitman et al., 2006; Lin, 2009; Denning and Hope, 2010).

Although there have been major advancements in antifungal therapy, the attributable mortality has essentially remained high due to the limited armamentarium of antifungal agents, diagnostic challenges and the emergence of widespread drug resistance (Gürcüoğlu et al., 2010). The formation of fungal biofilms has contributed to the intrinsic resistance among the fungal species and thus cause important clinical repercussions (Rogers, 2006). The biofilm-associated infections adversely impact the patient's health and also result in significant increases in patient morbidity, hospital costs and difficulties in treating fungal infections (Wilson et al., 2002; Gürcüoğlu et al., 2010).

Only a few classes of antifungal drugs namely polyenes, pyrimidine analogues, allylamines, azoles and echinocandins are used orally or intravenously for the treatment of fungal infections, and each of them has therapeutic limitations, ranging from toxicity, to limited routes of administration, solubility, stability and drug resistance (Denning and Hope, 2010; Roemer et al., 2011). These may become increasingly crucial determinants of the outcome of antifungal treatments and led to heightened interest to search for novel antifungal agents of plant origin. Fungal infections also represent a special challenge in the development of antifungal drugs as fewer pathogen-specific targets are available due to the high degree of phylogenetic relatedness between fungi and human in which both kingdoms are classified as eukaryotes (Heitman et al., 2006).

Plants are the main sources in the search of new pharmaceuticals as comparative analysis of structural diversity in natural products and combinatorial libraries suggest that nature still has an edge over the current synthetic chemistry (Koch et al., 2005; Schmidt et al., 2008). Molecules with diverse structures and multiple stereocenters not only serve as new drugs themselves but also as starting materials for new synthetic compounds (Balunas and Kinghorn, 2005).

Combination of structural features of natural products with combinatorial chemistry is necessary to improve the quality of natural products in drug development. Further modifications of the molecules by synthetic chemistry techniques are required to produce natural-based synthetic analogues with lower toxicity, improved efficacy, yield and water solubility (Phillipson, 2007). Only pure phytochemical compounds which been subjected to the same tests as synthetic drugs can be considered as conventional drugs (Schmidt et al., 2008).

In Malaysia, the favourable equatorial climate and soil conditions enable largely evergreen tropical rain forest to flourish and supported a great array of plant species, many of which are unique and contain of medicinal potentials. Malaysia's rainforests are recognized as one of 17 mega-diversity countries in the world and estimated to have 17,600 species of plants (Valera, 2003). It is believed that a great, yet still not fully revealed, therapeutic potential exists in the tropical rainforests, as only a few percent out of 250,000 plant species in

the world has been screened with regard to their medicinal properties (Waksmundzka-Hajnos et al., 2008; Brahmachari, 2009).

According to Maridass and De Britto (2008), only about 6% of plants have been investigated for biological activity, and only 15% have been pharmacologically screened. Plants compounds have provided many clues for the development of novel drug compounds, which these bioactive compounds are used directly as therapeutic agents, starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Ahmad et al., 2006).

After 1945, a few discoveries of new drugs from higher plants with notable the exception of reserpine from the *Rauwolfia* species, vinblastine and vincristine from *Catharanthus roseus* proved that plants are excellent resources for drug development (Dewick, 2002). The essential oil from *Melaleuca alternifolia* (tea tree) and *Cymbopogon citratus* (lemongrass) are also well-known as natural topical antiseptics (Hammer et al., 2002; Silva et al., 2008). Since plants demonstrated promising medicinal potentials, this study undertook to evaluate the antifungal properties of selected medicinal plants based on their traditional therapeutic uses as antimicrobial agents in which scientific studies on their antifungal activities are limited.

The objectives of this study are:

1. To obtain various extracts of medicinal plants by using sequential solvent extraction method,
2. To evaluate the plant extracts for antifungal activities against pathogenic fungi,
3. To determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the bioactive extracts against the fungi using colorimetric broth microdilution method,
4. To isolate, purify and characterize the antifungal compound(s) using high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), preparative thin layer chromatography (PTLC), Fourier transform infrared spectroscopy (FT-IR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance spectroscopy (NMR),
5. To evaluate the cytotoxicity of the isolated antifungal compound(s) against a mammalian cell line (LLC-MK2).

CHAPTER 2

LITERATURE REVIEW

2.1 Pathogenic Fungi

Kingdom fungi classified under the eukaryote domain is a taxon of enormous diversity with various ecological niches, life cycle patterns as well as morphologies. There are over 1.5 million different fungal species in the world but about 5% were officially classified and only several dozen of them can consistently cause disease in humans (Heitman et al., 2006; Vandeputte et al., 2012).

Most fungi are terrestrial and many of them are parasites of plants, animals and human. There are two major morphological forms of fungal pathogens which including the filamentous fungi (molds) and the yeasts. Majority of the primary pathogens are filamentous fungi such as dermatophytes. Meanwhile the yeasts and some species of filamentous fungi such as *Aspergillus* spp. are known as opportunistic pathogens (Vandeputte et al., 2012). Table 2.1 shows the characteristics of main fungal infections.

Fungal pathogens are either from the endogenous host flora or are acquired from the host's immediate environment. Fungal infections related to the commensal flora are usually associated with immune suppression or

immunodeficiency. However, fungi originated from the environment have the capacity to establish themselves in the mammalian host through their natural niche selection of characteristics with intact immunity (Heitman et al., 2006).

Table 2.1: Characteristics of main fungal infections worldwide (Vandeputte et al., 2012).

Body location	**Pathogen type**	**Organ**	**Most frequent genus**
Superficial	Primary	Skin and hair	*Malassezia*
Cutaneous	Primary	Skin and hair	*Trichophyton*, *Epidermophyton*, *Microsporum*
Mucosal	Opportunistic	Vagina, digestive tract and eye	*Candida*, *Aspergillus*, *Fusarium*
Systemic	Opportunistic	Any organ (lungs, brain, bloodstream etc.)	*Candida*, *Aspergillus*, *Cryptococcus*, *Histoplasma*, *Pneumocystis*, *Coccidioidomyces*

2.1.1 Fungal Infections

The understanding of most systemic fungal diseases was established in the early 1900s when a drastic rise in fungal infections was observed and they are currently represent a global health threat (Heitman et al., 2006; Vandeputte et al., 2012). Many fungi are parasites which able to cause extensive damage and losses to agriculture and forestry, serious diseases in human and animals. Their spores are a cause of allergies (Vandeputte et al., 2012).

Fungal infections can be classified according to the tissues they infect (Table 2.1). These including the superficial mycoses which are the external part of the skin and hair, cutaneous and subcutaneous mycoses affect the keratinized structures of the body. Mucosal infections of the vagina, digestive tract, urinary tract and eye may occur, and the systemic mycoses may affect the whole body (Vandeputte et al., 2012).

Opportunistic pathogens belonging to the genera *Aspergillus*, *Candida* and *Cryptococcus* cause mucosal infections or invasive fungal infections such as aspergillosis, candidiasis and cryptococcosis (Pereira Gonzales and Maisch, 2012; Vandeputte et al., 2012). The dermatophytic and keratinophilic fungi of the genera *Trichophyton*, *Epidermophyton* and *Microsporum* infect eyes, nails, hair, skin and cause local infections including ringworm, onychomycosis and athlete's foot (Woodfolk, 2005).

2.1.2. *Candida* spp.

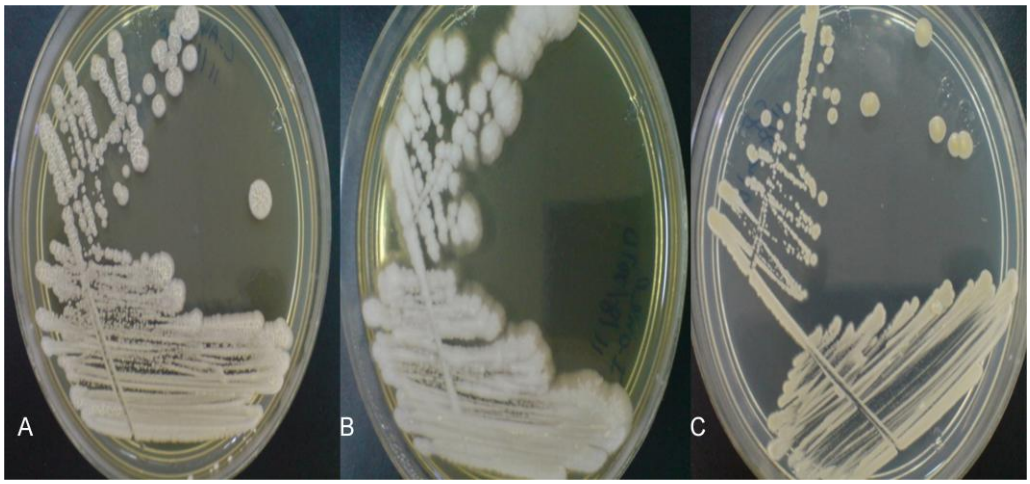
Issatchenkia orientalis formerly known as the *Candida krusei* (synonym) is usually found in two basic morphological forms (yeast and pseudohyphae) in growing cultures (Samaranayake and Samaranayake, 1994). *C. krusei* is asexual while it is very closely related to a sexual species and a sexual form, *I. orientalis*, has been proposed for this organism (Kurtzman et al., 1980; Barnett et al., 1983).

All *Candida* species (*C. albicans, C. parapsilosis* and *I. orientalis*) form round to oval budding yeast cells singly, in chain or loose clusters (Larone, 2002). When invading tissues, they form both pseudohyphae and true hyphae using chains of blastoconidia. They are rapid growers and form cream colored colonies (Figure 2.1).

Candida spp. are normal commensals of the healthy human biota in the gastrointestinal tract, oral and vaginal mucosa (Endo et al., 2010). However, it becomes pathogenic when there is an imbalance in their ecological niche or in the host's resistance to infection (Miceli et al., 2011). Clinical manifestations of candidiasis range from localized superficial invasion of mucosal surfaces to systemic infections with organ involvement and disseminated infection (Miceli et al., 2011).

Candida spp. has several virulence attributes such as adherence to host surfaces, production of phospholipases and proteinases, formation of hyphae that help in the evasion of host immune defences, the formation of biofilms that enhance its ability to penetrate epithelium and increased tolerance to stresses in adaptation in various habitats (Samaranayake and Samaranayake, 1994; Miceli et al., 2011). *C. albicans* is the predominant cause of invasive fungal infections (Gürcüoğlu et al., 2010; Miceli et al., 2011). *C. parapsilosis* is frequently cause of candidal endocarditis and onychomycoses (Larone, 2002; Silva et al., 2008). *I. orientalis* is the main cause of invasive fungal infections in patients who

undergoing bone marrow or stem-cell transplantation, and in patients with malignant hematological disease (Trifilio et al., 2007; Miceli et al., 2011).



A, *Candida albicans*; B, *Issatchenkia orientalis*; C, *Candida parapsilosis*

Figure 2.1: Pictures of *Candida albicans, Candida parapsilosis* and *Issatchenkia orientalis* grown on potato dextrose agar.

2.1.3 *Cryptococcus neoformans*

Cryptococcus neoformans is known as the "sugar-coated killer". It has an encased polysaccharide capsule and able to cause infection that associated with substantial morbidity and mortality (Heitman et al., 2006). They are characterized by oval or round encapsulated yeast with cream colored, smooth, shinny, moist and usually mucoid, with smooth edges on agar plate (Figure 2.2). *C. neoformans* can differentiate into several morphological forms such as yeast (during infections), chlamydospores, pseudohyphae, and hyphae under certain conditions (Alspaugh et al., 2000; Lin and Heitman, 2006).

Cryptococcus neoformans is the best-known basidiomycete human pathogen for the agent of cryptococcosis including pulmonary diseases and meningoencephalitis (Heitman et al., 2006). They display a wide host range for infection, ranging from small invertebrates to the water-dwelling porpoises and commonly causes secondary disease in the immunosuppressed hosts (Heitman et al., 2006). The common infection sites of *C. neoformans* are the bloodstream, central nervous system, skin, eyes, bones, lungs, gastrointestinal tract, prostate gland and urinary tract (Miceli et al., 2011). Clinical isolates of *C. neoformans* originated from the environment are usually associated with bird droppings, especially from pigeons (Lin, 2009). Virulence phenotypes of *C. neoformans* are suggested by the formation of the protective capsule, melanin formation and its ability to grow well at high temperature especially at 37°C (Heitman et al., 2006).

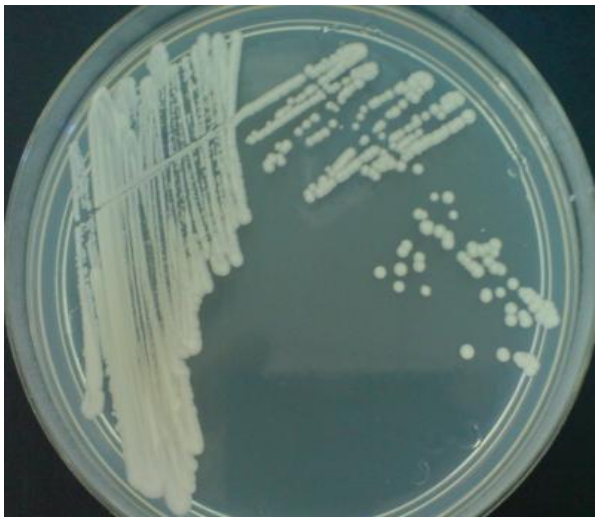


Figure 2.2: *Cryptococcus neoformans* grown on potato dextrose agar.

2.1.4 *Aspergillus brasiliensis*

The genus *Aspergillus* is one of the most difficult groups in classification and identification. There is a high biodiversity within the genus and taxa are difficult to be confirmed solely on their unstable phenotypic characters (Samson et al., 2007). *A. brasiliensis* can be distinguished from other black *Aspergilli* based on the intergenic transcribed region, β-tubulin and calmodulin gene sequences, by amplified fragment length polymorphism analysis, by extrolite profiles and by detecting sequence variations appeared in the calmodulin gene (Susca et al., 2007; Varga et al., 2007). *A. brasiliensis* is a biseriate species which is closely related to *Aspergillus niger* and *Aspergillus tubingensis* (Varga et al., 2007; Manikandan et al., 2010).

The surface morphology of *A. brasiliensis* is at first white colony then dark brown to black with cottony texture appearance (Figure 2.3) with branched conidiophores of round black conidia. *Aspergillus* spp. can cause aspergillosis that range in severity from mild allergic rhinitis to life-threatening invasive pulmonary and disseminated infection (Heitman et al., 2006). The common infection sites of *Aspergillus* spp. including lung, paranasal sinus, ear, eye, skin, mucous membranes and multiple systemic sites (Larone, 2002). Recently, *A. brasiliensis* was reported to cause keratitis, suggesting that this species may be responsible for a significant proportion of corneal infections caused by black *Aspergilli* (Manikandan et al., 2010).

Virulence factors of *Aspergillus* spp. including high sporulating capacity, thermotolerance, melanin formation as well as secretion of damaging products such as enzymes, toxins, exoproteases and other cytoplasmic fungal products which play a significant role in their infectivity (Heitman et al., 2006; Kniemeyer et al., 2011).

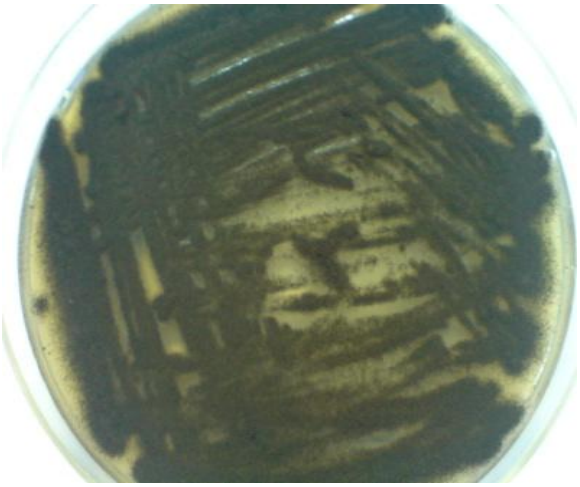


Figure 2.3: *Aspergillus brasiliensis* grown on potato dextrose agar.

2.1.5 *Trichophyton mentagrophytes*

The genus of *Trichophyton* is known as dermatophytes which have the capacity to invade keratinized tissue such as skin, hair and nails of vertebrates (Perea et al., 2001). *T. mentagrophytes* appeared as white and downy, buff to tan colony (Figure 2.4) with sparse aerial mycelium of round microconidia clustered on branched conidiophores (Larone, 2002).

Trichophyton mentagrophytes is a zoophilic fungus that originally recovered from soil (Oyeka, 2000; Mendez-Tovar, 2010). In rural areas, rabbits were found to be the most frequently implicated in the transmission of *T. mentagrophytes* to humans (Mantovani, 1979). This organism has been isolated from foot wears, shoes and shower stalls. It is a common cause of clinical tinea pedis and onychomycosis (Oyeka, 2000). Indeed, in some places, *Trichophyton* infection is considered a major public health problem highly prevalent in adults (Woodfolk, 2005). Potential virulence factors of *T. mentagrophytes* suggested by its keratinolytic capability that release enzymes keratinases, lipases and ceramides, as well as its non-fastidious characteristic which are readily germinates in the high humidity conditions, worldwide distribution and wide range of hosts (Oyeka, 2000; Mendez-Tovar, 2010).

Figure 2.4: *Trichophyton mentagrophytes* grown on potato dextrose agar.

2.2 Antifungal Drugs

Antifungal drugs can be categorized into five classes, namely polyenes, pyrimidine analogues, allylamines, azoles and echinocandins. Figure 2.5 shows the name and year of antifungal agents approved for clinical use by the United States Food and Drug Administration (FDA) from 1955 to 2010. Caspofungin is the first member of the class of drug echinocandins, which received FDA approval in 2001 followed by micafungin in 2005 and anidulafungin in 2006.

2.2.1 Development of New Antifungal Drugs

Much effort has been expended to plant-based antifungal drug discovery. However, no studies so far have discovered compounds suitable for the clinical trials (Vandeputte et al., 2012). Azoles are chemically synthesized antifungal drugs that are intensively studied by pharmaceutical companies to improve their efficacy (Vandeputte et al., 2012). Isavuconazole is a novel second-generation triazole currently in phase III studies (Muñoz et al., 2010; Livermore and Hope, 2012). It is a new water-soluble triazole with extended spectrum and is available as an oral and intravenous formulation (Thompson III and Wiederhold, 2010; Livermore and Hope, 2012). Deoxymulundocandin which was isolated from *Aspergillus sydowii* is a new water-soluble echinocandin which showed potent *in vitro* and *in vivo* activity against *Candida* and *Aspergillus* spp. (Warn et al., 2010; Mishra and Tiwari, 2011). An ambruticin analog which interferes with the osmoregulatory system was found to be effective in both murine models of coccidioidomycosis and pulmonary aspergillosis (Chiang et al., 2006; Shubitz et al., 2006).

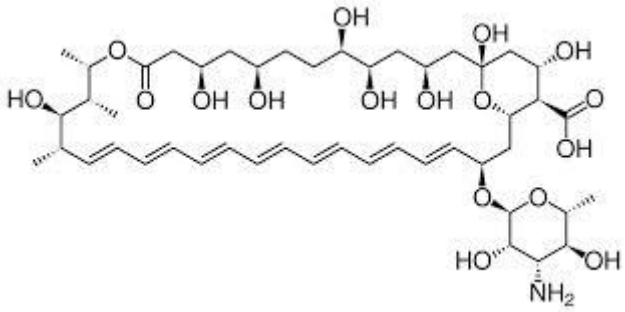
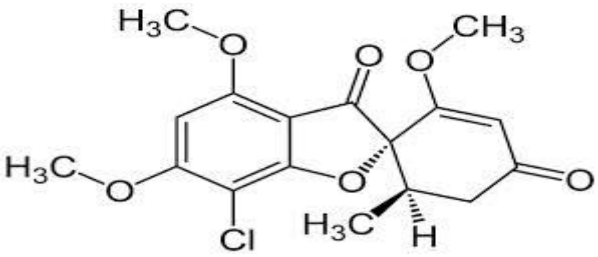
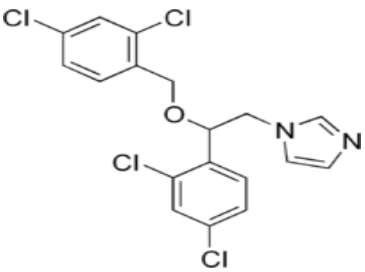
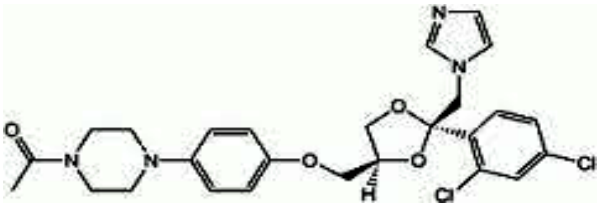
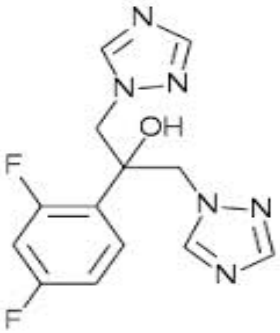
Year	Antifungal Agents	Compound Structure
1958	Amphotericin B	
1959	Griseofulvin	
1971	Flucytosine	
1979	Miconazole	
1981	Ketoconazole	
1990	Fluconazole	

Figure 2.5: Name and year of antifungal agents approved from year 1955 to 2010 (Lewis, 2011).

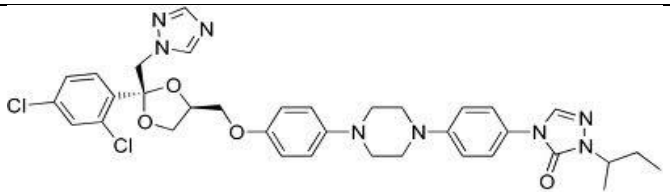
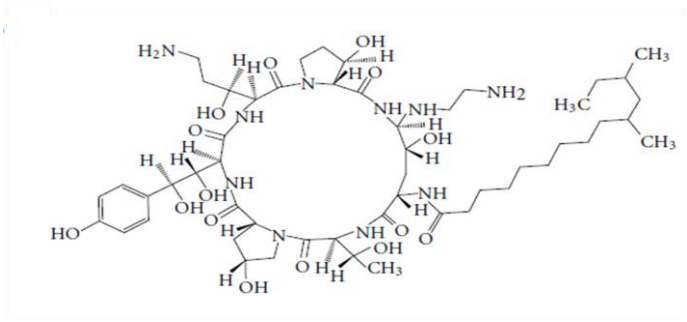
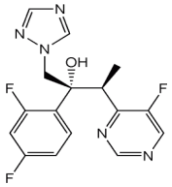
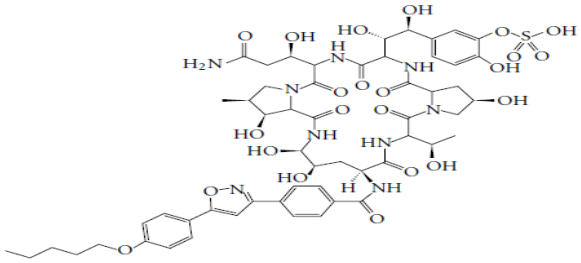
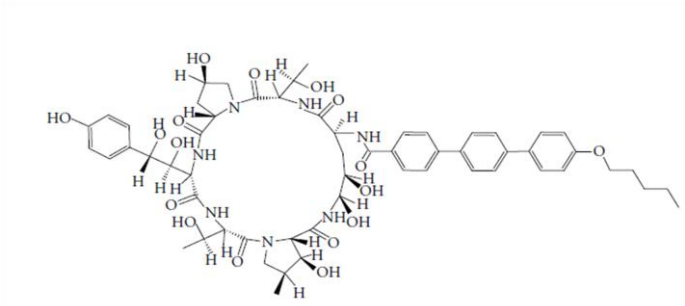
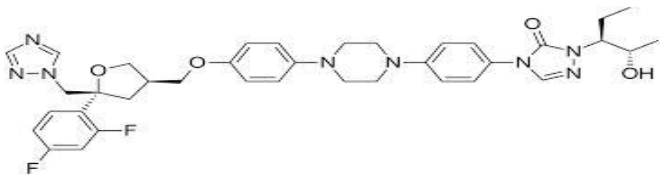
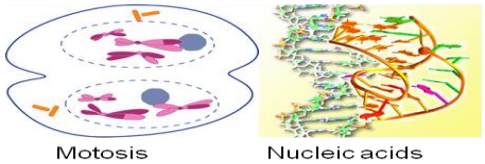
Year	Antifungal Agents	Compound Structure
1992	Itraconazole	
2001	Caspofungin	
2002	Voriconazole	
2005	Micafungin	
2006	Anidulafungin	
2006	Posaconazole	

Figure 2.5 continued

2.2.2 Mode of Action, Toxicities and Resistance of Antifungal Agents

Each class of antifungal drug has a specific mode of action, drug interactions and toxicity profile. Microorganisms have developed resistance mechanisms to counteract the effects of drugs. Figure 2.6 shows the mode of action and fungal resistance mechanisms of the major antifungal drugs. Figure 2.7 shows the toxicities of antifungal agents.

Resistance to antifungal drugs in the clinical setting is defined as the persistence or progression of the fungal infection despite the application of antifungal therapy (Rogers, 2006). The vast majority of clinical cases of antifungal resistance have been reported and certain fungal species are identified as inherited resistance to one or more antifungal agents (Sanglard, 2003). Examples of these are *Aspergillus terreus*, *Scedosporium apiospermum* and *Fusarium* spp. against amphotericin B; *I. orientalis* and *Aspergillus* spp. against fluconazole; *C. neoformans* and *Zygomycetes* against caspofungin (Rogers, 2006). Table 2.2 shows the applications of some antifungal agents for invasive fungal pathogens.

Site of action	Mode of action and cellular target	Antifungal agent (class)	Mechanism of resistance
Cell membrane 	Inhibition of 14-α-demethylase	Azoles	Efflux mediated by multidrug transporters Alteration in ergosterol biosynthetic pathway Mutations (decrease of affinity and in Erg11p and upregulation of ERG 11)
	Binding to ergosterol	Polyenes	Absence/ decrease of ergosterol content in cells
	Inhibition of squalene epoxidase	Allylamines	Unknown
Cell wall 	Inhibition of β-1,3 glucan synthesis	Echinocandins	Alteration of affinity of echinocandins for β-1,3 glucan synthase
Intracellular 	Nucleic acids synthesis and mitotic inhibitors	Pyrimidine analogs	Defect in cytosine permease Deficiency of enzymes implicated in the metabolism of the drug Deregulation of the pyrimidine biosynthetic pathway.

*A, FKS1; B, FKS2 (the putative target binding site of echinocandins are catalytic subunits of the glucan synthase complex); C, Rho (cell wall-regulating protein)

Figure 2.6: Mode of action and fungal resistance mechanisms of the major antifungal drugs used in clinical practice (Lewis, 2011; Vandeputte et al., 2012).

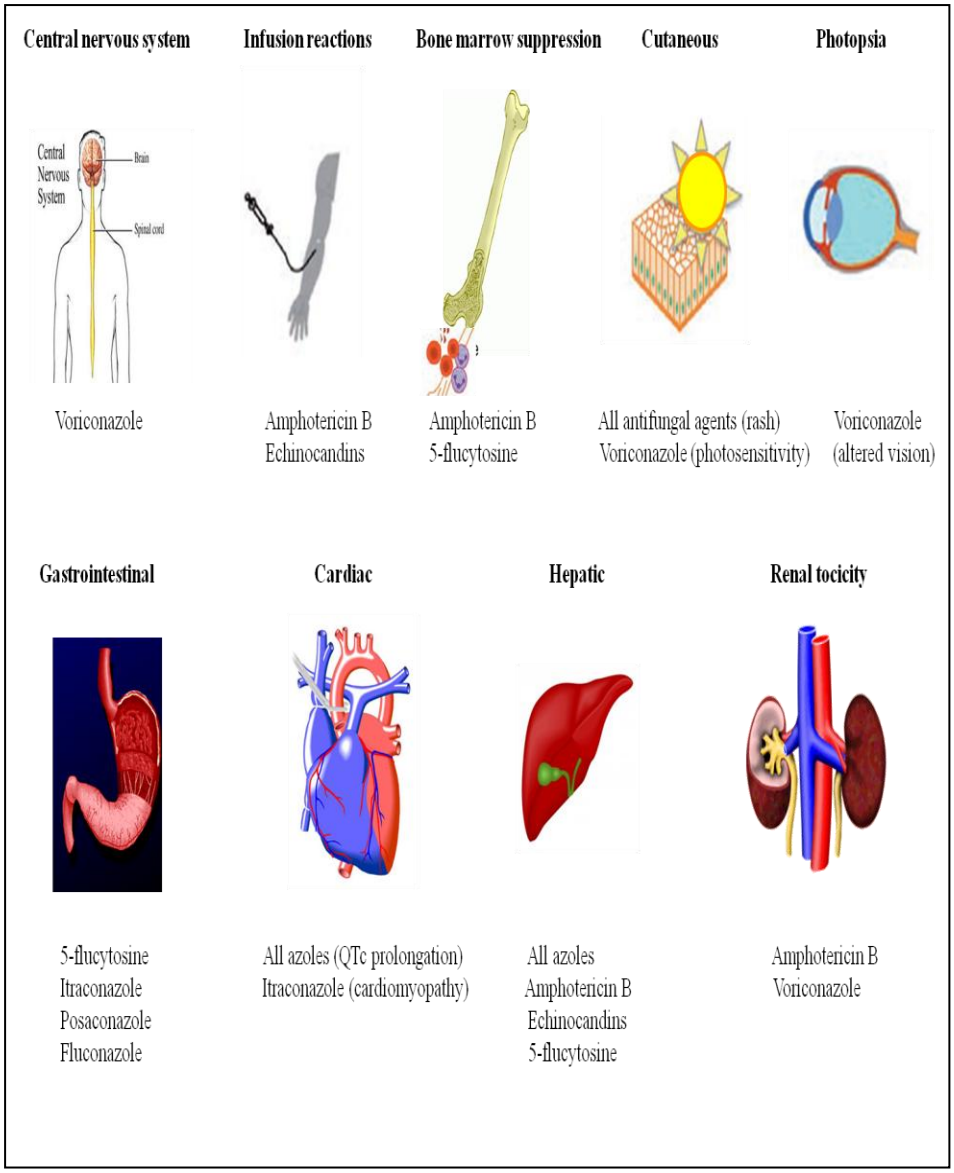


Figure 2.7: Toxicities of antifungal agents (Denning and Hope, 2010; Vandeputte et al., 2012).

Table 2.2: Relative *in vitro* and clinical activity of antifungal agents for more common and important invasive fungal pathogens[a] (Denning and Hope, 2010).

Species	**Polyenes**	**Triazoles**				**Echinocandins**		
	Amphotericin B	Fluconazole	Itraconazole	Voriconazole	Posaconazole	Caspofungin	Micafungin	Anidulafungin
Candida spp.								
C. albicans	++	++	++	++	++	++	++	++
C. glabrata	++	+/-	N	+	+	++	++	++
C. tropicalis	++	++	++	++	++	++	++	++
C. parapsilosis	++	++	++	++	++	+	+	+
C. krusei	+	-	+/-	+	+	++	++	++
C. neoformans	++	+	+	++	++	-	-	-
Trichosporon spp.	-	++	N	N	N	-	-	-
Aspergillus spp.								
A. fumigatus	++	-	++	++	++	+	+	N
A. flavus	++	-	++	++	++	+	+	N
A. terreus	-	-	++	++	++	+	+	N
A. niger	++	-	++	++	++	+	+	N
A. nidulans	-	-	++	++	++	+	+	N
Scedosporium prolificans	-	-	N	N	N	-	-	-
Scedosporium apiospermum	-	-	+/-	+	N	-	-	-
Fusarium spp.	+	-	-	+	+	-	-	-
Mucorales	+	-	+/-	-	+	-	-	-

[a]Most fungi causing cutaneous and allergic disease have been excluded.
Relative activity is indicated as follows: -, no activity; +/-, slight activity; +, modest activity; ++, good activity; N = no data.

2.3 Medicinal Plants

Observations on the use and efficacy of medicinal plants significantly contributed to the disclosure of their therapeutic properties (Silva and Fernandes, 2010). Pharmacognostic studies of medicinal plants led to the discovery of early drugs such as cocaine, atropine, hyoscine, codeine, digitoxin, quinine and morphine (Phillipson, 2001; Balunas and Kinghorn, 2005).

2.3.1 Discovery and Development of New Pharmaceuticals from Plants

Medicinal plants continue to play invaluable role in the drug discovery process (Brahmachari, 2009). A total of 20 natural products based drugs have undergone clinical evaluation and were approved for marketing worldwide in between the years of 2005 to 2010 (Table 2.3), among which four are isolated from plants (Mishra and Tiwari, 2011).

2.3.2 Antifungal Activities of Medicinal Plants

Medicinal plants represent a rich source of antimicrobial agents. A wide range of medicinal plant parts such as root, stem, flower, fruit, twigs, exudates and modified plant organs are used to treat common infectious diseases (Mahesh and Satish, 2008). Some of these plants have been screened scientifically for the antifungal activity of bioactive compounds as shown in Table 2.4.

Table 2.3: Natural products (NP) derived drugs from 2005 to 2010; lead compounds, classification, types of natural products, and therapeutic area (Mishra and Tiwari, 2011).

Year	Generic name (trade name)	Lead compound	Classification	Source of Natural products	Disease area
2005	Dronabinol /Cannabidol (Sativex®)	Dronabinol **1**/cannabidol **2**	NP	Plants	Pain
2005	Fumagillin (Flisint®)	Fumagillin **3**	NP	Microorganism	Antiparasitic
2005	Doripenem (Finibax®/Doribax®)	Thienamycin **4**	NP-derived	Microorganism	Antibacterial
2005	Tigecycline (Tygacil®)	Tetracycline **5**	Semi-synthetic NP	Microorganism	Antibacterial
2005	Ziconotide (Prialt®)	Ziconotide **6**	NP	Marine organism	Pain
2005	Zotarolimus (Endeavor^{TM}stent)	Sirolimus **7**	Semi-synthetic NP	Microorganism	Cardiovascular surgery
2006	Anidulafungin (Eraxis^{TM}/Ecalta^{TM})	Echinocandin B **8**	Semi-synthetic NP	Microorganism	Antifungal
2006	Exenatide (Byetta®)	Exenatide-4 **9**	NP	Animal	Diabetes
2007	Lisdexamfetamine (Vyvanse^{TM})	Amphetamine **10**	NP-derived	Plant	ADHD
2007	Retapamulin (Altabax^{TM}/Altargo^{TM})	Pleuromutilin **11**	Semi-synthetic NP	Microorganism	Antibacterial
2007	Temsirolimus (Torisel^{TM})	Sirolimus **7**	Semi-synthetic NP	Microorganism	Oncology
2007	Trabectedin (Yondelis^{TM})	Trabectedin **12**	NP	Marine organism	Oncology
2007	Ixabepilone (Ixempra^{TM})	Epothilone B **13**	Semi-synthetic NP	Microorganism	Oncology
2008	Methylnaltrexone (Relistor®)	Naltrexone **14**	Semi-synthetic NP	na	Pain
2009	Everolimus (Afinitor®)	Sirolimus **7**	Semi-synthetic NP	Microorganism	Oncology
2009	Telavancin (Vibativ^{TM})	Vancomycin **15**	Semi-synthetic NP	Microorganism	Antibacterial
2009	Romidepsin (Istodax®)	Romidepsin **16**	NP	Microorganism	Oncology
2009	Capsaicin (Qutenza®)	Capsaicin **17**	NP	Plant	Pain
2010	Monobactam aztreonam (Cayston^{TM})	Aztreonam **18**	Semi-synthetic NP	Microorganism	Antibacterial

All the structure of lead compounds is shown in Appendix A; "ADHD" indicates Attention-Deficit Hyperactivity Disorder; "na" denotes data not available.

Table 2.4: Medicinal plants and identified antifungal compounds (Cowan, 1999; Ahmad et al., 2006; Dzoyem et al., 2007; Endo et al., 2010; Hu et al., 2014).

Common name	Scientific name	Class of Compound	Compound
African ebony	*Diospyros crassiflora*	naphthoquinone	plumbagin
Allspice	*Pimenta dioica*	Essential oil	Eugenol
Apple	*Malus sylvestris*	Flavonoid derivative	Phloretin
Ashwagandha	*Withania somniferum*	Lactone	Withafarin A
Betel pepper	*Piper betel*	Essential oils	Catechols, eugenol
Black pepper	*Piper nigrum*	Alkaloid	Piperine
Brazilian pepper tree	*Schinus terebinthifolius*	Terpenoids	Terebinthone
Burdock	*Arctium lappa*	Polyacetylene, tannins, terpenoids	na
Buttercup	*Ranunculus bulbosus*	Lactone	Protoanemonin
Caraway	*Carum carvi*	Coumarins	na
Clove	*Syzygium aromaticum*	Terpenoid	Eugenol
Gamboge	*Garcinia hanburyi*	Resin	na
Garlic	*Allium sativum*	Sulfoxide, terpenoids	Allicin, ajoene
Glory lily	*Gloriosa superba*	Alkaloid	Colchicine
Grapefruit peel	*Citrus paradisa*	Terpenoid	na
Green tea	*Camellia sinensis*	Flavonoid	Catechin
Hops	*Humulus lupulus*	Phenolic acids, (Hemi) terpenoids	Lupulone, humulone
Horseradish	*Armoracia rusticana*	Terpenoids	na
Mountain tobacco	*Arnica montana*	Lactones	Helanins
Olive oil	*Olea europaea*	Aldehyde	Hexanal
Onion	*Allium cepa*	Sulfoxide	Allicin
Orange climber	*Toddalia asiatica*	Alkaloid	Benzo[*c*]phenanthridine, secobenzo[*c*]phenantridine
Orange peel	*Citrus sinensis*	Terpenoid	na
Peppermint	*Mentha piperita*	Terpenoid	Menthol
Periwinkle	*Vinca minor*	Alkaloid	Reserpine
Peyote	*Lophophora williamsii*	Alkaloid	Mescaline
Pomegranate peel	*Punica granatum*	Polyphenols	Punicalagin
Poppy	*Papaver somniferum*	Alkaloids	Opium
Purple prairie clover	*Petalostemum*	Flavonol	Petalostemumol
Rauvolfia, chandra	*Rauvolfia serpentina*	Alkaloid	Reserpine
Sacaca	*Croton cajucara*	Essential oil	Linalool
St. John's wort	*Hypericum perforatum*	Anthraquinone	Hypericin, others
Tree bard	*Podocarpus nagi*	Lactone	Negilactone
Winter savory	*Satureja montana*	Terpenoid	Carvacrol

"na" denotes data not available

2.3.3 Medicinal Plants under Investigation

Twelve species of medicinal plants from the family Araliaceae (*Hydrocotyle sibthorpioides*), Arecaceae (*Cocos nucifera*), Asteraceae (*Acmella ciliata*), Athyriaceae (*Diplazium esculentum*), Cucurbitaceae (*Sechium edule*), Fabaceae (*Parkia speciosa*), Nepenthaceae (*Nepenthes gracilis*), Orchidaceae (*Arundina graminifolia*), Passifloraceae (*Passiflora edulis*), Polygonaceae (*Muehlenbeckia platyclada*), Rubiaceae (*Diodia sarmentosa*) and Solanaceae (*Solanum muricatum*) were selected and screened for their antifungal properties. Figure 2.8 shows the pictures of the medicinal plants used in this study.

In Malaysia, these plants are commonly used either as food or folk remedies. The selected plant species are listed in Table 2.5 with their common name, plant part used, and the traditional therapeutic uses. A list of biochemical studies and the bioactive compounds isolated from the species selected for antifungal investigation are given in Table 2.6.

A, *Acmella ciliata* (Kunth) Cass.; **B**, *Hydrocotyle sibthorpioides* Lamk.; **C**, *Parkia speciosa* Hassk.; **D**, *Passiflora edulis* Sims.; **E**, *Arundina graminifolia* (D.Don) Hochr.; **F**, *Nepenthes gracilis* Korth.; **G**, *Muehlenbeckia platyclada* (F. Muell.) Meisn.; **H**, *Solanum muricatum* Ait.; **I**, *Cocos nucifera* L.; **J**, *Diodia sarmentosa* Sw.; **K**, *Sechium edule* (Jacq.) Swartz.; **L**, *Diplazium esculentum* (Retz) Sw.

Figure 2.8: Pictures of medicinal plants selected for the antifungal screening.

Table 2.5: The traditional therapeutic uses of plant species selected for antifungal investigation.

Plant Species	Common name	Part Used	Medicinal Usage	References
Acmella ciliata	Toothache plant	Leaves and flowers	To treat toothache, stomatitis, stammering, gum infections and throat complaints.	Holetz et al. (2002); Saraf and Dixit (2002).
		Aerial parts and roots	To treat inflammation and diarrhea.	
Arundina graminifolia	Bamboo orchid	Rhizomes	To treat body ache and bacterial infections.	Hossain (2011).
Cocos nucifera	Malayan dwarf' coconut palm	Flowers	To treat fever and promote urination.	Coconut Research Centre (2004).
		Juice	Applied to quench thirst, gangrenous ulcers, skin boils and to treat heart conditions, dysentery, fever, pain, digestive and bladder problems.	
		Oil	Used as antiseptic and soothing on skin problems.	
		Roots	Used as an infusion for sore throat gargles.	

Table 2.5 continued

Plant Species	Common name	Part Used	Medicinal Usage	References
Diodia sarmentosa	Tropical buttonweed	Aerial parts	To treat dysentery and used as a laxative and oxytocic agent, and also enhance erection and ejaculatory processes in the elderly males.	Onuaguluchi and Nwafor (1999); Soladoye et al. (2010).
Diplazium esculentum	Vegetable fern	Aerial parts	To treat expectoration of blood and coughs.	Roosita et al. (2008); Ong (2011).
		Rhizomes	To treat diarrhea, dysentery and coughs.	
		Leaves	To treat fever, dermatitis, measles, coughs and used as tonic by woman after childbirth.	
		Roots	To treat fever, dermatitis and used as a hair tonic.	
Hydrocotyle sibthorpioides	Lawn marshpennywort	Juice	Used as a diuretic and febrifuge and to treat typhoid fever.	Rai (2004); Yu et al. (2007); Au et al. (2008); Dangol (2008); Ong (2011).
		Leaves	To treat asthma, poulticing scrotal skin ailments.	
		Whole plant	To treat fever, dysentery, edema, detoxication, cardiovascular disorders, throat pain, colds, coughs, lymphadenitis, herpes zoster, acute hepatitis and cholecystitis.	
		Roots	To treat heart diseases.	

Table 2.5 continued

Plant Species	Common name	Part Used	Medicinal Usage	References
Muehlenbeckia platyclada	Centipede plant	Fruits and seeds	To treat oral ulceration.	Houghton and Manby (1985); Villegas et al. (1997); Siriwatanametanon et al. (2010); Susidarti et al. (2011).
		Sap	To treat wart.	
		Leaves and stems	Used for treatment of poisonous snake bites, bacterial infections, alleviating fever, detoxification, used as diuretic, antihaemorragic, sedative, antirheumatic, abortive, cicatrizing, antiulcerogenic, anti-inflammatory and antihelmintic agents.	
		Aerial parts	Applied externally for skin swelling, sore, and insect bites	
Nepenthes gracilis	Monkey cups	Fluid in unopened pitchers	Used as an eye-wash and to soothe inflamed skin.	Burkill (1966); Fan et al. (2010).
		Leaves and roots	Used as an astringent.	
Parkia speciosa	Stink bean	Leaves	To treat dermatitis and jaundice.	Ong (2008); Roosita et al. (2008).
		Seeds	Used in the treatment of diabetes, kidney pain, hepatalgia, edema, nephritis, cholera, hypertension, and as an antihelmintic.	

Table 2.5 continued

Plant Species	Common name	Part Used	Medicinal Usage	References
Passiflora edulis	Passion fruit	Fruits	Used as a heart tonic, stimulant, mild diuretic, digestive stimulant, a treatment for urinary infections and constipation.	Zibadi and Watson (2004); Ripa et al. (2009); Sunitha and Devaki (2009).
		Flowers	To treat asthma, bronchitis and whooping cough.	
		Leaves	To treat epilepsy, ulcers, hemorrhoids and also symptoms of alcoholism, anxiety, migraine, nervousness and insomnia.	
Sechium edule	Chayote	Leaves	To treat fever, arteriosclerosis, calcifications in the urinary system and hypertension.	Ong (2008); Roosita et al. (2008).
		Fruits and leaves	To relieve urine retention and burning during urination or to dissolve kidney stones.	
		Fruits	To treat hypertension, pulmonary ailments, used as a diuretic, poulticed on inflammations and wounds.	
		Seeds	To treat intestinal inflammations.	
		Roots	Used as a diuretic, to treat pulmonary ailments.	
Solanum muricatum	Melon Pear	Fruits	Consume as to quench thirst, refreshing and used as a diuretic, to treat hypotension.	Redgwell and Turner (1986); Sánchez-Vega (1994).

Table 2.6: Biochemical studies and bioactive compounds of the plants under investigation.

Plant Species	Biochemical Studies	Isolated Bioactive Compounds	References
Acmella ciliata	Antibacterial, antioxidant, cytotoxic activities of the aerial parts extracts.	Vanillic acid, trans-ferulic acid, trans-isoferulic acid, scopoletin, 3-acetylaleuritolic acid, β-sitostenone, stigmasterol, stigmasteryl-3-*O*-β-D-glucopyranosides and β-sitosteryl-3-*O*-β-D-glucopyranosides	Prachayasittikul et al. (2009)
	Antifungal (yeast) and antibacterial activities of the leaves extracts.	na	Holetz et al. (2002)
	Ovicidal, larvicidal and pupicidal activity in *Anopheles culicifacies* Giles, *Culex quinquefaciatus* Say, and *Aedes aegypti* Linn of the flowers extract.	Spilanthol	Saraf and Dixit (2002)
Arundina graminifolia	Antitumor activity of the tuber extract.	2,7-dihydroxy-1-(*p*-hydroxylbenzyl)-4-methoxy-9, 10-dihydrophenanthrene, 4,7-dihydroxy-1-(*p*-hydroxylbenzyl)-2-methoxy-9, 10-dihydrophenanthrene, 3,3′-dihydroxy-5-methoxybibenzyl, (2*E*)-2- propenoic acid-3-(4-hydroxy-3-methoxyphenyl)-tetracosyl ester, (2*E*)-2-propenoic acid-3-(4-hydroxy-3-methoxyphenyl)-pentacosyl ester and pentadecyl acid	Liu et al. (2012)

Table 2.6 continued

Plant Species	Biochemical Studies	Isolated Bioactive Compounds	References
Cocos nucifera	Antibacterial, antiviral, antileishmanial, antinociceptive and free radical scavenging properties of the husk fiber extract.	Polyphenolic compounds (catechin and epicatechin) together with condensed tannins (B-type procyanidins)	Esquenazi et al. (2002); Alviano et al. (2004); Mendonça-Filho et al. (2004)
	Antioxidant and antimicrobial properties of the mesocarp extract.	Chlorogenic acid, dicaffeoylquinic acid, caffeoylshikimic acids	Chakraborty and Mitra (2008)
Diodia sarmentosa	Anti-ulcerogenic properties of the whole plant extracts.	na	Akah et al. (1998)
Diplazium esculentum	Antioxidant, antimicrobial and cytotoxic (cancer cell lines) activities of the leaves extracts.	na	Mackeen et al. (1997); Wong et al. (2006); Zakaria et al. (2010); Sakunpak and Panichayapukaranant (2012)
	Antioxidant and cytotoxicity activities (cancer cell lines) of the shoots extracts.	na	Rahmat et al. (2004)

Table 2.6 continued

Plant Species	Biochemical Studies	Isolated Bioactive Compounds	References
Hydrocotyle sibthorpioides	Antioxidant and antiproliferative activities of whole plant extracts.	na	Huang et al. (2008b)
	Antitumor effect of whole plant extract on mice.	2-ethylacridine, 2 methyl-3-*O*-tolyl-6-hydroxy-4(3H)-quinazolinone, 3-(-4-hydroxy-methylphenyl)-2-methyl-4(3H)-quinazolinone, 9,10-dihydro-9,9,10-trimethyl-anthracene and demecolcine	Yu et al. (2007)
	Cytotoxicity activity against human tumor cell lines of whole plant extract.	Oleanane-type triterpene saponin	Huang et al. (2008a)
Muehlenbeckia platyclada	Anti-inflammatory, anticancer and antioxidant activities of the aerial parts extracts.	na	Yen et al. (2009); Siriwatanametanon et al. (2010)
	Antinociceptive and anti-inflammatory activities of the leaves extract.	Flavanol glycosides (morin-3-*O*-α-rhamnopyranoside, flavonols, kaempferol 3-*O*-α-rhamnopyranoside, kaempferol 3-*O*-β -glucopyranoside, quercetin 3-*O*-α-rhamnopyranoside and (+)- catechin)	Fagundes et al. (2010)
	Antibacterial activity of the leaves extracts.	Quercetin (3,3′,4′,5,7-pentahydroxyflavone)	Susidarti et al. (2011)
	Spermicidal effect of the plant extracts.	na	Gallego et al. (2012)

Table 2.6 continued

Plant Species	Biochemical Studies	Isolated Bioactive Compounds	References
Nepenthes gracilis	na	na	na
Parkia speciosa	Antibacterial activity of the seeds extracts.	na	Sakunpak and Panichayapukaranant (2012)
	Antidiabetic activity of the seeds extracts	na	Ali et al. (2006)
	Hypoglycemic effect of the seeds extract.	β-sitosterol and stigmasterol	Jamaluddin et al. (1994)
	Hemagglutinating activity of proteins isolated from the seeds extract.	Hypothetical proteins	Chankhamjon et al. (2010)
	Mitogenic activity of seeds extract on human lymphocytes.	Lectin	Suvachittanont and Jaranchavanapet (2000)
	Antioxidant activity of the leaves extract.	na	Razab and Aziz (2010)
	Antioxidant activity of the shoots and fruits extracts.	Polyphenolics	Wong et al. (2006)
	Antiangiogenic and antioxidant properties of the empty pods extracts.	Phenols	Aisha et al. (2012)
	Hypoglycemic effect of the pods extracts	β-sitosterol and stigmasterol	Jamaluddin et al. (1995)

Table 2.6 continued

Plant Species	Biochemical Studies	Isolated Bioactive Compounds	References
Passiflora edulis	Inhibition activity of the fruit decoction on enzymes that involved in tumor invasion, metastasis and angiogenesis.	na	Puricelli et al. (2003)
	Antibacterial, cytotoxic and antioxidant activities of the leaves and stems extracts.	na	Ripa et al. (2009)
	Anxiolytic effect of the leaves extracts.	na	Petry et al. (2001)
	Antioxidant and antiglycation properties of the leaves extracts.	na	Rudnicki et al. (2007); Sunitha and Devaki (2009).
Sechium edule	Antioxidant activity of the leaves, stem and seed extracts.	na	Ordoñez et al. (2006)
	Antibacterial, antifungal and antioxidant activities of the leaves extracts.	luteolin-7-*O*-glucoside	Ordoñez et al. (2009)
	Cytotoxicity, mutagenicity and antimutagenicity of the plant extracts.	na	Yen et al. (2001)

Table 2.6 continued

Plant Species	Biochemical Studies	Isolated Bioactive Compounds	References
Solanum muricatum	Antioxidative, anti-inflammatory and antiglycative effects of the fruits extracts.	Ascorbic acid, phenolic acids and flavonoids	Hsu et al. (2011)
	Antioxidant activity of the fruits extract.	Polyphenols	Sudha et al. (2011)
	Antitumor activity of the fruits extract.	na	Ren and Tang (1999)

"na" denotes data not available

2.4 Antimicrobial Susceptibility Testing

Generally, *in vitro* susceptibility tests are meant to provide a correlation between *in vitro* activity and therapeutic outcome, a measure of the relative activities of antimicrobial agents, and a mean to monitor the development of drug resistance (Liao and Dunne, 2003). Antimicrobial susceptibility test (AST) is commonly used for the detection of potential antimicrobials from biological extracts against different microorganisms (Ncube et al., 2008).

AST standard tests fall into two groups, i.e. diffusion and dilution methods. Diffusion methods include agar well diffusion, agar disc diffusion and bioautography which are known as qualitative techniques that will provide an indication of the presence or absence of bioactive compounds (Valgas et al., 2007). The dilution methods include agar dilution, broth microdilution and broth macrodilution which are considered as quantitative assays to distinguish between microbiostatic and microbicidal effects; and generate quantitative results with the minimum inhibitory concentration (MIC) values (Valgas et al., 2007; Ncube et al., 2008).

There are other commercial custom-prepared methods such as the agar screen plate, Epsilometer test (Etest), Sensititre YeastOne colorimetric test and the Vitek system which is used for antimicrobial sensitivity determination especially when the MIC value is needed. However these commercially

available kits are not commonly used for routine AST and testing activity of plant extracts (Joyce et al., 1992; Ncube et al., 2008).

Broth microdilution method has become the most popular susceptibility testing as the miniaturization and mechanization afforded by the use of disposable microplates gives reproducible results, requires small quantities of sample, can be used for examining multiple concentrations and large number of samples simultaneously (Eloff, 1998; Jorgensen and Ferraro, 1998). End-point determination of broth microdilution method uses colorimetric indicator or spectrophotometry to facilitate inspection of each of the wells for evidence of growth. A potential drawback of the spectrophotometric end-point determination, however, is that the application demands agitation of the plates to ensure homogeneity in the wells before reading, which could affect the reproducibility (Liu et al., 2007). The use of colorimetric indicators (tetrazolium salts or resazurin dye) requires less instrumentation and avoids the ambiguity associated with visual comparison (Liu et al., 2007; Ncube et al., 2008).

2.5 Purification, Isolation and Structural Characterization Techniques

Plant bioactive compounds of interest are usually isolated following a fractionation procedure. Bioactivity-guided fractionation techniques are often used as an option to isolate bioactive compounds in the systematic drug

development programs from natural resources in multinational drug companies (Jantan, 2004).

The complex composition of plant extracts is generally a limiting obstacle to the isolation of bioactive compounds (Ahmad et al., 2006). However, bioactivity-guided fractionation permits the detection of active compounds which responsible for the targeted biological activity in the crude plant extract (Jantan, 2004). The complexity of matrix can be reduced with pretreatment sample preparation steps (separation, purification and concentration) such as liquid-liquid partition, solid phase extraction, and preparative thin layer chromatography (PTLC) or thin layer chromatography (TLC) (Harborne, 1998; Waksmundzka-Hajnos et al., 2008). Complete identification of bioactive compounds depends on the measuring of its properties include melting points and spectral characteristics such as ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR) and mass spectral (MS) (Harborne, 1998).

In modern pharmacopoeias, advances in separations technology are associated with high performance chromatography methodologies such as the one or two dimensional thin layer chromatography, high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) (Foucault, 1995; Waksmundzka-Hajnos et al., 2008). TLC techniques are applied for qualitative analysis of organic compounds, isolation of pure compound, quantitative analysis and preparative-

scale isolation (Waksmundzka-Hajnos et al., 2008). The era of using digital photography for TLC documentation has facilitated the production of high resolution TLC image and capable to record weak fluorescence (Hahn-Deinstrop, 2007). Direct optical evaluation of TLC enables quantitative results to be obtained through the densitometry created by using a TLC scanner (Hahn-Deinstrop, 2007).

Advances in the structure elucidation technology further expanded the capabilities for structural identification and characterization. Hyphenated techniques such as HPLC coupled to photodiode array detection and to mass spectrometry is a strategic tool to provide on-line structural information (Waksmundzka-Hajnos et al., 2008). The acquisition of high field NMR with two dimensional NMR experiments such Nuclear Overhauser Effect Spectroscopy (NOESY), Heteronuclear Multiple Quantum Correlation (HMQC), Heteronuclear Multiple Bond Correlation (HMBC) and *J*-modulated, liquid chromatography-mass spectrometry (LC-MS) coupled with mass analyzers such as triple quadrupole, ion trap and quadrupole time-of-flight (Q-TOF), Fourier transform infrared spectroscopy (FT-IR), gas chromatography-mass spectrometry (GC-MS) and X-ray crystallography allow rapid and straightforward assignment of structure to complex natural products (Jantan, 2004; McChesney et al., 2007).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

Following chemicals and reagents were purchased from the manufacturers as listed in Table 3.1.

Table 3.1: Chemicals and reagents used in the study.

Chemical/ reagents	Manufacturers
Acetone, hexane (AR grade)	Mallinckrodt Chemicals, USA
Acetonitrile, dimethyl sulphoxide (DMSO), ethyl acetate, hexane and methanol (HPLC grade), deuterated methanol-d_4, potassium bromide (KBr), potato dextrose agar (PDA), potato dextrose broth (PDB) and sodium hydroxide pellets	Merck, Germany
Chloroform, ethyl acetate (AR grade)	System, USA
Ethanol, methanol, glacial acetic acid (AR grade)	Prochem, USA
Glycerol (AR grade)	GeneChem, Canada
p-iodonitrotetrazolium violet, amphotericin B, Dulbecco's modified eagle medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), neutral red solution (3.3 g/L in DPBS), fetal bovine serum (FBS), penicillin-streptomycin solution (100x), trypsin-EDTA solution (1x), sodium bicarbonate, sodium chloride	Sigma-Aldrich, USA
RPMI-1640 medium supplemented with glutamine and phenol red, without bicarbonate	MP Biomedicals, France
Tween 20	Amresco, USA
3-(*N*-morpholino) propanesulfonic acid (MOPS)	Calbiochem, Germany

*****AR, Analytical reagent

3.1.2 Instruments and Labwares

The instruments and labwares used and their brands are shown in Table 3.2.

Table 3.2: Instruments, labwares and their brands.

Instruments/ Labwares	Brand
Autoclave machine	HICIAVE Hvt-50
Bunsen burner	Campinguz
Bar blender	Waring Commercial
Disposable syringes (1 mL/3 mL)	Terumo
Electronic balance	Adventurer Pro AV 812
Micropipette tips	Axygen scientific
Centrifuge tube (5 mL/15 mL/50 mL)	BD Bioscience
Freeze-dryer	Martin Christ Alpha 1-4, LD plus
Fourier transform infrared (FT-IR) spectrophotometer	Perkin-Elmer Spectrum RX1
Gas chromatography-mass spectrometer (GC-MS)	Shimadzu, QP2010 Plus
GC-MS capillary column (0.25 mm x 30 m)	SGE, BPX5
High performance liquid chromatography (HPLC)	Shimadzu, Prominence UFLC 20AD
HPLC injection syringe	Hamilton
HPLC RP-18 column, HPTLC, TLC & PLC plates	Merck
Incubator	Memmert
Laminar air flow	Edamix series
Liquid chromatography-Tandem mass spectrometer	AB Sciex
Melting point apparatus	Stuart, SMP 10
Micropipette (100-1000 µL)	ViPR, Treff Lab
Microplate reader	Tecan Infinite, M200
Multi-channel pipette (20-200 µL)	Transferpette®
Nuclear magnetic resonance (NMR) spectrometer	JEOL JNM-ECX 400
NMR tubes (400 mHz, 178 mm length)	Kimble Chase
Orbital shaker	IKA- Werke KS 501, Gainson HZQ
Pasteur pipettes, GC-MS auto sampler vials (1.5 mL)	Fisher Scientific
Petri dish,U-shaped/ flat bottom microplate	Greiner bio-one
pH meter	Sartorius
Rotary evaporator set	BÜCHI Rota-vapor R205
Sample vial (soda glass specimen tube)	SAMCO
Sonicator	S 100H Elmasonic
Spectrophotometer	Thermo Scientific, Genesys 20
Sterile collection swab	Pan Mate
Stirrer	Harmony HTS-1003
Syringe filter nylon membrane (0.45 µm), Cellulose acetate filter membrane (0.20 µm)	Sartorius, Minisart
TLC cutter, TLC double-trough chamber, TLC semi-automatic sample applicator, TLC scanner, TLC visualizer	CAMAG
Ultra low temperature freezer	Haier
Vacuum concentrator	Eppendorf, Concentrator plus
Vortex mixer	VELP® SCIENTIFICA

3.1.3 Plant Materials

There were a total of 12 species of medicinal plants being selected and screened for their antifungal properties. These medicinal plants were selected based on their traditional therapeutic uses as antimicrobial agents and scientific studies on their antifungal activities are limited. The enthnopharmacological uses of certain part of these plants as antifungal agents are listed in Table 2.5. The extracts of eight species of medicinal plants were provided in this study as cited in Appendix B. The identification of the species was confirmed by Professor Ong Hean Chooi, an ethnobotanist of the University of Malaya, Malaysia. The plant names with the parts investigated, voucher specimen number, collection date, location and weight of the plants are shown in Table 3.3. Voucher specimens of the plants were deposited at Faculty of Science, Universiti Tunku Abdul Rahman, Perak Campus. Labels with information about the plants were placed at the back of the herbarium sheet (Appendix C).

3.1.4 Fungal Strains

The fungal strains that were used in this study were yeasts (*Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019, *Issatchenkia orientalis* ATCC 6258, *Cryptococcus neoformans* ATCC 90112) and molds (*Aspergillus brasiliensis* ATCC 16404 and *Trichophyton mentagrophytes* ATCC 9533). All fungal strains used were purchased from American Type Culture Collection. The microorganisms were maintained on potato dextrose agar (PDA) at 4°C for short term storage. The freezing technique for long term storage as described by Larone (2002) was applied (Appendix D).

Table 3.3: Plant samples with their voucher specimen number, date of collection, location, and weight.

Plant Samples (Part of plant investigated)	Voucher Number	Date	Location of plant collection	Fresh Weight (g)
Acmella ciliata (Kunth) Cass. (leaves)	UTAR/FSC/08/001	Oct 2009	Sungai Buloh, Selangor.	100
Arundina graminifolia (D. Don) Hochr. (leaves)	UTAR/FSC/10/011	May 2010	Cameron Highlands, Pahang.	370
Cocos nucifera L. (leaves)	UTAR/FSC/08/010	Oct 2008	Setapak, Wilayah Persekutuan Kuala Lumpur.	600
Diodia sarmentosa Sw. (aerial parts)	UTAR/FSC/10/018	Oct 2010	Nibong Tebal, Penang.	800
Diplazium esculentum (Retz.) Sw. (aerial parts)	UTAR/FSC/10/023	Nov 2010	Kampar, Perak.	1214
Hydrocotyle sibthorpioides Lamk. (whole plant)	UTAR/FSC/10/019	Oct 2010	Klang, Selangor.	1000
Muehlenbeckia platyclada (F. Muell.) (stems)	UTAR/FSC/10/017	Oct 2010	Cameron Highlands, Pahang.	535
Nepenthes gracilis Korth. (leaves)	UTAR/FSC/10/016	Sep 2010	Cameron Highlands, Pahang.	237
Parkia speciosa Hassk. (pods)	UTAR/FSC/10/015	Sep 2010	Cameron Highlands, Pahang.	1000
Passiflora edulis Sims. (whole fruit without the peel)	NA	Nov 2008	Sungai Siput, Perak.	890
Sechium edule (Jacq.) Swartz. (aerial parts)	UTAR/FSC/10/022	Oct 2010	Cameron Highlands, Pahang.	1200
Solanum muricatum Ait. (whole fruit without the seeds)	NA	Sep 2010	Cameron Highlands, Pahang.	1000

*NA: No voucher specimen prepared for fruit samples (*P. edulis* and *S. muricatum*)

3.1.5 Cell Line

The Rhesus monkey kidney epithelial cell line (LLC-MK2) was a gift from Professor Shamala Devi (Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia). LLC-MK2 was propagated in Dulbecco's modified eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2. For maintenance medium, the FBS concentration was reduced to 1%.

3.2 Extraction of Plant Materials

The sequential extraction method as described by Ncube et al. (2008) was applied with modifications. The plant samples were washed thoroughly under running tap water to remove dirt, dust and insects. The fresh samples were weighed and cut into small pieces, blended and sequentially extracted using hexane, chloroform, ethyl acetate, ethanol, methanol and water at room temperature with agitation (120 rpm) using an orbital shaker. The plant materials were extracted in two cycles per each solvent, which the first cycle being two days and second cycle being one day. The solvents were filtered and evaporated using a rotary evaporator at 40°C with the pressure set according to the type of solvent used (Table 3.4). After the evaporation process, the extracts were transferred into vials and were dried in a vacuum concentrator at 37°C until constant weight measurements were obtained. Meanwhile, aqueous extracts were lyophilized by freeze-dryer. The extracts were stored in the freezer at -20^{o}C prior use. Dry weights of each extract were recorded and percentage of yield was calculated using the formula below.

$$\text{Percentage of yield of each extract (\%)} = \frac{\text{Dry weight of extract obtained}}{\text{Weight of fresh plant material used}} \text{ x } 100\%$$

Table 3.4: Pressure applied for each solvent during rotary evaporation

Solvent	Pressure (mbar)
Hexane	335
Chloroform	474
Ethyl acetate	240
Ethanol	175
Methanol	337

3.3 Medium, Reagents, Plant Extracts and Fungal Inocula Preparation

3.3.1 Preparation of Broth Medium and Reagents

The preparation of broth medium and reagents were based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2002a; CLSI, 2002b). The method is shown in Appendix D.

3.3.2 Preparation of Plant Extracts

The samples were dissolved in methanol:water mixture (2:1, v/v) at a concentration of 10 mg/mL for the crude extracts, 1 mg/mL for the fractions and 0.5 mg/mL and 2.5 mg/mL for the isolated compound during the antifungal and cytotoxicity assay, respectively. The extracts were sonicated for 30 min at 30^{o}C, then filtered through 0.45 µm syringe filters and stored at -20°C prior to analyses.

3.3.3 Preparation of Fungal Inocula

The preparation of inoculum suspensions were based on CLSI guidelines (CLSI, 2002a; CLSI, 2002b). Inoculum suspensions were prepared from fresh, mature cultures grown on PDA. Table 3.5 summarizes the incubation period, incubation temperature, absorbance range, cell initial and final working densities for the respective fungi during the colorimetric broth microdilution assay. The yeast inocula were prepared by transferring several colonies of yeast into a sterile round bottom tube which contained 3 mL of saline solution. Inoculum suspensions of the mold species were prepared by covering the fungal colonies with 3 mL of saline solution for *T. mentagrophytes* and saline solution supplemented with 0.1% Tween 20 (wetting agent) for *A. brasiliensis.*

The mold suspensions were prepared by gently probing the surface with a sterile loop. The resulting mixture of conidia and hyphal fragments was transferred to a sterile round bottom tube. Both yeast and mold suspensions were then mixed by using a vortex mixer at 25 Hz for 15 s. Heavy particles in the mold suspensions were allowed to sediment for 10 to 15 min. The upper homogeneous suspensions were collected and transferred into a sterile round bottom tube. The densities of these suspensions (yeast and mold) were subsequently adjusted to the optimal absorbance (A) at 530 nm and further dilutions in RPMI broth medium were performed to obtain the final working inocula (Table 3.5).

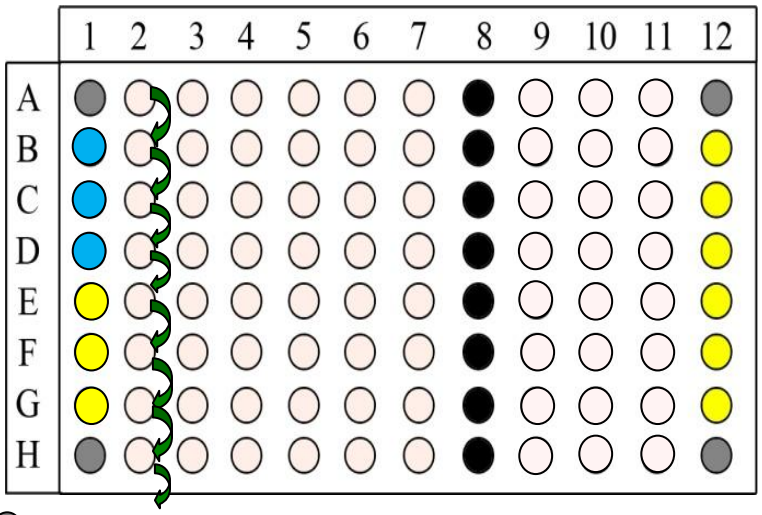
Table 3.5: Fungal inocula preparation conditions for the respective fungi (CLSI, 2002a; CLSI, 2002b).

Fungi	Incubation period (days)	Incubation Temperature (°C)	Absorbance range (A) at 530 nm	Cell initial densities (CFU/mL)	Cell final working densities (CFU/mL)
Candida spp.	2	35	0.12-0.15	1-5 x 10^6	1-5 x 10^3
C. neoformans	3	35	0.12-0.15	1-5 x 10^6	1-5 x 10^4
A. brasiliensis	3	35	0.09-0.11	0.4-5 x 10^6	0.4-5 x 10^4
T. mentagrophytes	7	28	0.15-0.18	1.2-6 x 10^6	1.2-6 x 10^4

3.4 Antifungal Assay

3.4.1 Determination of Minimum Inhibitory Concentration (MIC)

The colorimetric broth microdilution method of Eloff (1998) was modified and employed for the antifungal screening of plant extracts. This test utilized two-fold descending concentrations of extracts and a reference antibiotic in 96-well microplate (U-shaped), with concentrations ranging from 0.02 to 2.5 mg/mL for the plant extracts, 0.002 to 0.25 mg/mL for the fractions, 0.98 to 125 µg/mL for the isolated compounds, 0.063 to 8 µg/mL for amphotericin B and 0.2% to 25% (v/v) for methanol. The toxicity of methanol towards the fungi was assessed to ensure that the diluents were applied at the non-toxic level during the bioassays. Growth, sterility and negative controls were included (Figure 3.1). One hundred microliters of broth medium was added into the four corner wells as sterility controls. Negative control was prepared by adding 25 µL of extract and 75 µL of broth medium. Growth control contained 50 µL of broth medium and 50 µL of fungal inocula.



[Crude Extract][a]	[Fraction][b]	[Isolated Compound][c]	[Diluents][d]
2.50 mg/mL	0.250 mg/mL	125.00 µg/mL	25.00%
1.25 mg/mL	0.125 mg/mL	62.50 µg/mL	12.50%
0.63 mg/mL	0.063 mg/mL	31.25 µg/mL	6.25%
0.31 mg/mL	0.031 mg/mL	15.63 µg/mL	3.13%
0.16 mg/mL	0.016 mg/mL	7.81 µg/mL	1.56%
0.08 mg/mL	0.008 mg/mL	3.90 µg/mL	0.78%
0.04 mg/mL	0.004 mg/mL	1.95 µg/mL	0.39%
0.02 mg/ mL	0.002 mg/mL	0.98 µg/mL	0.20%

Sterility control (100 µL RPMI broth medium)
Growth control (50 µL RPMI broth medium + 50 µL working inoculum suspension)
Negative control (75 µL RPMI broth medium + 25 µL extract or methanol)
Positive control (50 µL serial two-fold dilution of amphotericin B + 50 µL working inoculum suspension)
Tested extracts or methanol (50 µL serial two fold dilution of extract or methanol + 50 µL working inoculum suspension)
Serial two-fold dilution by multichannel pipette (Column 2-11)
a Tested concentration ranges of crude extracts (hexane, chloroform, ethyl acetate, ethanol, methanol & aqueous extract)
b Tested concentration ranges of fractions (fraction 1, fraction 2 & fraction 3)
c Tested concentration ranges of isolated compounds (band 1 & band 2)
d Tested concentration ranges of diluent (plant extract diluent: methanol: water, 2:1, v/v)

Figure 3.1: An overall picture of a 96-well microplate preparation for the colorimetric broth microdilution assay.

The 96-well microplates were covered with lids and agitated at 60 rpm for 10 min using an orbital shaker. The 96-well microplates were incubated in an aerobic environment stacked no more than four plates high at 35°C for 48 h for *Candida* spp.; 72 h for *C. neoformans* and *A. brasiliensis*; and at 28°C for 7 days for *T. mentagrophytes.* After incubation, 20 µL of INT (0.40 mg/mL) was added and growth was determined by the formation of the dense insoluble red formazan crystals through visual assessment. The fungal growth inhibition was indicated by the absence of the dense insoluble red formazan crystals in the testing well. The lowest concentration of the extract at which no formation of the dense insoluble red formazan crystals was recorded as the MIC value. The tests were performed in triplicates. Mean or range values were recorded. Total activity, percent activity and fungal susceptibility index (FSI) were calculated as the equations shown below **(**Bonjar, 2004; Eloff, 2004).

$$\text{Percent activity (\%)} = \frac{\text{Number of susceptible fungal strains to a specific extract}}{\text{Total number of tested fungal strains}} \text{ x } 100\%$$

$$\text{Total activity (mL/g)} = \frac{\text{Quantity of material extracted from 1 g of plant material}}{\text{Minimum inhibitory concentration } (\frac{\text{g}}{\text{mL}})}$$

$$\text{FSI (\%)} = \frac{\text{Number of extracts effective against each fungal strain}}{\text{Total number of extracts}} \text{ x } 100\%$$

3.4.2 Determination of Minimum Fungicidal Concentration (MFC)

The MFC was obtained by inoculating 20 µL of the preparation that showed no evidence of growth in the MIC determination assays onto PDA and spread by using a sterile cotton swab. The lowest concentration at which growth was not observed was recorded as the MFC value. The tests were performed in triplicates.

3.5 Isolation of Bioactive Compounds

Among 72 extracts obtained from 12 species of medicinal plants, the most active extract was the hexane extract of *N. gracilis.* Bioactivity-guided fractionation was performed using liquid-liquid partitioning technique and preparative thin layer chromatography (PTLC) in order to isolate the antifungal compound(s). The collected fractions were subjected to the colorimetric broth microdilution assay to keep track of the bioactive compounds. *C. neoformans* and *T. mentagrophytes* were selected as indicative microorganisms, representing the yeast and mold strains, respectively, during the bioactivity-guided fractionation.

3.5.1 Liquid-liquid Partitioning of the Active Extract from *N. gracilis*

The hexane extract (1.12 g) of *N. gracilis* was dissolved in 150 mL of hexane and partitioned with an equal volume of water. The mixture was shaken vigorously in a 500 mL separating funnel and two distinct layers were formed after 30 min. Each partition was performed thrice. The water layer was

extracted using ethyl acetate. The collected hexane and ethyl acetate fractions were evaporated to dryness using a rotary evaporator while the water fraction was lyophilized. These yielded three fractions, i.e., hexane fraction (F_1), ethyl acetate fraction (F_2), and water fraction (F_3). The fractions were re-dissolved in a mixture of methanol:water (2:1, v/v) for the antifungal screening.

3.5.2 Isolation and Purification of the Active Compound from *N. gracilis*

The hexane fraction (F_1) exhibited the strongest antifungal activity and was further purified by PTLC using hexane: ethyl acetate (9:1, v/v) as the mobile phase. F_1 was redissolved in hexane (2 mg/mL) and applied as a thin even layer on a 10x10 cm PTLC plate (Kieselgel 60 F_{254}, 1 mm, glass-backed) by a semi-automatic sample applicator (volume: 190 µL, speed: 250 nL/s, spray gas: nitrogen gas).

The development of the PTLC plate was performed at room temperature. The solvent front was set at 8 cm. Following chromatographic development, the solvent was removed from PTLC plate by evaporation in the fume hood for 5 min. Each band was scraped off with the packing material (silica gel 60 F_{254}) by using a spatula. The resulted silica powder was extracted twice with sterile distilled water followed by partition twice with equal volume of hexane. The hexane layer obtained was evaporated using a rotary evaporator and the band was screened for antifungal activity. After recovery of the most active band (B_1), further purification was achieved by recrystallization in hexane.

3.5.3 HPLC Analysis on the Isolated Antifungal Compound from *N. gracilis*

The purity of the isolated bioactive compound was analyzed by using HPLC (Shimadzu, Prominence UFLC, LC-20AD, Japan) fitted with a RP-18 column (Merck Purospher STAR, 250 mm, 4.6 mm i.d., 5 µm, endcapped) and operated with a mobile phase consisted of deionized water (phase A) and acetonitrile (phase B). Elution was performed at a flow rate of 0.7 mL/min with a gradient elution program (t= 0 min, 0% B; t= 1 min, 10% B; t= 5 min, 50% B; t= 10 min, 100% B; t= 20 min, 100% B) and 20 µL (0.1 mg/mL) of the isolated compound was injected into the system. The detection of peaks was facilitated by a photodiode array detector set at 200 to 800 nm.

3.5.4 HPTLC Analysis on the Isolated Antifungal Compound from *N. gracilis*

HPTLC analysis was performed using HPTLC plate (Kieselgel 60 F_{254}, 0.2 mm, aluminium-backed) using hexane: ethyl acetate (9:1, v/v) as the mobile phase. The isolated compounds, fraction and hexane crude extract were sprayed (5 µL, 0.1 mg/mL) on the plate using a semi-automatic sample applicator with a band length of 6.0 mm. The plates were photographed using a 12 bit charge-couple device digital camera equipped with WinCATS software (Planar Chromatography Manager version 1.4.4). The TLC visualizer captured images under a uniform illumination of visible light and ultraviolet light (254 nm and 366 nm).

The spots on the HPTLC plate were quantified by using a TLC scanner and the data of absorption signal spectra (200 nm to 700 nm) were processed with the WinCATS software. The scanning procedure was programmed at the speed of 20 mm sec^{-1} and with a slit width of 6.0 x 0.45 mm. The retention factor (*Rf*) and percentage peak area (%) was automatically calculated by the software.

3.6 Characterization and Structure Elucidation of the Isolated Antifungal Compound from *N. gracilis*

3.6.1 Measurement of Melting Point

Melting point of the isolated bioactive compound was measured by using a melting point apparatus (Stuart, SMP 10, UK). The sample (1 mg) was inserted into a glass capillary tube and placed in the aluminium block of the sample chamber. The block was heated slowly (2°C per min) and the sample was observed through the magnifying lens until melting was observed. The melting point temperature was recorded from the light-emitting diode (LED) display. The measurement of melting point was performed thrice.

3.6.2 FT-IR Analysis

Infrared (IR) spectra of the isolated compound were recorded as KBr pellets on a FT-IR spectrometer (Perkin-Elmer RX1, USA). The KBr pellet (13 mm diameter) was prepared by mixing the finely ground solid sample and powdered KBr (1:100, w/w) and pressed under high pressure, 4000 pound per square inch (psi) by a hydraulic pellet press (ICL, USA). IR spectra were

recorded from an accumulation of 40 scans at the wavenumbers from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}.

3.6.3 GC-MS Analysis

The mass spectrum of the isolated compound was obtained by a GC-MS (Shimadzu, QP2010 Plus, Japan) using helium (1.46 mL/min) as carrier gas, fitted with 5% phenyl polysilphenylene-siloxane (BPX5) capillary column (0.25 mm x 30 m, film thickness 0.25 μm); the injector port was maintained at 250°C; the oven temperature gradient was 10°C/min from 80°C to 310°C with a total program time of 37 min; electron impact ionization method was used, the ion source and interface temperatures were set at 200°C and 310°C, respectively. The isolated sample was dissolved in methanol (HPLC grade) and filtered through a syringe filter (0.22 μm) prior to injection. The sample injection volume was 1 μL (20 μg/mL).

3.6.4 LC-MS/MS Analysis

The ion mass spectrum of the isolated compound was obtained by a LC-MS/MS (AB Sciex, USA) using nitrogen (purity= 99.9995%) as collision gas. The separation of LC-MS/MS was achieved using a C18 column (Phenomenex Aqua, USA, 50 x 2.0 mm i.d., 5 μm) with mobile phase comprised water-acetonitrile (10:90, v/v). The sample injection volume was 20 μL (20 μg/mL). Ionization was achieved using electron spray in the negative ionization mode with 2.93 kV of capillary voltage. The desolvation gas heated to 500°C and the

source block temperature was 80°C. The declustering potential was 40 V and the collision energy voltage was 10 V. The LC-MS/MS data was processed by the Analyst data acquisition software (AB Sciex, USA).

3.6.5 Nuclear Magnetic Resonance Spectroscopic Analysis

^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), HMBC and HMQC spectra of the isolated compound were recorded on a NMR spectrometer (JEOL JMN-ECX 400, Japan). Fifteen milligram of sample was dissolved in deuterated methanol-d_4, filtered and transferred into a NMR tube (Kimble Chase-400 MHz, Vineland) until it achieved a height of 4 cm. All chemical shifts (δ) were stated in parts per million (ppm) and coupling constants (*J*) were expressed in Hz.

3.7 Cytotoxicity Determination of the Isolated Antifungal Compound from *N. gracilis*

3.7.1 Determination of Optimum Cell Seeding Density

Confluence monolayer LLC-MK2 cells were trypsinized using 1 mL of trypsin-EDTA solution (1x). Cells were pelleted by centrifugation at 1,500 rpm for 15 min. The pellet was resuspended with the cell maintenance medium (DMEM with 1% FBS) and the total numbers of cells were counted using a hemocytometer. A working density of $8x10^5$ cell/mL was prepared and two-fold serial dilution was carried out to obtain six final densities ($8x10^5$, $4x10^5$, $2x10^5$, $1x10^5$, $5x10^4$ and $2.5x10^4$ cell/mL). One hundred microliters of cell suspension from the respective density was seeded into a 96-well flat bottom

microplate and added with 100 µL of cell maintenance medium. Figure 3.2 shows an overall microplate design of the 96-well microplate for the determination of optimum cell seeding density.

The 96-well microplate was incubated at 37°C in a humidified atmosphere with 5% CO_2 for 72 h. The cell viability was evaluated by Neutral Red Uptake assay. The absorbance value representing cell viability was measured at 540 nm. The optimum cell seeding density was assessed through the plotted absorbance value-cell density curve. Seeding density falls within the late transitional and the early stationary phase of the graph was used as seeding density for the cytotoxicity assay. The tests were performed in triplicate.

3.7.2 Neutral Red Uptake Assay

The cell viability of tested plates was evaluated by Neutral Red Uptake (NRU) assay according to the method of Repetto et al. (2008) with modifications. After 72 h of incubation, medium from the plates was removed and rinsed with 150 µL of DPBS twice. Maintenance medium (100 µL) containing neutral red (40 µg/mL) was added into each well. After 2 h of incubation at 37°C in a humidified atmosphere with 5% CO_2, the neutral red medium was removed from each well and rinsed twice with 150 µL of DPBS.

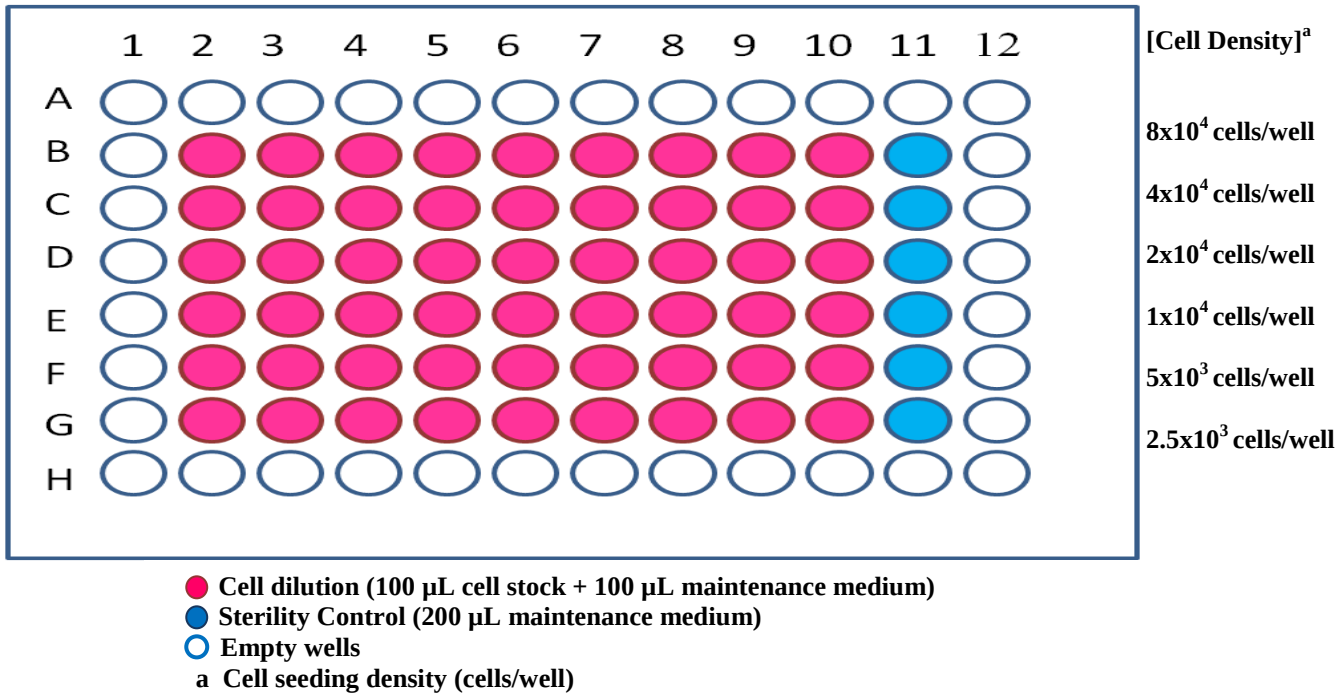


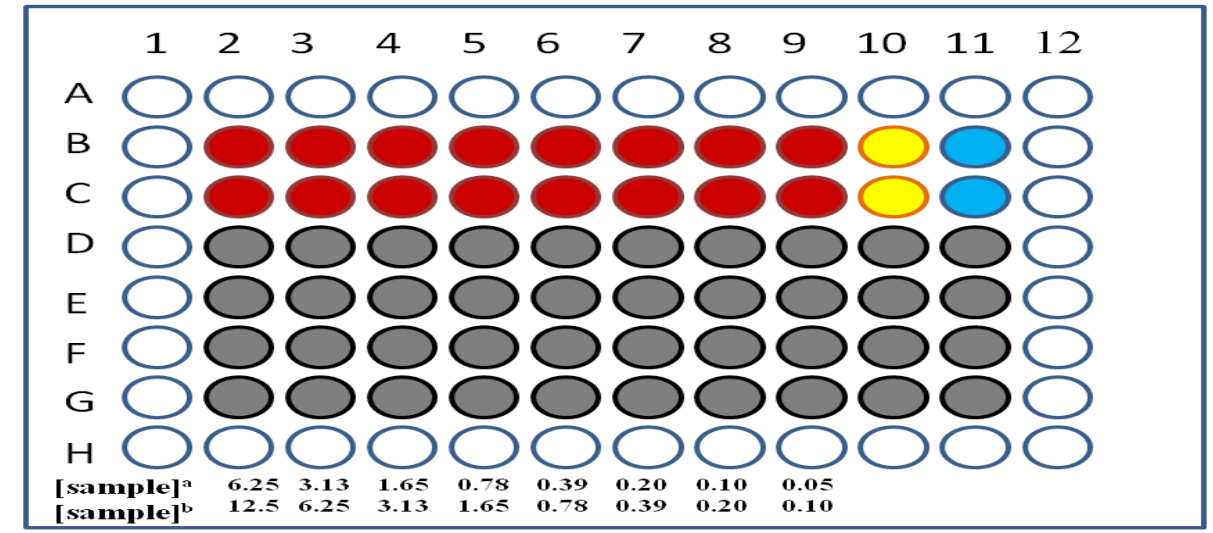
Figure 3.2: Design of a 96-well microplate for the determination of optimum cell seeding density of LLC-MK2 cells.

One hundred fifty microliters of neutral red destain solution was added into each well to extract the incorporated neutral red dye in the viable cells. The microplates were shook for 15 min with an orbital shaker at 60 rpm. The absorbance of each well was measured at 540 nm with a microplate reader to determine cell viability. The result was expressed through a concentration-cell viability curve. Mean value was determined from the triplicate results and the percentage of cell viability was calculated using the formula shown below (A= absorbance value measured at 540 nm):

$$\text{Cell viability (\%)} = \frac{(A_{sample} - A_{mediumcontrol})}{(A_{cellcontrol} - A_{mediumcontrol})} \times 100\%$$

3.7.3 Cytotoxicity Assay

To investigate the cellular toxicity of the diluent (methanol:water mixture in the ratio of 2:1, v/v) or isolated pure compound against the mammalian cells (LLC-MK2), 100 µL of cell suspension with the density of $3x10^4$ cells per well was seeded in 96-well flat bottom microplates. After 24 h incubation at 37°C in a humidified atmosphere with 5% CO_2, the confluent cultures were subjected to the cytotoxic screening (Repetto et al., 2008). The test was performed by two-fold serial dilution with the cell maintenance medium to obtain eight final working concentrations of diluent or the isolated pure compound (Figure 3.3). One hundred microliters



Testing wells (100 µL of cell suspensions + 100 µL of two-fold serial dilution of isolated compound or diluent)
Cell growth control (100 µL of cell suspensions + 100 µL of maintenance medium)
Sterility control (200 µL of maintenance medium)
Empty wells
Non-testing outer peripheral wells
a Tested concentration range of the isolated compound (µg/mL)
b Tested concentration range of diluent (%, v/v)

Figure 3.3: Design of a 96-well microplate for the cytotoxicity assay of diluent and isolated compound against the LLC-MK2 cells.

of the prepared working solutions was added to the testing wells accordingly except for the sterility and cell growth controls.

Two hundred and one hundred microliters of cell maintenance medium were added to the sterility control (Column 11) and cell growth control (Column 10), respectively. The microplate was incubated at 37°C in a humidified atmosphere with 5% CO_2 for 72 h. The cell viability was then evaluated by NRU assay. The tests were performed in triplicates.

The 50% cytotoxicity concentrations (CC_{50}) of the isolated antifungal compound and diluents were determined from the plots of cell viability against concentration. The data were analyzed by one-way ANOVA using Statistical Package for the Social Sciences (SPSS) software (Version 15.0 for Windows, SPSS Inc, Illinois USA) and the significance level was set at $p<0.05$. The selectivity index (SI) of the isolated antifungal compound was calculated as follows:

$$SI = \frac{CC_{50}}{\text{Minimum inhibitory concentration}}$$

3.8 Summary of the Antifungal Screening and the Bioactive Compound Isolation Process

The summary of the antifungal screening and the bioactive compound isolation process are shown in Figures 3.4 and 3.5 respectively.

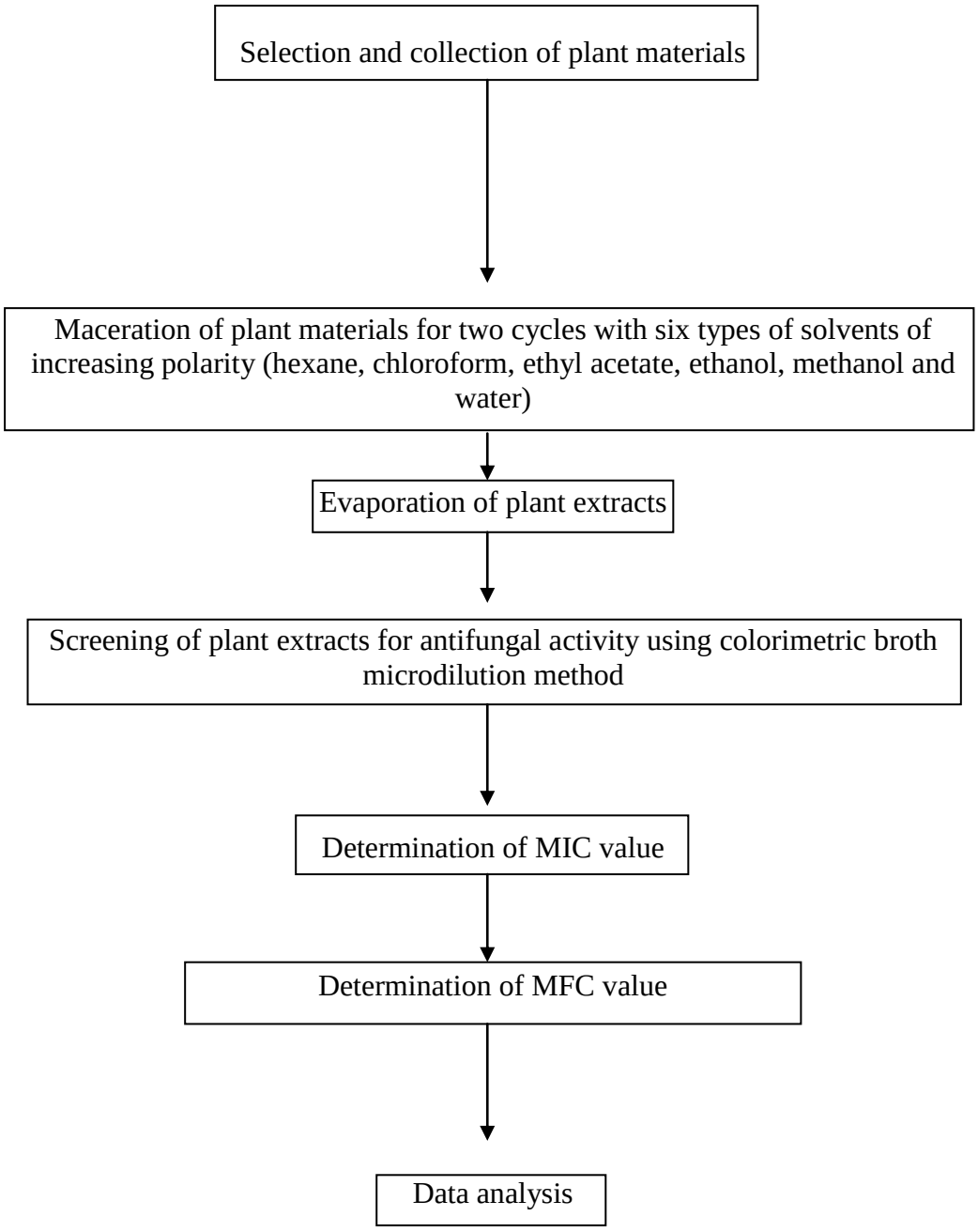


Figure 3.4: Methodology flow diagram of the antifungal screening of selected medicinal plants against fungi of medical important.

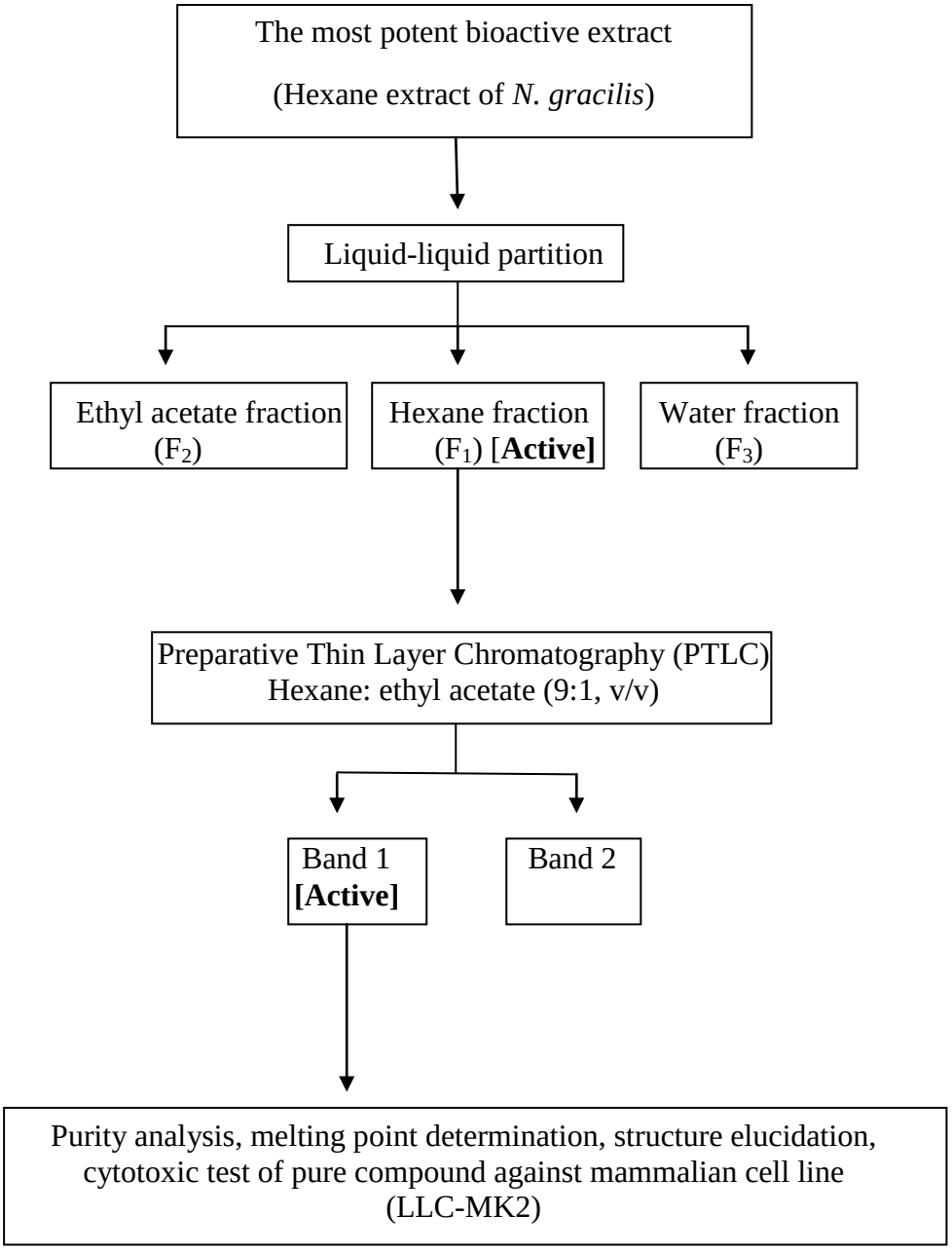


Figure 3.5: Flow chart of the isolation and structure elucidation of the antifungal compound from the leaves of *N. gracilis.*

CHAPTER 4

RESULTS

4.1 Plant Extraction

Twelve medicinal plants were selected and screened for antifungal properties. The extracts of eight species of medicinal plants were provided in this study. The respective plant species with the total percentage of yields are shown in Appendix B. Figure 4.1 shows the percentage of yields of extracts obtained from the leaves of *A. ciliata*, *A. graminifolia*, *C. nucifera* and *N. gracilis*. The highest percentage of yield extracted from each plant was the ethanol extracts which was 2.80%, 2.63%, 4.11% and 1.21% for *A. ciliata*, *A. graminifolia*, *C. nucifera* and *N. gracilis*, respectively. The lowest percentage of yield extracted from each plant was 0.53%, 0.24%, 0.33% and 0.05% from the hexane extract of *A. ciliata*, the ethyl acetate of *A. graminifolia*, the hexane extract of *C. nucifera* and the aqueous extract of *N. gracilis*, respectively. The highest total percentage of yield was 9.26%, extracted from the leaves of *C. nucifera*, followed by 8.37%, from the leaves of *A. ciliata* and 6.29%, from the leaves of *A. graminifolia*. The lowest total percentage of yield at 2.88% was obtained from the leaves of *N. gracilis*.

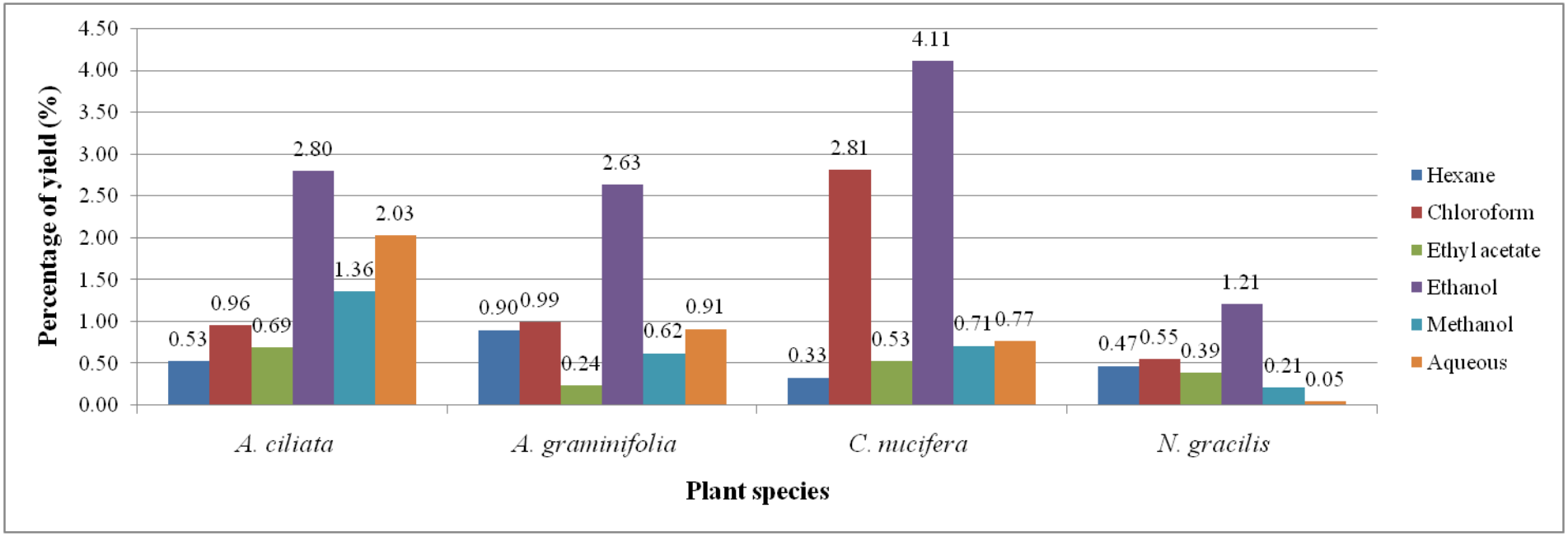


Figure 4.1: Percentage of yields of extracts obtained from the leaves of *A. ciliata*, *A. graminifolia*, *C. nucifera* and *N. gracilis*.

Footnote: The figure shows the percentage of yields extracted based on the fresh weight (w/w) of plant materials; the total percentage of yields of the extracts obtained from the respective 12 species of medicinal plants is shown in Appendix B.

4.2 Screening of Plant Extracts for Antifungal Activity

A total of 72 extracts obtained from 12 medicinal plants were tested for antifungal activity against four species of yeasts and two species of molds using the colorimetric broth microdilution method. All the plant extracts were evaluated for the fungistatic effect by the minimum inhibitory concentration (MIC) and the fungicidal effect by the minimum fungicidal concentration (MFC).

4.2.1 Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of Plant Extracts

The diluent, methanol:water mixture (2:1, v/v) did not show any growth inhibition effect (fungistatic) against all the fungi up to the highest concentration tested at 25% (v/v). The MIC and MFC results of various plant extracts against the fungal strains are shown in Table 4.1. All the fungi were susceptible to amphotericin B with MIC values equal or lower than 0.008 mg/mL.

By considering one extract against one type of fungus as one bioassay in the screening of plant extracts for antifungal activity, all the plant extracts demonstrated antifungal activity with 69.21% and 41.20% of the bioassays demonstrating fungistatic and fungicidal activities, respectively. The lowest MIC and MFC values (both with 0.02 mg/mL) were exhibited by the hexane extract of *N. gracilis* against *T. mentagrophytes*, *C. albicans* and *I. orientalis*, as well as the ethyl acetate, ethanol and aqueous extracts of *P. speciosa* against

I. orientalis. The hexane, chloroform and ethyl acetate extracts of *D. sarmentosa*, *H. sibthorpioides*, *M. platyclada*, and *S. muricatum*, the hexane and chloroform extracts of *N. gracilis* and *S. edule*, the hexane extract of *A. ciliata*, the chloroform extract of *D. esculentum*, demonstrated fungistatic activity against all the fungi with the MIC values ranged from 0.02 to 2.50 mg/mL (Table 4.1). Among the extracts, hexane extract of *N. gracilis* showed the lowest ranges of fungistatic and fungicidal activities against all the fungi with the MIC and MFC values ranged from 0.02 to 0.08 mg/mL and 0.02 to 0.16 mg/mL, respectively. The ethyl acetate, ethanol and methanol extracts of *A. graminifolia*, the aqueous extract of *C. nucifera*, the ethanol and methanol extracts of *N. gracilis*, and the methanol extract of *P. edulis* showed selective inhibitory activity against the yeasts (*C. albicans*, *C. parapsilosis*, *I. orientalis* and *C. neoformans*), with the MIC values ranged from 0.04 to 2.50 mg/mL.

None of the polar extracts (ethanol, methanol and aqueous) showed inhibitory activity against *A. brasiliensis* (Table 4.1). The MIC values for most of the extracts against *A. brasiliensis* ranged from 0.31 to 2.50 mg/mL and some with values as low as 0.04 to 0.16 mg/mL, included the chloroform and ethyl acetate extracts of *M. playclada* and the hexane extract of *N. gracilis*. Figure 4.2 shows the antifungal activity of plant extracts against *C. albicans*. Figure 4.3 indicates the fungicidal activity of plant extracts on agar plates by using the MFC assay.

Table 4.1: Minimum inhibitory concentration and minimum fungicidal concentration of the plant extracts against six fungi.

Plant species	Extracts	MIC (mg/mL)						MFC (mg/mL)					
		Molds		Yeasts				Molds		Yeasts			
		A.b	T.m	C.a	C.p	I.o	C.n	A.b	T.m	C.a	C.p	I.o	C.n
A. ciliata (leaves)	Hexane	0.31-0.63	0.02	0.16-0.31	0.63-1.25	0.16-0.31	0.02	2.50	0.08	1.25	NA	0.31	0.08
	Chloroform	NA	NA	1.25-2.50	NA	1.25-2.50	0.63-1.25	-	-	NA	-	NA	2.50
	Ethyl acetate	NA	0.31	0.63	1.25-2.50	0.31	0.16	-	0.31	1.25	NA	0.63	0.31
	Ethanol	NA	1.25-2.50	2.50	NA	1.25-2.50	0.31-0.63	-	2.50	2.50	-	NA	0.63
	Methanol	NA	2.50	2.50	NA	NA	0.63	-	2.50	2.50	-	-	NA
	Aqueous	NA	NA	NA	NA	2.50	0.63-1.25	-	-	-	-	NA	NA
	Amphotericin B	0.004	0.008	0.002	0.002	0.004	0.0001						
A. graminifolia (leaves)	Hexane	NA	NA	NA	NA	NA	1.25	-	-	-	-	-	NA
	Chloroform	NA	NA	NA	NA	1.25-2.50	1.25	-	-	-	-	NA	2.50
	Ethyl acetate	NA	NA	0.16	0.31	0.08	0.16	-	-	NA	NA	NA	0.31
	Ethanol	NA	NA	0.63-1.25	0.63-1.25	0.16-0.31	0.16-0.31	-	-	NA	NA	NA	0.63
	Methanol	NA	NA	1.25	2.50	0.31	0.16-0.31	-	-	NA	NA	NA	0.63
	Aqueous	NA	NA	NA	NA	NA	0.16-0.31	-	-	-	-	-	NA
	Amphotericin B	0.004	0.008	0.002	0.002	0.004	0.0001						

Mean or range of triplicates; NA, no activity; "-", not tested since no MIC value was obtained; C.a, *C. albicans*; C.p, *C. parapsilosis*; I.o, *I. orientalis*; C.n, *C. neoformans*; A.b, *A. brasiliensis*; T.m, *T. mentagrophytes*.

Table 4.1 continued

Plant species	Extracts	MIC (mg/mL)						MFC (mg/mL)					
		Molds		Yeasts				Molds		Yeasts			
		A.b	T.m	C.a	C.p	I.o	C.n	A.b	T.m	C.a	C.p	I.o	C.n
C. nucifera (leaves)	Hexane	NA	0.63	0.63	2.50	0.63	0.08-0.16	-	0.63	2.50	NA	2.50	0.16
	Chloroform	NA	1.25-2.5	0.08-0.16	0.63	0.04	0.08	-	2.5	NA	NA	2.50	0.31
	Ethyl acetate	NA	0.63-1.25	0.16	0.31-0.63	0.04-0.08	0.08	-	1.25	2.50	NA	2.50	0.31
	Ethanol	NA	2.50	0.16	0.63	0.02	0.08	-	NA	NA	NA	NA	0.63
	Methanol	NA	2.50	0.16	0.63	0.02	0.08	-	NA	NA	NA	NA	NA
	Aqueous	NA	NA	0.08	0.63	0.04	0.16	-	-	NA	NA	NA	NA
	Amphotericin B	0.004	0.008	0.001	0.002	0.004	0.0001						
D. sarmentosa (aerial parts)	Hexane	2.50	0.63	0.63-1.25	1.25	0.31	0.08	NA	1.25	1.25	NA	0.31	0.08
	Chloroform	2.50	0.63-1.25	0.63-1.25	1.25	0.31-0.63	0.16	NA	1.25	NA	NA	0.63	0.16
	Ethyl acetate	2.50	0.63-1.25	0.63-1.25	1.25-2.50	0.31-0.63	0.16	NA	1.25	2.50	NA	0.63	0.16
	Ethanol	NA	NA	0.63	NA	0.63	0.16-0.31	-	-	NA	-	0.63	NA
	Methanol	NA	NA	0.63-1.25	NA	0.63	0.31	-	-	NA	-	0.63	NA
	Aqueous	NA	NA	0.63	NA	0.63	0.31	-	-	NA	-	0.63	NA
	Amphotericin B	0.002	0.004	0.001	0.002	0.004	0.0001						

Mean or range of triplicates; NA, no activity; "-", not tested since no MIC value was obtained; C.a, *C. albicans*; C.p, *C. parapsilosis*; I.o, *I. orientalis*; C.n, *C. neoformans*; A.b, *A. brasiliensis*; T.m, *T. mentagrophytes*.

Table 4.1 continued

Plant species	Extracts	MIC (mg/mL)						MFC (mg/mL)					
		Molds		Yeasts				Molds		Yeasts			
		A.b	T.m	C.a	C.p	I.o	C.n	A.b	T.m	C.a	C.p	I.o	C.n
D. esculentum (aerial parts)	Hexane	NA	1.25	1.25	NA	0.31	0.08	-	1.25	NA	-	0.31	0.16
	Chloroform	2.50	0.63-1.25	0.63-1.25	2.50	0.31	0.08-0.16	NA	1.25	NA	NA	0.31	0.31
	Ethyl acetate	NA	NA	1.25	NA	0.31	0.16	-	-	NA	-	0.31	0.31
	Ethanol	NA	1.25	0.63-1.25	1.25-2.50	0.31	0.08-0.16	-	2.50	2.50	NA	0.31	0.16
	Methanol	NA	2.50	1.25-2.50	NA	0.31	0.16	-	NA	NA	-	0.31	0.31
	Aqueous	NA	NA	NA	NA	0.16-0.31	0.16	-	-	-	-	0.31	0.31
	Amphotericin B	0.002	0.008	0.002	0.002	0.004	0.0001						
H. sibthorpioides (whole plant)	Hexane	0.63-1.25	0.16	0.63	0.63-1.25	0.08	0.08	NA	0.16	1.25	NA	0.08	0.08
	Chloroform	1.25	0.31-0.63	0.63-1.25	1.25	0.31	0.16-0.31	1.25	0.63	1.25	NA	0.31	0.31
	Ethyl acetate	2.50	0.63	0.63-1.25	1.25	0.31	0.16-0.31	NA	1.25	NA	NA	0.31	0.31
	Ethanol	NA	NA	NA	NA	1.25	0.31-0.63	-	-	-	-	1.25	0.63
	Methanol	NA	NA	NA	NA	0.63	0.31-0.63	-	-	-	-	0.63	0.63
	Aqueous	NA	NA	NA	NA	0.63	0.63-1.25	-	-	-	-	0.63	2.50
	Amphotericin B	0.002	0.008	0.002	0.002	0.004	0.0001						

Mean or range of triplicates; NA, no activity; "-", not tested since no MIC value was obtained; C.a, *C. albicans*; C.p, *C. parapsilosis*; I.o, *I. orientalis*; C.n, *C. neoformans*; A.b, *A. brasiliensis*; T.m, *T. mentagrophytes*.

Table 4.1 continued

Plant species	Extracts	MIC (mg/mL)						MFC (mg/mL)					
		Molds		Yeasts				Molds		Yeasts			
		A.b	T.m	C.a	C.p	I.o	C.n	A.b	T.m	C.a	C.p	I.o	C.n
M.	Hexane	1.25	0.63	0.63-1.25	2.50	0.63	0.16	NA	1.25	1.25	NA	0.63	0.16
platyclada	Chloroform	0.08-0.16	0.08-0.16	1.25-2.50	2.50	0.63	0.31	0.16	0.31	NA	NA	0.63	0.63
(stems)	Ethyl acetate	0.16	0.31	0.16-0.31	0.63	0.08	0.08-0.16	0.31	0.31	NA	NA	0.16	0.31
	Ethanol	NA	0.63-1.25	0.31	0.31	0.08-0.16	0.16	-	1.25	2.50	NA	0.16	0.31
	Methanol	NA	1.25-2.50	0.31	0.31	0.08-0.16	0.16	-	2.50	NA	NA	0.16	0.31
	Aqueous	NA	2.50	0.16	0.16	0.08-0.16	0.16	-	2.50	NA	NA	0.16	0.63
	Amphotericin B	0.004	0.008	0.002	0.002	0.004	0.0001						
N. gracilis	Hexane	0.04-0.08	**0.02**	**0.02**	0.02	**0.02**	0.02	0.16	**0.02**	**0.02**	0.16	**0.02**	0.16
(leaves)	Chloroform	1.25	0.31	0.31-0.63	0.63-1.25	0.31	0.31-0.63	NA	0.31	0.63	2.50	0.31	1.25
	Ethyl acetate	NA	1.25-2.50	0.63	0.63	0.31	0.31	-	2.50	NA	NA	NA	2.50
	Ethanol	NA	NA	0.31	0.31-0.63	0.16	0.16	-	-	NA	NA	NA	2.50
	Methanol	NA	NA	0.31	2.50	0.08-0.16	0.04-0.08	-	-	NA	NA	NA	NA
	Aqueous	NA	NA	2.50	NA	0.04-0.08	0.04	-	-	NA	-	NA	NA
	Amphotericin B	0.002	0.008	0.002	0.004	0.004	0.0001						

Mean or range of triplicates; NA, no activity; "-", not tested since no MIC value was obtained; C.a, *C. albicans*; C.p, *C. parapsilosis*; I.o, *I. orientalis*; C.n, *C. neoformans*; A.b, *A. brasiliensis*; T.m, *T. mentagrophytes*.

Table 4.1 continued

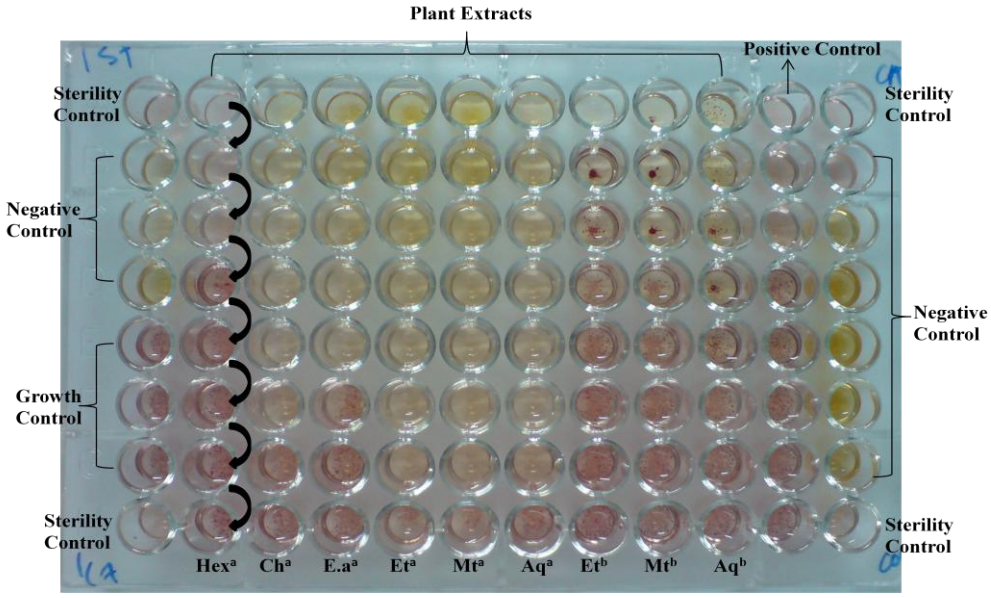
Plant species	Extracts	MIC (mg/mL)						MFC (mg/mL)					
		Molds		Yeasts				Molds		Yeasts			
		A.b	T.m	C.a	C.p	I.o	C.n	A.b	T.m	C.a	C.p	I.o	C.n
P. speciosa	Hexane	NA	0.63	2.50	1.25	0.31-0.63	0.16-0.31	-	0.63	NA	1.25	0.63	0.31
(pods)	Chloroform	NA	0.63	0.63	1.25	0.16	0.16-0.31	-	1.25	NA	1.25	0.31	0.63
	Ethyl acetate	NA	0.16	0.31	0.04-0.08	**0.02**	0.02	-	0.63	NA	NA	**0.02**	NA
	Ethanol	NA	1.25	0.63	0.31	**0.02**	0.04	-	2.50	NA	NA	**0.02**	NA
	Methanol	NA	1.25-2.50	1.25	0.16-0.31	0.04	0.04	-	NA	NA	NA	0.04	NA
	Aqueous	NA	0.63-1.25	2.50	0.63-1.25	**0.02**	0.02	-	1.25	NA	NA	**0.02**	NA
	Amphotericin B	0.004	0.008	0.004	0.004	0.004	0.0001						
P. edulis	Hexane	NA	0.63	0.63	1.25	0.31-0.63	0.04	-	0.63	1.25	1.25	0.63	0.16
(whole fruit	Chloroform	NA	NA	2.50	NA	NA	0.63-1.25	-	-	NA	-	-	2.50
without the	Ethyl acetate	NA	1.25-2.50	0.31	0.63-1.25	0.08	0.08	-	2.50	NA	NA	NA	0.31
peel)	Ethanol	NA	NA	NA	NA	NA	0.31	-	-	-	-	-	0.63
	Methanol	NA	NA	0.31	1.25-2.50	0.16	0.08-0.16	-	-	NA	NA	NA	0.31
	Aqueous	NA	NA	NA	NA	NA	0.31	-	-	-	-	-	2.50
	Amphotericin B	0.004	0.008	0.002	0.002	0.004	0.0001						

Mean or range of triplicates; NA, no activity; "-", not tested since no MIC value was obtained; C.a, *C. albicans*; C.p, *C. parapsilosis*; I.o, *I. orientalis*; C.n, *C. neoformans*; A.b, *A. brasiliensis*; T.m, *T. mentagrophytes*.

Table 4.1 continued

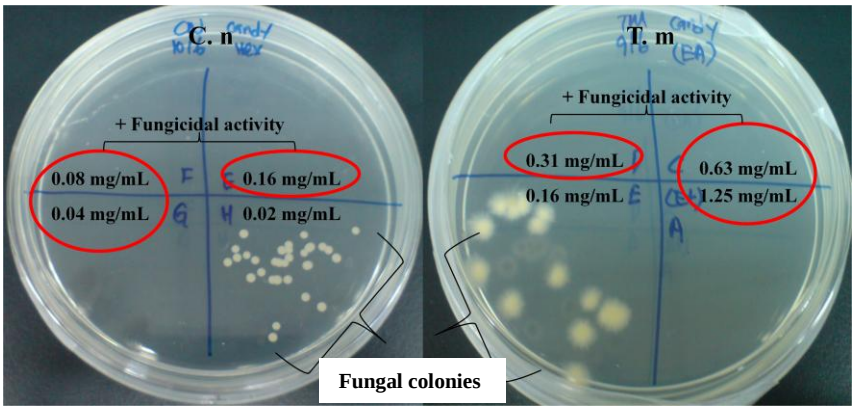
Plant species	Extracts	MIC (mg/mL)						MFC (mg/mL)					
		Molds		Yeasts				Molds		Yeasts			
		A.b	T.m	C.a	C.p	I.o	C.n	A.b	T.m	C.a	C.p	I.o	C.n
S. edule	Hexane	1.25	0.31-0.63	0.31-0.63	0.63	0.31	0.08	2.50	0.63	NA	NA	0.31	0.08
(aerial parts)	Chloroform	2.50	0.63-1.25	0.63-1.25	1.25	0.63	0.16	2.50	1.25	2.50	NA	0.63	0.31
	Ethyl acetate	NA	0.63-1.25	0.63-1.25	1.25	0.63	0.16-0.31	-	1.25	2.50	NA	0.63	0.31
	Ethanol	NA	NA	NA	NA	0.63	0.31	-	-	-	-	0.63	NA
	Methanol	NA	NA	NA	NA	0.63	0.31-0.63	-	-	-	-	0.63	NA
	Aqueous	NA	NA	NA	NA	0.63	0.31-0.63	-	-	-	-	0.63	0.63
	Amphotericin B	0.002	0.008	0.002	0.002	0.004	0.0001						
S. muricatum	Hexane	0.31	0.16	0.63	1.25	0.63	0.08	0.31	0.31	0.63	1.25	0.63	0.08
(whole fruit	Chloroform	0.63-1.25	0.31-0.63	0.63-1.25	2.50	1.25	0.08-0.16	1.25	0.63	NA	2.50	1.25	0.31
without the	Ethyl acetate	2.50	0.63-1.25	0.63	1.25	0.63-1.25	0.08-0.16	2.50	1.25	1.25	NA	1.25	0.31
seeds)	Ethanol	NA	NA	NA	NA	0.63	0.31-0.63	-	-	-	-	0.63	0.63
	Methanol	NA	NA	NA	NA	0.63	0.63-1.25	-	-	-	-	0.63	NA
	Aqueous	NA	NA	NA	NA	0.63-1.25	2.50	-	-	-	-	1.25	NA
	Amphotericin B	0.002	0.008	0.002	0.002	0.004	0.0001						

Mean or range of triplicates; NA, no activity; "-", not tested since no MIC value was obtained; C.a, *C. albicans*; C.p, *C. parapsilosis*; I.o, *I. orientalis*; C.n, *C. neoformans*; A.b, *A. brasiliensis*; T.m, *T. mentagrophytes*.



➲ Serial two-fold descending concentration of extract; [a]*C. nucifera*; [b]*D. sarmentosa*; Hex, Hexane; Ch, Chloroform; E.a, Ethyl acetate; Et, Ethanol; Mt, Methanol; Aq, Aqueous.

Figure 4.2: Antifungal activity of *C. nucifera* and *D. sarmentosa* extracts against *C. albicans* using colorimetric broth microdilution assay.



⬭ Concentration of extracts exhibited fungicidal activity against the tested strain; C. n, *C. neoformans*; T. m, *T. mentagrophytes.*

Figure 4.3: Fungicidal activity of *N. gracilis* extracts against *C. neoformans* and *T. mentagrophytes* on potato dextrose agar plates using the MFC assay.

4.2.2 Total Activity of Plant Extracts

The total activities of plant extracts active against the fungi are shown in Table 4.2. The ethanol extract of *P. speciosa* showed the highest total activity (2080 mL/g) against *I. orientalis*, followed by the ethanol extract of *C. nucifera* (2055 mL/g) against *I. orientalis* and the ethanol extract of *P. speciosa* (1040 mL/g) against *C. neoformans*. The hexane extract of *N. gracilis* was the most active extract against fungi with total activities of 58.75 mL/g and 235 mL/g against *A. brasiliensis* and the other five fungi (*T. mentagrophytes*, *C. albicans*, *C. parapsilosis*, *I. orientalis* and *C. neoformans*), respectively. The lowest total activity was found in the hexane extract of *S. muricatum* against *C. parapsilosis* at 0.03 mL/g.

4.2.3 Percent Activity of Plant Extracts

Eight species of the medicinal plants that were investigated, yielded 18 extracts with the highest percent activity of 100% (Figure 4.4). This broad-spectrum antifungal activity were illustrated by the hexane, chloroform and ethyl acetate extracts of *D. sarmentosa*, *H. sibthorpioides*, *M. platyclada*, and *S. muricatum*, the hexane and chloroform extracts of *N. gracilis* and *S. edule*, the hexane extract of *A. ciliata*, and the chloroform extract of *D. esculentum*. All the extracts of *M. platyclada* and *P. speciosa* demonstrated antifungal properties with the percent activity values ≥83%. The lowest percent activity value obtained was 16.67% which were found in the hexane and aqueous extracts of *A. graminifolia*, as well as the ethanol and aqueous extracts of *P. edulis*.

Table 4.2: Total activity of plant extracts against the fungi.

Plant species	Plant extracts	Total activity (mL/g)					
		Fungal strains					
		A.b	**T.m**	**C.a**	**C.p**	**I.o**	**C.n**
A. ciliata (leaves)	Hexane	8.41	26.50	17.10	4.24	17.10	26.50
	Chloroform	NA	NA	3.84	NA	3.84	7.68
	Ethyl acetate	NA	22.26	10.95	2.76	22.26	43.13
	Ethanol	NA	11.20	11.20	NA	11.20	44.44
	Methanol	NA	5.44	5.44	NA	NA	21.59
	Aqueous	NA	NA	NA	NA	8.12	16.24
A. graminifolia (leaves)	Hexane	NA	NA	NA	NA	NA	7.20
	Chloroform	NA	NA	NA	NA	3.96	7.92
	Ethyl acetate	NA	NA	15.00	7.74	30.00	15.00
	Ethanol	NA	NA	21.04	21.04	84.84	84.84
	Methanol	NA	NA	4.96	2.48	20.00	20.00
	Aqueous	NA	NA	NA	NA	NA	29.35
C. nucifera (leaves)	Hexane	NA	5.24	5.24	1.32	5.24	20.63
	Chloroform	NA	11.24	175.63	44.60	702.50	351.25
	Ethyl acetate	NA	4.24	33.13	8.41	66.25	66.25
	Ethanol	NA	16.44	256.88	65.24	**2055.00**	513.75
	Methanol	NA	2.84	44.38	11.27	355.00	88.75
	Aqueous	NA	NA	96.25	12.22	192.50	48.13
D. sarmentosa (aerial parts)	Hexane	0.16	0.63	0.32	0.32	1.29	5.00
	Chloroform	0.40	0.80	0.80	0.80	1.59	6.25
	Ethyl acetate	0.68	1.36	1.36	0.68	2.70	10.63
	Ethanol	NA	NA	10.48	NA	10.48	21.29
	Methanol	NA	NA	3.12	NA	6.19	12.58
	Aqueous	NA	NA	7.46	NA	7.46	15.16
D. esculentum (aerial parts)	Hexane	NA	0.72	0.72	NA	2.90	11.25
	Chloroform	0.16	0.32	0.32	0.16	1.29	2.50
	Ethyl acetate	NA	NA	0.40	NA	1.61	3.13
	Ethanol	NA	2.72	2.72	1.36	10.97	21.25
	Methanol	NA	1.36	1.36	NA	10.97	21.25
	Aqueous	NA	NA	NA	NA	10.97	21.25

The total activity was expressed as the yield extracted from 1 g of plant material divided by the highest concentration of the MIC range of triplicates; NA, no activity; C.a, *C. albicans*; C.p, *C. parapsilosis*; I.o, *I. orientalis*; C.n, *C. neoformans*; A.b, *A. brasiliensis*; T.m, *T. mentagrophytes*.

Table 4.2 continued

Plant species	Plant extracts	Total activity (mL/g)					
		Fungal strains					
		A.b	**T.m**	**C.a**	**C.p**	**I.o**	**C.n**
H. sibthorpioides (whole plant)	Hexane	0.72	5.63	1.43	0.72	11.25	11.25
	Chloroform	1.44	2.86	1.44	1.44	5.81	5.81
	Ethyl acetate	0.80	1.59	0.80	0.80	3.23	3.23
	Ethanol	NA	NA	NA	NA	3.52	6.98
	Methanol	NA	NA	NA	NA	12.22	12.22
	Aqueous	NA	NA	NA	NA	8.73	4.40
M. platyclada (stems)	Hexane	2.32	4.60	2.32	1.16	4.60	18.13
	Chloroform	29.38	29.38	1.88	1.88	7.46	15.16
	Ethyl acetate	28.13	14.52	14.52	7.14	56.25	28.13
	Ethanol	NA	55.76	224.84	224.84	435.63	435.63
	Methanol	NA	3.64	29.35	29.35	56.88	56.88
	Aqueous	NA	2.16	33.75	33.75	33.75	33.75
N. gracilis (leaves)	Hexane	**58.75**	**235.00**	**235.00**	**235.00**	**235.00**	**235.00**
	Chloroform	4.40	17.74	8.73	4.40	17.74	8.73
	Ethyl acetate	NA	1.56	6.19	6.19	12.58	12.58
	Ethanol	NA	NA	39.03	19.21	75.63	75.63
	Methanol	NA	NA	6.77	0.84	13.13	26.25
	Aqueous	NA	NA	0.20	NA	6.25	12.50
P. speciosa (pods)	Hexane	NA	0.95	0.24	0.48	0.95	1.94
	Chloroform	NA	1.75	1.75	0.88	6.88	3.55
	Ethyl acetate	NA	12.50	6.45	25.00	100.00	100.00
	Ethanol	NA	33.28	66.03	134.19	**2080.00**	**1040.00**
	Methanol	NA	9.84	19.68	79.35	615.00	615.00
	Aqueous	NA	5.20	2.60	5.20	325.00	325.00
P. edulis (whole fruit without the peel)	Hexane	NA	2.38	2.38	1.20	2.38	37.50
	Chloroform	NA	NA	0.96	NA	NA	1.92
	Ethyl acetate	NA	2.84	22.90	5.68	88.75	88.75
	Ethanol	NA	NA	NA	NA	NA	41.29
	Methanol	NA	NA	25.81	3.20	50.00	50.00
	Aqueous	NA	NA	NA	NA	NA	12.26

The total activity was expressed as the yield extracted from 1 g of plant material divided by the highest concentration of the MIC range of triplicates; NA, no activity; C.a, *C. albicans*; C.p, *C. parapsilosis*; I.o, *I. orientalis*; C.n, *C. neoformans*; A.b, *A. brasiliensis*; T.m, *T. mentagrophytes*.

Table 4.2 continued

Plant species	Plant extracts	Total activity (mL/g)					
		Fungal strains					
		A.b	T.m	C.a	C.p	I.o	C.n
S. edule (aerial parts)	Hexane	0.32	0.63	0.63	0.63	1.29	5.00
	Chloroform	0.40	0.80	1.59	0.80	1.59	6.25
	Ethyl acetate	NA	0.72	1.43	0.72	1.43	2.90
	Ethanol	NA	NA	NA	NA	11.75	23.87
	Methanol	NA	NA	NA	NA	6.19	6.19
	Aqueous	NA	NA	NA	NA	7.78	7.78
S. muricatum (whole fruit without the seeds)	Hexane	0.13	0.25	0.06	0.03	0.06	0.50
	Chloroform	0.08	0.16	0.08	0.04	0.08	0.63
	Ethyl acetate	0.08	0.16	0.32	0.16	0.16	1.25
	Ethanol	NA	NA	NA	NA	40.16	40.16
	Methanol	NA	NA	NA	NA	14.76	7.44
	Aqueous	NA	NA	NA	NA	0.32	0.16

The total activity was expressed as the yield extracted from 1 g of plant material divided by the highest concentration of the MIC range of triplicates; NA, no activity; C.a, *C. albicans*; C.p, *C. parapsilosis*; I.o, *I. orientalis*; C.n, *C. neoformans*; A.b, *A. brasiliensis*; T.m, *T. mentagrophytes*.

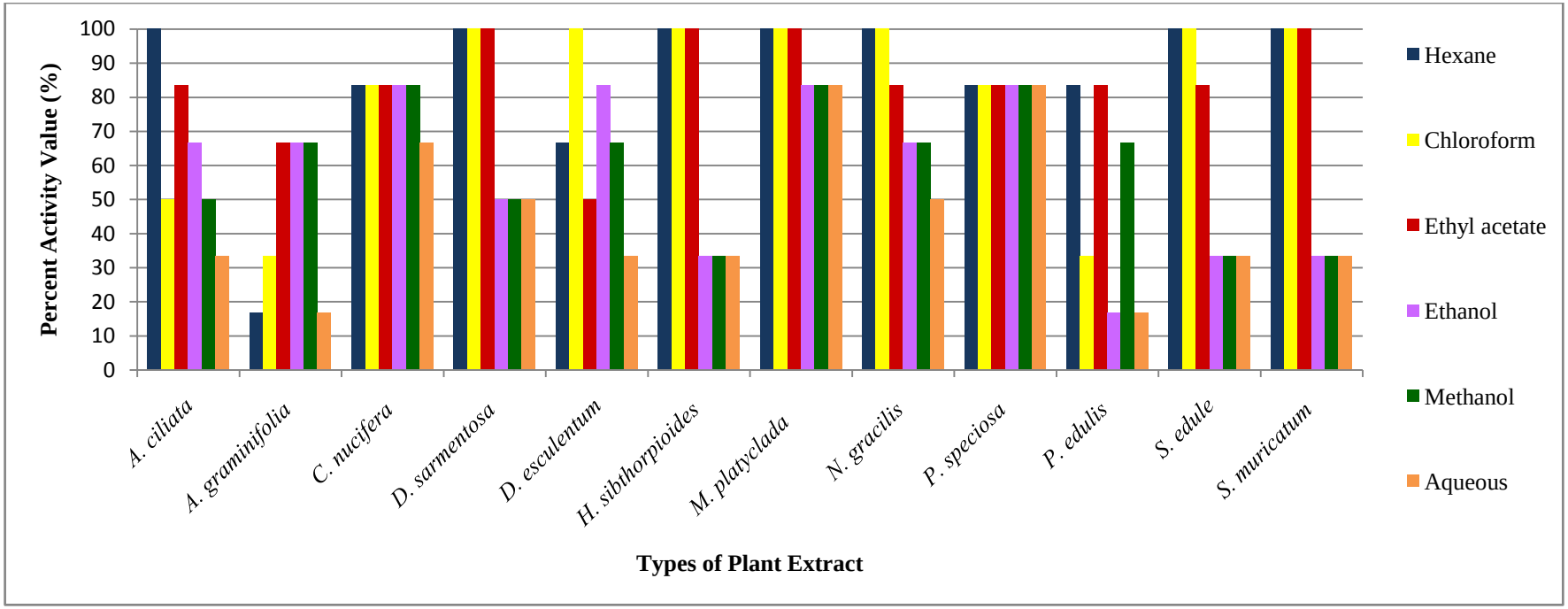


Figure 4.4: Percent activity values of various plant extracts against the fungi.

Footnote: The figure shown is based on data from three independent experiments, total number of fungal strains tested, n= 6.

4.2.4 Fungal Susceptibility Index (FSI)

Figure 4.5 shows the FSI values of the fungi to various plant extracts. The most susceptible fungal strain was *C. neoformans* which was susceptible to all the plant extracts with the susceptibility index of 100%, followed by *I. orientalis*, *C. albicans* and *C. parapsilosis* with FSI values of 91.67%, 77.78% and 62.50%, respectively. The molds were found to be more resistant to the plant extracts compared to the yeasts. The lowest FSI value (25%) was demonstrated by *A. brasiliensis*. *T. mentagrophytes* possessed only 58.33% of susceptibility to the tested extracts (Figure 4.5).

4.3 Bioactivity-guided Fractionation of the Extract with the Highest Antifungal Activity

The hexane extract of *N. gracilis* was selected for fractionation and bioactivity-guided isolation of the antifungal compound(s). The hexane extract had the lowest MIC and MFC values of 0.02 mg/mL, with 100% of percent activity value and broad spectrum of antifungal activity.

4.3.1 Liquid-liquid Partitioning of the Hexane Extract from *N. gracilis*

Two hundred thirty-seven grams of fresh leaves of *N. gracilis* was extracted with hexane. The resulting hexane extract (1.12 g, 0.47%) was fractionated to give three fractions: hexane fraction (F_1), ethyl acetate fraction (F_2), and water fraction (F_3) with yields (w/w) of 0.78 g (0.33%), 0.25 g (0.10%), and 0.028 g (0.01%), respectively.

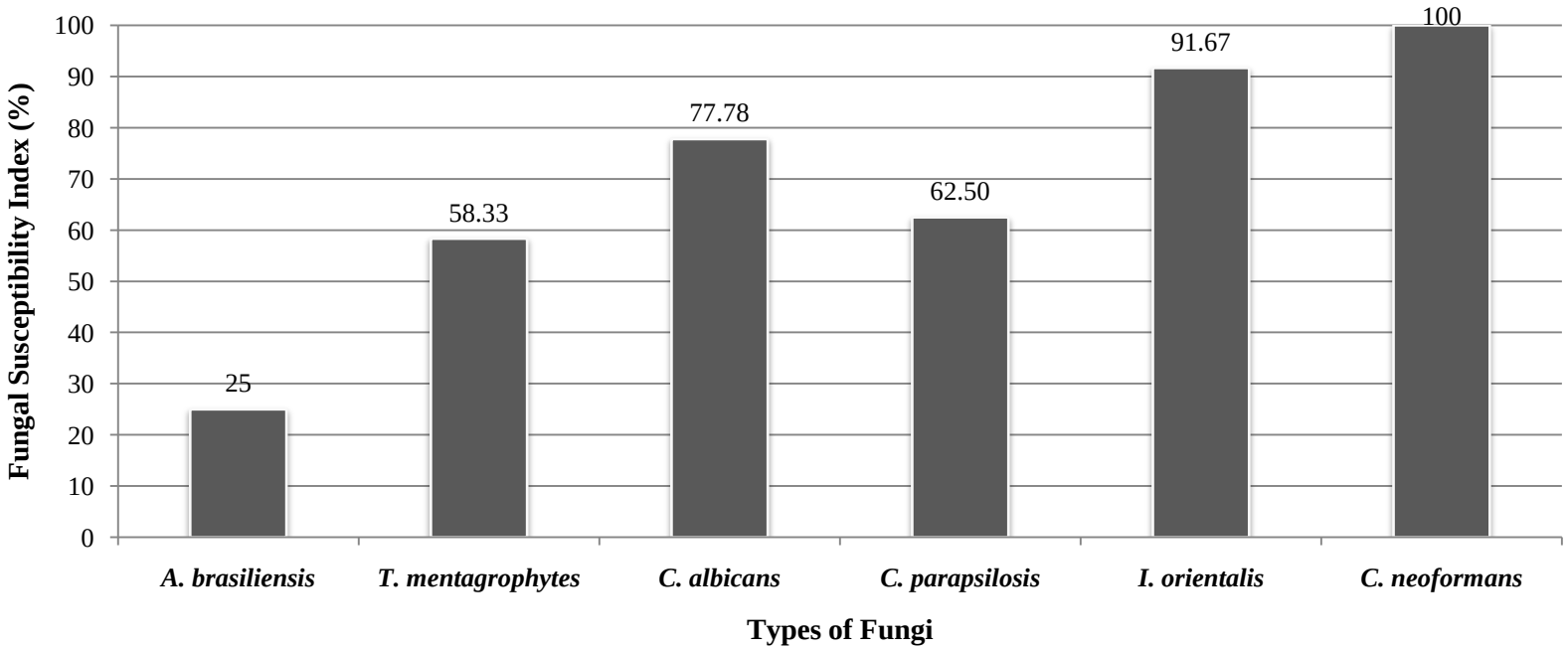


Figure 4.5: Fungal susceptibility index of fungi to various plant extracts by using the colorimetric broth microdilution assay.

Footnote: The figure shown is based on data from three independent experiments.

4.3.2 Evaluation of Antifungal Activity on the Fractions obtained from Liquid-liquid Partitioning

The antifungal activity of the collected fractions was monitored by the colorimetric broth microdilution assay using *C. neoformans* and *T. mentagrophytes* as the indicative microorganisms, representing the yeast and mold, respectively. Two fractions, F_1 and F_2 showed antifungal activities on the tested strains with the MIC and MFC values ranged from 8 to 31 µg/mL (Table 4.3). The lowest fungistatic (MIC) and fungicidal (MFC) concentration were exhibited by F_1 against *C. neoformans* and *T. mentagrophytes* at 16 and 8 µg/mL, respectively. F_3 did not show any antifungal activity against the tested fungi. Among the active fractions, F_1 resulted in the higher percentage of yield and stronger antifungal activity as compared to F_2, and thus F_1 was selected for further isolation of the bioactive compound(s).

Table 4.3: Minimum inhibitory concentration and minimum fungicidal concentration of three fractions obtained from the hexane extract of *N. gracilis* against *C. neoformans* and *T. mentagrophytes*.

Fraction	MIC (µg/mL)		MFC (µg/mL)	
	T. mentagrophytes	*C. neoformans*	*T. mentagrophytes*	*C. neoformans*
F_1	**8**	**16**	**8**	**16**
F_2	16	31	16	31
F_3	NA	NA	-	-
Amphotericin B	8	0.125		

Mean of triplicates; NA, no activity; "-", not tested since no MIC value was obtained

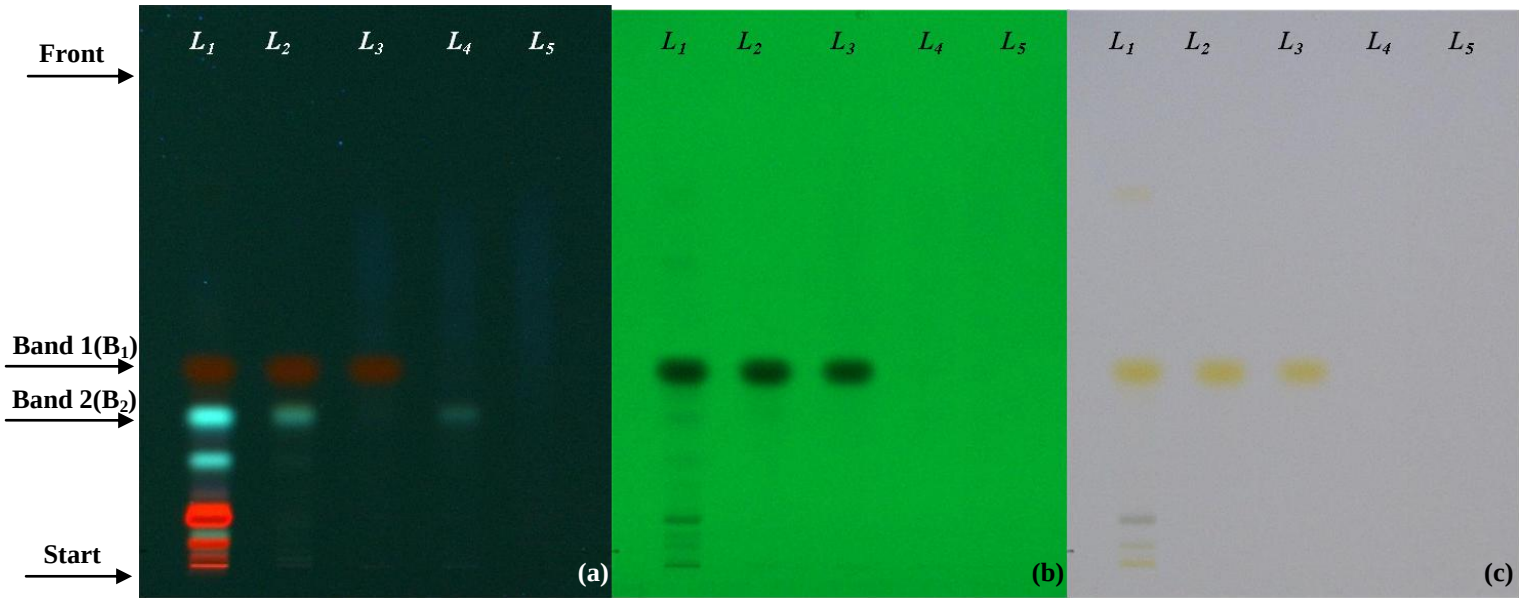
4.4 Bioactivity-guided Fractionation of the Bioactive Fraction of *N. gracilis*

The bioactive compounds from F_1 (the most active fraction) were separated and isolated by PTLC plates with hexane:ethyl acetate (9:1, v/v) as the mobile phase. Figure 4.6 shows the TLC chromatograms of the two compounds (B_1 and B_2) in F_1 with different absorption properties in visible and UV lights. A dark red band and a dark brown band of compound B_1 at R_f 0.34 were resolved on TLC in 366 nm and 254 nm, respectively. A brilliant light blue band of compound B_2 at R_f 0.21 was resolved on TLC in 366 nm UV light (Figure 4.6). Following PTLC, 0.29 g of B_1 and 0.012 g of B_2 were obtained from F_1 fraction. The yield was 0.12% and 0.005% for B_1 and B_2, respectively, based on the weight of the fresh leaves used (w/w). The antifungal activities of the isolated compounds, B_1 and B_2 against the indicative microorganisms were assessed. As shown in Table 4.4, compound B_1 showed strong antifungal activity with MIC and MFC values ranging from 2 to 8 μg/mL. Compound B_2 did not show fungicidal (MFC) activity against *C. neoformans*, and with MIC values of 63 and 125 μg/mL against *C. neoformans* and *T. mentagrophytes*, respectively.

Table 4.4: Minimum inhibitory concentration and minimum fungicidal concentration of compounds isolated from the fraction F_1 of hexane extract of *N. gracilis* against *C. neoformans* and *T. mentagrophytes*.

Compound	MIC (μg/mL)		MFC (μg/mL)	
	T. mentagrophytes	*C. neoformans*	*T. mentagrophytes*	*C. neoformans*
B_1	**4**	**2**	**8**	**4**
B_2	125	63	125	NA
Amphotericin B	8	0.125		

Mean of triplicates; NA, no activity



(a) Shot of the TLC plate in UV-light 366 nm after vertical development in saturated chamber.
(b) Shot of the TLC plate in UV-light 254 nm after vertical development in saturated chamber.
(c) Shot of the TLC plate in white light after vertical development in saturated chamber.

Sorbent: TLC precoated plate silica gel 60 $F_{254,}$ 200 µm , 10 x 10 cm
Solvent system: Hexane:ethyl acetate (9:1, v/v)
Chamber: Double-trough 10 x 10 cm chamber with glass lid
Migration distance: 8 cm
Samples (10 µL): *L1*, Hexane crude extract (0.5 mg/mL); *L2*, F_1 (0.3 mg/mL); *L3*, Band 1 (0.1 mg/mL); $L_{4,}$ Band 2 (0.1 mg/mL); L_5, Negative Control (Hexane only)

Figure 4.6: Documentation of thin layer chromatograms on the separation of F_1 and isolated compounds from *N. gracilis*.

4.5 Purification of Compound B_1 from *N. gracilis*

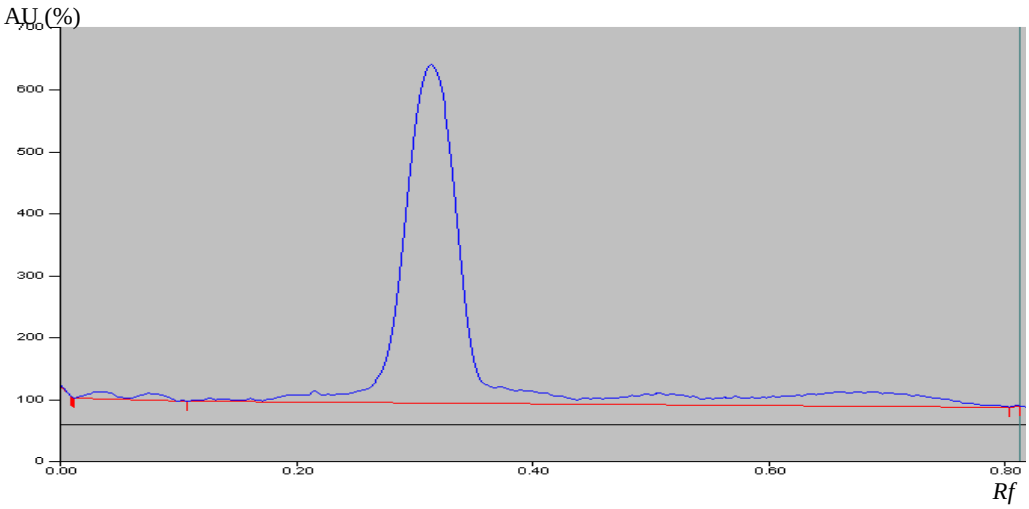
Between the two isolated compounds, B_1 had a higher percentage of yield and showed the stronger antifungal activity which was selected for further purification and structural characterization. The purity of the isolated compound B_1 was analyzed using a HPLC and a HPTLC coupled with a scanner and a visualizer.

4.5.1 HPTLC Analysis of the Isolated Antifungal Compound B_1

The TLC chromatogram showed a single peak (R_f 0.34) scanned at 425 nm (Figure 4.7) with a percentage peak area of over 93.62%, showing a bright yellow coloration under visible light (Figure 4.6, c, *L3*) and faint quenching under UV light at 254 nm (Figure 4.6, b, *L3*). The UV/VIS absorption spectrum of B_1 at multiple wavelengths (200 to 700 nm) was recorded at start, middle and end position of the band and exhibited three absorption maxima at 210 nm, 260 nm and 425 nm (Figure 4.8).

4.5.2 HPLC Analysis of the Isolated Antifungal Compound B_1

The peaks in the HPLC chromatograms were detected by a photodiode array detector at 365 nm, 425 nm and between 200 to 800 nm (Figure 4.9), with a major peak at retention time of 13.82 min and a percentage peak area of over 98.5%.



Peak num: 1
Start position (R_f): 0.23
Start height (AU): 12.70
Maximum position (R_f): 0.34
Maximum height (AU): 645.30
Maximum percentage (%): 90.73
End position (R_f): 0.37
End height (AU): 25.50
Area (AU): 21549.80
Peak area (%): 93.62

Figure 4.7: Direct optical evaluation of compound B_1 on HPTLC, measured at 425 nm.

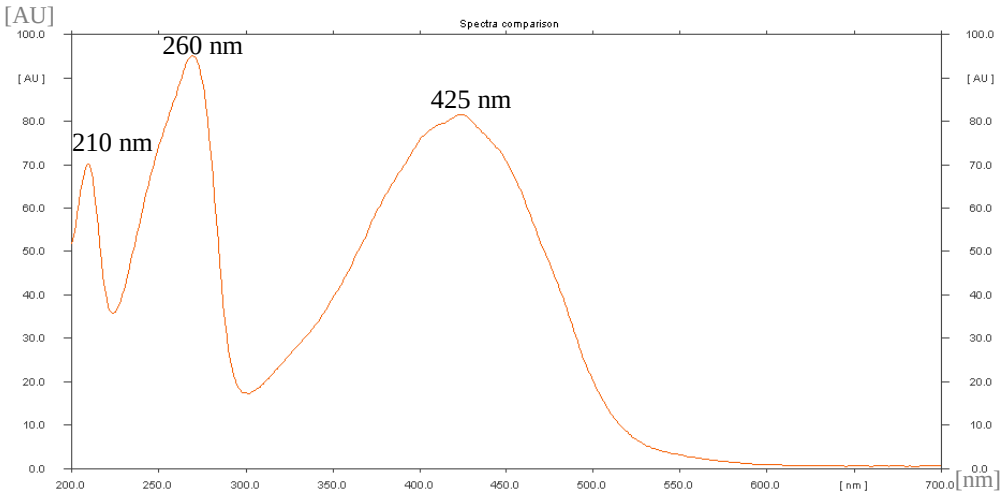
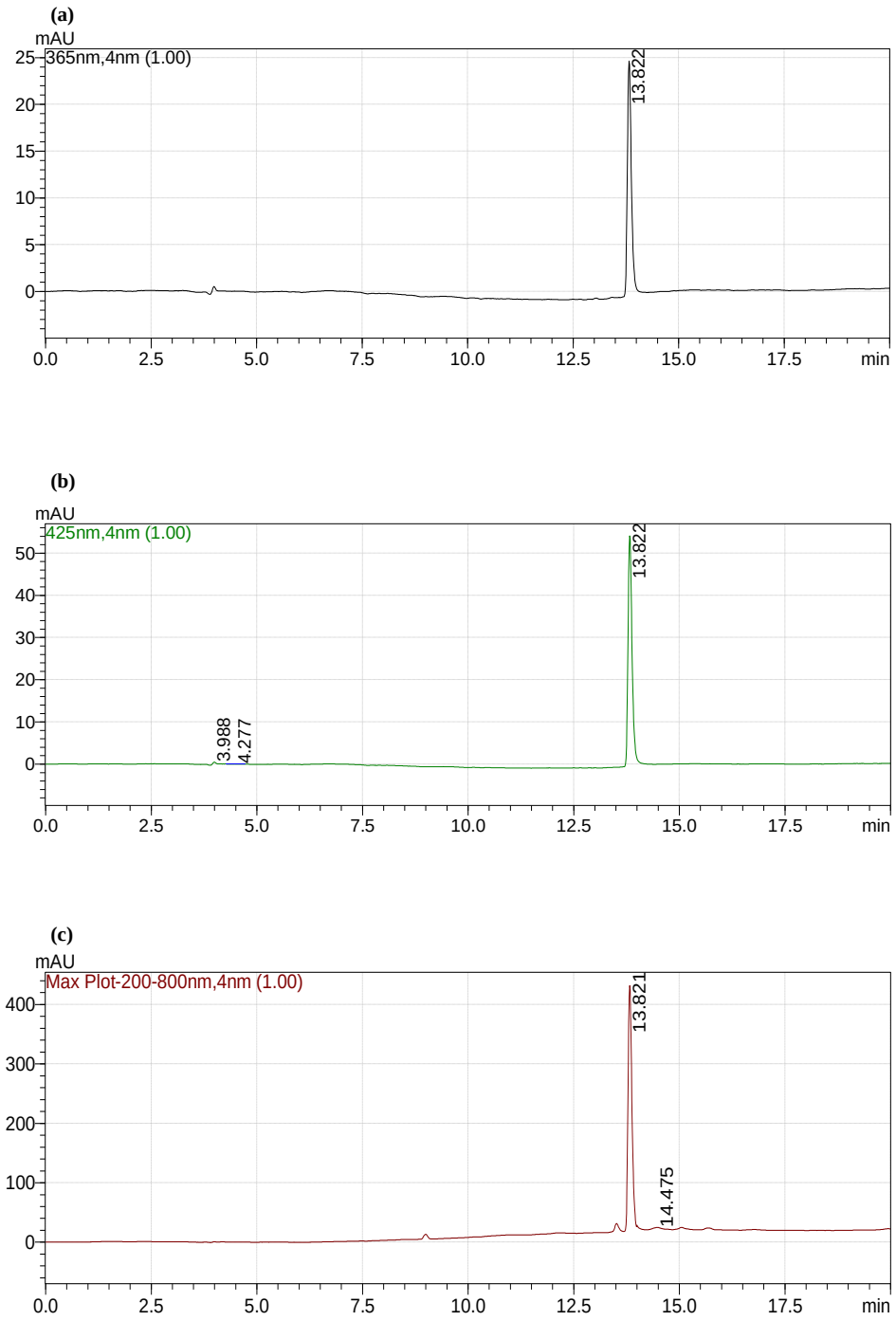


Figure 4.8: The UV/VIS absorption spectrum of compound B_1 measured at 200 to 700 nm using a TLC scanner.



(a) HPLC chromatogram detected at 365 nm.
(b) HPLC chromatogram detected at 425 nm.
(c) HPLC chromatogram detected at 200 to 800 nm.

Figure 4.9: HPLC chromatograms of compound B_1.

4.6 Characterization and Structure Elucidation of Compound B_1

4.6.1 Physical Appearance and Melting Point of Compound B_1

After recrystallization from hexane, compound B_1 appeared as yellow needle-shaped crystals (Figure 4.10). The melting point of compound B_1 was recorded as 78-79°C.

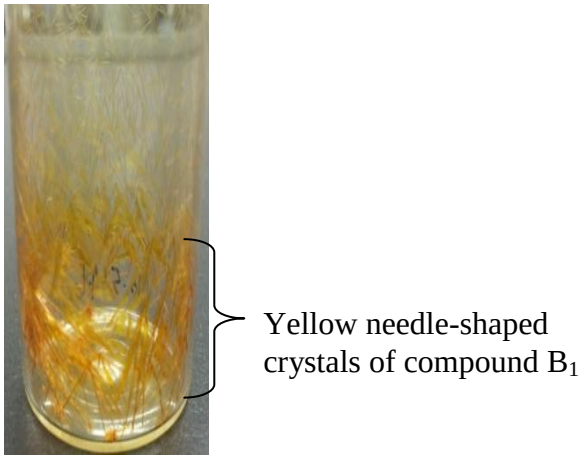


Figure 4.10: Crystallized compound B_1.

4.6.2 FT-IR Spectrum of Compound B_1

The infra-red (IR) spectrum of compound B_1 showed several intense bands in the region of 4000 and 600 cm^{-1}. The IR spectrum (in KBr) indicated the presence of –OH group at 3450 cm^{-1}, conjugated carbonyl group at 1640 cm^{-1}, along with other absorption bands at 2367, 2345, 1261 and 803 cm^{-1} (Figure 4.11).

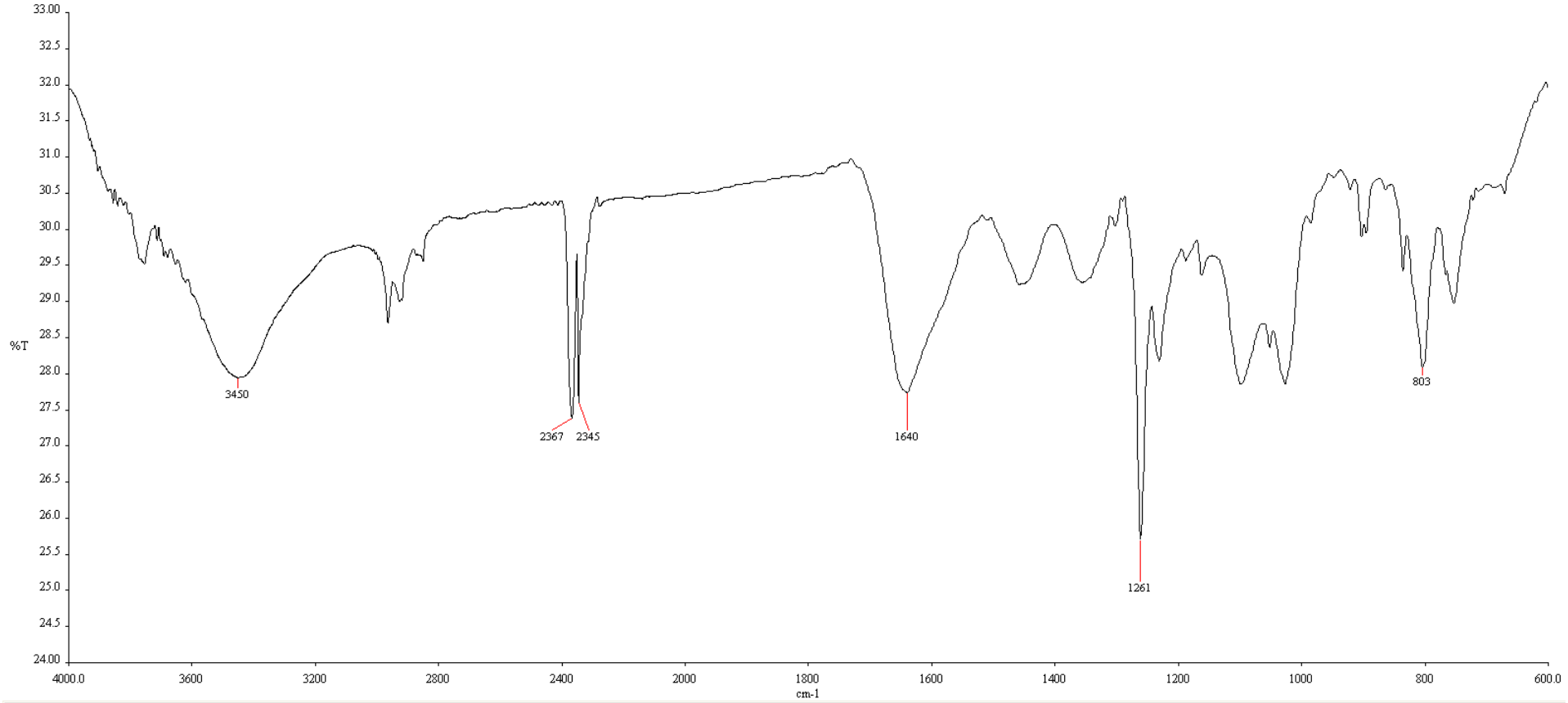


Figure 4.11: FT-IR spectrum of compound B_1.

4.6.3 GC-MS Analysis of Compound B_1

The total ion chromatogram revealed compound B_1 as a single peak at the retention time of 10.85 min (Figure 4.12). The molecular ion peak of compound B_1 was observed at *m/z* 188.05. Other major peaks are observed at *m/z* 173, 160 and 131 (Figure 4.13).

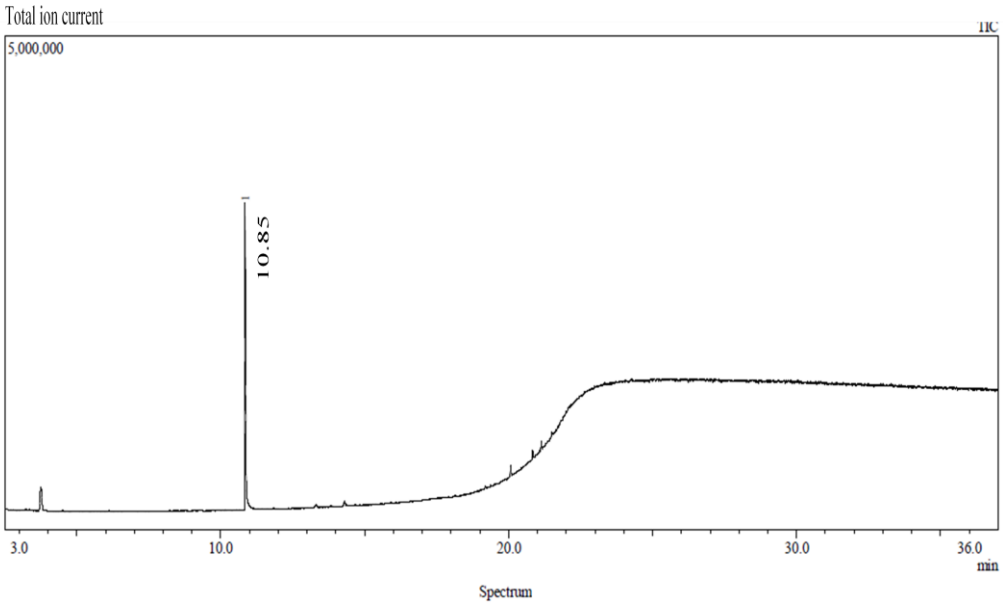
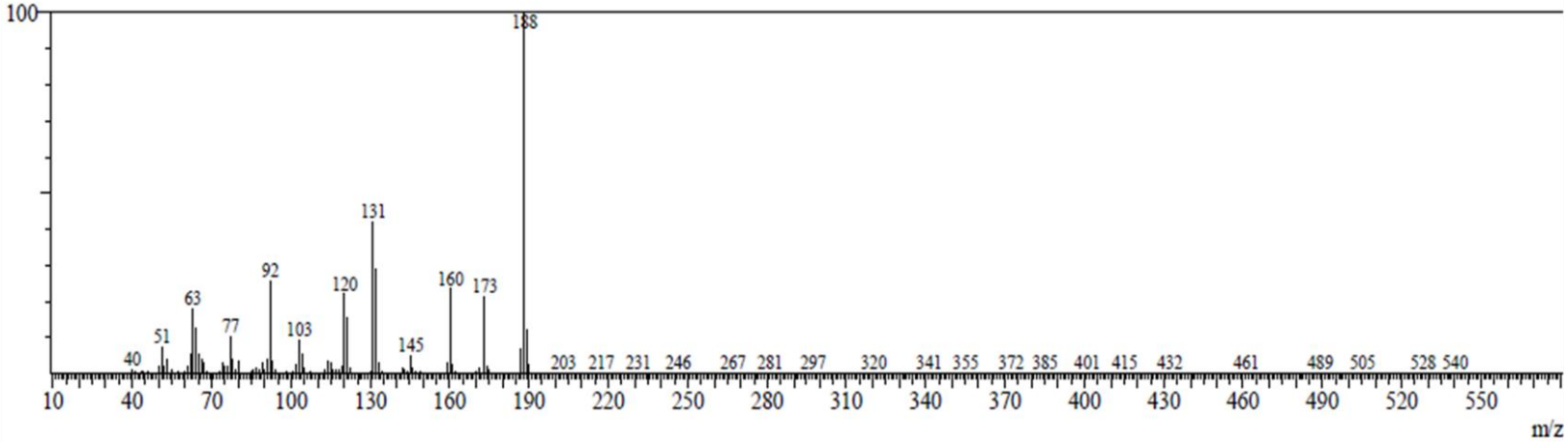


Figure 4.12: Total ion chromatogram of compound B_1.



Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height
1	1,4-Naphthalenedione, 5-hydroxy-2-methyl-	10.846	188.05	1363347	653477

Figure 4.13: Electron impact ionization mass spectrum of compound B_1.

4.6.4 LC-MS/MS Analysis of Compound B_1

The mass of compound B_1 was further confirmed by using LC-MS/MS analysis. The sample was analyzed in the negative mode by monitoring the precursor-product combination in multiple-reaction monitoring (MRM) mode. The total ion chromatogram showed an analyte peak with retention time of 5.17 min and there were no other interfering peaks detected (Figure 4.14). The negative product ion mass spectrum of the deprotonated molecule of compound B_1 showed a precursor ion *m/z* 186.9 and a product ion *m/z* 159.0 (Figure 4.15).

4.6.5 NMR Spectra Analysis of Compound B_1

In the ^{1}H-NMR spectrum (Figure 4.16) of compound B_1, the presence of a chelated hydroxyl group in the compound was indicated by a downfield broad singlet at δ_H 11.89 (OH, s). Meanwhile, proton signals observed at δ_H 7.19 (1H, d, J = 8.6 Hz), 7.60 (1H, t, J = 7.9 Hz), 7.50 (1H, d, J = 6.7 Hz) and 6.79 (1H, s) were assignable to three aromatic protons and one vinylic proton, on top of an upfield singlet observed at δ_H 2.10 (3H, s) for a methyl group. The spectral data described above was indicative of a disubstituted napthaquinone structure.

The ^{13}C-NMR (Figure 4.17) and HMQC (Figure 4.18) spectra showed a total of 11 carbon signals revealing the compound to have a total of 11 carbons including two carbonyl carbons observed at δ_C 184.5 (C-1) and 190.4 (C-4), a methyl carbon at δ_C 15.1 (2-CH_3), four methine carbons at δ_C 118.6 (C-8), 123.6 (C-6), 135.0 (C-3) and 136.0 (C-7), and four quaternary carbons at

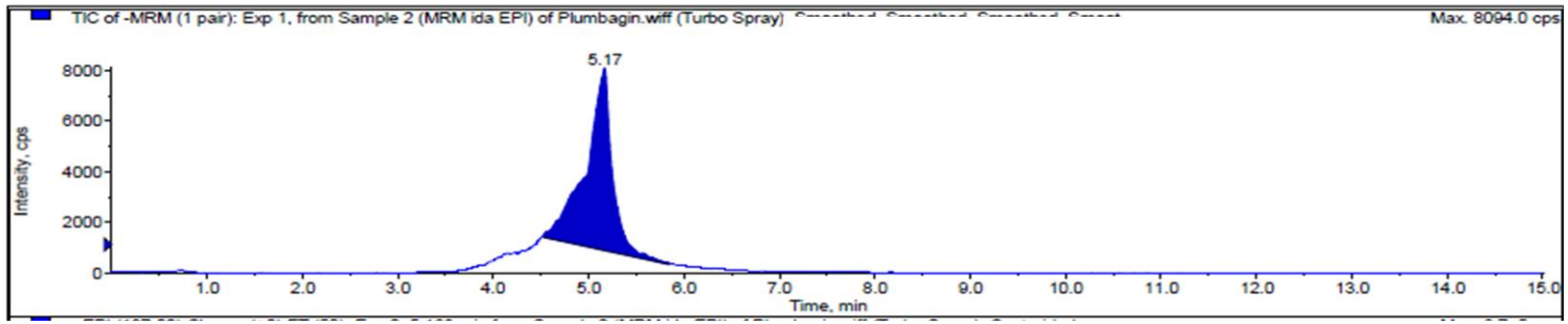


Figure 4.14: LC-MS/MS chromatogram of compound B_1.

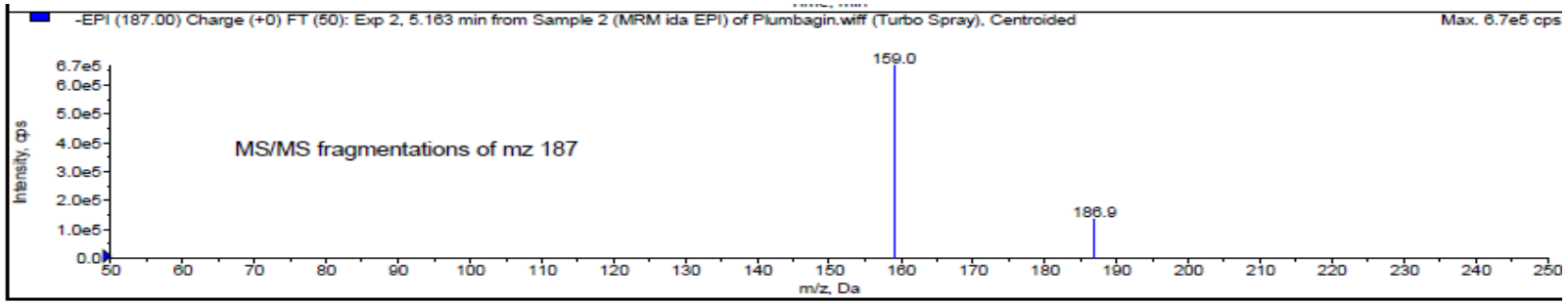


Figure 4.15: Negative product ion mass spectrum of the deprotonated molecule of compound B_1.

δ_C 115.0 (C-4a), 132.2 (C-8a), 149.7 (C-2) and 161.1 (C-5). The ^{1}H and ^{13}C NMR spectral data are consistent with those reported by Chen et al. (2011) as shown in Table 4.5 and Table 4.6, respectively.

Interpretation of the two dimensional (2D) NMR data from HMQC and HMBC spectra of compound B_1 has facilitated the proton and carbon correlations, and establishment of the final structure. Correlations that observed in the HMQC spectrum (Figure 4.18) of compound B_1 are shown Table 4.7. The presence of an exchangeable proton (δ_H 11.89) without any HMQC correlation suggests the presence of a hydroxyl group within the structure as supported by the IR spectrum (Figure 4.11) and the ^{1}H-NMR spectrum (Figure 4.16).

The assignments of quaternary carbons were based on the HMBC experiment (Figure 4.19). The assignment of methyl group to the carbon position C-2 was supported by HMBC correlations observed between 2-CH_3 (δ_H 2.10) and C-2 (δ_C 149.7), C-3 (δ_C 135.0), and C-1 (δ_C 184.5). Apart from that, cross peaks between H-8 at δ_H 7.50 to the carbonyl carbon (δ_C 184.5), C-4a (δ_C 115.0) and C-6 (δ_C 123.6), between H-3 (δ_H 6.79) with 2-CH_3 (δ_C 15.1), C-4a (δ_C 115.0) and C-1 (δ_C 184.5), between H-6 (δ_H 7.19) with C-4a (δ_C 115.0) and C-8 (δ_C 118.6) and between H-7 (δ_H 7.60) with C-8a (δ_C 132.2) and C-5 (δ_C 161.1) were also observed. Based on the above ^{1}H-NMR, ^{13}C-NMR, HMQC and HMBC spectral evidence, compound B_1 is therefore identified as plumbagin with a molecular formula of $C_{11}H_8O_3$.

Table 4.5: ^{1}H NMR spectral data of plumbagin and those reported in the literature.

Position	Plumbagin (δ, CD_3OD, 400 MHz)	Reference (δ, $CDCl_3$, 500 MHz)[a]
2-CH_3	2.10 (3H, s)	2.22 (3H, s)
H-3	6.79 (1H, s)	6.83 (1H, s)
H-6	7.19 (1H, br d, *J*= 7.9 Hz)	7.28 (1H, d, *J*= 8.5 Hz)
H-7	7.60 (1H, t, *J*= 7.9 Hz)	7.66 (1H, d, *J*= 7.5 Hz)
H-8	7.50 (1H, br d, *J*= 7.9 Hz)	7.60 (1H, d, *J*= 6.2 Hz)
5-OH	11.89 (1H, s)	11.99 (1H, s)

[a] Chen et al., 2011

Table 4.6: ^{13}C NMR spectral data of plumbagin and those reported in the literature.

Position	Plumbagin (δ, CD_3OD, 100 MHz)	Reference (δ, $CDCl_3$, 125 MHz)[a]
2-CH_3	15.1	16.5
C-1	184.5	184.8
C-2	149.7	149.6
C-3	135.0	136.1
C-4	190.4	190.3
C-4a	115.0	115.2
C-5	161.1	161.2
C-6	123.6	124.2
C-7	136.0	135.5
C-8	118.6	119.3
C-8a	132.2	132.0

[a] Chen et al., 2011

Table 4.7: HMQC spectral data of plumbagin.

Position	^{13}C (δ)	^{1}H (δ)
2-CH_3	15.1	2.19
C-1	184.5	-
C-2	149.7	-
C-3	135.0	6.79
C-4	190.4	-
C-4a	115.0	-
C-5	161.1	-
C-6	123.6	7.19
C-7	136.0	7.60
C-8	118.6	7.50
C-8a	132.2	-

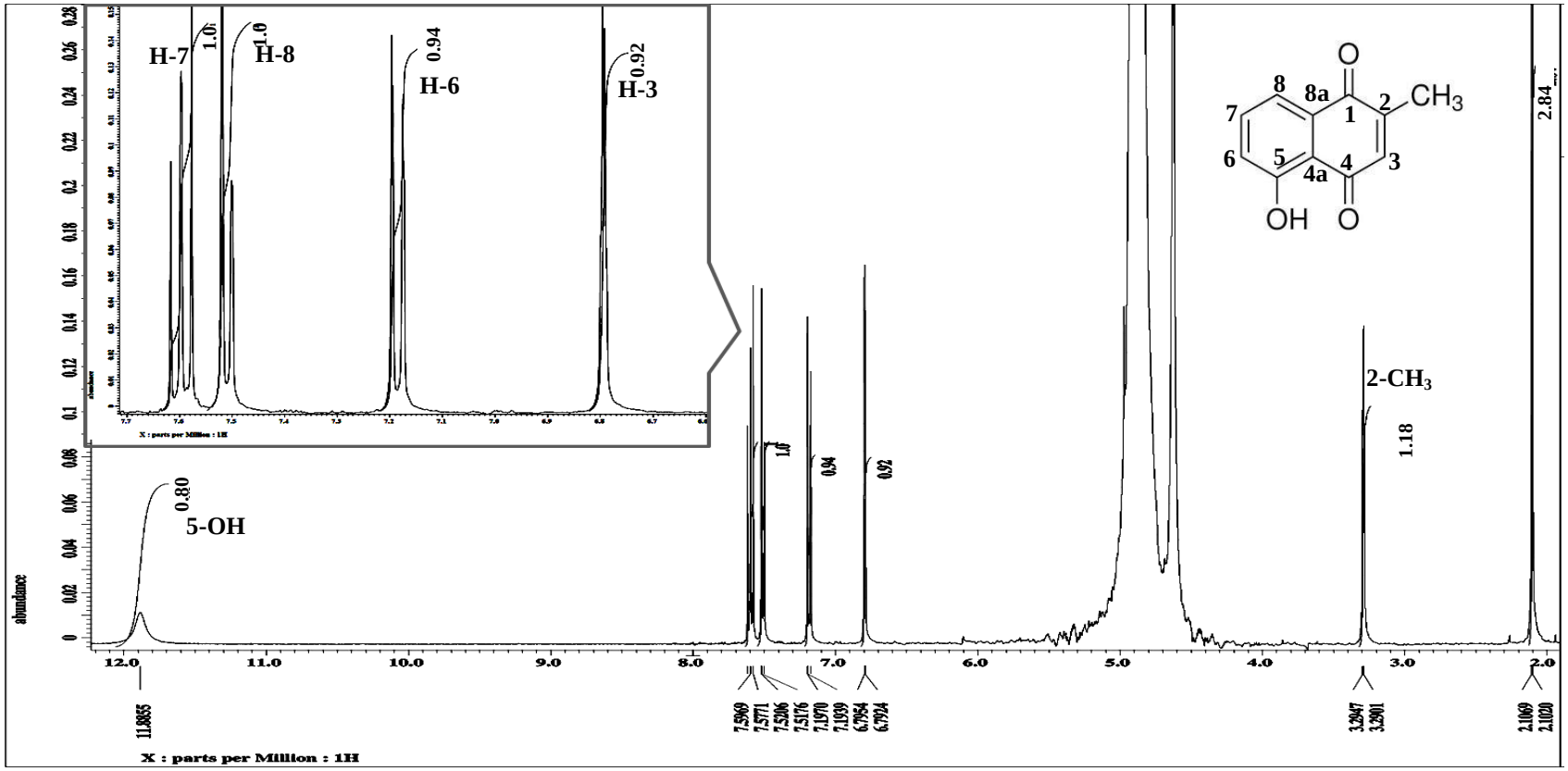


Figure 4.16: ^{1}H NMR (400 MHz) spectrum of compound B_1.

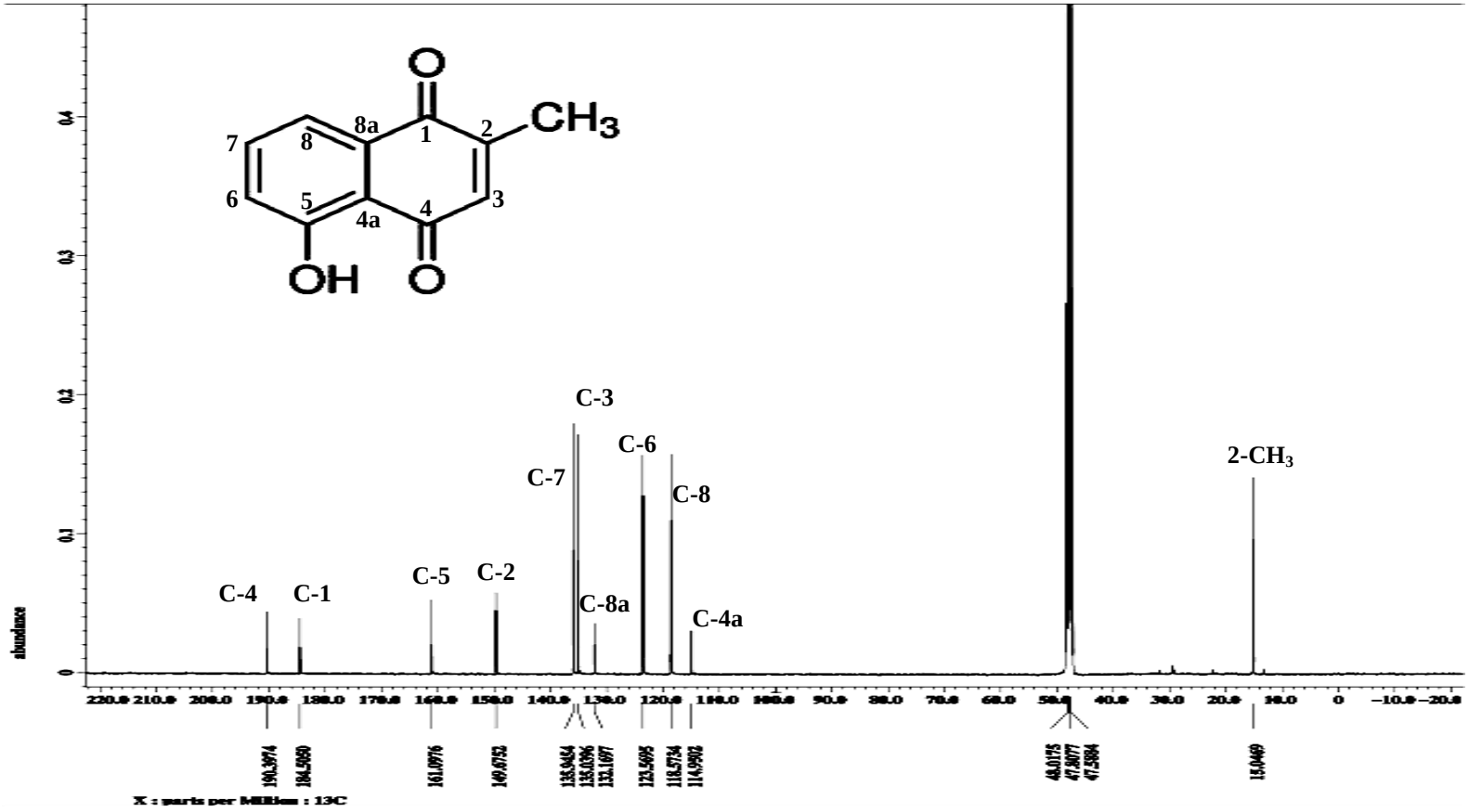


Figure 4.17: ^{13}C NMR (100 MHz) spectrum of compound B_1.

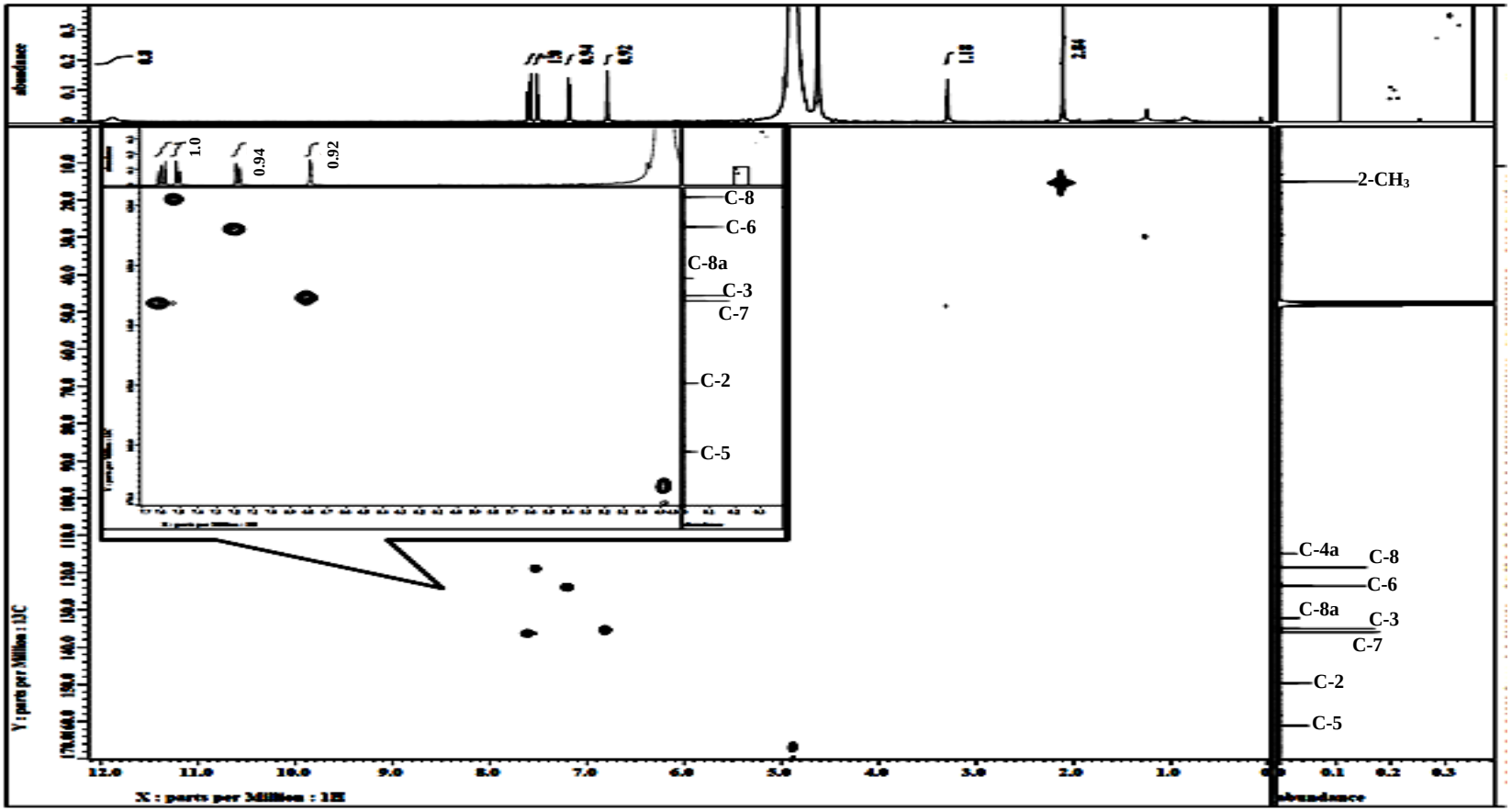


Figure 4.18: HMQC spectrum of compound B_1.

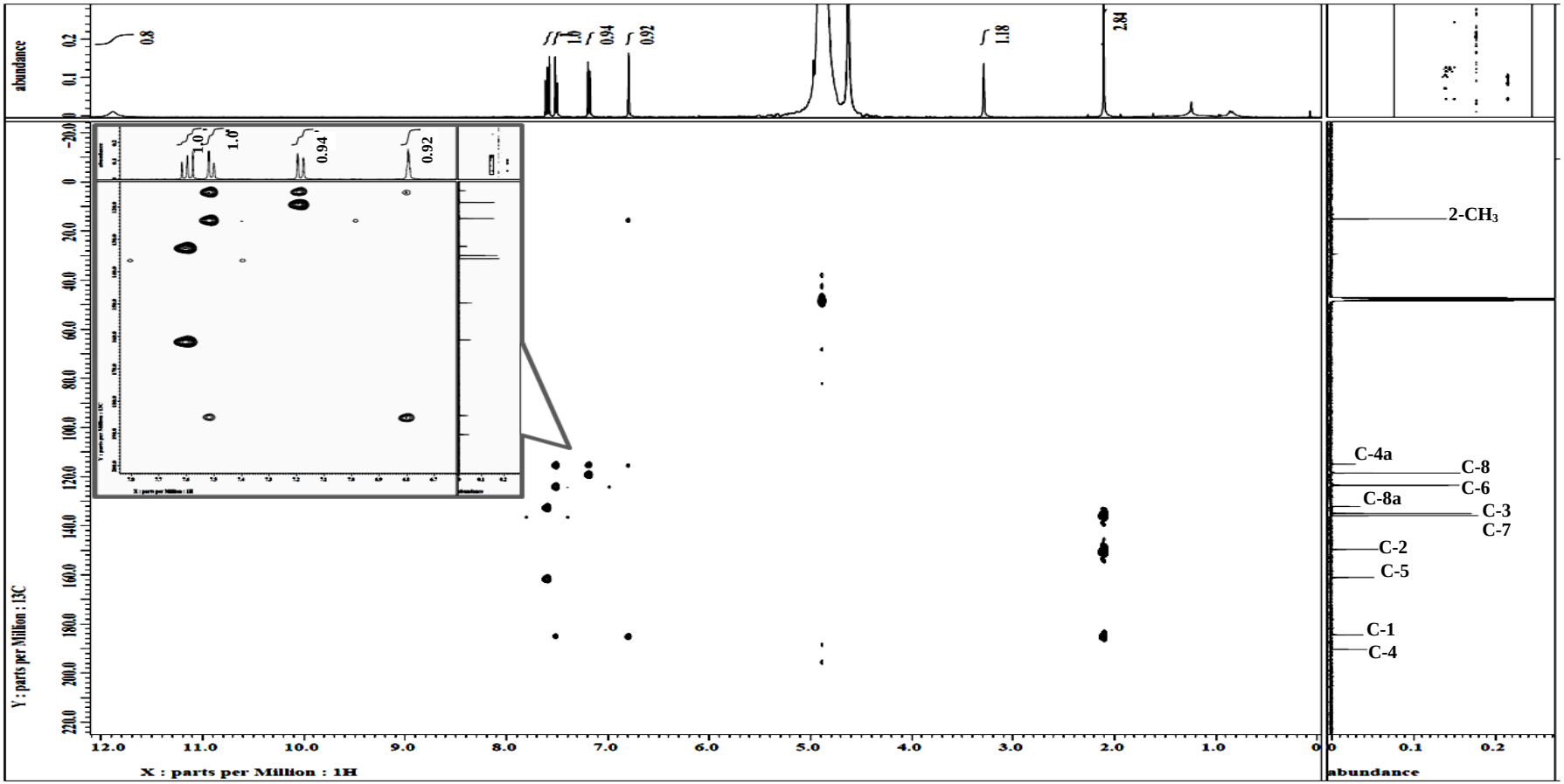


Figure 4.19: HMBC spectrum of compound B_1.

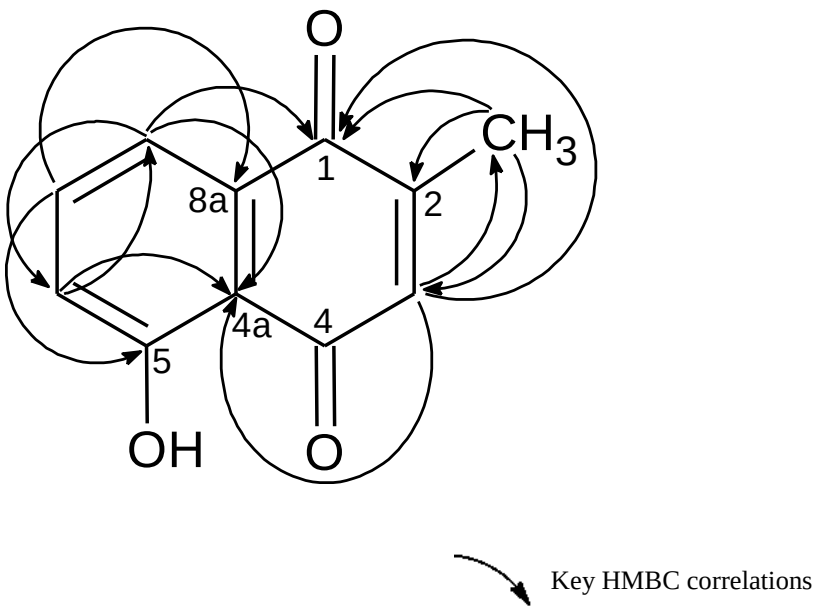


Figure 4.20: Key HMBC correlations of plumbagin (5-hydroxy-2-methyl-naphthalene-1,4-dione).

4.7 Antifungal Activity of Plumbagin

The antifungal activity of the purified plumbagin isolated from *N. gracilis* against six types of fungi was evaluated using the colorimetric broth microdilution method. The MIC and MFC of the isolated plumbagin are shown in Table 4.8. The MIC values ranged from 2 to 31 μg/mL for plumbagin and from 0.125 to 8 μg/mL for amphotericin B. Plumbagin effectively inhibited the growth of all the fungi and exerted a fungicidal effect, with the MFC values ranging from 2 to 63 μg/mL. *C. albicans,* and *I. orientalis* being the most susceptible species to plumbagin with the lowest MIC and MFC values of 2 μg/mL. Concentration of plumbagin that inhibited the growth of *I. orientalis,* and *T. mentagrophytes* was about 2-fold and 4-fold lower, respectively, than that required for amphotericin B (Table 4.8).

Table 4.8: Minimum inhibitory concentration and minimum fungicidal concentration of plumbagin against all the fungi.

Fungal Strains	MIC (µg/mL)		MFC (µg/mL)
	Plumbagin	Amphotericin B	Plumbagin
C. albicans	**2**	2	**2**
C. parapsilosis	8	2	16
I. orientalis	**2**	4	**2**
C. neoformans	4	0.125	8
A. brasiliensis	31	2	63
T. mentagrophytes	2	8	4

The results are the mean values of triplicates.

4.8 Cytotoxicity Determination of Plumbagin

4.8.1 Determination of Optimum Cell Seeding Density

The optimum cell seeding density of LLC-MK2 cell line was assessed through the plotted absorbance value-cell density curve (Figure 4.21). Cell growth consists of four distinct phases: (a) exponential growth phase; (b) transitional phase; (c) stationary phase and (d) death phase. The seeding density of $3x10^4$ cells/well falls within the late transitional and the early stationary phase of the curve and thus was used as seeding density for the cytotoxicity assay.

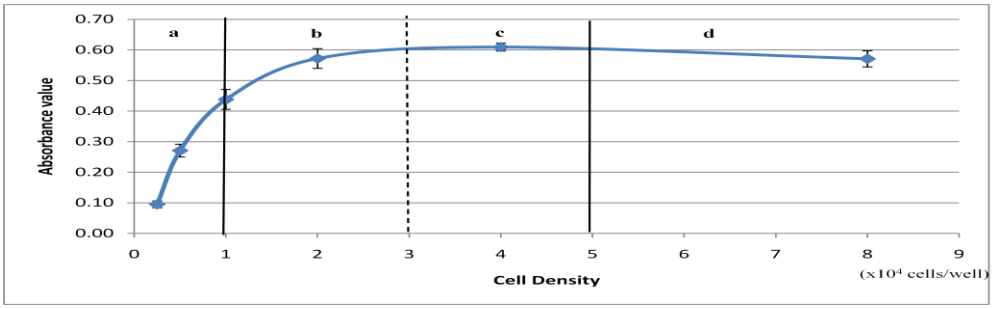


Figure 4.21: Analysis of optimum cell seeding concentration of Rhesus monkey kidney epithelial cells (LLC-MK2) in 96-well microplate by using the NRU assay.

Footnote: a, Exponential growth phase; b, transitional phase; c, stationary phase; d, death phase; - - - - period within the late transitional and the early stationary phase. Each data point represents mean ± S.D of triplicate assays.

4.8.2 Determination of 50% Cytotoxic Concentration (CC_{50})

4.8.2.1 Cytotoxic Effects of Diluent on LLC-MK2 Cell Line

Various concentrations of the diluents, methanol:water mixture (2:1, v/v) versus percentages of LLC-MK2 cell viability were plotted in the graph (Figure 4.22). At higher concentrations of diluent (0.78-12.5%, v/v) were found to be significantly cytotoxic ($p< 0.05$) to the cell line as the percentages of cell viability after treatment for 72 h were less than 10%. The CC_{50} value of diluent obtained was 0.60% (v/v) on the LLC-MK2 cell line. At the concentration ≤ 0.39% (v/v), no cytotoxicity was observed as the cell viability percentages were more than 90% (Figure 4.22). The final percentage of the methanol: water mixture (2:1, v/v) contained in the testing well during the cytotoxic assay for plumbagin was at the nontoxic concentration (< 0.25%, v/v).

4.8.2.2 Cytotoxic Effects of Plumbagin on LLC-MK2 Cell Line

The percentage viability of LLC-MK2 cell treated with various concentrations of plumbagin from *N. gracilis* as measured by the NRU assay is given in Figure 4.23. Plumbagin was found to be significantly cytotoxic ($p< 0.05$) to the cell line at the concentrations ranging from 0.78 to 6.25 μg/mL with the percentage of cell viability values less than 10% after 72 h treatment. According to Figure 4.23, the 50% cytotoxicity concentration (CC_{50}) of plumbagin on the cell line was 0.60 μg/mL. No cytotoxicity was observed at concentrations less than 0.20 μg/mL as the percentages of cell viability were approximately 100%.

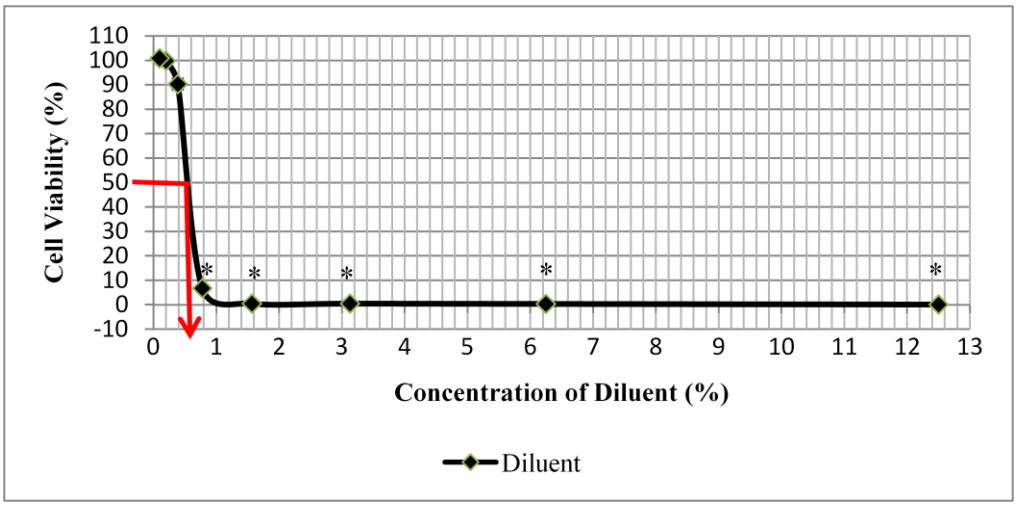


Figure 4.22: Cytotoxic effects of the diluent, methanol:water mixture (2:1, v/v) on LLC-MK2 cell line measured by the NRU assay.

Footnote: → $CC_{50;}$ LLC-MK2 cells were treated with various concentrations (0.01-12.50 %) of the diluents, methanol:water mixture (2:1, v/v) for 72 h. The results represent mean ± standard deviation (S.D.) of triplicate assays;*statistically significant ($p<0.05$).The data were analyzed with one-way ANOVA using Statistical Package for the Social Sciences (SPSS) software and the significance level was set at $p<0.05$.

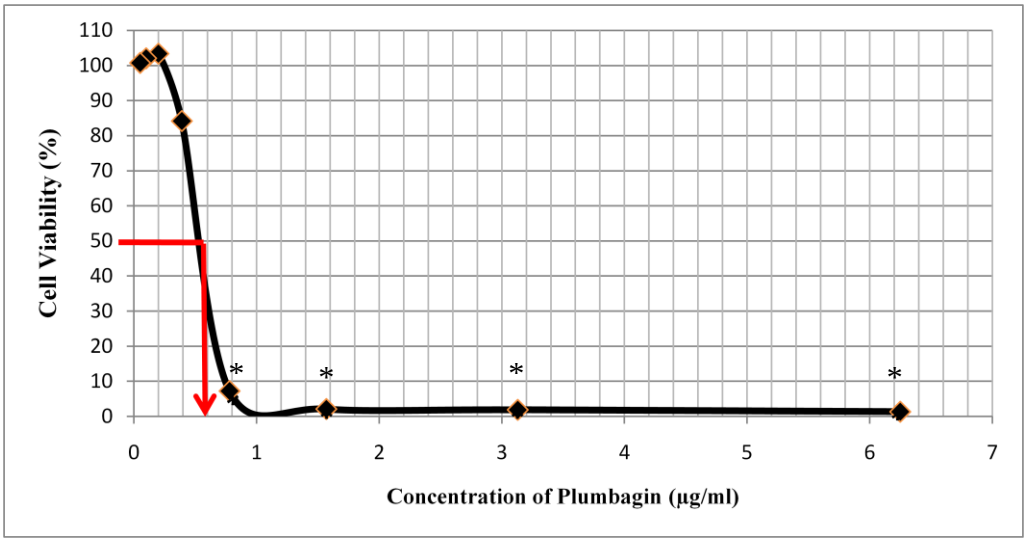


Figure 4.23: Cytotoxic effects of plumbagin on LLC-MK2 cell line measured by the NRU assay.

Footnote: → CC_{50}; LLC-MK2 cells were treated with various concentrations (0.05-6.25 µg/mL) of plumbagin for 72 h. The results represent mean ± standard deviation (S.D.) of triplicate assays, *statistically significant ($p<0.05$).The data were analyzed with one-way ANOVA using Statistical Package for the Social Sciences (SPSS) software and the significance level was set at $p<0.05$.

4.8.3 Selectivity Index of Plumbagin against Fungi

The toxicity of plumbagin to LLC-MK2 cell and the antifungal activity against the tested fungi were compared by using selectivity index (SI). The selectivity indices ($SI=CC_{50}/MIC$) of plumbagin against all the fungi were ranged from 0.02 to 0.30 (Table 4.9). A value greater than 1 is considered more selective for activity against the tested fungi (the compound showed specific bioactivity against fungi without affecting the tested cell line), and a value less than 1 is considered more selective for activity against LLC-MK2 cell (the compound showed general toxic effects on both tested fungi and cell line) (Tiuman et al., 2005).

Table 4.9: Selectivity index (SI) of plumbagin against fungi.

Fungal Strains	Selectivity Index
C. albicans	0.30
C. parapsilosis	0.08
I. orientalis	0.30
C. neoformans	0.15
A. brasiliensis	0.02
T. mentagrophytes	0.30

The results are the mean values of triplicates.

CHAPTER 5

DISCUSSION

5.1 Plant Sample Preparation and Extraction Method

There are four main approaches available for the selection of plants for a screening program to seek medicinal properties or the new bioactive chemical entities. These includes random collection followed by mass screening, exploitation of ethno-pharmacological knowledge in the selection, follow-up of existing literature leads or chemotaxonomic approaches (Suffness and Douros, 1979; Ghosh et al., 2011). However, selection of plants based on a combined analysis of ethnomedicinal, phytochemical, taxonomical and toxicological data are preferable due to the limited screening capacities of researchers (Ghosh et al., 2011).

In this study, fresh plant materials were used as the starting material for the extraction. The choices of plant parts used for the antifungal screening were guided by the ease of handling and availability of the different plant parts like the leaves, fruits, stems and etc. (Ncube et al., 2008). Although the selected medicinal plants (*A. ciliata*, *A. graminifolia*, *C. nucifera*, *D. esculentum*, *D. sarmentosa*, *H. sibthorpioides*, *M. platyclada*, *N. gracilis*, *P. edulis*, *P. speciosa*, *S. edule* and *S. muricatum*) have been used traditionally as herbal remedies, to

date, there have been relatively little or no reports examining their antifungal activities against medically important fungi.

Biological activity of plant extract is mostly dependent on the plant material, choice of solvent used and the extraction procedure (Das et al., 2010). Fresh or dried plant materials can be used as a source for the extraction of secondary metabolites. However, the use of dried plant material is not desirable for the extraction of compounds from plants due to degradation of natural metabolites during the drying process (Kaufman et al., 1999). Oven-drying at high temperature (> 60°C) and dehydration during the drying process (ambient air-, oven- and freeze-drying) might cause the loss of oil, volatile components, hydrocarbons, polyphenols, condensed tannins and antioxidant activity of plants (Wiart, 1984; Bos et al., 2002; Nindo et al., 2003; Bodo et al., 2004; Abascal et al., 2005). On the other hand, traditional or tribal healers prepare a wide range of healing juices, crude extracts and paste from various fresh plant materials (Das et al., 2010).

Fresh plant materials which used in this study were blended to finer sizes prior to extraction. Size reduction increases the surface area for extraction which in turn enhances the mass transfer of active components from plant material to the solvent (Ncube et al., 2008; Ghosh et al., 2011). Thus increases the rate of extraction thereby shortened the extraction period (Das et al., 2010). In addition, to increase the extraction efficiency and the quantity of extracts, plant

materials were extracted in two cycles per each solvent. Replacement of fresh solvent with the solvent that is in equilibrium will change the concentration gradient of solvent and eventually enhances the mass transfer of the remaining active components into the fresh solvent (Ghosh et al., 2011).

A variety of solvents are applied as extractants for their ability to solubilize secondary metabolites from plants. In this study, sequential solvent extraction method was used to obtain various plant extracts for the antifungal screening. This extraction method involves successive extraction with solvents of increasing polarity from a non-polar to polar solvent (hexane, chloroform, ethyl acetate, ethanol, methanol and aqueous) to extract a wider polarity range of active compounds from the plant materials. As the purpose of this study is to screen the selected plant for a variety of active compounds against medically important fungi, this extraction method is more appropriate to be used in order to achieve this purpose.

In this study, both ethanol and methanol were used as polar organic solvents in extraction as some compounds are obtained only in specific solvent such as xanthoxyllines, totarol, quassinoids, lactones and phenones are extracted by methanol; polyacetylenes, sterols and propolis are extracted by ethanol; starches, polypeptides and lectins are commonly obtained by water (Cowan, 1999). Though there is a wide diversification in the usage of solvents, the choice of solvent also depends on the main purpose of study. For instance,

study of general phytochemical analysis or preliminary screening of bioactive compounds, the wider the variety of compounds the solvent will extract the better as there is a higher chance that biologically active compounds will be discovered (Eloff, 1998).

5.2 Total Percentage of Yield

The total percentage of yields extracted from the 12 medicinal plants using solvents of varying polarity are shown in Appendix B. The total percentage of yields obtained from the leaves of *A. ciliata*, *A. graminifolia*, *C. nucifera* and *N. gracilis* ranged from 2.88% to 9.26%. The distribution of secondary metabolites in different plants varies depending upon the organs and tissues (Sheen, 1969; Hariram et al., 2011). Phytochemicals derived from the leaf and stem materials are the main component of many botanical drugs, classical examples are the tea tree (*Melaleuca alternifolia*), balm (*Melissa officinalis*), ginkgo (*Ginkgo biloba*), peppermint (*Mentha x piperita*), ephedra (*Ephedra sinica*), hawthorn (*Crataegus monogyna* and *Crataegus oxyacantha*) and wormwood (*Artemisia absinthium*) (Waksmundzka-Hajnos et al., 2008). The beneficial medicinal effects of plants are unique to a particular plant species or family. However, the combination of secondary metabolites in a particular plant is varying between organs or tissues, among species from tree to tree and from season to season (Gottlieb, 1990; Parekh et al., 2005). According to Figure 4.1, ethanol extract of all the four plants (*A. ciliata*, *A. graminifolia*, *C. nucifera* and *N. gracilis*) showed the highest percentage of yield as compared

to the other extracts. These indicate there are more polar compounds distributed in the leaves of these plants than non-polar compounds.

5.3 Antifungal Susceptibility Testing

5.3.1 Panel of Test Fungi

In this study, the five species of opportunistic fungal pathogens including *C. albicans, C. parapsilosis, I. orientalis, C. neoformans, A. brasiliensis* and a pathogenic dermatophyte, namely *T. mentagrophytes* were selected in the screening for antifungal activity of the medicinal plant extracts.

The choice of test microorganisms depends on the main purpose of study. In a primary investigation, drug-sensitive reference strains are preferably used and should also represent medically important species of different classes (Cos et al., 2006). A small set of proposed panel of test microorganisms for primary antifungal *in vitro* screening for natural products includes *C. albicans* as representatives of the yeast, *T. mentagrophytes* and *Epidermophyton floccosum* for the dermatophytes, *Aspergillus niger* and *Fusarium solani* for the filamentous fungi (Cos et al., 2006).

5.3.2 Colorimetric Broth Microdilution Method

The antifungal activity of plant extracts were assessed by the colorimetric broth microdilution technique which is a quick, sensitive, quantitative test that

produces reproducible results with decreased cost, space and labour (Eloff, 1998; Lee, 2009). Amphotericin B was applied as reference antibiotic for positive control in the susceptibility testing. All the fungi (*C. albicans, C. parapsilosis, I. orientalis, C. neoformans, A. brasiliensis* and *T. mentagrophytes*) tested had MIC values of ≤8 μg/mL, which the MIC ranges were similar to those previously reported in the *in vitro* activity of amphotericin B (Malheiros et al., 2005; Masoko et al., 2007; Shai et al., 2008; Thompson III and Wiederhold, 2010).

5.3.3 Antifungal Activity of Plant Extracts

The fungistatic and fungicidal effects of plant extracts were evaluated by MIC and MFC, respectively. The diluent, methanol:water mixture (2:1, v/v) used to dissolve the plant samples was devoid of any antifungal activity with the highest concentration tested, 25% (v/v). A methanol:water mixture prevents rapid evaporation of solvent and this helps to maintain the stated concentration of stock solutions (Cos et al., 2006).

All the plant extracts showed antifungal activity with 69.21% and 41.20% of the bioassays demonstrating fungistatic and fungicidal activities, respectively. Aligiannis et al. (2001) categorized the plant extracts to be strong inhibition by MIC value up to 0.5 mg/mL; moderate inhibition by MIC between 0.6 and 1.5 mg/mL and weak inhibition with MIC above 1.6 mg/mL for the antimicrobial susceptibility testing. The MIC values obtained in this study indicate that 30%

of the bioassays showed strong inhibition against the fungal strains; with MIC values ranging from 0.02 to 0.31 mg/mL.

The hexane extract of *N. gracilis* showed the lowest concentration ranges of fungistatic and fungicidal activities against all the fungal pathogens evaluated. The results suggested that the active components presence in this extract not only exerted potent fungistatic or fungicidal effects, but also demonstrated broad spectrum of antifungal activity. Other members of the family Nepenthaceae have shown antimicrobial activity against several microorganisms. Methanol extract of *N. mirabilis* (whole plant) was active against *Staphylococcus aureus* (Wiart et al., 2004). The hexane extract of *N. ventricosa* x *maxima* leaf exhibited strong antifungal activity against all the phytopathogenic fungi with MIC values ranging from 0.007 to 0.04 mg/mL, especially against *A. niger* with MIC of 0.02 mg/mL (Shin et al., 2007a). Although the medically-important fungi used in this study are different from the phytopathogenic fungi, a comparable MIC of 0.02 mg/mL against *A. brasiliensis* was demonstrated by the hexane extract of *N. gracilis*. The antifungal properties could be due to the same phytochemicals that were present in the hexane extract from the *N. ventricosa x maxima* leaf (Shin et al., 2007b).

Endogenous flavonoid gallate esters and several phenolic constituents, such as plumbagin, isoshinanolone, epishinanolone, shinanolone, quercetin and

kaempferol were identified from the leaves of *N. gracilis* (Aung et al., 2002; Fan et al., 2010). However, reports of pharmacological and biological investigation of *N. gracilis* are scarce and no studies have been reported on the antifungal activity of *N. gracilis* against medically-important fungi. At the preliminary stage of this study, it is yet not known what constituent is responsible for the antifungal activity. Isolation and purification of the biological active compounds from the hexane extract of *N. gracilis* to further confirm the antifungal potential is warranted.

The total activity is an arbitrary measure of the quantity of antifungal compounds present in the extract and indicates the largest volume to which the biologically active compounds in 1 g of the plant material can be diluted and still effective to inhibit the growth of the fungi (Eloff, 2004). The total activities of plant extracts against the fungi indicated that the ethanol extract of *P. speciosa* was the most active extract as it showed the highest total activity against *I. orientalis* (2080 mL/g) and *C. neoformans* (1040 mL/g). This implies that the ethanol extract of *P. speciosa* prepared from 1 g of plant material could be diluted to a volume of 2080 mL and 1040 mL, still retain fungistatic activity against *I. orientalis* and *C. neoformans*, respectively. It indicates the possible commercial value of such an extract if the mammal and environmental toxicity is within an acceptable level (Mahlo et al., 2010). Besides MIC and MFC results, the total activity and percent activity of *P. speciosa* also highlighted the antifungal potency of this plant.

P. speciosa is locally known as "petai" which is traditionally used to apply externally for wounds and ulcers, as well as to treat dermatitis (Ong, 2008; Roosita, 2008). Previously reported biological activities of the seed extracts of *P. speciosa* were attributed to the presence of some bioactive agents such as the isolation of several cyclic polysulfides are used for the treatment of antibacterial activity on kidney, ureter and urinary bladder infections (Gmelin et al., 1981), thiazolidine-4-carboxylic acid for anticancer activity (Suvachittanont et al., 1996) and β-sitosterol and stigmasterol for hypoglycemic effect (Jamaluddin et al., 1994). There were no previous reports on the antimicrobial activity of the pod extracts of *P. speciosa*. In facts, the extracts were found to contain high levels of phenolic compounds which contributed to its antiangiogenic and antioxidant properties (Aisha et al., 2012). The presence of high level of phenolic compounds in the *P. speciosa* pod extracts might explain the observed antifungal activities in this study.

All the plant extracts showed antifungal activity and 18 extracts obtained from 8 species of medicinal plants demonstrated 100% antifungal potency (Figure 4.4). These medicinal plants (except *S. muricatum*) are used in traditional medicine for the treatment of microbial infections such as to treat gum and throat infections (*A. ciliata*), dermatitis and measles (*D. esculentum*), and etc as listed in Table 2.5. The data reported herein show that these medicinal plants have substantial antifungal activity against medically-important fungi, which corroborates their use in traditional medicine for the treatment of microbial infections.

Previous report has demonstrated that the presence of ascorbic acid, phenolic acids and flavonoids in the extracts of *S. muricatum* possess antioxidative, anti-inflammatory and antiglycative effects that could attenuate the progression of diabetes in a mouse model (Hsu et al., 2011). The polyphenols of the fruits of *S. muricatum* were also shown to possess good scavenging activity (Sudha et al., 2011). These bioactive compounds are known to be synthesized by plants in response to microbial infections that may disrupt the microbial membrane through enzyme inhibition by the oxidized compounds as well as to complex with extracellular and soluble proteins (Mason and Wasserman, 1987; Tsuchiya et al., 1996). Such occurrences may explain the antifungal activity observed with the extracts obtained from the fruits of *S. muricatum* in this study.

All of the six extracts obtained from the stems of *M. platyclada* showed percent activity values of equal or more than 83% (Figure 4.4). Fagundes et al. (2010) reported that the antinociceptive and anti-inflammatory activities of *M. platyclada* could be related to the synergistic activity of some bioactive compounds presence in the leaves such as flavonoids, saponins, tannins, terpenoids, sterols, coumarins, volatile oils, triterpenes and steroids. Antimicrobial properties of flavonoids, saponins, tannins and terpenoids for examples are well documented (Cowan, 1999).

Literature review on the phytochemical constituents of *M. platyclada* revealed that the antibacterial compound isolated from the leaves extract was a flavonol glycoside with quercetin as an aglycone (Susidarti et al., 2011). It appears that there is no report in the literature concerning the antifungal activities of the stems extracts of *M. platyclada.* However, the present work revealed that the stems of this species have effective antifungal activity against *Candida* spp., *C. neoformans*, *A. brasiliensis* and *T. mentagrophytes*, which also confirms some of its ethnomedicinal uses for the treatment of microbial infections such as bacterial infection, wound infection, sores, skin swelling, oral ulceration, wart and curing bruise (Siriwatanametanon et al., 2010; Susidarti et al., 2011).

In vitro assays may provide a guideline for the selection of highly active plant extracts for subsequent isolation and characterization of potentially useful bioactive compounds (McGaw et al., 2007). To determine which plants have the highest potential for further development, not only the MIC value is important, the quantity of the bioactive compounds extracted from the plant material is also one of the factors to be considered (Eloff, 1999). The availability of quantities of pure chemical substances sufficient for lead optimization, lead development, and clinical trials is the main consideration in natural product for pharmaceutical discovery and development (McChesney et al., 2007).

The results of the antifungal screenings indicate that the hexane extract of *N. gracilis* possess potent antifungal properties. Thus, the extract was selected for fractionation and bioactivity-guided isolation of the antifungal compound(s) based on the lowest MIC and MFC (0.02 mg/mL), the high total activities values (≥58.75 mL/g) as well as the highest percent activity value (100%) against all the fungi. It appears that there is no report in the literature concerning the antifungal activity against human pathogens for *N. gracilis*. Generally, none of the crude extracts showed MIC values comparable to those of the reference antibiotic (amphotericin B) as the active compound(s) in a crude extract may constitute only a small proportion of the array of compounds in the extract and the lowest concentration tested was at 0.02 mg/mL.

5.3.4 Fungal Susceptibility Index (FSI)

Fungal susceptibility index is expressed as percentage value that is used to compare the relative susceptibility among the fungal strains, values range from 0% (resistant to all plant extracts) to 100% (susceptible to all plant extracts) (Bonjar, 2004). The antifungal activity of the plant extracts in this study was more pronounced on the yeast strains, particularly on *C. neoformans* with FSI of 100% (Figure 4.5).

The results indicate that all of the plant extracts are able to exhibit fungistatic effect against the heterobasidiomycetous fungus, *C. neoformans* even it possesses a unique structural feature, a protective capsule which is not common

with other fungi. In spite of that, *C. neoformans* is also one of the yeasts frequently responsible for biofilm-associated infections (Vandeputte et al., 2012). The fungal biofilms are resistant to almost all the currently used antifungals (exceptions of echinocandins and lipid formulation of amphotericin B) and is also known to withstand host immune defences (Chandra et al., 2005; Heitman, 2006). As a result, they constitute a nonnegligible source of nosocomial fungal infections, essentially through the use of medical devices (Vandeputte et al., 2012). Therefore biofilm-associated infections represent a mean of survival within the host, constituting a persistent source of infections (d'Enfert, 2009).

The CLSI guidelines on broth microdilution techniques for antifungal susceptibility testing of yeasts are using the planktonic populations in the inocula preparation and this will not allow a prediction of antifungal efficacy against the fungal biofilms (CLSI, 2002a). However, data from the present study provides essential information of plants which exhibited fungicidal effects against these medically-important fungi. This may ultimately be useful in disclosing the medicinal plants with better therapeutic value and in the search for novel antifungal agents.

The molds were found to be more resistant as *T. mentagrophytes* and *A. brasiliensis* possessed only 58.33% and 25% of susceptibility to the tested plant extracts, respectively. The findings that *Aspergillus* species is the least

inhibited fungus to plant extracts are agreed with the susceptibility of this genus to different plants extracts reported by several studies. Tadeg et al. (2005) reported *Aspergillus niger* was found to be virtually insensitive to all plant extracts obtained from eight species of medicinal plants. Among the five fungal pathogens evaluated, *Aspergillus fumigatus* was found to be the least sensitive fungus to all the extracts obtained from 24 *Combretum* species and also the leaf extracts of seven South African plant species (Masoko et al., 2007; Shai et al., 2008). The conidia of *A. niger* were comparatively less susceptible to tea tree oil than the tested yeast cells (Hammer et al., 2002).

It is important to note that according to the CLSI guidelines on broth microdilution techniques for antifungal susceptibility testing of filamentous fungi, the fungal inocula are predominantly conidia. However, during incubation period with suitable conditions, these conidia will germinate and grow into hyphae (Manavathu et al., 1999; CLSI, 2002b). The thickness and density of the conidial wall might responsible for the reduced susceptibility of conidia to antifungal agents (Cheng and Levin, 1970). Moreover, germination of hyphae might also contributed to the reduced susceptibility of filamentous fungi during the antifungal susceptibility testing as the thickness of hyphae is greater than conidial wall (Hammer et al., 2002). One of the key features of *Aspergillus* spp. morphology is their hyphae will grow with tendency to form short, distorted complex hyphae that may be as wide as 12 µm with appearance nearly parallel to one another and form the mycelium (Larone, 2002). This

might subsequently contribute to the reduced susceptibility of *A. brasiliensis* against the plant extracts.

5.4 Isolation and Structure Elucidation of Antifungal Compound from *N. gracilis*

5.4.1 Bioactivity-guided Fractionation of the Hexane Extract from *N. gracilis*

The hexane extract of *N. gracilis* was further fractionated through liquid-liquid partitioning to give three fractions, the hexane fraction (F_1), ethyl acetate fraction (F_2), and water fraction (F_3). Among the fractions, F_1 afforded maximum yield (0.33%) followed by F_2 (0.10%) and F_3 (0.01%). The F_1 and F_2 fractions showed antifungal activities on the fungi with MIC and MFC values ranged from 8 to 31 μg/mL, while F_3 did not show any antifungal activity (Table 4.3). Comparison of the fungistatic and fungicidal activities of the fractions with that of the hexane crude extract indicated that the F_1 fraction showed stronger in activity than the crude extract against *C. neoformans* and *T. mentagrophytes*. The antifungal potency increased upon fractionation which supports the fact that active compounds are concentrated more in the fraction than crude extract.

Theoretically the activity shown by a mixture in a crude extract is due to the sum of the activities of the individual constituents (Houghton et al., 2007). Following fractionation, leading to the concentration and isolation of individual compounds, should result in aliquots having a stronger activity than the

original crude extract. This is the basic of standardizing extracts for predictable activity, based on the correlation of content of a particular compound with the desired pharmacological effect (Houghton et al., 2007). Furthermore, it is important that the most active compound is present in sufficiently high amounts in the particular fraction to give activity and adequate for further isolation and characterization. Thus, the F_1 fraction was selected for further fractionation and isolation of the bioactive compound(s) as it showed the highest percentage of yield and the strongest antifungal activity among the analyzed fractions from the hexane extract of *N. gracilis*.

5.4.2. Isolation and Structure Elucidation of Compound B_1 from *N. gracilis*

The TLC preparative-scale isolation was used to obtain the compounds present in the F_1 fraction. It is a conventional method of separation and isolation of small quantities of mixture components. It can also be applied for the isolation of individual compounds before their identification by various physicochemical methods (Waksmundzka-Hajnos et al., 2008). The separation of F_1 fraction revealed that there were two major compounds (B_1 and B_2) with different absorption properties are present in the F_1 fraction (Figure 4.6). The first compound (B_1) with higher R_f value of 0.34 was detected in visible light and UV light (254 nm and 366 nm) as yellow, dark red and dark brown band, respectively. The second compound (B_2) was resolved under 366 nm UV-light as a brilliant light blue band at R_f 0.21. The results also confirmed that compounds B_1 and B_2 are originally contained in the hexane extract. Compound

B_1 exhibited a stronger antifungal potency with MIC and MFC values ranged from 2 to 8 μg/mL against the indicative microorganisms (Table 4.4).

The UV/VIS absorption spectrum of compound B_1 in the region 200–700 nm showed the present of two characteristic bands, an intensive band at 260 nm and a broad band at 425 nm (Figure 4.8). The absorption spectrum revealed that the isolated plumbagin was completely in agreement with the reference standard from the published data (Bothiraja et al., 2011; Chen et al., 2011). The melting point of compound B_1 was recorded as 78-79°C is consistent with those reported by Bothiraja et al. (2011) as the reported melting point for the isolated plumbagin was at 78.4°C while for the reference standard plumbagin was at 78.6°C. The GC-MS mass spectrum obtained matched with the reference standard plumbagin reported by Shin et al. (2007b). The negative product ion mass spectrum of the deprotonated molecule of plumbagin showed a precursor ion *m/z* 186.9 and a product ion *m/z* 159.0, while the plumbagin isolated from the roots of *Plumbago zeylanica* was reported at *m/z* 187 and *m/z* 159, respectively (Hsieh et al., 2005). The ^{13}C NMR and ^{1}H NMR suggested that compound B_1 was a napthoquinone structure with the molecular formula $C_{11}H_8O_3$, which was further supported by the HMBC and HMQC spectra. On the basis of the above spectroscopic analyses and comparing with published data (Hsieh et al., 2005; Bothiraja et al., 2011; Chen et al., 2011), the structure of plumbagin isolated from the hexane extract of *N. gracilis* was deduced.

In this study, the plumbagin yield obtained from the fresh leaves of *N. gracilis* was 0.12%. The types of solvents and plant tissues used may affect the overall yield of plumbagin. Babula et al. (2009) reported that the highest yield of naphthoquinones was obtained by using methanol and the lowest using hexane. However, hexane was found to result in high recovery with highest degree of naphthoquinones purity (Grevenstuk et al., 2008). In addition, Babula et al. (2009) also quantified and compared the amount of plumbagin presence in the extracts of different tissues from *N. spectabilis* and *N. ventricosa*. The authors concluded that the content of plumbagin varied in different plant tissues as the highest amount was determined in apex and the lowest ones in petiole. Generally, different extraction methods, botanical varieties, geographical origin, seasonal variation, and many extrinsic factors such as soil, light, water, temperature and nutrients may affect the quality and quantity of phytochemical accumulation in plants (Ahmad et al., 2006).

5.5 Antifungal Activity and Cytotoxicity of Plumbagin

Several naphthoquinone derivatives such as plumbagin, isoshinanolone, epishinanolone, shinanolone, droserone, hydroxydroserone, nepenthones and 2-methylnaphthazarin were identified as secondary metabolites produced by *Nepenthes* spp., including the leaves from *N. gracilis*, roots from *N. rafflesiana* and *N. thorelli*, as well as the whole plant extracts of *N. insignis* (Bringmann et al., 2000; Aung et al., 2002; Rischer et al., 2002). Among these, plumbagin is the most efficient secondary metabolite isolated so far from carnivorous plants (Eilenberg et al., 2010). Pavela (2013) studied the acute toxicity of seven

structurally similar naphthoquinones including plumbagin, lawsone, juglone, naphthazarin, menadione, 1,2-naphthoquinones and 1,4-naphthoquinones against the adults house fly (*Musca domestica*), of which plumbagin was the only one to show good acute toxicity. Plumbagin is the most active compound among the eight quinones evaluated against spider mite and aphids which may have potential as commercial insecticides and miticides (Akhtar et al., 2012).

Plants with naphthoquinone and other related quinine content are widely used in the traditional medicine systems, as these phytochemicals are one of the major natural product classes with varied biological activities (Thomson, 1971; Matysik et al., 2008; Babula et al., 2009). Plumbagin is considered to be a potential antifungal drug (Eilenberg et al., 2010) which is produced by many vascular plants from families that belong to the Caryophyllales order (Rischer et al., 2002) such as Droseraceae (Budzianowski, 2000), Drosophyllaceae (Bringmann et al., 1998), Plumbaginaceae (Bothiraja et al., 2011), Ebenaceae (Evans et al., 1999) and Nepenthaceae (Aung et al., 2002).

The *in vitro* antifungal activity of the isolated plumbagin from *N. gracilis* showed that it is as effective as the clinically used antifungal agent. The MIC values ranged from 2 to 31 μg/mL for plumbagin and from 0.125 to 8 μg/mL for amphotericin B against all the fungi. The plumbagin concentration that inhibited the growth of *I. orientalis*, and *T. mentagrophytes* was about 2-fold and 4-fold lower, respectively, than that required for amphotericin B.

Plumbagin has a wide range of biological properties such as anti-cancer (Kawiak et al., 2007), antiviral (Perez-Sacau et al., 2003), anti-inflammatory, antiplatelet, antiallergic (Lien et al., 1996), antimalarial (Biot et al., 2004), antibacterial (Cai et al., 2000) and antifungal (Dzoyem et al., 2007). Despite there are numerous documentation about the therapeutic potential of naphthoquinones, their exact mode of action still remains unclear (Eilenberg et al., 2010).

Plumbagin exhibited potent antifungal activity as compared with amphotericin B, it could be considered as a promising antifungal agent. However, the desirable properties of an antifungal agent are not only to exert potent fungistatic or fungicidal effects, but also with minimum toxicity on normal mammalian cells. In this study, the toxicity of plumbagin to Rhesus monkey kidney epithelial cells (LLC-MK2) and the antifungal activity against the tested fungi were compared by using the selectivity index (SI). A value greater than 1 is considered more selective for activity against the fungi, and a value less than 1 is considered more selective for activity against LLC-MK2 cell (Tiuman et al., 2005). A compound with SI values greater than 1 is preferable as it shows specific antifungal activity against the fungal cells without affecting the mammalian cells. According to Table 4.9, the isolated plumbagin was more selective against the mammalian cells (LLC-MK2) than the fungi, with SI values ranged from 0.02 to 0.30. The results also indicate that plumbagin is more toxic to mammalian cells than to fungal cells.

Two major mechanisms have been proposed for plumbagin cytotoxic action in various biological systems. First mechanism is associated with the excessive generation of reactive oxygen species (ROS) such as superoxide radicals, singlet oxygen and hydrogen peroxide (Seung et al., 1998; Castro et al., 2008). Second mechanism involved the redox and oxidation cycle of quinones namely "redox cycles" which lead to their ability to act as potent electrophiles to inhibit the electron transportation, as uncouplers of oxidative phosphorylation, as intercalating agent in the DNA double helix and as bio-reductive alkylating agents in the bio-molecules (Babula et al., 2009). The redox cycling and free radical forming was identified as the main mode of actions for plumbagin (Castro et al., 2008; Eilenberg et al., 2010).

Long-term use of medications containing quinine derivatives may cause serious side effects and diseases such as neoplasms (Matysik et al., 2008). In animal studies, plumbagin was reported with many toxic side effects including diarrhea, skin rashes, increases in white blood cell and neutrophil counts, increases in serum phosphatase and acid phosphatase levels, and hepatic toxicity (Singh and Udupa, 1997). It has been noted that treatments with plumbagin can cause reproductive toxicity (Premakumari et al., 1977; Bhargava, 1984). In addition, animal studies suggest that plumbagin with its high lipophilicity and insolubility in water has produced low bioavailability for oral administration (Hsieh et al., 2006). Consequently, a large and frequent dose is necessary to achieve the optimum therapeutic efficacy and thus may lead to severe side effects (Singh and Udupa, 1997; Bothiraja et al., 2012).

The most widespread naphthoquinones in nature originated from shikimic acid are plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) and juglone (5-hydroxy-1,4-naphthoquinone) (Babula et al., 2009). Juglone along with plumbagin is exuded by the roots of the black walnut (*Juglans nigra* L.) into surrounding and soil which can be attributed to allelopathy and phytotoxicity (Babula et al., 2009). The cytotoxic effect of plumbagin in human peripheral blood lymphocytes was at least two-fold higher than juglone (Seshadri et al., 2011). Plumbagin at 5 µM was proven to be non-cytotoxic for the resting mouse lymphocytes, but at the concentration of 2 µM, plumbagin was found to induce apoptosis in human resting lymphocytes, indicating the species-specific differences in the activity of plumbagin (Checker et al., 2009). Thus its high cytotoxicity and relatively low therapeutic selectivity are the major challenge that limits its medical application (Kayser et al., 2003).

Modifications in the naphthoquinones core structure produced a variety of quinones with different substituents lead to marked differences in activity and consequently also in toxicity (Ryu et al., 2005; Eilenberg et al., 2010). Several plumbagin derivatives (5-hydroxy-1,4-naphthoquinone derivatives) have been synthesized and did not show significant cytotoxic against human lung adenocarcinoma epithelial cell line (A549) but showed selective antifungal activities (Ryu et al., 2005). *In vitro* assays showed that several 2,3-di-substituted 1,4-naphthoquinones are as effective as fluconazole and amphotericin B against human fungal pathogens (Tandon et al., 2009).

Plumbagin may be considering as natural product lead for the discovery of novel analogs. More analogs of the compound can be synthesized to discover a promising antifungal compound which is able to reduce selectable pressure for potentially evolved resistance as well as showing an effective antifungal action without affecting preparation stability, having high safety, quality to be used by patients is of great concern. Furthermore, the assessment of cell damage by plumbagin remains unknown and the antifungal activity of plumbagin on the fungal biofilm was not tested in this study.

5.6 Suggestions for Future Studies

It is important to design *in vitro* experiments to approximate as closely as possible to the clinical case or disease. Understanding the relationship between fungus and host during infection may lead to a better clinical outcome. Future study of antifungal susceptibility testing should also include the testing of biofilm rather than the planktonic populations, particularly investigate compounds which could inhibit or retard biofilm formation. Screening the antifungal assay at a specific time interval (month) is necessary to determine the stability of compounds in the extracts. It is very important to ensure the antimicrobial potential will not degrade when times goes on.

Pharmacological evaluation and toxicity testing involving other cell lines, mutagenic as well as carcinogenic responses of plumbagin and its closely related derivatives would be useful in further elucidating the cytotoxic effects

of these compounds. Further study may also include the assessment of cell damage by plumbagin (and probably other similar derivatives) through scanning electron microscope (SEM), transmission electron microscope (TEM), and atomic force microscope (AFM) observations. Such investigations may further explore the effect of plumbagin on morphological, surface features and ultra-structure of the fungi. There is a need to continuously improve the quality, efficacy, and quantity of bioactive compounds that enter the lead identification process as to keep pace with other drug discovery efforts.

The methods available for extraction of natural products are commonly utilized the hazardous organic solvents, alternative environmental friendly extraction technique such as the supercritical fluid extraction (SFE) technique can be used. SFE using the supercritical carbon dioxide eliminates time-consuming process of concentration and uses no or minimum organic solvent. Supercritical carbon dioxide is a solvent with lowest toxicity and inflammability for the extraction of natural compounds, especially thermolabile secondary metabolites (Waksmundzka-Hajnos et al., 2008). Further phytochemical studies should continue on isolating biologically active compounds from those medicinal plants demonstrating good activity in the initial screening included the extracts of *P. speciosa* and *M. platyclada.* The plant extracts can be further studied by working with a broader range of microorganisms such as resistance microbial strains and viruses. In addition, different parts of plants can be extracted and compared for their levels of secondary metabolites and potential antimicrobial activity.

CHAPTER 6

CONCLUSIONS

In this study, a total 72 extracts from 12 medicinal plants were evaluated for antifungal activity against human fungal pathogens (four yeasts and two molds) using the colorimetric broth microdilution method. All the plant extracts showed antifungal activity with 69.21% and 41.20% of the bioassays demonstrating fungistatic and fungicidal activities, respectively. Eighteen extracts from eight medicinal plants exhibited antifungal activity against all the fungi. All the plant extracts possessed antifungal activity against at least one strain of fungi. The yeast, *C. neoformans* was found to be 100% susceptible to all the plant extracts with MIC values ranging from 0.02 to 2.50 mg/mL. In contrast, *A. brasiliensis* was found to be the most insensitive among the tested fungal strains with a susceptibility index of 25%.

Overall results of the antifungal susceptibility screenings revealed that the hexane extract of *N. gracilis* possessed strong antifungal properties with low MIC and MFC and total activities values more than 58.70 mL/g against all the fungi. Bioactivity-guided fractionation, isolation and structural elucidation of the hexane extract of the leaves of *N. gracilis* yielded plumbagin (5-hydroxy-2-methyl-naphthalene-1,4-dione) with potent antifungal activity; MIC values

ranged from 2 to 31 μg/mL against all the fungi. The antifungal property of *N. gracilis* against human fungal pathogens is being reported for the first time.

Cytotoxic testing using LLC-MK2 cell indicated the isolated plumbagin is more toxic in mammalian cells than in fungal cells. The data reported herein are important, taking into account the medical importance of the studied fungi and that this plant species is traditionally consumed or applied externally as astringent, without taking into account the presence of highly toxic component such as plumbagin. Thus, pharmacological study of medicinal plants remains important to provide evidence with scientific basis for the continued traditional application of plants and understanding of a plant's efficacy as well as toxicity.

The results obtained in this study are in line with the traditional uses of the medicinal plants as natural source of antifungal agents. There is not much detailed documentation on the antifungal properties of Malaysian medicinal plants. This study will open up a scope for future utilization of these plants for therapeutic purposes especially towards those disease caused by pathogenic microorganisms. The scientific values of medicinal plants in term of quality, safety and efficacy have yet to be established together with the search of new lead structures in the drug development. The screening of plants for potential antifungal agents is important as it provides society with potential sources of new, effective and safer drugs and offering a great help in solving the problem of increasing emergence of antifungal resistance.

The oldest known living eukaryotic organism, turning 4778 years old in 2013, is a specimen of a bristlecone pine, *Pinus longaeva* from the White Mountains of Inyo County, California (Flanary and Kletetschka, 2005). It is reasonable to assume that some of these bioactive compounds that have enabled plants to survive may also be useful to maintain the health and well being of humans. In conclusion, the results obtained from the present study strongly support further investigation into bioactive agents of plant as they are considered an abundant resource of novel chemotypes and pharmacophores in the drug discovery. The future of drug discovery and development from plants thus appears to be a tale of justifiable hope.

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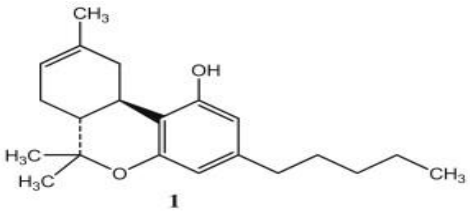
APPENDIX A

Struture of the lead compounds from natural products derived drugs from 2005 to 2010 (Mishara and Tiwari, 2011)**.**



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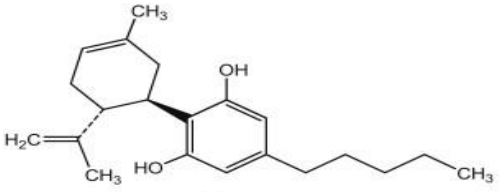

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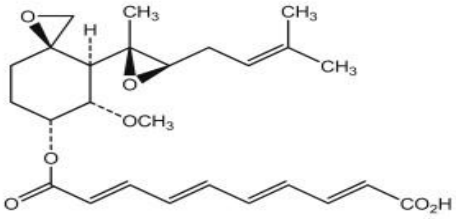

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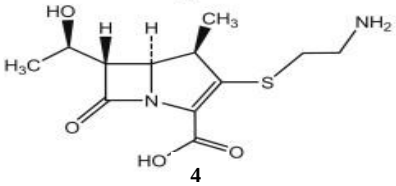

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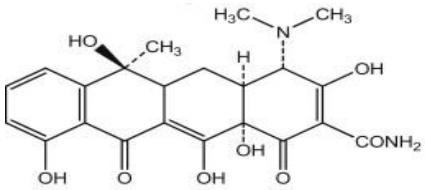

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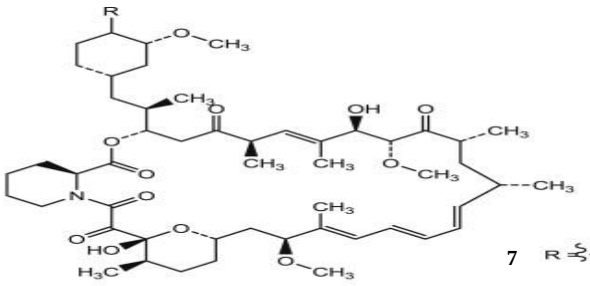

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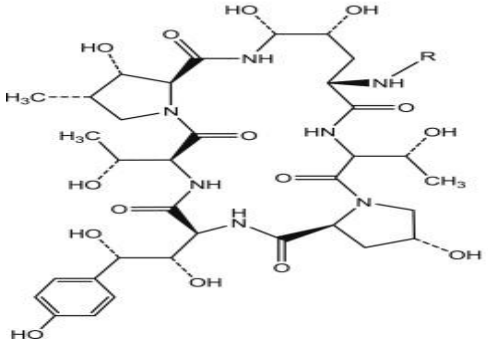

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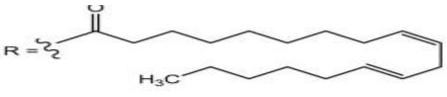

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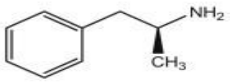

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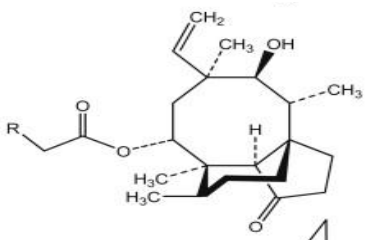

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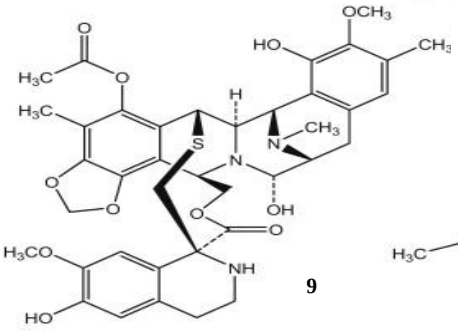

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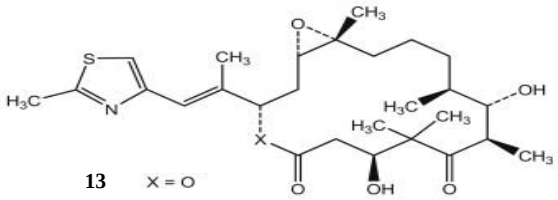
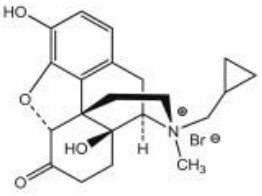


13 X = O



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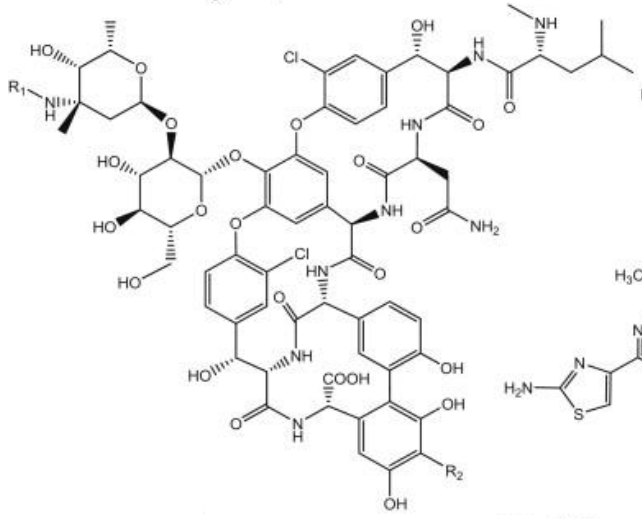

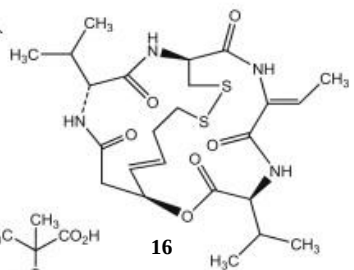

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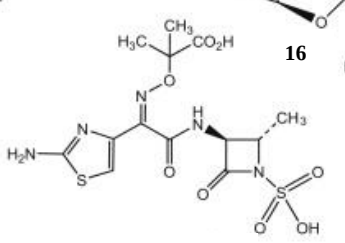

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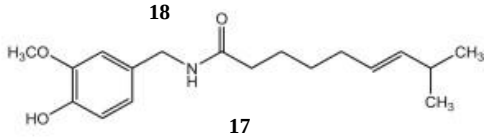

17

15 $R_1 = H, R_2 = H$

APPENDIX B

Plant species and the percentage of yield of each extact

Plants	Fresh weight (g)	Extracts	Percentage of yield (%)	References
Acmella ciliata (leaves)	100	Hexane	0.53	-
		Chloroform	0.96	
		Ethyl acetate	0.69	
		Ethanol	2.80	
		Methanol	1.36	
		Aqueous	2.03	
		Total	8.37	
Arundina graminifolia (leaves)	370	Hexane	0.90	-
		Chloroform	0.99	
		Ethyl acetate	0.24	
		Ethanol	2.63	
		Methanol	0.62	
		Aqueous	0.91	
		Total	6.29	
Cocos nucifera (leaves)	600	Hexane	0.33	-
		Chloroform	2.81	
		Ethyl acetate	0.53	
		Ethanol	4.11	
		Methanol	0.71	
		Aqueous	0.77	
		Total	9.26	
Diodia sarmentosa (aerial parts)	800	Hexane	0.04	Chung (2011)
		Chloroform	0.10	
		Ethyl acetate	0.17	
		Ethanol	0.66	
		Methanol	0.39	
		Aqueous	0.47	
		Total	1.83	
Diplazium esculentum (aerial parts)	1214	Hexane	0.09	Tan (2011b)
		Chloroform	0.04	
		Ethyl acetate	0.05	
		Ethanol	0.34	
		Methanol	0.34	
		Aqueous	0.34	
		Total	1.20	

Plants	Fresh weight (g)	Extracts	Percentage of yield (%)	References
Hydrocotyle sibthorpioides (whole plant)	1000	Hexane	0.09	Tan (2011a)
		Chloroform	0.18	
		Ethyl acetate	0.10	
		Ethanol	0.44	
		Methanol	0.77	
		Aqueous	0.55	
		Total	2.13	
Muehlenbeckia platyclada (stems)	535	Hexane	0.29	Beh (2011)
		Chloroform	0.47	
		Ethyl acetate	0.45	
		Ethanol	6.97	
		Methanol	0.91	
		Aqueous	0.54	
		Total	9.63	
Nepenthes gracilis (leaves)	237	Hexane	0.47	-
		Chloroform	0.55	
		Ethyl acetate	0.39	
		Ethanol	1.21	
		Methanol	0.21	
		Aqueous	0.05	
		Total	2.88	
Parkia speciosa (pods)	1000	Hexane	0.06	Tan (2011a)
		Chloroform	0.11	
		Ethyl acetate	0.20	
		Ethanol	4.16	
		Methanol	2.46	
		Aqueous	0.65	
		Total	7.63	
Passiflora edulis (fruits)	890	Hexane	0.15	Yee (2009)
		Chloroform	0.24	
		Ethyl acetate	0.71	
		Ethanol	1.28	
		Methanol	0.80	
		Aqueous	0.38	
		Total	3.56	
Sechium edule (aerial parts)	1200	Hexane	0.04	Chen (2011)
		Chloroform	0.10	
		Ethyl acetate	0.09	
		Ethanol	0.74	
		Methanol	0.39	
		Aqueous	0.49	
		Total	1.85	

Plants	Fresh weight (g)	Extracts	Percentage of yield (%)	References
Solanum muricatum (fruits)	1000	Hexane	0.004	Chen (2011)
		Chloroform	0.01	
		Ethyl acetate	0.02	
		Ethanol	2.53	
		Methanol	0.93	
		Aqueous	0.04	
		Total	3.54	

APPENDIX C

Plant Vouchering Form

UNIVERSITI TUNKU ABDU RAHMAN
FACULTY OF SCIENCE
Jalan Universiti, Bandar Barat,
Kampar, 31900 Perak.

COLLECTOR: _____

DATE COLLECTED: _____

FAMILY: _____

SCIENTIFIC NAME: _____

VERBAL: _____

LOCALITY: (STATE) _____

(DISTRICT) _____

(TOWNSHIP) _____

ELEVATION: _____

HABITAT:
_____

OCCURRENCE: (COMMON) ___ (OCCASIONAL)____ (RARE) ____

PLANT HEIGHT: _____

FLOWER COLOUR: _____

SEED: _____

FRUIT: _____

EDIBLE / NON-EDIBLE: _____

MEDICINAL PLANT (USAGE):_____

APPENDIX D

Preparation of medium, reagents and stock fungal cultures

Potato dextrose agar (PDA)

Thirty eight grams of powder was dissolved with 1 L of distilled water in a glass bottle. The medium was mixed well and sterilized by autoclaving at temperature 121°C and pressure of 15 Ibs. for 15 min. After autoclaved, the sterile liquid agar was allowed to cool in 55°C water bath. The medium was poured into sterile petri dishes in a laminar air flow cabinet. The solidified agar plates were stored at 4°C.

Potato dextrose broth (PDB)

Twenty one gram of powder was dissolved with 1 L of distilled water in a glass bottle. The medium was mixed well and sterilized by autoclaving at temperature 121°C and pressure of 15 Ibs. for 15 min.

Preparation of Cell Growth Indicator: p-iodonitrotetrazolium Violet (INT) Solution

The concentration of INT was prepared at 0.4 mg/mL by dissolving 40 mg of INT powder in 100 mL of distilled water. The solution was then sonicated and filtered using 0.45 μm syringe filter inside a laminar flow cabinet into sterile centrifuge tubes that covered with aluminium foil and kept at -20°C.

Broth medium

To prepare 0.5 L of broth medium, 10.4 g of RPMI-1640 medium supplemented with glutamine and phenol red, without bicarbonate and 34.53 g of 3-(*N*-morpholino) propanesulfonic acid (MOPS) were dissolved in 400 mL of distilled water. The pH was adjusted to 7.0 at 25°C with 1 mol/L sodium hydroxide. The medium was topped up to 0.5 L and filter sterilized by 0.20 μm cellulose acetate filter membrane and dispensed 100 mL volume into sterile glass bottle. The bottles were labelled with the preparation and expiration dates and stored at 4°C until required (3-months shelf life).

Preparation of Reference Antibiotic: Amphotericin B

The concentration of Amphotericin B was prepared at 32 μg/mL by dissolving 3.2 mg of amphotericin B in 1 mL of DMSO and 99 mL of RPMI broth medium. The antibiotic solution was sonicated and filtered using 0.45 μm syringe filter inside laminar flow cabinet into sterile centrifuge tubes and kept at -20°C.

Preparation of Cell Culture Medium: Dulbecco's Modified Eagle Medium (DMEM)

DMEM (1 L) was supplemented with 100 units penicillin, 10 μg streptomycin, 3.7 g/L sodium bicarbonate and 5% (for cell culture) or 1% (for maintenance medium) of fetal bovine serum (FBS). The pH was adjusted to 7.4 at 25°C with 1 mol/L hydrochloric acid (HCl). The medium was filter sterilized by 0.20 μm cellulose acetate filter membrane and stored at 4°C.

Preparation of Neutral Red (NR) Medium

The neutral red solution (3.3 g/L) was filtered (0.22 μm syringe filter) and diluted by the maintenance medium to achieve the required working concentration (40 μg/mL). The NR medium was stored at room temperature the day before use.

Preparation of Neutral Red Destain Solution

The neutral red destain solution contained the mixture of glacial acetic acid, distilled water and ethanol in the ratio of 1:49:50 (v/v/v). The solution was mixed well and kept at room temperature.

Freezing technique (Long term storage)

Fungus was cultured on a slant of PDA in a screw-cap 50 mL centrifuge tube or into 1.5 mL of PDB in a screw-cap cryopreservation vial. The fungus was incubated until it reached maturity (produced conida or spores), glycerol was added (20%) and the tube was stored in a -80°C freezer.