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**ETHNOBOTANY AND ANTIFUNGAL ACTIVITY OF TROPICAL
GINGERS (ZINGIBEREACEAE)**

by

Christine Elizabeth Ficker

**Thesis submitted to the
School of Graduate Studies and Research
in partial fulfillment of the requirements for the
Masters of Science Degree in Biology.**

Carleton University

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**The undersigned hereby recommend to the
Faculty of Graduate Studies and Research
acceptance of this thesis,**

**"Ethnobotany and antifungal activity of tropical
gingers (Zingibereaceae)"**

submitted by

Christine Elizabeth Ficker

**in partial fulfillment of the requirements
for the degree of Master of Science**

A handwritten signature in black ink, appearing to be 'P. H. V.', written over a horizontal line.

Chair, Department of Biology

A handwritten signature in black ink, appearing to be 'my m', written over a horizontal line.

Thesis Supervisor

Abstract

This thesis identifies plant families, species and plant-derived compounds suitable for development as antifungal phytomedicines. A broad-spectrum antifungal screening of 29 plant species used in traditional medicines worldwide was performed using 13 important human fungal pathogens. From this screening, *Zingiber officinale* was identified as the species tested with the broadest range of antifungal activity suitable for development as a phytomedicine. The antifungal activity of this species was further investigated and 2 antifungal compounds were isolated from *Z. officinale*, which were determined to be [6]-gingerol and [6]-gingerdiol. The antifungal activity of the Zingibereaceae family was investigated by examining the relationship between the use of Zingibereaceae species in traditional Kenyah antifungal remedies and their antifungal activity. Although a correlation between the antifungal activities of the Zingibereaceae species and their use in Kenyah traditional antifungal remedies was not found, it was clear that the Zingibereaceae had high antifungal activity and are potential sources of potent antifungal compounds.

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General Introduction

General Introduction

A dramatic increase in the number of patients with AIDS and other immunocompromised conditions has resulted in greater rates of opportunistic infections of human hosts by fungi (DeMuri and Hostetter, 1995; Perfect and Schell, 1996; White *et al.*, 1998). Antifungal drugs such as amphotericin B and the azole group of antibiotics, have thus far been crucial to the effective treatment of pathogenic fungal infections.

Amphotericin B, a polyene antibiotic, was first isolated in 1953 from *Streptomyces nodosus*, a soil actinomycete (Figure I1.1) (DeMuri and Hostetter, 1995). This antibiotic is believed to act by binding irreversibly to ergosterol, the major sterol component in the fungal cell membrane. This interaction results in the formation of channels through which cellular components, particularly potassium ions, leak, thereby destroying the cellular proton gradient and causing cell death (Vanden Bossche *et al.*, 1994). Amphotericin B has the broadest activity spectrum of any clinically useful antifungal; however several undesirable side effects such as impaired renal function, anaphylaxis, anorexia, convulsions, generalized pain, chills, fever and headache have resulted in the restriction of its use in clinical treatments (Kerridge, 1986).

The azole group of antifungals, first discovered in the late 1960s, is entirely synthetic and very important in the treatment of fungal infections. The azole antibiotics act by inhibiting the fungal enzyme lanosterol demethylase, a cytochrome P450-dependent enzyme involved in the conversion of lanosterol to ergosterol (DeMuri and Hostetter, 1995). Fungi exposed to azoles accumulate methylsterols, which induce a variety of functional alterations including membrane leakiness, changes in membrane bound enzymes and inhibition of growth (Vanden Bossche *et al.*, 1987). Ketoconazole was the first azole

Figure I1.1. Molecular structure of amphotericin B.

derivative found to be orally active against dermatophytic and dimorphic fungi in clinical studies (Figure II.2) (Van Cutsem and Janssen, 1987). Due to the relative lack of side effects, ketoconazole has been used extensively in clinical treatments, predominantly in the treatment of *Candida* infections. However, ketoconazole tends to be fungistatic and has very little activity against many filamentous fungi (Alexander and Perfect, 1997).

With the widespread use of amphotericin B and azole antibiotics there has been an increasing frequency of reports of resistance to these antibiotics (DeMuri and Hostetter, 1995; Jensen-Pergakes *et al.*, 1998). Law *et al.* (1994) reported that the development of antibiotic-resistant *C. albicans* occurred in patients receiving long-term therapy. Twenty-five percent of the *C. albicans* strains isolated from AIDS patients were found to be azole resistant, whereas only 4.2% of *C. albicans* isolated from other patients demonstrated resistance. Fungal strains with cross-resistance to different azole antibiotics as well as between azole and polyene antibiotics have also been reported (Alexander and Perfect, 1997; White *et al.*, 1998). It is expected that increased use of amphotericin B and azole antibiotics will favour the development of antibiotic resistant fungal strains and the occurrence of cross-resistance will become an increasing problem when treating immunocompromised patients.

Included in the antibiotic cross-resistant fungi are the clinically derived *C. albicans* strains D10 and CN1A (Bard *et al.*, 1987; Broughton *et al.*, 1991; Kelly *et al.*, 1997). These amphotericin B- and azole-resistant strains are characterized by mutations in the P-450 ergosterol biosynthetic pathway. D10 is characterized by a deficiency in the enzyme lanosterol demethylase (Bard *et al.*, 1987) and CN1A is characterized by a deficiency in the enzyme sterol isomerase (Lees, N. D., personal communications). These P-450 mutations

Figure I1.2. Molecular structure of ketoconazole.

reduce the amphotericin B and azole binding sites and results in the cross-resistance of these fungal strains.

Increasingly, fungi with low virulence, previously considered to be contaminants or harmless colonizers, are emerging as pathogens associated with significant morbidity and mortality in immunocompromised patients (Anaissie *et al.*, 1989; Vartivarian *et al.*, 1993). Included in these emerging pathogens are species of *Fusarium* (Richardson *et al.*, 1988, Guarro and Gené, 1995), *Alternaria* (Wiest *et al.*, 1987; Anaissie *et al.*, 1989), *Trichosporon* (Vartivarian *et al.*, 1993; Gupta and Summerbell, 1999), *Pseudoallescheria* (Walsh *et al.*, 1992), *Wangiella* (Fothergill, 1996), *Saccharomyces* (Anaissie *et al.*, 1989) and *Rhizopus* (Ribes *et al.*, 2000). Many of the emerging human pathogens have been documented to be innately resistant to the presently available antifungal drugs (Anaissie *et al.*, 1989; Walsh *et al.*, 1992; Graybill, 1996; Pujol *et al.*, 1997). Numerous potential reasons exist for the emergence of novel fungal pathogens including the extensive use of antifungal antibiotics encouraging the colonization by fungi that are intrinsically drug resistant and the decrease of selection pressure due to the immunocompromised conditions of many hosts (Alexander and Perfect, 1997).

Due to the development of resistance in known fungal pathogens and the emergence of fungal pathogens intrinsically resistant to the currently available drugs, the next generation of antifungal agents must be developed. The field of ethnobotanical research has expanded greatly in recent years as the value of this type of research in identifying novel therapeutics has come to be more widely recognized (McCutcheon *et al.*, 1992). Analyses of traditional medicines have reported significant correlations between plant use and activity (McCutcheon *et al.*, 1992; Leaman, 1996; Jones *et al.*, 2000; Omar *et al.*, 2000). Novel,

safe and effective plant species and compounds can be found through consultation with traditional healers from around the world (Jones *et al.*, 2000).

The objectives of this thesis were to, 1) identify plant species used in traditional medicines that demonstrate significant antifungal activities and are appropriate for development as phytomedicines, 2) isolate and characterize antifungal compounds from the plant species identified in objective 1 and 3) focusing on one plant family, examine the relationship between the antifungal activities of plant species used in traditional remedies for the treatment of diseases of fungal origin and the antifungal activities of species used in traditional remedies for the treatment of diseases not of fungal origin.

In Chapter 1, a general survey of antifungal activities of 29 tropical and temperate plant species used in traditional medicines worldwide was conducted. Fungi used in this study were important human pathogens that represented both yeast-like and filamentous growth forms, demonstrated a wide range of antibiotic susceptibility and included the amphotericin B- and ketoconazole-resistant *C. albicans* isolates D10 and CN1A.

Zingiber officinale Roscoe, identified in Chapter 1 as having broad-spectrum antifungal activity, was further investigated in Chapter 2. Using a bioassay guided isolation, active antifungal compounds from *Z. officinale* were isolated and characterized. Minimum lethal concentrations of the isolated compounds were determined for 13 strains of important human pathogenic fungi

The ethnobotany of the Zingibereaceae family was investigated in Chapter 3. The Zingibereaceae plant family is very important in many traditional medicines including that of the Kenyah People, an indigenous group inhabiting the island of Borneo (Sofowora, 1980; Hirschhorn, 1983; Coe and Anderson, 1996; Leaman, 1996). A study

site was established in the interior of Indonesian Borneo and Zingibereaceae species used in Kenyah traditional remedies for the treatment of diseases likely to be of fungal origin and in the treatment of diseases not likely to be of fungal origin were collected. The antifungal activity of these two groups of plant species were tested and compared.

Chapter One

Inhibition of diverse human pathogenic fungi by ethnobotanically selected plant extracts

1.1. Introduction

During the past decade, there has been an increase in the group of patients with alterations in immune status associated with HIV, cancer chemotherapy and organ and bone marrow transplantations (Perfect and Schell, 1996; White *et al.*, 1998). Coinciding with this rise in the immunocompromised patient population has been an increase in the incidence of human systemic mycoses (Graybill, 1996; Alexander and Perfect, 1997). Amphotericin B and the azole group of antifungal agents have been used extensively in the treatment of these fungal infections. Unfortunately, the widespread use of these antibiotics has led to the development of drug resistance in several common pathogenic fungi (Alexander and Perfect, 1997; Jensen-Pergakes *et al.*, 1998). The immunocompromised condition of these patients results in a decrease in host selection pressure. With this alteration in selection pressure, fungi with low virulence, previously considered to be contaminants or harmless colonizers, have emerged as important human pathogens, some of which are innately resistant to the presently available antifungal drugs (Anaissie *et al.*, 1989; Walsh *et al.*, 1992; Vartivarian *et al.*, 1993; Graybill, 1996).

Due to the emergence of antibiotic resistant human pathogenic fungi it is of great importance to develop the next generation of antifungal agents. The field of ethnobotanical research has expanded greatly in recent years as the value of this type of research in identifying novel therapeutics has come to be more widely recognized (McCutcheon *et al.*, 1992).

Using an ethnobotanical approach, this study identifies plant species with significant antifungal activities that are appropriate for further study and development as antifungal phytomedicines. The inhibitory characteristics of 29 plant species used worldwide as

traditional medicines against 13 fungal strains are reported. An analysis of published literature using the NAPALERT database (obtained from the University of Illinois) revealed that although the traditional uses of most of the plant species used in this study were well documented, few of these species have been tested for antifungal activity against a broad range of fungi. The antifungal activities of the plant extracts are compared to those of amphotericin B, ketoconazole and berberine, a known antifungal derived from a species of *Berberis* (Stermitz *et al.*, 2000). A diverse group of human pathogenic fungi were used to test for antifungal activities of the plant extracts. Included in this group are clinically derived yeast-like and filamentous fungi in addition to the *Candida albicans* strains D10 and CN1A, which are known to be resistant to amphotericin B and azole antifungal compounds (Bard *et al.*, 1987; Broughton *et al.*, 1991; Kelly *et al.*, 1997).

1.2. Materials and Methods

1.2.1. Fungal strains and inoculum preparation

Yeast-like and filamentous fungal strains (*Candida albicans*, *Candida krusei*, *Candida kefyr*, *Candida glabrata*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, *Wangellia dermatitides*, *Alternaria alternata*, *Aspergillus fumigatus*, *Fusarium oxysporum*, *Microsporum gypseum*, *Pseudallescheria boydii*, *Rhizopus mucor* and *Trichophyton mentagrophytes*) that are opportunistic human pathogens were used in this study. The source and culture number of the fungal strains are given in Table 1.2. Fungal strains were cultured on Sabouraud's dextrose agar medium at 30°C and are maintained at Carleton University, Ottawa, ON. All transfers of fungal strains were done under a biological containment hood (Bioclone2, Microzone, Ottawa, ON).

Inoculum of yeast-like fungi were prepared in liquid Sabouraud's broth and adjusted to an OD₆₀₀ of approximately 2.0. Inoculum of filamentous fungi were prepared by placing a 1 x 3 cm block of agar medium containing actively growing mycelium into 10 ml of Sabouraud's dextrose broth and fragmenting the hyphae for 1.5 minutes at high speed in a water-cooled Waring blender.

1.2.2. Plant material and extract preparation

Extracts from 29 plants (Table 1.1) representing 16 families used in traditional remedies were obtained from the University of Ottawa Herbarium; 11 were from Togo, West Africa, 12 were from Costa Rica, Central America, and six were from Eastern Ontario. Plants were collected and preserved in alcohol in the field, then transported to the lab for processing. The preserved plant material was ground in a blender with ethanol (1:3 w/v), then filtered and the solvent was rotoevaporated at 45°C to near dryness, whereupon the extract was freeze-dried for 48 hours to achieve complete dryness. Two hundred milligrams of the dried extract was reconstituted in 1 ml ethanol for a final concentration of 2 mg/10 µl. More than one plant part of some of the species was extracted, resulting in a total of 36 extracts.

1.2.3. Disc diffusion bioassays

One hundred fifty microliters of fungal inoculum was spread onto Sabouraud's dextrose agar plates with a bent glass rod. Three sterile 3M paper discs, 7.5 mm in diameter, were impregnated with 10 µl (2 mg) ethanolic plant extract, allowed to air dry and

Table 1.1. Country of origin of plant species tested and their uses in traditional medicine.

Species	Family	Voucher #	Traditional medicinal usage
Togo, West Africa			
<i>Euphorbia hirta</i> L	Euphorbiaceae	UO-18554	asthma, cathartic, bronchitis, laxative, anthelmintic, dysentery, intestinal pain, wounds, antimicrobial
<i>Euphorbia odoratum</i> L	Euphorbiaceae	UO-18557	malaria, astringent, antispasmodic, antihypertensive, antiinflammatory
<i>Harungana madagascariensis</i> (Lam.) Poir.	Hypericaceae	UO-18555	emmenagogue, abortive, aphrodisiac, facilitate birth, interrupt menses, antidiarrheal
<i>Mormodica charantia</i> L	Cucurbitaceae	UO-18552	laxative, diseases of the spleen and liver, gout, rheumatism, diabetes
<i>Phyllanthus amarus</i> L	Euphorbiaceae	UO-18550	jaundice, stomach ache, skin diseases, colds
<i>Pleiocarpa mutica</i> Benth.	Burseraceae	UO-18558	fever, malaria
<i>Psidium guajave</i> L	Myrtaceae	UO-18556	malaria, diarrhea, dysentery,
<i>Trema guineensis</i> (Schum. & Thonn.) Ficalho	Ulmaceae	UO-18551	bronchial congestion, asthma, anthelmintic, antispasmodic, female infertility
<i>Veronia conferta</i> von Schreber	Asteraceae	UO-18560	increase lactation, aphrodisiac, sleeping sickness, stomach ache, antiparasitic, cough,
<i>Ximenia americana</i> L.	Olacaceae	UO-18559	anthelmintic
<i>Zingiber officinale</i> Roscoe	Zingiberaceae	UO-17722	coughs, tuberculosis, bronchitis, malaria, antimicrobial, contraceptive

Table 1.1. Continued

Species	Family	Voucher #	Traditional medicinal usage
Costa Rica, Central America			
<i>Cabralea</i> spp.	Meliaceae	UO-19049	antimicrobial
<i>Cedrela odorata</i> L	Meliaceae	UO-19089	wounds, abdominal pain, chills, cough, malaria
<i>Cedrela salvadorensis</i> L	Meliaceae	UO-19025	gastrointestinal pain, chills, abortive
<i>Piper tuberculatum</i> L	Piperaceae	UO-19061	regulate menstrual cycle, bronchial infections, cough, syphilis, gonorrhea, female infertility,
<i>Protium glabrum</i> (Rose) Engl	Burseraceae	UO-19053	anti-inflammatory, analgesic
<i>Protium panamensis</i> (Rose) I. M. Johnston	Burseraceae	UO-19054	anti-inflammatory
<i>Renalmia aromatica</i> (Aubl.) Griseb	Zingiberaceae	UO-19052	febrifuge
<i>Swietenia mahagoni</i> L	Meliaceae	UO-19046	malaria, diabetes, postpartum hemorrhage, hypertension, diarrhea
<i>Trichilia americana</i> Browne, P	Meliaceae	UO-19013	antiprotozoal
<i>Trichilia glabra</i> L	Meliaceae	UO-19020	antiprotozoal
<i>Trichilia pleena</i> (A. Juss.) C. DC.	Meliaceae	UO-19002	antimalarial
<i>Trichilia trifolia</i> Browne, P	Meliaceae	UO-18464	purgative, abortive, emmenagogue
Ontario, Eastern Canada			
<i>Acer rubrum</i> L	Aceraceae	UO-18500	antimicrobial
<i>Acer saccharum</i> Marsh.	Aceraceae	UO-18501	antimicrobial
<i>Betula papyrifera</i> Marsh.	Betulaceae	UO-18502	antimicrobial
<i>Juglans cinerea</i> L	Juglandaceae	UO-18507	antimicrobial
<i>Prunus serotina</i> Ehrh.	Rosaceae	UO-18508	antimicrobial
<i>Quercus rubra</i> L	Fabaceae	UO-18511	antimicrobial

placed face down on the inoculated agar surface. A negative control consisting of 10 μ l DMSO and a positive control consisting of 2 mg berberine, a known antifungal derived from a species of *Berberis* (Stermitz *et al.*, 2000), administered in 10 μ l ethanol were also prepared. The petri plates were then sealed with parafilm.

To test for ultraviolet light effects on antifungal activity, dark and light treatments were performed. For ultraviolet treatments, plates were irradiated with 10 W/m² for 2 hours using three 20 W black light blue tubes (320-400 nm range). For dark treatments, plates were wrapped in aluminum foil prior to incubation. All treatments were incubated at 30°C for 48 hours at which time the inhibition zone was marked on the petri plate and the diameter was measured with a ruler. An extract was classified as active when the diameter of the inhibition zone was equal to or larger than 8 mm, 0.5 mm larger than the diameter of the paper disc.

Fungi showing growth inhibition when treated with 9 (25%) or more of the plant extracts were considered extract sensitive. This value is somewhat arbitrary but serves as a frame of reference against which the antibiotic resistance or susceptibility of the fungi can be compared.

1.2.4. Amphotericin B and ketoconazole minimum lethal concentration (MLC) bioassays

Minimum lethal concentration (MLC) bioassays were performed in sterile, disposable microtiter plates (Costar, 96 V-shaped well). Fungal inoculum, as describe above, was diluted with Sabouraud's dextrose broth (1:500). One hundred microliters of the diluted fungal inoculum was dispensed into each microtiter well within a row. A 3-fold dilution was performed from an initial amphotericin B (Sigma, Oakville, ON) or

ketoconazole (Sigma, Oakville, ON) concentration of 90 µg/ml in the first microtiter well to a final concentration of 0.005 µg/ml in the second to last microtiter well. The last well in each row served as a drug free control. Microtiter plates were sealed with parafilm and maintained stationary in the dark at 30°C for 48h at which time they were examined for fungal growth. The MLC was recorded as the lowest tested concentration of antibiotic that resulted in an absence of fungal growth.

Using the 3-fold dilution series resulted in a large gap of untested antibiotic concentrations from 30 µg/ml to 90 µg/ml. Therefore, to define MLC values between 30 µg/ml and 90µg/ml, bioassays were repeated using a 0.25 step-wise dilution. The initial antibiotic concentration was again 90 µg/ml in the first microtiter well and the lowest concentration was 6.8 µg/ml. The lowest concentration of antibiotic with total fungal growth inhibition was recorded as the MLC.

Fungi with MLC values above 10 µg/ml were considered antibiotic resistant. This value corresponds with resistance end-points previously reported in the literature (Alexander and Perfect, 1997; White *et al.*, 1998)

1.3. Results and Discussion

Of the 23 fungal strains tested against amphotericin B and ketoconazole, using the measure of resistance described above, three (*C. albicans* strains al-3 and al-5 and *R. mucor*) displayed resistance to ketoconazole only and seven (*C. albicans* strains CN1A and D10, *W. dermatitides*, *F. oxysporum*, *M. gypseum*, *P. boydii*, and *T. mentagrophytes*) displayed resistance to both antifungals (Table 1.2). Notably, all filamentous fungi tested, except *A. alternata* and *A. fumigatus* were resistant to at least one of the two antifungals. The

Table 1.2. Infection types and minimum lethal concentration (MLC) values of fungal strains used in this study.

Fungal species/strain	Source ¹	Infection types	MLC values (µg/ml)	
			Amphotericin B	Ketoconazole
yeast-like				
<i>Candida albicans</i> isolates				
D10	N. D. Lees	systemic and subcutaneous infections (Kwon-Chung and Bennett, 1992)	90	>90
CN1A	N. D. Lees		90	90
al-1	OGH-308-1329		1.1	0.37
al-2	OGH-311-0628		1.1	0.122
al-3	OGH-312-0305		3.3	30<MLC≤90
al-4	OGH-316-0588		1.1	0.13
al-5	OGH-316-0504		1.1	30<MLC≤90
al-6	OGH-316-433		3.3	10
al-7	OMH-FR2853			
<i>Candida krusei</i> isolates				
kr-1	OGH-238-0967	systemic and subcutaneous infections (Kwon-Chung and Bennett, 1992; Perfect and Schell, 1996; Abbas <i>et al.</i> , 2000)	3.3	10
kr-2	OMH-FR2428		1.1	3.3
<i>Candida kefyr</i>				
	OGH-301-0788	systemic and subcutaneous infections (Kwon-Chung and Bennett, 1992)	3.3	0.13
<i>Candida glabrata</i>				
	OGH-299-378	systemic and subcutaneous infections (Kwon-Chung and Bennett, 1992; Fidel <i>et al.</i> , 1999)	3.3	0.13
<i>Cryptococcus neoformans</i>				
	OMH-FR2704	(Anaisse <i>et al.</i> , 1989; Kwon-Chung and Bennett, 1992)	0.04	0.04
<i>Saccharomyces cerevisiae</i>				
	ATCC 48252	systemic infections in immunocompromised patients (Anaisse <i>et al.</i> , 1989; Kwon-Chung and Bennett, 1992)	0.37	1.1
<i>Wangellia dermatitides</i>				
	OMH-FR2236	(Anaisse <i>et al.</i> , 1989; Kwon-Chung and Bennett, 1992)	>90	30

Table 1.2. Continued

Fungal species/strain	Source ¹	Infection types	MLC values (µg/ml)	
			Amphotericin B Ketoconazole	
filamentous				
<i>Alternaria alternata</i>	OMH-FR9884	cutaneous and subcutaneous infections, (Anaïsse <i>et al.</i> , 1989; Kwon-Chung and Bennett, 1992; Vartivarian <i>et al.</i> , 1993)	0.37	0.37
<i>Aspergillus fumigatus</i>	OMH-FR2837	respiratory and subcutaneous infections	1.1	10
<i>Fusarium oxysporum</i>	OMH-FR6448	keratomycosis, subcutaneous infections (Anaïsse <i>et al.</i> , 1989; Kwon-Chung and Bennett, 1992; Vartivarian <i>et al.</i> , 1993)	90	>90
<i>Microsporum gypseum</i>	OMH-FR2385	tinea corporis, tinea pedis (Kwon-Chung and Bennett, 1992, Romano <i>et al.</i> , 2000)	30	30
<i>Pseudallescheria boydii</i>	OMH-FR2625	sinusitis, central nervous system colonizer (Sheftel <i>et al.</i> , 1987; Kwon-Chung and Bennett, 1992)	67.5	90
<i>Rhizopus mucor</i>	OMH-FR2874	invasion of tissue and surrounding vessels	0.014	30
<i>Trichophyton mentagrophytes</i>	OMH-T2379	tinea corporis, tinea pedis (Kwon-Chung and Bennett, 1992)	67.5	67.5

¹ N.D. Lees

ATCC=American Type Culture Collection

amphotericin B and ketoconazole MLC values given in Table 1.2 are compatible with values reported for the fungal strains examined (Del Poeta *et al.*, 1994; DeMuri and Hostetter, 1995; Franzot and Hamadan, 1996; Pujol *et al.*, 1997; Wildfeuer *et al.*, 1998; Dannaoui *et al.*, 1999).

Thirteen fungal strains were selected (*C. albicans* strains al-1, D10, and CN1A, *C. neoformans*, *S. cerevisiae*, *W. dermatitides*, *A. alternata*, *A. fumigatus*, *F. oxysporum*, *M. gypseum*, *P. boydii*, *R. mucor* and *T. mentagrophytes*) which showed a wide range of susceptibility to the antibiotics and represented both yeast-like and filamentous fungal growth forms to test for antifungal activities of the plant extracts.

For each of the 13 fungal strains, the number of plant extracts that exhibited antifungal activity was compared to amphotericin B and ketoconazole MLC values (Fig 1.1). Figure 1.1 highlights a pattern with fungi falling into one of three groups: 1) antibiotic resistant and plant extract sensitive (*C. albicans* strains CN1A and D10, *T. mentagrophytes*, *W. dermatitides*, *M. gypseum*), 2) antibiotic resistant and plant extract resistant (*F. oxysporum*, *P. boydii*, *R. mucor*), and 3) antibiotic sensitive and plant extract resistant (*A. alternata*, *A. fumigatus*, *C. albicans* strain al-1, *C. neoformans*, *S. cerevisiae*, *R. mucor*). As *R. mucor* was resistant to ketoconazole and susceptible to amphotericin B, it was the only isolate tested that fell into more than one group. Evidently, several fungal pathogens (group 1) that are resistant to commonly used antifungals may be inhibited by several plant-derived compounds. The *C. albicans* isolates D10 and CN1A that are highly resistant to amphotericin B and ketoconazole, for example, are inhibited by many of the extracts tested. This suggests that the antibiotics and the active compounds in the extracts have different modes of action. The mutations in the P-450 ergosterol biosynthetic pathway in CN1A and

Figure 1.1. Sensitivity (MLC values) to amphotericin B (open squares) and ketoconazole (closed triangles) compared to the number of plant extracts that inhibit each of 13 pathogenic fungal strains.

Group 1=antibiotic resistant and plant extract sensitive; **Group 2**=antibiotic resistant and plant extract resistant; **Group 3**=antibiotic sensitive and plant extract resistant.

Aa=*Alternaria alternata*; Af=*Aspergillus fumigatus*; al-1, D10, CN1A=*Candida albicans* strains; Cn=*Cryptococcus neoformans*; Fo=*Fusarium oxysporum*; Mg=*Microsporum gypseum*; Pb=*Pseudoallescheria boydii*; Rm=*Rhizopus mucor*; Sc=*Saccharomyces cerevisiae*; Tm=*Trychophyton mentagrophytes*; Wd=*Wangellia dermatitides*.

D10 that result in a decreased cell membrane ergosterol content and resistance to ketokonazole and amphotericin B may increase the permeability of the cell membrane to the growth inhibitors in the plant extracts. If this is the case, then the resistance to amphotericin B and ketoconazole displayed by the fungi in group 2 would be achieved by a different mechanism than alterations in the cell membrane ergosterol content as these fungi are also resistant to inhibition by the plant extracts.

The results of testing the 36 ethanolic plant extracts against the six yeast-like and seven filamentous fungi are summarized in Tables 1.3 and 1.4. Of the 36 extracts tested, all but six (*E. hirta* leaves, *P. amarus* leaves, *V. conferta* leaves, *Cabralea* spp. bark, *C. odorata* fruit and *T. pleena* bark) were inhibitory to the growth of at least one fungal strain. Effects (positive or negative) on antifungal activities by the UV-light treatment were not observed, except in four cases. UV-light treatment was observed to enhance the inhibitory effect of *J. cinerea* bark extract on *C. neoformans* and *S. cerevisiae*. However, the UV-light treatment of *J. cinerea* wood extract caused a reduction in the zone of inhibition in disk assays with *P. boydii*. Similarly, lower inhibitory activities were observed following UV light treatment of *P. serotina* wood and *A. saccharum* bark extracts when tested against *C. albicans* strain D10.

Plant extracts with activity against five or more fungal strains included *M. charantia* leaves, *Z. officinale* rhizome, *J. cinerea* bark, *Q. rubra* bark and *P. serotina* wood. Of the extracts tested, *J. cinerea* bark and *Z. officinale* rhizome demonstrated the broadest range of antifungal activity, measured as either the number of fungi inhibited or as the average diameter of the zones of inhibition. Fungi inhibited by *J. cinerea* bark and *Z. officinale* rhizome included those categorized as susceptible as well as resistant to amphotericin B and

Table 1.3. Growth inhibition of yeast-like fungi by plant extracts.

Agent tested	Mean diameter (mm) of growth inhibition zone for fungal species											
	Cn			Sc			al-1			D10		
	+uv	-uv	+uv	+uv	-uv	+uv	+uv	-uv	+uv	+uv	-uv	-uv
Togo, West Africa												
<i>Euphorbia hirta</i> (l)												
<i>Euphorbia odoratum</i> (l)												10.2
<i>Harungana madagascariensis</i> (l)						11.5	9.5	8.8				9.5
<i>Mormodica charantia</i> (l)		9.5							9.5	11.5		9.5
<i>Phyllanthus amarus</i> (l)												
<i>Pleiocaria nutica</i> (l)												9.5
<i>Psidium guagave</i> (l)									11.5			
<i>Trema guineensis</i> (l)		9.5										9.5
<i>Vernonia conferta</i> (l)												
<i>Ximenia americana</i> (l)									9.5			
<i>Zingiber officinale</i> (r)									11.5			
Costa Rica, Central America												
<i>Cabralea</i> spp. (b)												
<i>Cedrela odorata</i> (b)												
<i>Cedrela odorata</i> (f)												
<i>Cedrela odorata</i> (l)												
<i>Cedrela salvadorensis</i> (w)									10.8	9.5		
<i>Piper tuberculatum</i> (l)	10.8								9.5		9.5	
<i>Protium glabrum</i> (l)											10.8	12.8
<i>Protium panamense</i> (l)												
<i>Renalmia aromatica</i> (l)									12.8	13.5		10.8
<i>Swietenia mahogoni</i> (w)									12.8	11.5	12.8	

Table 1.3. Continued

Agent tested	Mean diameter (mm) of growth inhibition zone for fungal species											
	Cn		Sc		al-1		D10		CN1A		Wd	
	+uv	-uv	+uv	-uv	+uv	-uv	+uv	-uv	+uv	-uv	+uv	-uv
<i>Trichilia americana</i> (f)			11.5									9.5
<i>Trichilia americana</i> (l)									9.5			
<i>Trichilia glabra</i> (f)	10.8										9.5	9.5
<i>Trichilia pleena</i> (w)									9.5			
<i>Trichilia pleena</i> (b)												
<i>Trichilia triflora</i> (w)	9.5						11.5					
Ontario, Eastern Canada												
<i>Acer rubrum</i> (b)							12.2					
<i>Acer saccharum</i> (b)							14.8					
<i>Betula papyrifera</i> (w)							9.5					
<i>Juglans cinerea</i> (b)	14.2		14.8		13.5	12.2	21.5	24.2	11.5	11.5	13.5	11.5
<i>Juglans cinerea</i> (w)							14.8	27.5				9.5
<i>Prunus serotina</i> (b)							12.8					
<i>Prunus serotina</i> (w)							19.5	17.5	9.5	10.5		
<i>Quercus rubra</i> (b)							11.5	23.5				
<i>Quercus rubra</i> (w)							8.5	9.5				
Berberine	31	25	25	17.5	22	24.2	21.5	17.5	27.5	25	17.5	16.2
DMSO												

Minimum diameter possible = 8.0 mm. Standard error on all values was < 15% of mean.

Cn= *Cryptococcus neoformans* ; Sc= *Saccharomyces cerevisiae* ; al-1, D10, CN1A= *Candida albicans* strains; Wd= *Wangellia dermatitides* .

(b)=bark; (f)=fruit; (l)=leaves; (r)=rhizome; (w)=wood

Table 1.4. Growth inhibition of filamentous fungi by plant extracts.

Agent tested	Mean diameter (mm) of growth inhibition zone for fungal species											
	Aa		Af		Fo		Mg		Pb		Rm	
	+uv	-uv	+uv	-uv	+uv	-uv	+uv	-uv	+uv	-uv	+uv	-uv
Togo, West Africa												
<i>Euphorbia hirta</i> (l)												
<i>Euphorbia odoratum</i> (l)							14.4	12.2	11.5		16.8	16.2
<i>Harungana madagascariensis</i> (l)							16.2	16.2				
<i>Mormodica charantia</i> (l)			13.2	20.2			11.5				11.5	
<i>Phyllanthus amarus</i> (l)												
<i>Pleiocaria mutica</i> (l)												
<i>Psidium guajava</i> (l)												
<i>Trema guineensis</i> (l)												
<i>Vernonia conferta</i> (l)												
<i>Ximenia americana</i> (l)												
<i>Zingiber officinale</i> (r)							17.5	20.8	11.5	12.2	8.8	24.8 18.2
Costa Rica, Central America												
<i>Cabralea</i> spp. (b)												
<i>Cedrela odorata</i> (b)									11.5			
<i>Cedrela odorata</i> (f)												
<i>Cedrela odorata</i> (l)												9.5
<i>Cedrela salvadorensis</i> (w)												
<i>Piper tuberculatum</i> (l)												
<i>Protium glabrum</i> (l)												
<i>Protium panamense</i> (l)							11.5					
<i>Renalmia aromatica</i> (l)									10.8	9.5		
<i>Swietenia mahogoni</i> (w)							13.5	13.5				

Table 1.4. Continued

Agent tested	Mean diameter (mm) of growth inhibition zone for fungal species											
	Aa		Af		Fo		Mg		Pb		Rm	
	+uv	-uv	+uv	-uv	+uv	-uv	+uv	-uv	+uv	-uv	+uv	-uv
<i>Trichilia americana</i> (f)												
<i>Trichilia americana</i> (l)												
<i>Trichilia glabra</i> (f)												
<i>Trichilia pleena</i> (w)												
<i>Trichilia pleena</i> (b)												
<i>Trichilia triflora</i> (w)												
Ontario, Eastern Canada												
<i>Acer rubrum</i> (b)							9.5	11.5			11.5	11.5
<i>Acer saccharum</i> (b)							12.8					
<i>Betula papyrifera</i> (w)												
<i>Juglans cinerea</i> (b)							24.2	27.5	10.8	9.5	16.8	17.5
<i>Juglans cinerea</i> (w)							17.5	13.5			15.5	14.2
<i>Prunus serotina</i> (b)							10.8	11.5				
<i>Prunus serotina</i> (w)							12.2		14.2		18.8	22.2
<i>Quercus rubra</i> (b)							11.5	10.8			9.5	
<i>Quercus rubra</i> (w)												
Berberine	10	9.5	10.2	10.2	9.5	10.2	24.8	18.8	16.2	17.5	24.8	24.2
DMSO												

Minimum diameter possible = 8.0 mm. Standard error on all values was < 15% of mean.

Aa= *Alternaria alternata* ; Af= *Aspergillus fumigatus* ; Fo= *Fusarium oxysporum* ; Mg= *Microsporium gypseum* ;

Pb= *Pseudallescheria boydii* ; Rm= *Rhizopus mucor* ; Tm= *Trichophyton mentagrophytes*

(b)=bark; (f)=fruit; (l)=leaves; (r)=rhizome; (w)=wood

ketoconazole. Of particular interest, the *Z. officinale* rhizome extract was the only extract to have activity against the *R. mucor* strain that was not inhibited by berberine, ketoconazole or any other plant extracts tested. Likewise, *J. cinerea* bark was the only extract to inhibit the growth of the amphotericin B- and ketoconazole-resistant *F. oxysporum* strain.

J. cinerea bark not only possesses high antifungal activity, but it has also been found to be a potent antibacterial agent (Omar *et al.*, 2000). The antibacterial and antifungal activity of *J. cinerea* is thought to be due to the presence of quinones (Mahoney *et al.*, 2000). However, the high toxicity of quinones renders this species potentially unsafe for human use and further investigation into the development of *J. cinerea* as a phytomedicine is not recommended.

Extracts of *Z. officinale* have been reported to possess numerous biological activities (Oloke *et al.*, 1989; Roth *et al.*, 1998; Goto *et al.*, 1990; Habsah *et al.*, 2000) and have been used extensively in antinausea clinical trials (Mowrey and Clayson, 1982; Wood *et al.*, 1988; Bone *et al.*, 1990), including trials conducted with pregnant women (Fischer-Rasmussen *et al.*, 1990). As adverse side effects have not been reported in any of the clinical trials, *Z. officinale* is generally regarded as safe (gras) for human use and further investigation into the antifungal activity of this species and its possible use as a phytomedicine is warranted. It was therefore determined that studies conducted for Chapter 2 of this thesis would focus on the isolation and characterization of active antifungal compounds from *Z. officinale*.

It was anticipated that isolating the antifungal compound would involve testing several fractions derived from *Z. officinale* for the presence of antifungal activity. Due to an expected limitation in the quantity of fractions as well as time constraints, it was determined

that all 13 human pathogenic fungi could not be used to screen the fractions for antifungal activity, as performed in this study. Therefore, using the results of this study, *M. gypseum* was selected on the basis of a general sensitivity to the plant extracts tested. The *M. gypseum* strain used was inhibited by the largest number of plant extracts and presented the largest overall inhibition zones.

Additional antifungal agents must be developed to successfully control the new and emerging human fungal pathogens resistant to antibiotics that are currently available. In this study, a survey of plant species important in traditional medicines was conducted to identify species suitable for development as antifungal phytomedicines. While none of the species tested had activity against all 13 fungal strains, the extracts of *J. cinerea* bark and *Z. officinale* rhizome were identified as demonstrating significant antifungal activities towards a wide range of fungi. Due to the possible human toxicity of *J. cinerea*, this species is not recommended for further study. *Z. officinale*, however, has extremely low human toxicity and is a candidate for development as an antifungal phytomedicine. The antifungal activity of *Z. officinale* will therefore be examined further in this thesis.

Chapter Two

Bioassay guided isolation and identification of antifungal compounds in *Zingiber officinale*

2.1. Introduction

In recent years there has been an increase in the reported incidences of human systemic mycoses resistant to the existing antibiotics (Law *et al.*, 1994; Graybill, 1996; Alexander and Perfect, 1997; White *et al.*, 1998). Novel antifungal agents need to be identified and developed to effectively treat these emerging antibiotic resistant human pathogenic fungi.

In Chapter 1 of this thesis an ethnobotanical approach was used to identify plant species suitable for development as antifungal phytomedicines. Extracts from plant species important in traditional medicines worldwide were tested for the presence of antifungal activity. *Zingiber officinale* Roscoe was found to have significant antifungal activity against several important human pathogens including the amphotericin B and ketoconazole resistant *Candida albicans* strain CN1A. Due to its extremely low human toxicity (Wood *et al.*, 1988; Bone *et al.*, 1990; Fischer-Rasmussen *et al.*, 1990) and significant antifungal activity, *Z. officinale* was selected as a candidate for further study and development as an antifungal phytomedicine.

Z. officinale has a long history of use in traditional medicines worldwide, including in Africa (Sofowora, 1980; Fleuretin and Pelt, 1982), Asia (Hirschhorn, 1983; Leaman, 1996; Yamahara *et al.*, 1985; Holdsworth and Rali, 1989; Yamahara *et al.*, 1989; Chan *et al.*, 2000), Indian Subcontinent (Pushpangadan and Atal, 1984; John, 1984), the Pacific (Whistler, 1985; Singh, 1986) and Central America (Coe and Anderson, 1996). The uses of *Z. officinale* in these traditional medicinal practices are summarized in Table 2.1.

Table 2.1. Uses of *Zingiber officinale* in traditional medicine.

Ailment	Location	Plant part	Remedy	Mode of preparation	References
Coughs, colds and fevers	Nicaragua	rhizome	ingested	decoction	Coe and Anderson, 1996
	Indonesia	rhizome	ingested	infusion made with <i>Ficus</i> spp.	Elliott and Brimacombe, 1987
	Nepal	rhizome	body massage	juice of rhizome mixed with cow's butter	Bhattari, 1990
	India	rhizome	ingested	rhizome prepared with coffee and black pepper	John, 1984
Muscle aches and sprains	Papua New Guinea	rhizome	chewed	chewed rhizome placed on aching tooth	Holdsworth and Rali, 1989
	Fiji	rhizome	ear drops	crushed with honey for earaches	Singh, 1986
	Nicaragua	rhizome	ingested	decoction	Coe and Anderson, 1996
	Papua New Guinea	rhizome	poultice	juice of chewed rhizome applied to ease migraines	Holdsworth and Rali, 1989
Postpartum and childbirth	Indonesia	rhizome	poultice	grated and applied to headache	Hirschhorn, 1983
	Nicaragua	rhizome	ingested	decoction made to ease childbirth and pregnancy	Coe and Anderson, 1996
	Indonesia	rhizome	body lotion	included in skin lotion used by mothers postpartum	Elliott and Brimacombe, 1987
				not documented	
Contraceptive and fertility agent	Yemen	rhizome	ingested	a sorcerer places a spell on one hundred rhizomes which are to be eaten by the woman, without the knowledge of her husband, at intervals and in quantities prescribed by the traditional practitioner to achieve female sterility	Fleurintin and Pelt, 1982
	Papua New Guinea	rhizome	ingested		Holdsworth and Wamoi, 1982

Table 2.1. Continued

Ailment	Location	Plant part	Remedy	Mode of preparation	References
Nausea and stomach pain	Papua New Guinea	rhizome	ingested	tea made from rhizome for stomachache	Holdsworth and Rali, 1989
	Fiji	rhizome	ingested	juice of crushed ginger and onions stops vomiting	Singh, 1986
				juice of crushed ginger with honey for stomachache	Elliott and Brimacombe, 1987
	Papua New Guinea	rhizome	ingested	rhizome chewed and juice swallowed to alleviate vomiting	Holdsworth and Rali, 1989
	India	rhizome	ingested	juice from rhizome is taken for stomachache	John, 1984
	China	rhizome	ingested	not documented	Yamahara <i>et al.</i> , 1989
	Africa	rhizome	ingested	not documented	Sofowora, 1980
Wounds, bites and stings	Indonesia	rhizome	poultice	pounded, add salt, apply to wound	Hirschhorn, 1983
	Papua New Guinea	rhizome	juice	stingray sting is extracted with a needle and Holdsworth and Rali, 1989 chewed rhizome juice is added to the cut	

Recently, a broad range of biological activities has been attributed to *Z. officinale*. These include antiemetic (Mowrey and Clayson, 1982; Wood *et al.*, 1988; Bone *et al.*, 1990; Endo *et al.*, 1990), insecticidal (Roth *et al.*, 1998; Sahayaraj, 1998; Agarwal *et al.*, 2001), antibacterial (Oloke *et al.*, 1989; Yamada *et al.*, 1992; Hiserodt *et al.*, 1998), antiprotozoal (Goto *et al.*, 1990), antioxidant (Habsah *et al.*, 2000; Nakatini, 2000; Sekiwa *et al.*, 2000), antirhinoviral (Denyer *et al.*, 1994), anti-inflammatory (Mascolo *et al.*, 1989) and antihepatotoxic (Hikino *et al.*, 1985) activities. Extracts from *Z. officinale* also have been shown to inhibit fungal growth (Roth *et al.*, 1998; Singh and Bai, 2000; Agarwal *et al.*, 2001).

The majority of studies evaluating the antifungal activity of *Z. officinale* have been performed using crude extracts (Guérin and Réveillère, 1984; Shetty *et al.*, 1987; Meena and Sethi, 1994) and only two studies have isolated and identified active compounds. Endo *et al.* (1990) isolated 4 diarylheptanoid compounds from the rhizome of *Z. officinale*, one of which inhibited hyphal growth and reduced appressoria formation of the plant pathogen *Pyricularia oryzae*. Agarwal *et al.* (2001) isolated five compounds from *Z. officinale* including gingerol, gingerone, dihydrogingerone, dehydrozingerone and dehydroshogaol, all of which were reported to significantly inhibit hyphal growth of the potato pathogen *Rhizoctonia solani*. Although these studies identified several compounds with antifungal activities, the number of fungal species tested was limited.

In the following study antifungal compounds from *Z. officinale* were isolated and characterized. Minimum lethal concentrations of the isolated compounds are reported for 13 important human pathogenic fungi, including azole and amphotericin B resistant strains.

2.2. Materials and Methods

2.2.1. Extract preparation

Z. officinale rhizome (388.3 g, dry weight), obtained from Togo, Africa was ground using a 2 mm diameter mesh in a Wiley mill. Ethanol was added to the ground plant material (3:1 v/w) and the mixture was placed in a sonicator for 30 minutes. The mixture was filtered and the process was repeated. The combined ethanol extract was dried using a rotoevaporator until the volume was halved. The ethanol extract was washed with hexane (1:2 w/w) and the resulting two fractions were separated. Water was added to the residue (1:2 w/w) and the solution was washed with ethyl acetate (1:2 w/w). Again, the resulting two fractions were separated. The three fractions were rotoevaporated at 45°C to near dryness, then freeze-dried for 48 hours to achieve complete dryness. Each fraction was tested for antifungal activity using the *Microsporum gypseum* strain described in Chapter 1 of this thesis.

2.2.2. Isolation of antifungal compounds using silica gel column chromatography and preparative thin plate chromatography

A chromatographic column (35cm x 5cm) was prepared by mixing approximately 150 g Davisil Silica Gel, 62-230 mesh, with 500ml hexane. After the slurry had settled, excess hexane was eluted and the active fraction, determined by disc diffusion bioassays, was placed on top of the slurry. To provide a buffer zone, ensuring minimal disturbance of the column upon addition of the mobile phase, inert sand was placed on top of the extract to a height of 1cm. The extract was eluted with 400ml of each of 2:1, 1:1 and 1:2 hexane:ethyl acetate, followed by 400 ml of ethylacetate. One hundred and sixty fractions

of 10 ml were collected and dried using a rotoevaporator. Disc diffusion bioassays were performed using *M. gypseum* and 0.5 mg of collected fraction per disc (99% ethanol as solvent).

To achieve further separation of the compounds, consecutive fractions with antifungal activity were combined and loaded on a 62-230 mesh Silica Gel column with ethyl acetate as the mobile phase. The extract was eluted with 100% ethyl acetate, and 100 5 ml fractions were collected. The collected fractions were dried and tested for antifungal activity using disc diffusion bioassays with *M. gypseum* and 0.5 mg of collected fraction per disc. Thin layer chromatography (TLC, on silica gel 72-230 mesh aluminum backed 20 cm² plates) was done with the active fractions and compounds with the same R_f values were combined. Approximately 50 mg of each TLC-fractionated compound was dissolved in 99% ethanol and subjected to an additional TLC purification. The TLC plates were set in the mobile phase, 3:2, hexane:ethylacetate for one hour, after which they were removed and examined under UV light. The fluorescing compound bands were marked with a pencil and the silica gel scraped off within that area, soaked in 50ml ethyl acetate for 20 minutes, filtered and dried using a rotoevaporator and weighed. The presence of antifungal activity was determined using disc diffusion bioassays with *M. gypseum* and 0.05 mg compound per disc.

2.2.3. Identification of isolated antifungal compounds

[6]-gingerol was identified at the Ottawa Carleton Institute of Biology by high performance liquid chromatography (HPLC), Varian, using purchased standards and at the Ottawa Carleton Institute of Chemistry by mass spectrometry (MS), Kratos. The

HPLC method, using a Waters reverse phase 100 mm column, 3 μm spherical particles and 5 μl injects at 30 minute intervals is as follows: flow rate 0.4 ml/min, 30% MeCN in H_2O to 95% MeCN in 15 minutes, hold at 95% MeCN for 5 minutes. [6], [8] and [10]-gingerol standards were purchased from Dalton Chemical Laboratories Inc., Toronto, Ontario. Of note is that the standards became available for purchase only after the [6]-gingerol isolation had been performed and that the standards were synthetically derived. The elution times of the [6], [8] and [10]-gingerols from the HPLC column were 7.2, 11.2 and 14.3 minutes respectively.

[6]-gingerdiol was identified at the Ottawa Carleton institute of Chemistry using nuclear mass resonance (NMR), Bruker 500 mhz, and MS, Kratos.

2.2.4. Minimum Lethal Concentration (MLC) bioassays of isolated antifungal compounds

MLC bioassays were performed as per Chapter 1 and were recorded as the lowest concentration of compound that resulted in a complete absence of fungal growth. Compound concentrations tested ranged from 1000 $\mu\text{g/ml}$ to 75 $\mu\text{g/ml}$. Amphotericin B was used as the positive control with concentrations ranging from 90 $\mu\text{g/ml}$ to 0.005 $\mu\text{g/ml}$. MLC tests were performed in duplicate.

2.2.5. Fungal strains and inoculum preparation

The fungal strains used in this study and the inoculum preparation was as described in Chapter 1 of this thesis.

2.3. Results and Discussion

Of the fractions obtained using solvents of different polarity, only the fractions extracted in ethyl acetate showed antifungal activity. Using column chromatography two compounds, labeled as A and B, demonstrated antifungal activity. Using TLC, mobile phase 7:3 ethylacetate:hexane, R_f values for compounds A and B were 0.72 and 0.59, respectively. Compound A was identified as [6]-gingerol based on the TLC R_f value, HPLC retention times and MS data comparable to literature values (Yamada *et al.*, 1992; Fleming *et al.*, 1999). Compound B was identified as [6]-gingerdiol based on MS and NMR spectral data comparable to literature values (Kikuzaki *et al.*, 1992; He *et al.*, 1998). Figures 2.1 and 2.3 show the MS spectra and Figures 2.2 and 2.4 show the molecular structures of [6]-gingerol and [6]-gingerdiol. The ^1H NMR and ^{13}C NMR spectral data is reported in Tables 2.2 and 2.3.

MLC bioassays were performed with the isolated compounds [6]-gingerol and [6]-gingerdiol, as well as with the synthetically derived [6], [8] and [10]-gingerol standards (Dalton Chemical Laboratories Inc., Toronto Ontario) against six yeast-like fungi and seven filamentous fungi. The results of the MLC bioassays are listed in Table 2.4. As the quantity obtained of [10]-gingerol was not sufficient to determine the MLC values of all 13 fungi, bioassays were performed with five of the fungi, *C. albicans* strains al-1, D10 and CN1A, *M. gypseum* and *P. boydii*. The MLC values obtained using the [6]-gingerol isolated from *Z. officinale* and the [6]-gingerol standard were identical and therefore are reported only once.

Figure 2.1. MS spectral data of [6]-gingerol.

Figure 2.2. Molecular structures of [6], [8] and [10]-gingerols.

[6]-gingerol, n=4
[8]-gingerol, n=6
[10]-gingerol, n=8

Figure 2.3. MS spectral data of [6]-gingerdiol.

Figure 2.4. Molecular structure of [6]-gingerdiol.

Table 2.2. ¹H NMR spectral data of [6]-gingerdiol.

H	[6]-gingerdiol spectral data
1a	2.60 <i>ddd</i> (7.0, 8.1, 13.8)
1b	2.71 <i>ddd</i> (6.5, 8.2, 13.5)
2a	1.72 <i>dddd</i> (4.1, 7.2, 9.8, 13.7)
2b	1.75 <i>dddd</i> (6.1, 8.2, 8.9, 13.8)
3	3.88 <i>m</i>
4a	1.50 <i>td</i> (10.1, 13.8)
4b	1.60 <i>td</i> (3.0, 13.9)
5	3.84 <i>m</i>
6	1.43 <i>m</i>
7, 8, 9	1.31 <i>m</i>
10	0.90 <i>t</i> (6.5)
2'	6.75 <i>dd</i> (2.0, 7.9)
5'	6.92 <i>d</i> (8.2)
6'	6.71 <i>dd</i> (1.6, 8.2)
3'-OMe	3.86 <i>s</i>

Table 2.3. ^{13}C NMR spectral data of [6]-gingerdiol.

C	[6]-gingerdiol spectral data
1	32.5
2	40.9
3	71.6
4	44.5
5	70.2
6	38.7
7	25.4
8	32.1
9	22.8
10	14.3
1'	135.2
2'	112
3'	147.8
4'	144.6
5'	115.2
6'	121.3
3'-OMe	56.1

Table 2.4. MLC values ($\mu\text{g/ml}$) of *Z. officinale* derived compounds and of amphotericin B.

Fungi tested	MLC values ($\mu\text{g/ml}$)				
	[6]-gingerol	[8]-gingerol	[10]-gingerol	[6]-gingerdiol	Amphotericin B
yeast-like					
<i>Candida albicans</i>					
al-1	1000	750	>1000	1000	1.1
CN1A	750	750	750	1000	90
D10	1000	750	750	1000	90
<i>Cryptococcus neoformans</i>	1000	750	not tested	1000	0.04
<i>Saccharomyces cerevisiae</i>	>1000	>1000	not tested	1000	0.37
<i>Wangellia dermatitides</i>	1000	750	not tested	563	>90
filamentous					
<i>Alternaria alternata</i>	1000	750	not tested	1000	0.37
<i>Aspergillus fumigatus</i>	750	563	not tested	750	1.1
<i>Fusarium oxysporum</i>	1000	750	not tested	1000	90
<i>Microsporium gypseum</i>	563	422	316	422	30
<i>Pseudoallescheria boydii</i>	750	563	563	1000	67.5
<i>Rhizopus mucor</i>	750	563	not tested	1000	0.014
<i>Trychophyton mentagrophytes</i>	750	563	not tested	750	90

The filamentous fungi on average had lower MLC values with all *Z. officinale* compounds than the yeast-like fungi. Following the trend observed in Chapter 1 of this thesis, *M. gypseum* was the most sensitive to the inhibitory effects of [6], [8] and [10]-gingerol and [6]-gingerdiol with MLC values of 563 µg/ml, 442 µg/ml, 316 µg/ml and 422 µg/ml respectively. Overall, the [6]-gingerdiol fungal MLC values were not significantly different than the [6]-gingerol MLC values. As well, the MLC values of [8]-gingerol were lower than those of [6]-gingerol for all fungi, except *S. cerevisiae*, which had very high MLC values (>1000 µg/ml) for both compounds. This correlates with the results documented by Hikino *et al* (1985) and Yamada *et al* (1992) where it was reported that antihepatotoxic and antibacterial activities of [n]-gingerols were dependant on the length of the carbon side chain, with [8]-gingerol exhibiting the highest activities. Increasing or decreasing the side chain resulted in a decrease of the reported activities.

Although [6], [8] and [10]-gingerols and [6]-gingerdiol exhibited antifungal activities, substantially higher concentrations of these compounds were required to arrest fungal growth when compared to the required concentrations of amphotericin B. However, the use of amphotericin B is restricted by adverse side effects (Kerridge, 1986). *Z. officinale* is a known therapeutic and has been used extensively as an antinausea agent in clinical trials, including studies performed with pregnant women and children (Mowrey and Clayson, 1982; Grøntved *et al.*, 1988; Fischer-Rasmussen *et al.*, 1990). Adverse side effects have not been documented in these trials, therefore, *Z. officinale*, as well as compounds derived from this plant are generally regarded as safe and can be administered in large doses.

This study, along with those conducted by Endo *et al.* (1990) and Agarwal *et al.* (2001) have identified several compounds in *Z. officinale* with antifungal properties. Although the amount of pure compounds obtained was not sufficient to perform a formal synergism study, it was clear that the crude ethyl acetate extract, containing many compounds had excellent activity. This was also reported by Agarwal *et al.* (2001) where ginger oleoresin, a residue of acetone and ethanol extracts containing only 4 to 7.5% gingerols and gingerol derivatives including shogaol, zingerone, paradols and gingerdiols was reported to have antifungal activity similar to that of pure [6]-gingerol. This suggests that *Z. officinale* can be used as an antifungal extractive enriched in these compounds. Not only might a therapeutic formulation of a blend of active constituents have higher antifungal activity than the pure compounds, but might also diffuse the selection pressure, mitigating the development of resistance compared to that expected with a formulation containing a single active ingredient (Feng and Iseman, 1995).

With the emergence of fungal pathogens resistant to the existing antibiotics, the discovery of new antifungal agents is becoming increasingly important. In this study, [6], [8] and [10]-gingerols and [6]-gingerdiol derived from *Z. officinale* were reported to possess antifungal activities against 13 important human pathogens. When compared to amphotericin B, the plant-derived compounds possessed only modest activity. However, the plant-derived compounds have a major advantage over the antibiotic in that side effects have not been recorded in clinical trials using *Z. officinale*. Therefore, compounds derived from this plant species are an important source of phytomedicines and can be used immediately as antifungal agents due to their low human toxicity.

Chapter Three

The ethnobotany of the Zingibereaceae family as used by the Kenyah of East Kalimantan, Indonesian Borneo

3.1. Introduction

Although the population of Indonesian Borneo is approximately 12.5 million, the majority is concentrated in large coastal cities. The interior of the island is sparsely populated with an estimated population density between 1 and 8 persons per km² (King, 1993). The inhabitants of interior Borneo are primarily indigenous and historically have been classified into three major groups: Dayak, Punan and Malay. The ethnicity, origins, customs and beliefs of the Borneo indigenous peoples, as well as the geography of the island are explained in detail in Appendix 1 of this thesis.

Since 1910 there has been a government appointed physician based at a clinic at Long Nawang (Leaman, 1996), the administrative capital of the interior of the province of East Kalimantan. More recently, intermittently staffed dispensaries have been established at the villages of Long Ampung and Long Sungai Barang (Leaman, 1996), the latter being the site of this study. Despite these few attempts by the Indonesian government to establish health care in the interior of Indonesian Borneo, the inhabitants of the region often cannot access the health services provided. An inhabitant of Long Sungai Barang who had received training in the health care profession reported that medicines and proper medical supplies are often unavailable or unaffordable. Seriously ill patients are flown by missionary planes to Samarinda or Tarakan, but the airfare for accompanying family members and costs of treatment are beyond the means of most residents (Leaman, 1996). Traditional medicine is therefore very important as the primary health care system of the indigenous peoples of Borneo.

The medicinal ethnobotany of the indigenous peoples of Borneo has been reviewed previously (Hirschhorn, 1983; Elliott and Brimacombe, 1987; Grosvenor *et al.*,

1995; Leaman, 1996; Wu *et al.*, 1996). The most extensive study to date, conducted by Leaman (1996), reported on the medicinal ethnobotany of the Kenyah, a Dayak group inhabiting the Apo Kayan Plateau region of Indonesian Borneo. This study documented the food, ritualistic and medicinal uses and their modes of preparation of over 240 plant species belonging to 76 families.

The present study focuses on members of the Zingibereaceae family used in Kenyah traditional medicine as antifungal remedies and their efficacy when tested in laboratory fungal bioassays. Leaman documented the use of 20 plant species belonging to the Zingibereaceae family in Kenyah traditional medicine, of which 4 were used as remedies for illnesses that could be attributed to fungal pathogens. These conditions included itchiness, skin infections, vitiligo, dog mange, weeping wounds and ringworm. For the majority of the remedies, only one plant part is used and is either directly applied to the affected area as a heated poultice, ointment, infusion or body rub, or a decoction or infusion is made and ingested. Analyses of the traditional medicinal remedies of North American Indigenous Peoples have reported significant correlations between plant use and activity (McCutcheon *et al.*, 1992; Jones *et al.*, 2000; Omar *et al.*, 2000). Therefore, in this study it was hypothesized that extracts derived from Zingibereaceae species used in traditional Kenyah remedies for ailments that could be attributed to fungal pathogens would possess greater antifungal activities than extracts derived from Zingibereaceae species used in remedies for ailments that could not be attributed to fungal pathogens.

3.2. Methods and Materials

3.2.1. The study site

The study site, the Kenyah village of Long Sungai Barang, is located at the headwaters of the Kayan river at an elevation of 800m on the Apo Kayan Plateau in the center of Borneo, approximately 2°N latitude, 115°E longitude (Leaman, 1996). The mean annual rainfall in this area is between 3,000 and 4,000mm with the driest months being July and August. The mean daily temperature is 20°C, fluctuating between 15°C and 25°C (Leaman, 1996). Figure 3.1 shows the location of the study site and the surrounding villages. A more detailed description of the study area is provided in Appendix 1.

3.2.2. Ethnobotanical data and plant collection

The research permit required for this study was obtained from the Indonesian National Institute of Science (LIPI) (Permit number 4488/V.3/KS/2001). The permit application and acquisition process was approximately one year in duration and involved many levels of the Indonesian government.

The plants and plant parts collected in this study were based on the ethnobotanical data documented by Leaman, 1996. For each plant species, Leaman reported the local Kenyah name, the plant parts used in the traditional remedies and the method of preparation and administration of the remedies. Leaman also assigned values to each remedy based on their importance in Kenyah traditional medicine.

Based on Leaman's information, 10 Zingibereaceae plants were collected using their local Kenyah names. Voucher specimens were collected for later taxonomic

Figure 3.1. Island of Borneo and location of the study site, the village of Long Sungai Barang and the surrounding villages.

identification. Four plants were identified as being used in remedies for ailments that could be attributed to fungal pathogens (itchiness, skin infections, vitiligo, dog mange, weeping wounds and ringworm). Six plants were identified as being used in remedies that could not be attributed to fungal pathogens (dysentery, arthritis, headache, general aches and pains, coughs and nose bleed).

For the plants used in possible antifungal remedies, the rhizome as well as the plant part used in the remedy was collected. For the plants used in remedies for ailments not attributed to fungal pathogens, the rhizome as well as the plant part used in the remedy with the highest importance value was collected. The rhizome was the only plant part collected when it was the only part used in the possible antifungal remedy. The rhizome was again the only plant part collected when it was the only part used in the remedy with the highest importance value.

Villagers knowledgeable in the Kenyah traditional medicine and the surrounding flora were recruited to help conduct the plant collections. All participants received compensation for their time spent away from village and family duties. The Kenyah villagers who were consulted in this study also had participated in the study conducted by Leaman. Although formal ethnobotanical interviews were not conducted in this study, informal discussions took place, with my Indonesian counterpart acting as a translator, regarding the uses of the collected plants in traditional medicine. The uses documented in this way correlated with those documented by Leaman.

The Zingibereaceae specimens were collected according to the method described by Burtt and Smith, 1976. The collected plant material was placed in plastic collecting bottles or wrapped in newspaper and placed in plastic bags. Sixty percent ethanol,

purchased at a pharmacy in Samarinda prior to travel to the study site, was used as the preserving agent. The taxonomic determinations were conducted at the Herbarium Bogoriense, Bogor, Indonesia, using taxonomic keys and in consultation with the herbarium staff. The identified specimens were compared with the collections previously deposited in the Herbarium Bogoriense. Voucher specimens were placed in the Herbarium Bogoriense and the University of Ottawa Herbarium, Ottawa, Ontario.

3.2.3. Fungal species and inoculum preparation

Fungal species and inoculum preparation was as per Chapter 1 of this thesis.

3.2.4. Extract preparation

Extracts were prepared by blending the preserved plant material, approximately 500 g fresh weight (Osterizer commercial blender), in 99% ethanol (1:3 w/v ratio). The mixture was filtered using a Beuchner, the filtrate was retained and the residue was again blended using the same amount of 99% ethanol as above. The filtrates were combined and the solvent was rotoevaporated at 45°C to near dryness, whereupon the extract was freeze-dried for 48 hours to achieve complete dryness. The fresh material yielded approximately 35 to 45 grams of extract. Dried extract (200 mg) was reconstituted in 1ml of 99% ethanol for a final concentration of 2 mg/10 µl. In total, 17 ethanol extracts were evaluated for antifungal activity.

3.2.5. Disc diffusion bioassays

The disc diffusion bioassays using 2 mg per disc were performed in triplicate as per Chapter 1 of this thesis. Ethanol (99%, 10 µl/disc applied and allowed to evaporate) was used as a negative control and berberine (2 mg/disc) was used as a positive control. All trials were incubated in the dark at 30° C for 48 h.

3.3. Results and Discussion

Ten species belonging to the Zingiberaceae family were collected, of which four species were used in traditional remedies for ailments that could be attributed to fungal pathogens (*Alpinia galanga*, *Etlingera eliator*, *Hedychium cylindricum*, *Zingiber pachysiphon*), and six species were used in traditional remedies for ailments that could not be attributed to fungal pathogens (*Alpinia mutica*, *Costus globosus*, *Curcuma longa*, *Etlingera littoralis*, *Hornstedia* spp., *Zingiber purpureum*). Table 3.1 lists the species and plant parts collected as well as their use in Kenyah traditional medicine. In addition to these collections, rhizomes of *Z. officinale* var. *officinale* and *Z. officinale* var. *rubrum* were purchased from the local market in Bogor, Indonesia. It is important to note that herbarium voucher specimens were not collected for the *Z. officinale* specimens and that the taxonomic identification of the purchased material is in question.

Four of the 10 Zingiberaceae species collected using their local Kenyah names were not the same species as reported by Leaman (1996). Table 3.2 lists the main morphological differences between the species as identified in this study (*Curcuma zedoaria*, *Hornstedia* spp., *Zingiber pachysiphon* and *Zingiber purpureum*) and as identified by Leaman (*Curcuma longa*, *Etlingera* spp., *Zingiber longipedunculatum* and

Table 3.1. The Zingiberaceae species and plant parts collected and their use in the traditional medicine of the Kenyah People.

Species	Voucher #	Local name ¹	Plant part	Remedy ¹	Ailment ¹
<i>Alpinia galanga</i> (L.) Sw.	UO-18901	Lia Buke' Borak	stalk	heated and ingested	heartburn
				heated with rhizome and ingested	vitiligo
			rhizome	decoction	stomach pain
<i>A. Ipinia mutica</i> Robx.	UO-18902	Kerapat Bai		heated, rubbed on skin	itchiness, vitiligo, ringworm
			stalk	decoction	dysentery
<i>Costus globosus</i> Bl.	UO-18903	Pe Luan	rhizome	no documented use	
			rhizome	heated poultice	arthritis
<i>Curcuma zedoaria</i> (Berg.) Roscoe	UO-18904	Lia Mit Pute'		decoction	bloody diarrhea
			rhizome	cold infusion	headache, general malaise
<i>Etilingera eliator</i> (Jack) R. M. Smith	UO-18905	Nyanding		ingested infusion	coughs, nose bleed
			fruit	endosperm applied as ointment	blowgun dart wounds, caterpillar stings, dog mange and wounds
				endosperm added to crushed snail shells and ingested	worms
<i>Etilingera linoralis</i> (König)	UO-18906	Ubut Ladyei	rhizome	no documented use	
			rhizome	decoction	cleanse urinary tract, worms, jaundice
<i>Hedychium cylindricum</i> Ridley	UO-18907	Tubo Sakai	rhizome	heated poultice	insect bites and stings, weeping wounds, infected sores, ringworm

Table 3.1. Continued

Species	Voucher #	Local name ¹	Plant part	Remedy ¹	Ailment ¹
<i>Hornstedtia</i> spp.	UO-18908	Lamei	stalk	endosperm applied as an ointment	insect stings and bites
			rhizome	no documented use	
<i>Zingiber pachysiphon</i> Burt & Smith	UO-18909	Ubut Bele Sua'	inflorescence	heated nectar applied as ointment	dog mange
			rhizome	no documented use	
<i>Zingiber purpureum</i> Roscoe	UO-18910	Lia Sukeng	rhizome	decoction infusion applied as body rub infusion ingested, or applied as a body rub	cough chest pain, tiredness general aches and pains, fever

¹ as reported by Leaman, 1996

Table 3.2. The main distinguishing characteristics between the Zingiberaceae species as identified in this study and as identified in the study conducted by Leaman, 1996.

Kenyah name	Taxonomically identified by Leaman, 1996		Taxonomically identified in this study	References
Lamei	<i>Elingera</i> sp.	inflouescence of varying shapes, not spindle shaped	<i>Hornstedtia</i> spp. inflouescence spindle shaped	Koorders, 1923; Backer and Van Den Brink, 1968; Smith, 1995; Beaman and Beaman, 1998; Larson et al., 1999
Lia Mit Pute'	<i>Curcuma longa</i> L.	long, cylindrical root tubers	<i>Curcuma zedoaria</i> (Berg.) Roscoe short, spherical root tubers	Koorders, 1923; Backer and Van Den Brink, 1968; Darwis <i>et al.</i> , 1992; Beaman and Beaman, 1998; Hadiah, 1999; Larson et al., 1999
Ubut Bele Sua'	<i>Zingiber longipedunculatum</i> Ridley	inflouescence held on prostrate peduncle	<i>Zingiber pachysiphon</i> Burt & Smith inflouescence held on erect peduncle	Smith, 1988
Li Sukeng	<i>Zingiber officinale</i> Rosc.	rhizome yellow, medium hardness	<i>Zingiber purpureum</i> Roscoe rhizome very bright yellow, soft	Backer and Van Den Brink, 1968; Smith, 1988; Hadiah, 1999

Zingiber officinale). As the morphological differences between the species in question are slight, it is most likely that the Kenyah villagers consulted were not able to distinguish between the species. It is also possible that the villagers were looking for phenological attributes, for example the presence of a particular colour of inflorescence, among other characteristics, which change over time, but are not very variable. Unfortunately, the lack of coherence between local Kenyah names and species was not immediately recognized in the field and further investigation of plant identification by the Kenyah should be undertaken. It is unlikely that the different identifications resulted from taxonomic errors as the plant material was relatively fresh and the identifications were confirmed by qualified taxonomic authorities at the Herbarium Bogorienses.

The results of testing the 17 Zingibereaceae species extracts on the six yeast-like and seven filamentous fungi are reported in Tables 3.3 and 3.4. Fungi with growth inhibition with five or more of the Zingibereaceae extracts included *W. dermatitides*, *M. gypseum*, *T. mentagrophytes* and *P. boydii*. Fungi exhibiting growth inhibition with four or less of the extracts included *A. alternata*, *A. fumigatus*, *F. oxysporum*, *C. neoformans*, *S. cerevisiae*, *R. mucor* and *C. albicans* strains al-1, D10 and CN1A. As in Chapter 1 and 2, *M. gypseum* was the most sensitive of the fungi tested. Seven of the Zingibereaceae extracts exhibited antifungal activities and produced large zones of inhibition when tested against *M. gypseum*.

The activity of the 17 extracts against the yeast-like and filamentous fungi was compared using the statistical package GLIM 4.0 to conduct an analysis of deviance (Figure 3.2). Success was recorded as fungal growth inhibition, regardless of the size of the inhibition zone. Errors were specified as binomial, a logit link-function was used and

Table 3.3. Mean diameter (mm) of fungal growth inhibition zones ($\pm$ s.e.) of yeast-like fungi.

Zingiberaceae species	C. neo	S. cer	al-1	CN1A	D10	W. derm
<i>A. galanga</i> (stalk) ¹						11.94 $\pm$ 0.80
<i>A. galanga</i> (rhizome) ¹	10.17 $\pm$ 0.67					27.28 $\pm$ 7.55
<i>A. mutica</i> (stalk)						8.17 $\pm$ 0.67
<i>A. mutica</i> (rhizome)						
<i>C. globosus</i> (rhizome)						
<i>C. zedoaria</i> (rhizome)	14.83 $\pm$ 2.14	13.28 $\pm$ 0.22	14.61 $\pm$ 1.46	13.5 $\pm$ 0.0	12.80 $\pm$ 0.70	11.5 $\pm$ 1.0
<i>E. elaitor</i> (fruit) ¹						
<i>E. elaitor</i> (rhizome)						
<i>E. littoralis</i> (rhizome)						
<i>H. cylindricum</i> (rhizome) ¹						
<i>Hornstedia</i> sp. (stalk)						
<i>Hornstedia</i> sp. (rhizome)						
<i>Z. officinale</i> var. <i>officinale</i> (rhizome)						
<i>Z. officinale</i> var. <i>rubrum</i> (rhizome)						
<i>Z. pachysiphon</i> (inflourescence) ¹						
<i>Z. pachysiphon</i> (rhizome)	11.39 $\pm$ 0.49			9.28 $\pm$ 0.97	8.39 $\pm$ 0.89	11.28 $\pm$ 1.18
<i>Z. purpureum</i> (rhizome)	15.72 $\pm$ 0.59	12.61 $\pm$ 1.23	19.94 $\pm$ 0.44	11.06 $\pm$ 0.44	25.06 $\pm$ 0.44	16.83 $\pm$ 1.15
berberine						
ethanol						

¹ Used in possible antifungal remedy

Minimum diameter possible = 8.0 mm. Standard error on all values was 14% of mean

C. neo= *Cryptococcus neoformans* ; S. cer= *Saccharomyces cerevisiae* ; al-1, CN1A, D10= *Candida albicans* strains;W. derm= *Wangiella dermatitides*

Table 3.4. Mean diameter (mm) of fungal growth inhibition zones ($\pm$ s.e.) of filamentous fungi.

Zingiberaceae species	A. alt	A. fum	F. ox	M. gyp	P. boy	R. muc	T. men
<i>A. galanga</i> (stalk) ¹				8.17 $\pm$ 0.67			
<i>A. galanga</i> (rhizome) ¹	9.5 $\pm$ 0.0	14.39 $\pm$ 1.60	11.94 $\pm$ 0.44	20.61 $\pm$ 1.60	31.06 $\pm$ 3.23	12.17 $\pm$ 1.33	16.61 $\pm$ 0.59
<i>A. mutica</i> (stalk)							
<i>A. mutica</i> (rhizome)		9.5 $\pm$ 0.0		25.33 $\pm$ 0.40	9.5 $\pm$ 0.0	14.17 $\pm$ 2.40	9.5 $\pm$ 0.0
<i>C. globosus</i> (rhizome)				27.94 $\pm$ 0.0	8.17 $\pm$ 0.67		26.39 $\pm$ 0.89
<i>C. zedoaria</i> (rhizome)				9.5 $\pm$ 0.0			
<i>E. elaitor</i> (fruit) ¹							
<i>E. elaitor</i> (rhizome)							
<i>E. littoralis</i> (rhizome)		8.83 $\pm$ 0.67					
<i>H. cylindricum</i> (rhizome) ¹							
<i>Hornstedtia</i> sp. (stalk)							
<i>Hornstedtia</i> sp. (rhizome)							
<i>Z. officinale</i> var. <i>officinale</i> (rhizome)				10.83 $\pm$ 0.67	8.39 $\pm$ 0.67		10.39 $\pm$ 1.55
<i>Z. officinale</i> var. <i>rubrum</i> (rhizome)				13.94 $\pm$ 0.44	9.5 $\pm$ 0.0	9.72 $\pm$ 0.22	12.61 $\pm$ 0.80
<i>Z. pachysiphon</i> (inflorescence) ¹							
<i>Z. pachysiphon</i> (rhizome)	11.05 $\pm$ 0.44	14.17 $\pm$ 0.77	9.5 $\pm$ 0.0	27.28 $\pm$ 0.22	17.05 $\pm$ 0.22	11.5 $\pm$ 1.15	20.61 $\pm$ 2.32
<i>Z. purpureum</i> (rhizome)	9.95 $\pm$ 0.22	13.94 $\pm$ 1.74	10.17 $\pm$ 0.38	19.94 $\pm$ 0.44	13.06 $\pm$ 0.44		21.28 $\pm$ 1.07
berberine							
ethanol							

¹ Used in possible antifungal remedy

Minimum diameter possible = 8.0 mm. Standard error on all values was $16 \leq$ % of mean

A. alt= *Alternaria alternata*; A. fum= *Aspergillus fumigatus*; F. ox= *Fusarium oxysporum*; M. gyp= *Microsporium gypseum*;

P. boy= *Pseudallescheria boydii*; R. muc= *Rhizopus mucor*; T. men= *Trichophyton mentagrophytes*

Figure 3.2. Proportion of fungi inhibited by ethanol extracts ($\pm$ s.e.) of Zingiberaceae species used in Kenyah traditional medicine.

* used as Kenyah antifungal remedy

1=*A. galanga* (stalk); 2=*A. galanga* (rhizome); 3=*A. mutica* (stalk); 4=*A. mutica* (rhizome); 5=*C. globosus* (rhizome); 6=*C. zedoaria* (rhizome); 7=*E. eliator* (fruit); 8=*E. eliator* (rhizome); 9=*E. littoralis* (rhizome); 10=*H. cylindricum* (rhizome); 11=*Hornstedia* spp. (stalk); 12=*Hornstedia* spp. (rhizome); 13=*Z. officinale* var. *officinale* (rhizome); 14=*Z. officinale* var. *rubrum* (rhizome); 15=*Z. pachysiphon* (inflorescence); 16=*Z. pachysiphon* (rhizome); 17=*Z. purpureum* (rhizome)

the binomial denominators were respectively the total number of fungi tested, the number of filamentous fungi tested and the number of yeast-like fungi tested. Presented values are back-transformed from the model estimates. There was significant variation in the antifungal activity of the various extracts ($P < 0.001$). Of the extracts, *A. galanga* (rhizome) and *Z. purpureum* (rhizome) inhibited growth of all filamentous fungi tested, but only inhibited growth of two (33%) and four (67%) of the fungi with a yeast-like growth form, respectively. *C. globosus* (rhizome), *Z. officinale* var. *officinale* (rhizome) and *Z. officinale* var. *rubrum* (rhizome) inhibited five (71.4%), two (28.6%) and four (57.1%) filamentous fungal species, respectively, but inhibited none of the yeast-like fungi. Of particular interest is the *C. zedoaria* (rhizome) extract, which inhibited the growth of all yeast-like fungi, but inhibited only two (28.5%) of the filamentous fungi.

For *E. eliator* and *Z. pachysiphon*, two of the four Zingibereaceae species identified as having possible antifungal properties, plant parts other than the rhizome were used in the traditional remedies. When the extracts of both the part used and the rhizome of these plants were tested against the yeast-like and filamentous fungi little to no fungal growth inhibition was reported. Similarly, the rhizome extract of *H. cylindricum* did not inhibit the growth of the fungi tested. *A. galanga* was the only plant used in possible antifungal remedies that demonstrated antifungal activity. Both the stalk and rhizome of *A. galanga* were used in possible antifungal remedies. However, when tested in this study, the stalk extract demonstrated little activity and inhibited the growth of only one fungus, whereas the rhizome extract was very active and inhibited the growth of nine fungi (Figure 3.2).

For the Zingibereaceae species where parts other than the rhizome were used in non-antifungal remedies (*A. mutica* and *Hornstedtia* spp.), the part used in the remedy, as well as the rhizome did not have significant antifungal activity. The majority of the plants where the rhizome was used in possible non-antifungal remedies (*C. globosus*, *C. zedoaria*, *E. littoralis* and *Z. purpureum*), the rhizome possessed significant antifungal activity, *E. littoralis* being the exception, where the rhizome inhibited only the growth of one fungus.

The antifungal activities of the plant parts used in possible antifungal remedies and the activities of the parts not used in the remedies with the highest importance value were compared, also using an analysis of deviance (Figure 3.3). The analysis was conducted again using the statistical package GLIM 4.0. Only the extracts of the plant parts that were used in the possible antifungal activities and the extracts of the plant parts that were used in the remedies with the highest importance value were included in this analysis. Success was again recorded as fungal growth inhibition, regardless of the size of the inhibition zone. Factors included plant part and use in traditional remedies and were first individually fitted to the model to determine their effect on deviance, and then in a step-wise fashion starting with the factor that accounted for the most deviance (plant part), followed by the factor that accounted for less deviance (used as a possible antifungal remedy). The results obtained from the antifungal bioassays using the *Z. officinale* var. *officinale* (rhizome) and *Z. officinale* var. *rubrum* (rhizome) extracts were excluded from this analysis, as their taxonomic identification is in question and therefore their use in Kenyah traditional medicine is uncertain.

Figure 3.3. Comparison of the antifungal activities of extracts of plant parts used in possible antifungal remedies and in non-antifungal remedies.

When each factor was fitted by itself to the model, although not statistically significant ($P > 0.05$), there was a tendency for plants not used as possible antifungal remedies to be more potent inhibitors of fungal growth than those that possibly were used as antifungal remedies (Figure 3.3). This was not the case when the factors were fitted after plant part had been added to the model (i.e., rhizome vs. other), indicating that these factors were interrelated, perhaps because plant parts not used as antifungal remedies tended to be from the rhizome, which also was significantly more potent in terms of antifungal activity ($P < 0.001$). The relatively higher antifungal activity of extracts derived from the rhizome is not surprising, as the concentrations of gingerols and gingerdiols, both active antifungal compounds as demonstrated in Chapter 2 of this thesis, are highest in these tissues.

Overall, the Zingibereaceae extracts tested in this study had higher success rates, measured as fungal growth inhibition, regardless of the size of the inhibition zone, when tested against the filamentous fungi than when tested against the yeast-like fungi. Nine (69.2%) extracts inhibited the growth of the filamentous fungi, whereas five (38.5%) extracts inhibited the growth of the yeast-like fungi. A similar trend was observed in both Chapter 1 and Chapter 2. The yeast-like fungi used in this study are phylogenetically diverse and belong to classes in both the Ascomycotina and Basidiomycotina subphylum (Figure 3.4). The filamentous fungi are not as diverse and except for *R. mucor* (phylum Zygomycota), belong to one class of the Ascomycotina subphylum. All of the fungi that were inhibited by five or more of the Zingibereaceae extracts, including *W. dermatitides*, the only yeast-like fungus sensitive to the extracts, belong to the class ascomycetes. It is possible that a common characteristic of the fungi

Figure 3.4. Phylogenetic diversity of the fungi used in this study.

in this class renders them more susceptible to the antifungal properties of the extracts than the fungi belonging to other classes. It is also possible that the yeast-like fungi chosen as representatives of the two other classes in this study were uncharacteristically resistant to the activities of the extracts and other fungi belonging in these classes would be more sensitive to the extracts. This phenomenon is demonstrated in this study by *F. oxysporum* and *A. alternata*. Although both fungi belong in the ascomyces class, they were inhibited by only one extract. Of note is that the *C. zedoaria* (rhizome) and *Z. purpureum* (rhizome) extracts inhibited fungi in all the classes represented in this study. This suggests that these extracts are very potent inhibitors of fungal growth.

The results presented in this study are contrary to the initial hypothesis which was that antifungal activities for those Zingibereaceae extracts used as antifungal remedies in Kenyah traditional medicine would be more pronounced than for those not used for these purposes. Several points should be considered when interpreting the lack of support for this hypothesis. First, it is possible that the Zingibereaceae species collected in this study on the premise that they are used in possible Kenyah antifungal remedies may not actually be used in this capacity. Several pathogenic agents including fungi, bacteria, protozoa and viruses can cause the symptoms of itchiness, vitiligo and infections of wounds. The antimicrobial potential of these extracts should therefore be further evaluated. Second, the mode of preparation of the remedies could increase their effectiveness against fungal pathogens. The use of ethanol extracts in this study and not fresh plant material, as is used in the traditional remedies could have affected the antifungal activity of some species. Third, healing of infected wounds involves complex host responses. A number of plant species used as phytomedicines including *Echinacea*

purpurea and *Panax ginseng* act by modulating the immune system (See *et al.*, 1997; Park *et al.*, 2001). It is possible that instead of having a direct effect on fungal growth, Zingibereaceae species also act to enhance the host natural defenses. Alternatively, those Zingibereaceae species not used as wound dressings, that exhibit significant antifungal activities, may be somewhat antagonistic to the host wound healing process.

Contrary to the findings in this study, previous studies conducted on the traditional medicine of North American Indigenous Peoples have demonstrated a strong correlation between the use of plants in traditional remedies and activity (McCutcheon *et al.*, 1992; Jones *et al.*, 2000; Omar *et al.*, 2000). In the studies conducted by Omar *et al.* (2000) and Jones *et al.* (2000), disc diffusion bioassays were performed as in this study, using the same fungal species and isolates. However, in those previous studies, the zones of fungal growth inhibition reported with plants used in antifungal remedies were inferior to the inhibition zones reported in this study with plants used in either antifungal or non-antifungal remedies. It is quite possible that tropical plants in general have higher antifungal activity than temperate plants. Therefore, North American Indigenous Peoples would have to search widely for plant species with the base level of activity suitable for use in antifungal remedies, regardless of other plant properties such as irritability, palatability and abundance. Conversely, the Kenyah have an abundance of plants with suitable activity that would allow for the selection of plants used in their antifungal remedies to be based more on other plant properties.

It should be emphasized that as a group, members of the Zingibereaceae family are important in Kenyah traditional medicine for the treatment of many illnesses. Clearly, some of the Zingibereaceae extracts examined in this study exhibited significant

antifungal activities. This study therefore reinforces the hypothesis that the Zingibereaceae family is a potential source of potent antifungal compounds and perhaps other compounds useful in treating infectious diseases.

General Discussion and Conclusions

General Discussion and Conclusions

The objectives of this study were to 1) identify plants used in traditional medicine with significant antifungal activity that are appropriate for development as phytomedicines, 2) isolate and characterize active antifungal compounds from the plant species identified in objective 1, and 3) focusing on one plant family, examine the relationship between the antifungal activities of plant species used in traditional remedies for the treatment of diseases of fungal origin and the antifungal activities of species used in traditional remedies for the treatment of diseases not of fungal origin.

From a broad spectrum screening of 23 tropical and 6 temperate plant species used in traditional medicines, *Zingiber officinale* was identified as having a wide range of antifungal activity measured as either the number of fungal species inhibited or the diameter of the fungal growth inhibition zone. *Z. officinale* has been used extensively in antinausea clinical trials (Mowrey and Clayson, 1982; Wood *et al*, 1988; Bone *et al*, 1990), including trials conducted with pregnant women (Fischer-Rasmussen *et al.*, 1990). As adverse side effects have not been reported in any of these trials, *Z. officinale* is considered safe for human use and is a good candidate for development as a phytomedicine. Further investigations into the antifungal activity of *Z. officinale*, as well as the activities of other members of the Zingibereaceae family, were conducted.

Two active antifungal compounds were purified from the rhizome of *Z. officinale* and were identified as [6]-gingerol and [6]-gingerdiol using MS, HPLC and NMR techniques. [8] and [10]-gingerols were purchased from Dalton Chemical Laboratories Inc., Toronto, Ontario, which were also found to have antifungal activities. This study, along with the studies conducted by Endo *et al* (1990) and Agarwal *et al* (2001), has

identified several active antifungal compounds from *Z. officinale*. As the crude ethyl acetate extract, containing a diversity of compounds demonstrated excellent activity, it is possible that the compounds have either additive or synergistic effects. It would therefore be interesting to determine the activity of the compounds as well as the mode of action of the compounds.

The Zingibereaceae family is important in the traditional medicines of many indigenous peoples including the Kenyah of East Kalimantan, Borneo. The relationship was examined between the antifungal activities of Zingibereaceae species used in traditional remedies for the treatment of diseases of possible fungal origin and the antifungal activities of species used in traditional remedies for the treatment of diseases not of fungal origin. Although the differences in activities between the 2 groups of Zingibereaceae species were not significant, species in both groups demonstrated high antifungal activities, inhibiting the growth of both filamentous and yeast-like as well as antibiotic resistant and sensitive fungal species. Future studies to determine the basis of plant selection and identification by the Kenyah for use in traditional remedies would be interesting. Of note is that of the Zingibereaceae plant parts collected and tested, which included the rhizome, fruit, inflorescence, and stalk, the rhizomes had significantly higher activity. It is therefore recommended to save time and effort, future studies of this sort focus only on the rhizome.

My results demonstrate that many plant species used in traditional medicines are excellent sources of antifungal compounds. Of the 40 plant species tested in this thesis, 32 (80%) inhibited the growth of at least one of the tested fungal strains and 20 (50%) inhibited either one or both of the antibiotic resistant *C. albicans* isolates D10 and CN1A.

As well, species belonging to the Zingiberaceae family are potent inhibitors of fungal growth and due to their lack of human toxicity are excellent candidates for development as phytomedicines.

In this thesis, I had the unique experience of working with remote peoples whose knowledge about plants is ancient and extensive. However, with the progression of Indonesian development, in particular the clearing of the rainforest, this knowledge is at risk of disappearing (Elliott and Brimacombe, 1987). Prior to the Suharto government which commenced in 1965, Kalimantan had over 120 million hectares of forested land (King, 1993). By 1990, the remaining forest covered only 63% of the former extent, with the average rate of deforestation estimated at 600 000 hectares per year (Potter, 1996). Dayak societies are being radically transformed as changes such as logging roads penetrating into the interior and the introduction of superior water way transportation are improving local mobility and opening up village societies to modern influences (King, 1993). The study site of this thesis, the village of Long Sungai Barang and the surrounding villages are expected to be connected by logging access roads to Malaysia and coastal Kalimantan in the next decade. One can only imagine the irreversible impact this will have not only on the livelihood, but also on the entire cultural complex of the Borneo indigenous peoples. Already there has been an important loss of the traditional knowledge of plant uses as younger generations are moving from their villages to larger city centers for work and education and are exposed to a more westernized society (King, 1993; Raja *et al.*, 1997). Studies such as this one are important and timely as they provide not only a written record of plant use practices of indigenous peoples whose plant

lore is fast disappearing (Coe and Anderson, 1996), but also highlight those species which can be incorporated into modern practices as phytomedicines.

Appendix One

The geography and Peoples of Borneo

A1.1. Geography of Borneo

Borneo, the third largest island in the world, covering approximately 746,000 km² is located within the biogeographical region known as Sundaland in South-East Asia. The island of Borneo is divided politically by the Republic of Indonesia, the federation of Malaysia and the sultanate of Brunei Darussalam (Figure A1.1). The largest, most southerly part of the island covering approximately 540,000 km² belongs to the Republic of Indonesia and is referred to as Kalimantan. This is further divided into the four provinces of West, Central, South and East Kalimantan. 200,000 km² in the north of Borneo belongs to the Federation of Malaysia and is divided into the two states of Sarawak and Sabah. The remaining small pocket of land, approximately 6,000 km², surrounded by Sarawak and Sabah is occupied by the sultanate of Brunei Darussalam.

In 1990, the total population of Borneo was 12.5 million with 9 million inhabiting Kalimantan and the majority of the population concentrated along the coast and navigable rivers (King, 1993). The 5 major rivers, Kayan, Barrito, Mahakam, Rejang and Kapuas, radiate from the center of the island and are punctuated in several points by impassable rapids. The relief of Borneo increases moving inland from the coast forming many plateaus and mountain ranges toward the center (Figure A1.2).

The island of Borneo supports some of the most diverse tropical habitats on earth and is a recognized center of biodiversity (MacKinnon and Sumardja, 1996). It covers less than 0.2% of the earth's land surface, yet 1 in 25 of all known plants are found here as well as 1 in 20 of all known birds and mammals. There is presently estimated to be 9,000 to 15,000 species of seed plants in Borneo (Burley, 1996), of which 34% of plant species and 59% of genera are endemic to the island (MacKinnon and Sumardja, 1996).

Figure A1.1. The main political and administrative boundaries in Borneo (adapted from King, 1993).

Figure A1.2. The relief of Borneo (adapted from King, 1993).

However, the flora of Borneo has been poorly documented up to this point with recent field collections throughout Borneo suggesting that around 15% of the flora remains uncollected (Burley, 1996). These findings suggest that the diversity of the island may be underestimated.

A1.2. The Peoples of Borneo

The indigenous peoples of Borneo have historically been classified into three major groups: Dayaks, Punan and Malay. The term Dayak includes all non-Muslim indigenous peoples of Borneo. Dayaks have traditionally lived in longhouse communities located at the confluence of two rivers and practice swidden rice agriculture, supplemented by hunting, fishing and gathering (Leaman, 1996; Rousseau, 1990). The term Punan includes those indigenous groups who are forest nomads and practice hunting and gathering as a means of subsistence (Leaman, 1996). Malay refers to those indigenous peoples of the Islamic religion. Most Malays live along the coast and cultivate wetland rice and plantation crops and practice sea fishing and trade (Leaman, 1996; Rousseau, 1990). These three main groups are subdivided into many smaller sub-groups by a confusing system relating to the origin of the People's homeland and dialects spoken. Due to this system of classification, the migration patterns and the interrelations of the indigenous peoples, many sub-groups have more characteristics in common with those of other major groups than with those belonging to the same major group. Figure A1.3 provides an overview of the ethnic taxonomy of the Bornean indigenous peoples.

Figure A1.3. Ethnic taxonomy of the Indigenous Peoples of Borneo (adapted from Rousseau, 1975).

A1.3. Origin of the Apo Kayan Kenyah

In 1978 it was determined that 40,000 Kenyah comprising over 40 named divisions were living in over 110 communities on the island of Borneo (Whittier, 1978). The majority were located in East Kalimantan where they were located along the Kayan, Mahakam and Melanau rivers and their tributaries (Whittier, 1978; Rosseau, 1990). Some Kenyah oral histories place their homeland at the headwaters of the Iwan, Bahou and Peyunan rivers, all tributaries of the Kayan River, or the neighboring Usun Apau Plateau (Whittier, 1978). However, it is most probable that the Kenyah occupied the highlands of Sarawak with other Dayak groups, but due to overpopulation and intergroup warfare migrated to the aforementioned tributaries of the Kayan River and the Usun Apau Plateau where they are presumed to have remained for approximately 50 years (Whittier, 1978; Rousseau, 1990). After this period of time, the Kenyah moved to Apao Datah in the headwaters of the Iwan and Bahau rivers. Due to agricultural overexploitation they began their migration to the Apo Kayan region, which at the time, was occupied by the Kayan People. Accounts disagree as to whether the out-migrations of the Kayan from the Apo Kayan region was due to the in-migrations of the Kenyah and the ensuing conflicts, or if the out-migration of the Kayan preceded the in-migration of the Kenyah (Rousseau, 1990). However, it has been suggested that the out-migration of the Kayan was already well underway when the Kenyah moved in and fought with the remaining groups (Rousseau, 1990). From the Apo Kayan, the Kenyah continued their migrations along the Kayan, Baluy and Baram Rivers.

The migrations of the Kenyah People, along with many indigenous peoples of Borneo continue today. Figure A1.4 shows the locations of the main ethnic groups throughout the island of Borneo

A1.4. Kenyah Tradition, Culture and Beliefs

Among the Kenyah, 3 social strata were clearly distinguished which included aristocrats, commoners and slaves (Hose and McDougall, 1912; Whittier, 1978; King, 1993). Although slaves were considered to be the property of their owners, Hose and McDougall (1912) reported that they were treated with such kindness and consideration that they had little to complain of and most possessed little desire to be freed.

The Kenyah lived along the banks of major rivers in multi longhouse communities that were governed by a council of aristocratic elders comprised of men knowledgeable in *adat* law and its rituals (Hong, 1987). *Adat* was the unwritten body of rules and principles that pertained to all aspects of Kenyah life, including relations to other humans as well as to the natural and supernatural worlds (Whittier, 1978; Hong, 1987; King, 1993). It included the dead and the living, the evil and the good, the sacred and profane with each of these elements possessing a soul, or life, of its own (Hong, 1987). As long as the Kenyah behaved appropriately in their relations to the elements, harmony and balance was maintained. Inappropriate behavior resulted in a disturbance in this balance and resulted in supernatural punishment of the individual, family or community in the form of illness, death, crop failure and flood.

Figure A1.4. Location of the main ethnic groups of Borneo (adapted from King, 1993).

— Rivers

--- Provincial/state boundaries

— International boundaries

0 500 Kms

The ritualistic activity of the Kenyah were focused around 5 points: fertility (especially of rice), the taking and use of heads, birds and the reading of omens, the treatment of the dead and the healing of the sick and troubled (Winzeler, 1993).

The Kenyah recognized a primary deity responsible for creation. This deity was comprised of two parts, one symbolizing the Upperworld, the heavens and skies, and the other part symbolizing the Underworld, the primeval waters and earth. The Upperworld was associated with maleness, birds and the fertility ritual of headhunting. The Underworld was associated with femaleness, serpents and agriculture. This creator deity, however, was not recognized as being omnipotent (King, 1993) and many other principal deities were recognized as being important in everyday life.

Aside from the deities and principal spirits, the Kenyah believed that the cosmos was inhabited by all kinds of spiritual entities, some benevolent, some malicious and some both, depending on their mood and the actions of humans (King, 1993). The supernatural beings would send messages or omens, usually in the form of the behavior, calls and appearance of forest birds, which special individuals among the Kenyah community were able to interpret. Spiritual guidance was also sought through the reading of livers of sacrificed pigs or chickens. By being mindful of the omens, the Kenyah maintained good relations with the spirit world and were assured of a good harvest or a successful hunting or fishing trip.

The Kenyah attitudes towards sickness and healing stemmed from the fundamental belief that a person consisted of both a body and a soul, the latter having a life of its own (Winzeler, 1993). When a person was asleep, the soul could wander from the body. If the soul wandered too far it was believed that it was in danger of being

caught by an evil spirit. If this was to occur, the body was deprived of its spiritual essence or energy (King, 1993) and sickness and eventual death ensued. Shamanistic healing focused on the retrieval or rescue of the captured soul and the driving away or even slaying of malevolent spirits (Winzeler, 1993).

Due to intense missionary influence from 1950 to 1970, almost all Dayak groups, including the Kenyah were converted to the Roman Catholic and Protestant churches. The inhabitants of Long Sungai Barang were of a Roman Catholic majority with a Protestant minority. With this move to Christianity the traditional ceremonies and rituals, which were the repository of tribal worldviews (King, 1993), have ceased to be performed. The customs, beliefs and rituals described above are unfortunately no longer practiced. Leaman reported that many Kenyah now speak of these traditional practices as *adat lama* (the old ways), and *adat waktu kafir* (ways of the pagan time).

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