

**ADDIS ABABA UNIVERSITY
FACULTY OF SCIENCE
SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF BIOLOGY**



**A BIOACTIVE COMPOUND EFFECTIVE AGAINST
CANDIDA ALBICANS FROM *SYNCEPHALASTRUM*
SPECIES**

BY

ALEMAYEHU AMARE

**A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF
ADDIS ABABA UNIVERSITY IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN
APPLIED MICROBIOLOGY**

July, 2009

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DECLARATION

I, the undersigned, declare that this thesis is my own work in design and in execution. It has never been submitted in any institution and that all sources of materials used for this thesis have been duly acknowledged.

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LIST OF ABBREVIATIONS USED

A.A.U.	Addis Ababa University
ADA	Agar Diffusion Assay
AIDS	Acquired Immunodeficiency Syndrome
ATP	Adenosine tri-phosphate
Bio Assay	Biological Assay
BMA	Broth Microdilution Assay
CDA	Czapek Dox Agar
CFU	Colony forming unit
°c	Degrees Celsius
Da	Dalton
DNA	Deoxyribonucleic acid
EP	Extracellular polymers
g	gram
hr	Hour
IR	Infra-red
l	Litre
MIC	Minimal Inhibitory Concentration
min	Minute
ml	10⁻³ litre
mm	10⁻³ metre
MEA	Malt Extract Agar

MEB	Malt Extract Broth
nm	10 ⁻⁹ metre
OD	Optical Density
PDA	Potato Dextrose Agar
P-TLC	Preparative Thin Layer Chromatography
Rf	Retention factor
RNA	Ribonucleic acid
rpm	Revolution Per Meter
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
SLF	Submerged Liquid Fermentation
SSF	Solid State Fermentation
TLC	Thin Layer Chromatography
μl	10 ⁻⁶ litre
U.V.	Ultra Violet
%	Percent

ABSTRACT

Synencephalastrum sp. (AFC003), the soil fungus, was isolated and investigated for the production of secondary metabolites on sterile moist rice. The sterilized moist rice was inoculated with young blocks of agar (10mm²) of the fungus culture and was harvested just after 28 days. The dried and finely ground moldy rice was extracted with only ethyl acetate. The crude extract was tested *in vitro* for activity against *Candida albicans* and inhibition diameter of 16mm was observed. Further purification was held and successive bioassays were conducted. Accordingly, the purified compound was separated with 1:8 solution of hexane: ethyl acetate following the use of several eluents in their increasing polarity. The bioactive fraction detected by TLC had a single band with R_f value of 0.53 when visualized under U.V. (254 and 366nm). The purified active compound also showed an inhibition diameter of 9mm when 10µg/ml was applied and comparison was made with the positive control (ketoconazole, 10µg/ml) and the negative control (hexane, 10µg/ml). A considerable inhibition zone was observed against *Candida albicans*. MIC determination of the potent compound was also attempted via broth microdilution method. MIC was defined as the lowest concentration of the substance that had no visible turbidity. Hence, MIC break point was ranging from 80µg/ml to 100µg/ml. Taxonomy of the compound producing organism was partially studied in the laboratory and the fungus is preliminarily identified to belong to the genus *Synencephalastrum*. Further analysis of the purified compound reveals its chemical structure.

Key words: Antifungal agents, antifungal Bioassay, *Candida albicans*, chromatography, MIC, secondary metabolite, Solid state fermentation

1. INTRODUCTION

Microfungi are defined as fungi with microscopic spore producing structures (Muller *et al.*, 2004). They grow on plant debris and in every conceivable habitat as saprophytes and parasites. Most microfungi are relatively long-lived, but sporulate only for short periods. The success of colonization, subsequent survival of individuals, and species composition of a community is influenced by the nature and quantities of nutrients together with the physical features of the substratum, water availability, humidity, and temperature (Muller *et al.*, 2004).

Soil is a very species-rich habitat containing all major groups of microorganisms like bacteria, algae, protists and fungi (Hagvar, 1998). The great majority of fungal species have at least some part of their life cycle in soil (Bridge and Spooner, 2001). Soil also contain a lot of biotechnologically and pharmaceutically important fungi such as *Chaetomium cupreum*, *Curvularia senegalensis*, *Fusarium oxysporum*, *Penicillium citrinum*, *Penicillium janthinellum*, *Penicillium paxilli*, *Penicillium sclerotiorum*, *Penicillium simplicissimum*, *Penicillium waksmanii*, *Rhizopus stolonifer*, *Syncephalastrum racemosum*, *Trichoderma koningii* and *Trichoderma reesei*.

Syncephalastrum is a filamentous fungus that is commonly isolated from soil and animal feces particularly in tropical and subtropical areas. It is a heterothallic fungus and requires a mating strain to produce zygospores. It is commonly considered as a contaminant and is very rarely associated with human disease (<http://www.doctorfungus.org/Thefungi/syncephalastrum.htm>).

Isolation and cultivation of fungi in pure culture requires preparation of sterilized media (Norris and Ribbon, 1969). Most culture media contain macro and micro nutrients. Since fungal growth is sensitive to the

composition of the medium, laboratory conditions, and the physical environment, these factors often varied to influence the growth form, life cycle, and metabolic products of fungi in culture (Muller *et al.*, 2004). For example, low nutrient or media with only polysaccharide carbon sources often favor sporulation and suppress mycelial growth, where as media with high concentration of monosaccharides or disaccharides tend to favor vegetative growth. High salt concentration may inhibit growth by reducing water activity (Muller *et al.*, 2004).

Growth kinetics of filamentous fungi can be determined using several methods such as dry weight measurement, packed cell volume, absorbance measurement, total protein (N), substrate or O₂ consumption, and CO₂ production (Burnett, 1976). However, many of these methods are impractical where fungi are growing on solid substrates with their mycelium intermingled with the substrate.

Certain adaptations more apparent in the microfungi involves the production of melanized fruiting structures as a means to protect enclosed spores during adverse conditions such as drought, low temperature, high ultraviolet rays, and so forth (Muller *et al.*, 2004). Vegetative structures such as chlamydospores, sclerotia, bulbils, appressoria, and setae may also be produced for dispersal or survival of cold or dry periods (Muller *et al.*, 2004).

A vast majority of substances can be found within the mycelia of the fungi such as a wide range of degradation products which arise from catabolic reactions. These are described as primary accumulated products and are associated with the maintenance and growth of the living cell and its structure (Burnett, 1976). On the other hand, there is a vast and ever-increasing number of secondary metabolites derived in various ways from the primary metabolites but differing in that they are highly specific

functionally or have unknown function while their production is often confined to one, or few, fungal species (Burnett, 1976).

SSF has been used for upgrading the feed values of waste cellulosic materials, for the production of enzymes, aflatoxins, oriental foods and single-cell proteins (Cannal and Moo-Young, 1980; Chahal *et al.*, 1983). SSF technique was employed as a source of culture extracts for the production of antifungal metabolites. In fact, SSF offers the advantage of cheap materials based on simple and locally available materials.

Candida albicans is a dimorphic fungus that can be either commensal or an opportunistic pathogen with the ability to cause a variety of infections, ranging from superficial to life threatening. Predisposing factors for *Candida albicans* infections include immunosuppressive therapy, antibiotic therapy, use of indwelling devices and intravenous catheters, HIV-infection, diabetes and old age (Odds, 1987). Nosocomial infections due to *Candida* are becoming increasingly important. The medical implications of device-related infections can be enormous such as increased resistance to antifungal therapy and failure of the medical implant (Raad, 1998).

The prolonged use of azole derivatives and amphotericin B against *Candida* has culminated in an emerging prevalence of drug resistant strains of *Candida albicans* (Law *et al.*, 1994; Kelly *et al.*, 1997). Thus, there is an exigent need for novel antifungal remedies, and fungi remain a vital source of these new substances (Iwu and Wootton, 2002).

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

- o To isolate, characterize and evaluate a wide range of soil microfungi grown on solid substrates for their production of effective anti-Candidal compounds

2.2 SPECIFIC OBJECTIVES

- o To isolate, characterize and maintain a wide range of soil microfungi from Menagesha Suba forest
- o To develop a solid state fermentation method for cultivation of fungi and production of secondary metabolites
- o To extract, purify and evaluate the *in vitro* activity of the antifungal compound
- o To determine the antimicrobial spectrum and the Minimal Inhibitory Concentration (MIC) of the secondary metabolite

3. Review Literature

3.1 Secondary Metabolites

Secondary metabolites may be defined as natural products that are not necessary for growth, are often produced only by specific groups of organisms, during part only of their life cycle, and are derived from a few precursors formed during primary metabolism (Bryce, 1992). Formerly, it was thought that production of secondary metabolites did not represent any advantage for the producing microorganism. Contrary, nowadays, it is considered that cell investment in secondary metabolite production is almost the confirmation of a function that should give the organism certain advantage against other members of the microbial community (Stone, 1992). Secondary metabolites are accepted to be essential for the producing cell as inhibitors of other organisms that compete for the same food supply or as regulators of cellular differentiation processes and are products of biosynthetic pathways (Demain, 1999; Demain and Fang, 2000).

The nature has provided a broad spectrum of structurally diverse secondary metabolites (Maier, 1999; Vandamme, 1994). Despite this great diversity, microbial secondary metabolites are synthesized from only a few precursors, in pathways with a relatively small number of reactions, which branch from just a limited number of reactions of the primary metabolism (Demain and Fang, 2000). The mentioned structure diversity is reflected in a variety of biological activities as, for instance, inhibitors of enzymes and anti-tumor, immunosuppressive and anti-parasitic agents (Carmichael, 1992). About 100,000 secondary metabolites have been characterised, among them approximately 50,000 are from microbial sources (Bezborodov, 1978). New microbial bioactive products continue to be discovered at an amazing pace: 200-300 per year in the late 70s, increasing to 500 per year by 1997 (Demain, 1999).

Antibiotics are perhaps the most widely studied type of secondary metabolites with 12,000 antibiotics known up to 1995 (Vining, 1992). Again, microbial cells are the most important source of this type of secondary metabolites. From the known antibiotics, 55% are produced by filamentous bacteria of the genus actinomyces, 11% from other actinomyces, 12% from non-filamentous bacteria and 22% from filamentous fungi (Vining, 1992).

Different alternatives for improving production of secondary metabolites with different activities for biotechnological applications have been extensively investigated and two methods have been applied; optimization of fermentation process and improvement of strains (Parekh, 2000). More over, the microbial production of secondary metabolites is extremely sensitive to environmental factors or culture conditions (Bunch and Harris, 1986).

Overproduction of secondary metabolites is a complex process and the successful development of improved strains requires knowledge of physiology of the microbial producer, pathway regulation and control of the product (Roessner, 1996). Recently, research efforts have been devoted to elucidate, at the molecular level, the regulatory mechanisms involved in the control of the biosynthesis of antibiotics (Roessner, 1996). Most of the work performed in this field has been focused in antibiotics produced by fungi and actinomycetes. Hence, a number of biochemical and genetic controls have been described to optimize the levels at which the antibiotics are produced in a specific media (Lorenzo, 1985).

3.1.1 History of Secondary Metabolites

Secondary metabolites with antimicrobial properties have been used for many years. Yet, it was not until 1870s, that Tyndall, Pasteur and Roberts reported the antagonistic effects of some organisms on others. In fact, the antibiotics era began in 1929 with the penicillin discovery by Fleming. However, antibiotic research and industry only flourished after the commercial production of penicillin in the 1940s (Demain and Fang, 2000; Kieslich, 1986). In spite of this, until 1970 only two classes of naturally occurring β -lactam antibiotics (penicillins and cephalosporins) were known, although a variety of β -lactam-containing molecules and other types of antibiotics have been identified with the advent of new screening and isolation techniques (Wells *et al.*, 1982).

Screening of microorganisms for production of antibiotics has provided the cornerstone of antibiotic research programs for the past thirty years. The great majority of such studies have been carried out with fungi and actinomycetes, which are capable of producing natural products with widely divergent chemical structures. Parallel to the screening for new antibiotics, efforts have been focused in finding low molecular weight secondary metabolites with other biological activities such as enzyme inhibitors, plant growth stimulators, herbicides, insecticides, antihelmintics and immunosuppressant have been obtained (Bentley, 1997).

3.1.2 Function and Importance of Secondary Metabolites

Several hypotheses were given as to the origin and function of secondary metabolites. The most accepted considers secondary metabolites as waste products that under the pressure of natural selection have evolved as messenger molecules which must endure long enough to shuttle between the

various components of the microbial community (Demain, 1999). This fact, would explain the secondary metabolites tendency to be small organic molecules, as a natural consequence of their functions (Jarvis, 1995). The explicit role of secondary metabolites in microbe-microbe interactions is unknown, especially how their production affects the community ecology (Verpoorte, 1998). In fact, the notion that microbial antibiotics are employed by the producing organisms to adversely affect their competitors is cited.

3.1.3 Genetics of Secondary Metabolites

According to Spizek *et al.* (1995), the genes regulating and ensuring synthesis of secondary metabolites and their expression can be grouped in to five classes: structural genes, that code for enzymes involved in the biosynthesis; regulatory genes, that determine the induction or repression of the structural genes; genes that determine the resistance of the producing organism; genes controlling the permeability to the compound, and genes that control primary pathways. The genetic regulation of all above mentioned genes is highly complicated because many environmental and microbial factors affect the production of these compounds.

3.1.4 Microorganisms Producing Secondary Metabolites

Secondary metabolites are common to organisms that lacks an immune system thus are rarely produced by higher animals (Maplestone *et al.*, 1992). Indeed, these compounds are mostly biosynthesized by bacteria, fungi, algae, corals, sponges, plants and lower animals.

3.1.5 Natural Product Drug Discovery Processes

The drug discovery process involves a number of steps until the natural product is proven to be effective under clinical trials and made available in the market (Fig 1).

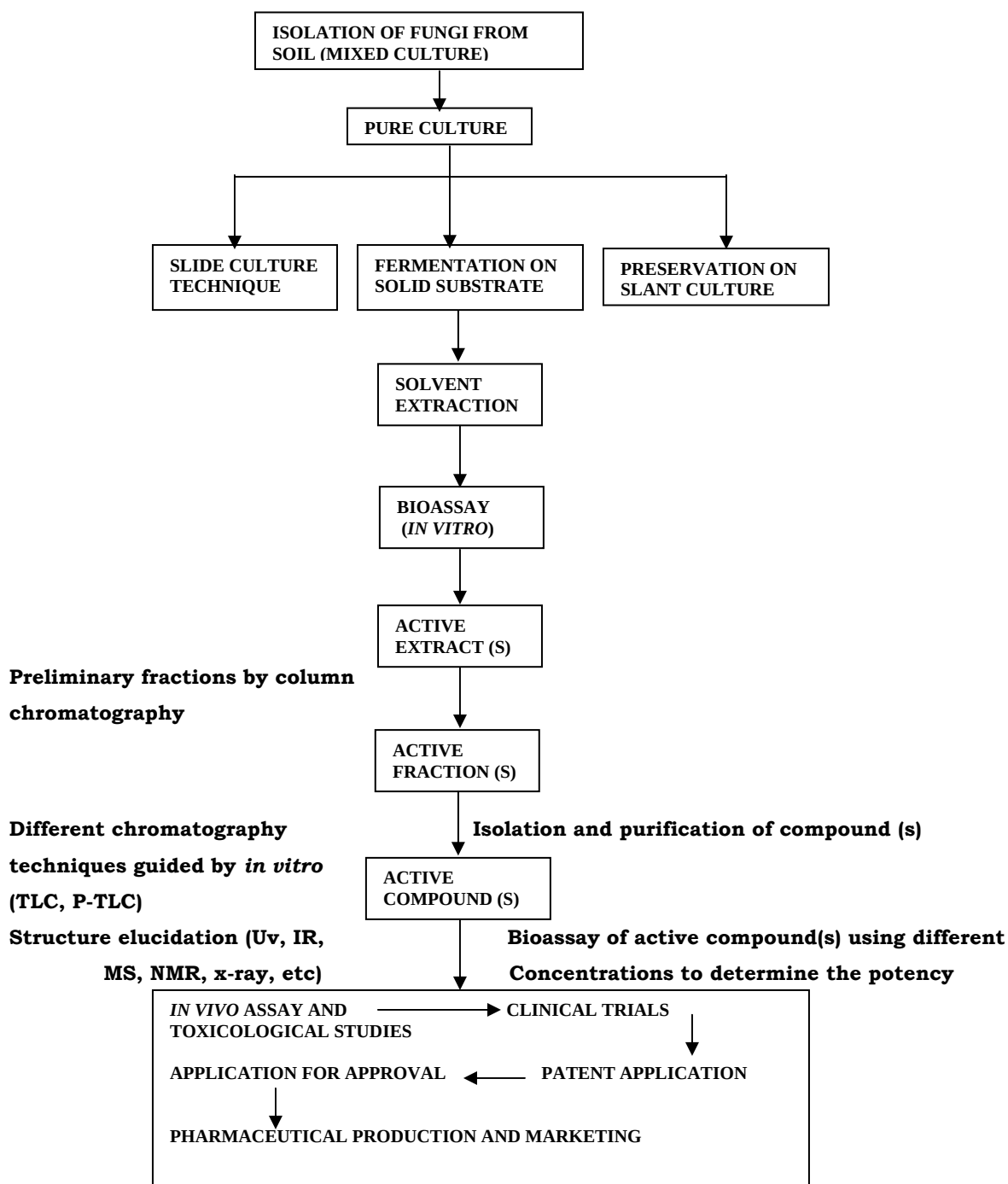


Fig.1 A flow chart for natural product discovery processes (bioassay guided approach). Adapted from Yarbrough *et al.* (1993).

3.1.6 Modes of Action of Antifungal Agents

Antimicrobials and specifically antibiotics are active against other microorganisms at low concentration (Demain, 1999). The main targets of drugs currently used for systemic therapy of invasive mycoses are well summarized in figure 2.

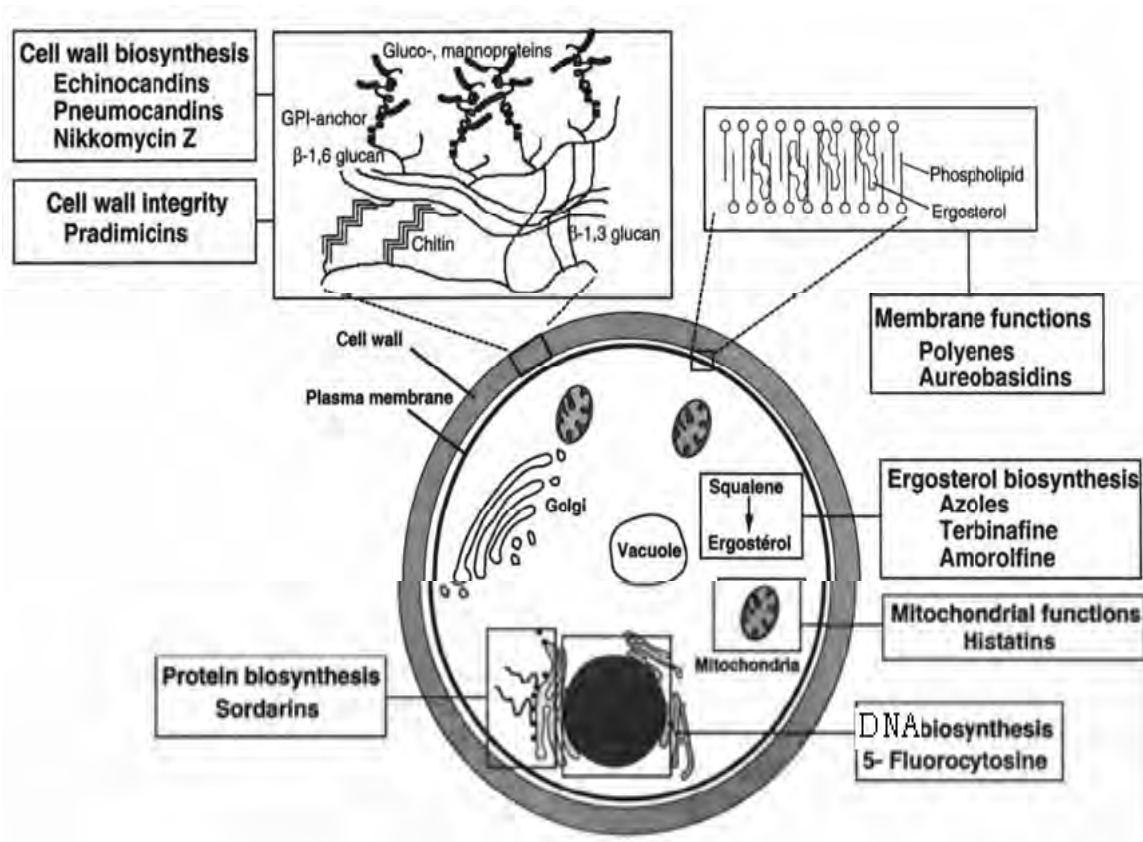


Fig.2 Schematic view of a fungal cell and the principal targets of antifungal agents. Adapted from Sanglard and Bille (2002).

3.1.6.1 Antifungal agents

The systemic antifungal compounds that are currently in clinical use can be divided into four major classes: the polyene antibiotics, the azole derivatives, the allylamines and the morpholine derivatives.

A) Polyenes

The polyenes affect the ergosterol in the plasma membrane, causing membrane disruption, increased permeability, leakage of cytoplasmic content, and cell death (Patel, 1998). The advantage of amphotericin B, a polyene derivative, is its much higher affinity for ergosterol-containing membranes than for cholesterol-containing membranes. The major drawbacks of amphotericin B use, on the other hand, are associated with its use and that intravenous administration is required for treatment of invasive mycoses.

Significant toxicities include fever, chills, arrhythmia, hypotension, respiratory distress, type IV renal tubular acidosis, renal failure, and anemia (Luber *et al.*, 1999). The significant toxicity of the medication led to the introduction of lipid-based products in the 1990s (Dupont, 2002). The two major indications for these lipid-based antifungals are intolerance to standard amphotericin B or refractory disease.

B) Azoles

Azole antifungal agents used in medicine are categorized into N-1 substituted imidazoles (ketoconazole, miconazole, clotrimazole) and triazoles (fluconazole, and itraconazole) (Sanglard and Bille, 2002). Azoles affect both cell and mitochondrial membranes. Azole antifungal agents are generally free from

serious toxicity; however, rare cases of fatal hepatotoxicity have been reported, particularly with ketoconazole (Dupont, 2002). The azoles inhibit cytochrome P-450 dependent 14 β -lanosterol demethylation, which is a critical step in fungal cell membrane ergosterol synthesis (Patel, 1998).

C) Allylamines

The most common compounds of this class are terbinafine and naftifine. Allylamines target squalene epoxidases, which is the first postsqualene enzyme of the ergosterols biosynthetic pathway. Although terbinafine is fungicidal against dermatophytes and filamentous fungi, it is only fungistatic against the majority of *Candida* species (Groll *et al.*, 2005).

D) Morpholine derivatives

Included in this group are amorolfine, fenpropimorph and tridemorph. These compounds are totally synthetic and, with the exception of amorolfine, are mostly used in agricultural fungicide preparations (Sanglard and Bille, 2002).

E) Others

I. Antimetabolites

Flucytosine is the only antimetabolite available for the treatment of systemic fungal infections. It is a fluorine analogue of cytosine that functions as an inhibitor of thymidylate synthetase. The major toxicities include bone marrow suppression, myocardial toxicity, and renal failure (Patel, 1998).

II. Echinocandins

Caspofungin and anidulafungin are the newest agents clinically available for use. They are water-soluble large amphipathic polypeptides that inhibit 1, 3-

β -glucan synthetase resulting in the reduction of cell wall 1, 3- β -glucans, the major glucan in many fungi (Michele and Villman, 2006). The major toxicities include drug interactions, phlebitis, and fever.

3.1.7 Combination Therapy

Recently, increased interest in combination therapy has developed. The goal of combination therapy is to increase efficacy, reduce toxicity, minimize resistance development, and extend the spectrum of activity (Walsh, 2002). Since experimental studies using combinations of polyenes and azoles have shown varied results from antagonism to synergy, concerns regarding potential antagonism in human mycoses have led clinicians to avoid their combination (Lomaestro and Piatek, 1998). As polyenes and azoles both target ergosterol, the fear is that the depletion of ergosterol by an azole will result in a reduction in target sites for amphotericin B thereby diminishing the potency of the polyene (Marr *et al.*, 2004).

3.1.8 Resistance to Antifungal Agents

A successful clinical response to therapy typically not only depends on the susceptibility of the pathogenic organism but also relies heavily on the host immune system, drug penetration and distribution, patient compliance, and absence of a protected or persistent focus of infection (Walsh, 2002). The use of antimicrobial drugs for prophylactic or therapeutic purposes in human and veterinary or for agricultural purposes has provided the selective pressure favoring the survival and spread of resistant organisms.

During the past two decades the frequencies and the types of life-threatening fungal infections have increased dramatically in immunocompromised patients (McCullough *et al.*, 1996). The acquired resistance to antifungal

agents has been one of the major problems of *Candida* infections once it allows the development of new strains that are resistant to these agents. Acquisition of resistance to azole compounds has been recorded with several organisms, in particular *Candida albicans*, in situations where the drugs have been given for long periods of time in the face of persistent infection. Although this is not a new problem, it appears to be increasing in prevalence and importance (Walsh, 2002). These antifungal agents are designed based on the fact that ergosterol biosynthesis is specific to fungi and is necessary for their growth.

One of the most successful polyene derivatives is amphotericin B. Microbial resistance to polyenes is associated with altered membrane lipids, particularly sterols. Other mechanism of resistance may involve altered phospholipids and increased catalase activity with decreased susceptibility to oxidative damage (Venkatakrisnan *et al.*, 2000).

The use of fluconazole, a drug that gained considerable importance as an alternative to amphotericin B, has resulted in the emergence of some *Candida* species as pathogens that are either inherently resistant to fluconazole or developed resistance to it (El-Yazigi *et al.*, 1997).

3.1.9 Adverse Side Reactions

Existing antifungal drugs, the natural polyene antibiotics, amphoterecin B and flucytosine, and the synthetic azoles, clorimazole, ketoconazole and micoconazole show adverse reaction and provide limited chemotherapeutic success. The adverse side reactions include liver necrosis, abnormal elevations of liver function, renal tubular damage, serious hepatotoxicity, irritation, gastrointestinal upsets, drowsiness, nausea, vomiting, diarrhea, headache, chilling and others (Groll *et al.*, 2005).

3.2 Genus *Candida*

The heterogeneous genera *Candida* belongs to the family Cryptococcaceae with in the division Deuteromycetes (Fungi Imperfecti). The genus contains approximately 200 species (Kreger-Van Rij, 1984). *Candida* species are ubiquitous, being found on many plants and as normal flora of the alimentary tracts of mammals and the mucocutaneous membranes of humans. Moreover, *Candida albicans* is taken as a representative of this genus and studied for its sensitivity to the antifungal compound being tested against it.

3.2.1 *Candida albicans*

It took 200 years before the etiological agent of thrush, the first type of candidiasis, was described and identified correctly in 1846 by Berg. He considered that thrush was caused by a fungus (Calderone and Gow, 2002). He also concluded that healthy children were less prone to thrush, although epidemics could occur associated with communal feeding bottles. After his studies it became clear that the disease had several different manifestations in addition to the oral infection. Robin was the first to use the term *albicans* in 1853 (which means “to whiten”) when he reclassified the fungus responsible for the thrush, already classified by Gruby in 1842 as a species of *Sporotrichum*, as *Oidium albicans* (Calderone, 2002). Only some years latter, the name *Candida* was used by Berkhout in 1923. The name was derived from the Latin phrase *toga Candida*, which was used to describe a special white robe worn by Candidates for the Roman Senate. The derived name is probably in reference to the whitish colonies on agar or the oral lesions of aphthae or thrush (Calderone, 2002).

The binomial *Candida albicans* was officially adopted in 1954 at the Eighth Botanical Congress (Calderone and Gow, 2002). The genus *Candida* has been described as a “taxonomic pit” into which the yeasts without a known sexual stage or other remarkable phenotypic character have been thrown (Odds, 1987). Molecular biological approaches to the study of *Candida albicans* were first published during the 1980s. The organism was proven to have a diploid genome, and natural heterozygosity was described in the isolates (Calderone and Gow, 2002; Whelan and Magee, 1981).

3.2.2 Strain Differentiation

The strain differentiation of *Candida albicans* can be achieved with phenotypic or genetic methods. The former can include the serotyping (Brawner *et al.*, 1992), the resistogram typing (Hunter and Fraser, 1987), the yeast “killer toxin” typing, morphotyping, biotyping and protein typing (McCullough *et al.*, 1996). Among the genetic methods are the use of species specific DNA probes (Elie *et al.*, 1998), detection of secreted aspartic proteinase genes (Flahaut *et al.*, 1998) and fluorescent *in situ* hybridization (Lischewski *et al.*, 1997). Several tests based on the growing factors of each species can also be included in the phenotypic differentiation, such as the API 32C, the Auxacolor system (Campbell *et al.*, 1998), BactiCard *Candida* and Murex *Candida albicans* (Crist *et al.*, 1996). These tests are more expedite, simple and as accurate as the ones described before. The most expedite and less expensive method commonly used to differentiate *Candida albicans* is the CHROMagar (Beighton *et al.*, 1995; Jabra-Rizk *et al.*, 2001), this solid medium allows a differentiation between several *Candida* species through the color of the colonies formed.

3.2.3 Virulence Factors

Candida albicans appears to possess a number of virulence attributes that may promote successful parasitism. These attributes include relatively rapid germination up on seeding tissue from the blood stream (Hazen *et al.*, 1991), protease production (Kwon-Chung *et al.*, 1992), surface integrin-like molecules for adhesion to extracellular matrix proteins (Gustafson *et al.*, 1991), complement binding receptor (Calderone and Fonzi, 2001), phenotypic switching (Soll, 1992), and surface variation and hydrophobicity (Hazen *et al.*, 1991).

3.2.3.1 Cell wall

The cell wall of *Candida albicans* is composed of 80 to 90% of carbohydrates, 6 to 25% of proteins and 1 to 7% of lipids (Chaffin *et al.*, 1998). The carbohydrates include branched polymers of glucose (β -glucans), unbranched polymers of *N*-acetyl-D glucosamine (chitin) and polymers of mannose (mannan), covalently associated with proteins (Chaffin *et al.*, 1998).

3.2.3.2 Proteinase Production

When hydrolysis of substrates or action of extracellular proteins affects the function and viability of the host, the enzymes may be considered virulence factors that contribute to the establishment of infection. One of these enzymes is the *Candidal* secreted aspartyl proteinase (SAP), belonging to the group of acid proteinases, that was first identified by Staib (1969). Besides SAP, phospholipase, esterase, glucoamylase, hemolytic factor, acid phosphatase, lipase, hyaluronidase, chondroitin sulfatase, metallopeptidase and trehalase are also included in this group of enzymes (Chaffin *et al.*, 1998). The first case can include enzymes as $\text{exo-}\beta$ -(1, 3)-glucanase, β -1, 3-

glucan transferase, chitinase, β -Nacetylglucosaminidase and transglutaminase.

3.2.3.3 Dimorphism

Candida albicans undergoes reversible morphological transitions between ovoid, unicellular budding cells (yeast cells or blastopores) and chains of filamentous cells (Calderone and Gow, 2002); as shown in figure 3. The contribution of dimorphic growth to virulence has been investigated in studies in which virulence was determined for mutants that can grow only in either the yeast or filamentous form (Prasad, 1991). There are evidences that suggest that yeast-hypha morphogenesis is coregulated with other virulence factors. For instances, the SAP4-6 genes, which are the members of a large family of secreted aspartyl proteinase genes, that promote the virulence of *Candida albicans* (Brown, 2002; Hube *et al.*, 1997), are expressed specifically during hyphal development.

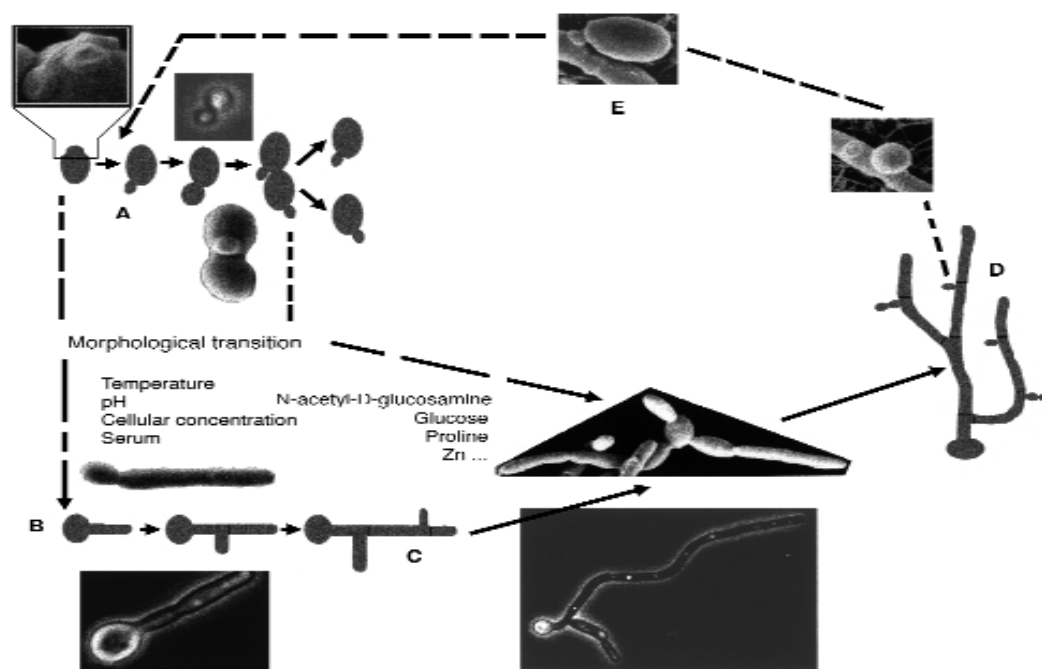


Fig. 3 Dimorphism in *Candida albicans*. (A) Blastospores (B) Germ tube (C) Germ tubes grow and septa are laid down behind the extending apical tip to form a hypha. (D) Hyphal branches (E) Secondary blastospores become separated from the filament. Adapted from Molero *et al.* (1998).

3.2.3.4 Adhesion and Biofilm Formation

One of the most important factors of virulence of *Candida* species is their ability to adhere using a variety of mechanisms, permitting the yeast to anchor at a site and the process of tissue colonization to commence (Cotter and Kavanagh, 2000). The components of the organism that promote host recognition and colonization are referred to as adhesins. The adhesins of *Candida albicans* are usually of polysaccharide or glycoprotein nature and are also called binding proteins or receptors (Calderone and Gow, 2002). Examples of adhesins are chitin, factor 6 oligomannosaccharide, 66-kDa fimbrial protein, fibronectin binding protein, iC3b binding protein, fucose binding protein, GlcNAc or glucosamine, SAP and ALS gene family (Cannon and Chaffin, 1999). So, *Candida albicans* possesses multiple adhesins and there may be more than one adhesion that recognizes a host ligand or cell. Included in the non-specific interactions, the mechanisms involved in the reversible adherence process, are the electrostatic interactions and the cell surface hydrophobicity (Cotter and Kavanagh, 2000).

The biofilm formation includes adhesion of planktonic cells to the surface, growth and secretion of extracellular polymers and cell detachment. Comparing the extracellular polymers (EP) of bacterial and yeast biofilms, the EP of yeast biofilm have significantly less amount of total carbohydrates and proteins, but higher proportion of glucose, also containing galactose, suggesting that the composition of the biofilm EP might be unique (McCourtie and Douglas, 1985). In contrast to the extensive literature describing bacterial biofilms, little attention has been paid to medically

relevant fungal biofilms. Two consequences of biofilm growth with profound clinical implications are the markedly enhanced resistance to microbial agents and protection from host defences (Ramage *et al.*, 2001).

3.2.3.5 Prevalence of Candidiasis

Candida albicans belongs to the normal microflora of the human GIT and is the most prevalent human pathogen in the genus (Odds, 1987). The prevalence of oral *Candida albicans* carriage is reported in 64-84% of HIV-infected patients (Torssander *et al.*, 1987; Bergbrant and Faergemann, 1997; Sangeorzan and Bradley, 1994) and in 28% of HIV-negative persons (Chave *et al.*, 1996).

In the beginning of AIDS epidemic, almost all *Candida* infections in AIDS patients were caused by *Candida albicans* (Odds *et al.*, 1987). Up to 90-95% of colonized patients will develop oropharyngeal candidiosis (thrush) as the AIDS progresses (Dupont *et al.*, 1992; Feigal *et al.*, 1991). In Africa, where the diagnostic procedures are limited, oral candidiasis is one of the most commonly encountered opportunistic infections and a sign for clinical case definition of AIDS (McCullough *et al.*, 1996). In Ethiopia, Woldeamanuel and Abate (1997) reported that there is no antigenic variation between isolates of *Candida albicans* from normal and AIDS patients showing that it is the same organism of the normal flora that also become infectious depending on the immunity status of individuals.

Opportunistic infections caused by pathogenic fungi such as *Candida albicans* increased recently and become a serious problem. Patients with compromised immune systems, those who receive organ transplants and cancer chemotherapy, and those infected by HIV, are particularly prone to such infections (Nishiyama and Yamaguchi, 2000). Although fluconazole is

one of the most commonly used antifungal agents, the isolation of fluconazole-resistant strains of *Candida albicans* has been reported (Maebashi *et al.*, 2003). In light of this, new antifungal agents with a different mode of action have been sought extensively.

3.3 Taxonomy of *Syncephalastrum*

Syncephalastrum is characterized by the formation of cylindrical merosporangia over the surface of a fertile vesicle. Zygosporangia have a rough- and dark-walled zygosporangium and more or less equal suspensors (Benjamin, 1959).

Two species of *Syncephalastrum* are known, *S. racemosum* with multisporangia (Benjamin, 1959) and *S. monosporum* with unispored merosporangia (Zheng *et al.*, 1988). *Syncephalastrum monosporum* has three varieties (Zheng *et al.*, 1988). Benjamin (1959), Zycha *et al.* (1969), and Benjamin and Tucker (1978) illustrated *S. racemosum*. Benjamin (1959), and Schipper and Stalpers (1983), presented synonyms for *S. racemosum*, which included *S. verruculosum* (Misra, 1975). The taxa of *Syncephalastrum* is outlined below.

Kingdom: Fungi

Phylum: Zygomycota

Class: Zygomycetes

Order: Mucorales

Family: Syncephalastraceae

Genus: *Syncephalastrum*

3.4 Solid State Fermentation (SSF)

Solid-state fermentation (SSF) processes can be defined as “the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free-flowing water (Moo-Young, *et al.*, 1983). These processes have been used for the production of value-added products (antibiotics, alkaloids, plant growth factors, etc), biofuel, enzymes, organic acids, aroma compounds and also for bioremediation of hazardous compounds, biological detoxification of agroindustrial residues, nutritional enrichment, biopulping, biopharmaceutical products, etc (Barrios-Gonzales *et al.*, 1988; Trejo-Hernandez *et al.*, 1992; Senez *et al.*, 1980). SSF is briefly associated with the production of traditional fermented foods such as “koji”, Indonesian “tempeh” or Indian “ragi” (Raimbault *et al.*, 1998).

Substrates that have been traditionally fermented by solid state include a variety of agricultural products such as rice, wheat, millet barley, grains, beans, corn and soybeans (Perez-Guerra *et al.*, 2003). This technology has gained renewed attention from industry because it has become a more attractive alternative to liquid fermentation for many productions. Thus, SSF was found to produce a more stable product, with less energy requirements, in smaller fermenters and smaller volumes of polluting effluents (Hesseltine, 1972).

3.4.1 Selection of Microorganisms

The ability of the microorganisms for growing on a solid substrate is a function of their requirements of water activity, their capacity of adherence and penetration into the substrate and their ability to assimilate mixtures of different polysaccharides due to the nature, often complex, of the substrates used (Perez-Guerra *et al.*, 2003). The filamentous fungi are the best-adapted

microorganisms for SSF owing to their physiological, enzymological and biochemical properties. The hyphal mode of fungal growth gives the filamentous fungi the power to penetrate into the solid substrates (Mitchell *et al.*, 1992). This also gives them a major advantage over unicellular microorganisms for the colonisation of the substrate and the utilisation of the available nutrients. In addition, their ability to grow at low water activity (a_w) and high osmotic pressure conditions (high nutrient concentration) makes fungi efficient and competitive in natural microflora for bioconversion of solid substrates (Mitchell *et al.*, 1992).

3.4.2 Factors Affecting Microbial Growth and Product Synthesis in SSF

3.4.2.1 Water Activity and Moisture Content of the Substrate

The role of the water content of the substrate has been widely described and reviewed by different authors. Moisture content is a critical factor on SSF processes because this variable has influence on growth and biosynthesis and secretion of different metabolites (Moo-Young *et al.*, 1983). Lower moisture content causes reduction in solubility of nutrients of the substrate, low degree of swelling and high water tension (Oriol *et al.*, 1988). On the other hand, higher moisture levels can cause a reduction in enzyme yield due to steric hindrance of the growth of the producer strain by reduction in porosity or interparticle spaces of the solid matrix, thus interfering oxygen transfer (Saucedo-Castañeda *et al.*, 1992).

Generally, the water content of the substrate oscillates between 30 and 75% (Cannal and Moo-Young, 1980). Lower values can induce the sporulation of the microorganism, whereas higher levels can reduce the porosity of the system, which can produce oxygen transfer limitation, and increase the risk of bacterial contamination (Oriol *et al.*, 1988). During fermentation, the water

level of the substrate can change due to evaporation and microbial activity. In general, the result of all these processes is the loss of humidity, being necessary to add water using humidificators or applying water saturated air flow.

The water requirements of microorganisms must be better defined in terms of water activity (a_w) rather than water content of the solid substrate (Moo-Young *et al.*, 1983). Water activity is defined as the relationship between the vapor pressure of water in a system and the vapor pressure of the pure water (Perez-Guerra *et al.*, 2003). From a microbiological point of view a_w indicates the available or accessible water for the growth of the microorganism.

The water activity affects the biomass development, metabolic reactions, and the mass transfer processes (Gervais *et al.*, 1988). Although water activity is a function of the concentration of the solutes, in those systems in which solutions are adsorbed in a matrix, the values of a_w also depend on the physical structure and the chemistry nature of the matrix (Gervais *et al.*, 1988). The adequate value of a_w depends on both the product and the requirements of the microorganism (Perez-Guerra *et al.*, 2003). Generally, a_w for metabolite production is higher than for growth (Perez-Guerra *et al.*, 2003).

3.4.2.2 Mass Transfer Processes: Aeration and Nutrients

In SSF, the mass transfer processes related to gases and nutrients diffusion are strongly influenced by the physical structure of the matrix and by the liquid phase of the system (Moo-Young *et al.*, 1983). In general, the gases diffusion increases with the pore size and decreases with the reduction of the diameter due to substrate packaging (Moo-Young *et al.*, 1983).

3.4.2.3 Temperature

The increase in temperature in SSF is a consequence of the metabolic activity when the heat removal is not enough. This affects directly spores germination, growth and product formation. The temperature level reached is a function of the type of microorganism, the porosity, the particle diameter and the depth of the substrate (Moo-Young *et al.*, 1983). Heat transfer in SSF is very low because of the limited heat transfer capacity of the solid substrates used. Control of temperature is more difficult in SSF than in submerged fermentation. Conventionally, aeration is the main method used to control the temperature of the substrate (Moo-Young *et al.*, 1983).

3.4.2.4 pH

The measurement and control of this variable in SSF is very difficult. Nevertheless, the substrates employed in SSF usually have buffering effect due to their complex chemical composition (Perez-Guerra *et al.*, 2003).

4. Materials and Methods

4.1 Isolation and Maintenance of Leaf Decomposing

Microfungi from Soil

Strains were isolated from soil or leaf litter using the method described by Bills and Polishook (1994). Soil samples were collected during the rainy seasons from the Menagesha Suba state forest which is located 30km southwest of Addis Ababa city (38°32'E, 08°56'N). The origin, the condition, and the location of the material in the habitat were considered while taking the samples. Samples were obtained from just around the roots of the indigenous trees such as *Hygenia abyssinica* (“**Kosso**”), *Juniperous procera* (“**Tid**”), *Afrocarpus falcatus* (“**Zigba**”), *Erythrina brucei* (“**Korch**”), and *Olea africana* (“**Woyira**”). The decomposed leaf material was then transferred to small sterile plastic bottles and was kept in the refrigerator for later use.

1g of soil sample from each sterile plastic bottle was measured on a Mettler balance and was immersed in to a 50ml Erlenmeyer flask containing 10 ml of sterile distilled water. Homogeneity was ensured by putting it on a shaker for 1 hr being adjusted at 120 rpm. The suspension was left for 30 min in the Laminar Air Flow (LAF) hood in order to settle. Consequently, 1 ml of the soil suspension was transferred to each of the four test tubes containing 9 ml of sterile water and vortex was applied. Further more, 1 ml from the fourth dilution was transferred to a sterile media (PDA) using a sterile micropipette tip.

The dilution method for single spore cultures was adapted from Onions *et al.* (1981). More over, homogeneity was made possible by using a sterile bent glass rod. Finally, the organism containing media was incubated at 25°C for 48 to 72 hrs until colonies began to emerge. Last, inoculation of a single visible colony in to a new media using a sterile loop (0.5 mm in diameter)

brought about a pure culture from which a slant culture was prepared and maintained at 4°C in a refrigerator for future use as it was mentioned by Smith and Onions (1994). Code was assigned (such as AFC003) for every entity to confirm the source of the organism, the colony morphology and pigmentation.

4.2 Media Preparation

Preparation of Malt Extract Agar (MEA) for isolation of soil and leaf litter fungi was adapted from Harry *et al.* (1972). Similarly, Potato Dextrose Agar (PDA) and Czapek Dox Agar (CDA) were prepared according to Norris and Ribbons (1969).

Autoclaving was employed to sterilize the media by heating to 120°C for 15 min. So as to prevent the denaturation of heat-labile components such as antibiotics, they were added after sterilization but before the media cools and solidifies. By virtue of adding antibiotics such as chloramphenicol (100 mg/l), the elimination of bacterial growth was ensured. Rapid inhibition of bacterial growth was brought about by acidifying the media to a pH of 4.5 with lactic acid. Antifungal antibiotics were also incorporated in order to isolate some fungi preferentially, to slow hyphal extension, and to secure longer incubation periods.

4.3 Slide Culture Technique

In order to maintain the hyphae or spore structure of the isolate for identification purpose, the slide culture technique was employed (Riddell, 1950). A small block (about 10 mm²) of PDA was cut from the Petri dish containing a thin layer of this agar using a sterile scalpel and was placed on the slide that rested on the bent glass rod in the Petri dish. From the freshly

prepared fungal culture, the isolate was inoculated on each side of the agar block using a heavy inoculating needle. A sterile cover slip was put on it and a small amount of sterile water was added to the bottom of filter paper containing Petri dish.

After incubation of 24 hr at 25°C, the cover slip was carefully removed from the agar block (usually dusted with spores) and a drop of 95% alcohol was added to wet and kill the fungus growing over it. When most of the alcohol had evaporated, the mountant (lactophenol cotton blue solution) was added after being fixed to a clean glass slide. Permanent mounts were actually made by sealing the edge of the cover slip to the slide with finger varnish. It was then made ready for microscopic examination.

4.4 Cultivation of the Selected Isolate (AFC003) using SSF

4.4.1 Sterile Rice Media Preparation

A method of preparation described by Abate (1999) was employed. A weight of 200 g of local rice grain was soaked in 200 ml of tap water and allowed to imbibe for 3 hr in 1000 ml conical flasks followed by boiling until the grain was soft. The rice was drained and autoclaved at 121°C for 15 min. The sterile moist rice was then left to cool before inoculation.

4.4.2 Inoculation and Incubation

A young culture of the selected isolate (AFC003) was taken as inoculum resulting from pure culture preparation. The sterile moist rice was then inoculated with agar culture blocks of almost equal size (about 10 mm²) from the Petri dish just under aseptic conditions and was rolled with hands to 360° to ensure homogenous distribution of the spores all over the bottle. Subsequently, the inoculated rice culture was placed on the table under

static condition whose plug being loosely held for aeration at room temperature.

4.5 Isolation of Bioactive compounds

4.5.1 Solvent Extraction

The extraction procedure was a bit modified from the procedure used to extract trichothecene from rice by Bullock and DeGomez (1990). The rice culture of the selected isolate (AFC003) was exposed to U.V. radiation for about 10 min so as to kill the spores and to get rid of inhalation or contact with the skin. It was then finely ground using mortar and pestle and transferred to a tray to get sun dried. The powdered rice culture was extracted with only 500 ml of ethyl acetate (100%). This solution was left on the shaker overnight at 120 rpm. It was then filtered with a funnel to which filter paper and cotton were placed.

The culture filtrate was collected on a clean and dry flask, whereas the solid mass or pellet was discarded. The culture filtrate was transferred to a round flask that fit the rota-vapor being set at 160 rpm with a water bath heated to 60°C just below the boiling point of ethyl acetate (77°C) provided that pressure was maintained (Fig.4). The creamy product was being recovered, collected and packed in to sterile vials and kept at 4°C in a refrigerator.

As the crude natural compound was literally a cocktail of compounds. It was difficult to apply a single separation technique to isolate one compound from this crude mixture. Hence, the crude extract was initially separated into various discrete fractions containing compounds of similar polarities or molecular sizes.



Fig.4 Extraction method: Rota-vapor set at 160 rpm and 60°C

4.6 Separation Techniques

4.6.1 Thin Layer Chromatography

This method was adapted from Ishikawa (1971) while separating and determining residues of some carbamate insecticides in rice grain as 4-Nitrobenzene Diazo derivatives of their phenolmoities. Accordingly, the plate was cut in to 5 cm x 10 cm and a penal line was drawn on the white-side up using a pencil and a ruler (Fig.5). Consequently, three dots were made along the length of the horizontal line 1.5 cm apart and 1 cm was left from the edges of the plate.

The dots were spotted with 10 μ l of the crude extract(s) using capillary tubes. When all drops were dried, the plate was immersed in to a beaker containing a 1:1 solvent mixture of ethyl acetate: chloroform or 50:50 (v/v). Hence, it stood vertically leaning against the side of the beaker with the pencil line at

the bottom. The level of solvent was adjusted about halfway between the bottom of the plate and the horizontal line.

Afterwards, the beaker was capped tightly with a watch glass and left for 30min up until the solvent move past to the top of the plate but not entirely run off. It was then carefully removed and placed on several thicknesses of paper towels to blot dry given that a line was earmarked for the distance moved by the solvent. A while later, the plate was visualized under U.V. light (254 and 366nm) and spots were encircled with a pencil. Moreover, the R_f (retention factor) value was computed for each spot using the formula given below.

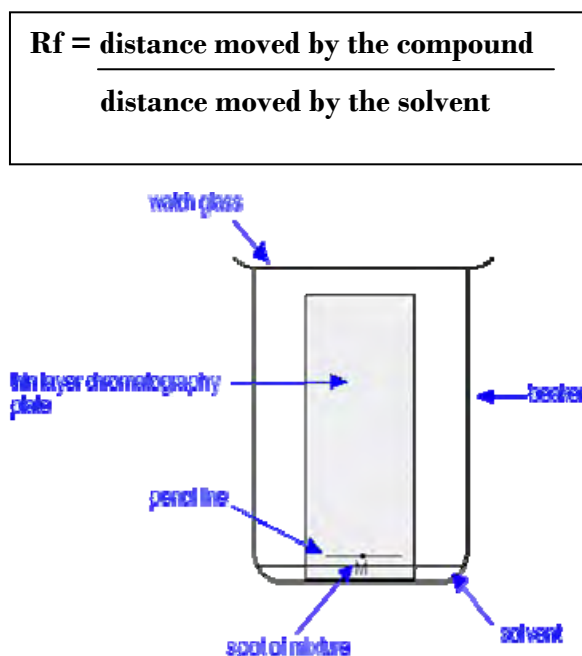


Fig.5 TLC plate soaked in a solvent mixture containing beaker

4.6.2 Preparative TLC

The very purpose of the preparative TLC was to scrape the compounds formed in the band with a lot of ease. It was prepared as follows: 100g of Merck silica gel GF254 powder was measured and suspended in 200ml of

deionized water. Being vigorously shaken for 45 seconds in rubber-stopper Erlenmeyer flask of 500ml capacity, the thickened slurry was poured in to trough of quickfit spreader. The spreader was the pulled with 1.0mm trailing edge to prepare 20x20cm plates. It was allowed to air dry (until it turned white) for 30 to 45 min and further activated by heating in an oven at 120°C for 30 min prior to use. Moreover, other procedures were much the same as one used in the TLC method. Preparation procedure for the P-TLC was adapted from (<http://hoivo.pharmacy.uiowa.edu/separation/preptlc.html>).

4.6.3 Column Chromatography

Column chromatography was one of the most important techniques practiced during natural product isolation. It was used to purify compounds based on their polarity with solvents given in Table 1. 1.724g of the active crude extract was eluted with ethyl acetate and was applied on a column of 8cm external diameter and 35 cm length. After coming up with the solvent mixture and the right proportion of crude extract to silica gel in just 1:30 ratio, the column was packed very tightly in order to achieve uniform separation as the compound moves down the column. The glass column was plugged with cotton wool directly above the stopcock to prevent the silica gel from escaping the column through the stopcock (Fig.6). The different fractions were collected from the column chromatography and TLC was performed on each fraction to see which fractions were identical or different, and from the TLC analysis like fractions were pooled together.

Table 1: Chemical properties of common solvents used in natural product isolation processes. Adapted from Ishikawa (1971).

Solvent	Polarity index	Boiling point (°C)	Viscosity (cPoise)	Solubility in water (% w/w)
<i>n</i> -Hexane	0.0	69	0.33	0.001
Dichloromethane	3.1	41	0.44	1.6
<i>n</i> -Butanol	3.9	118	2.98	7.81
iso-propanol	3.9	82	2.30	100
<i>n</i> -Propanol	4.0	92	2.27	100
Chloroform	4.1	61	0.57	0.815
Ethyl acetate	4.4	77	0.45	8.7
Acetone	5.1	56	0.32	100
Methanol	5.1	65	0.60	100
Ethanol	5.2	78	1.20	100
Water	9.0	100	1.00	100

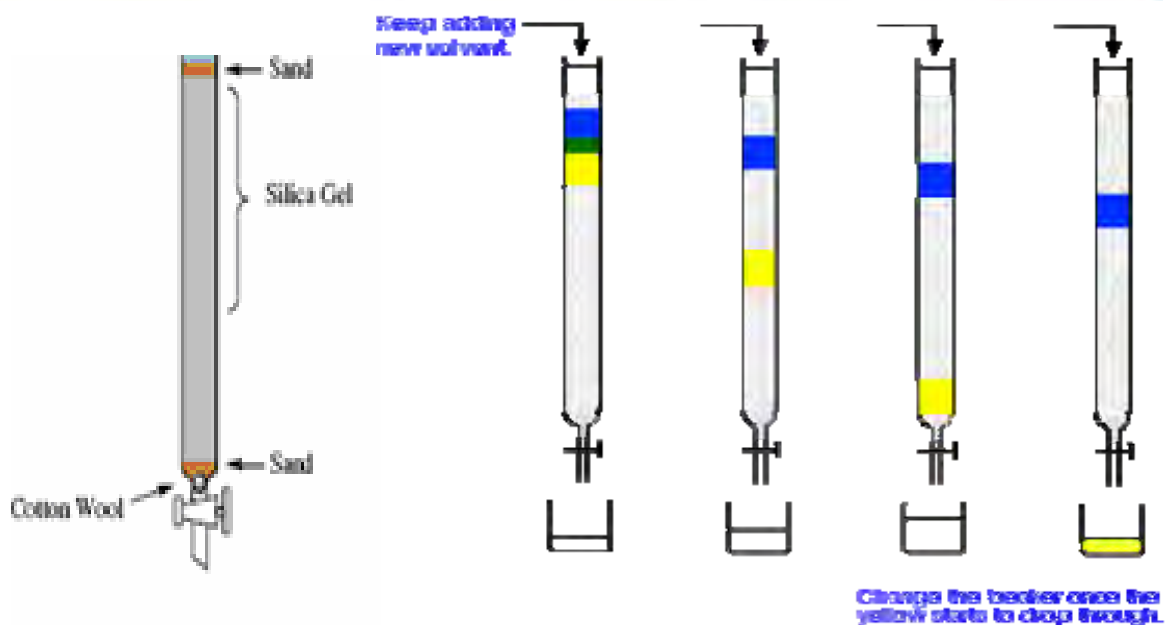


Fig. 6 The illustration of column chromatography

The crude extract was eluted with ethyl acetate, chloroform, petroleum ether, hexane and acetone. Fractions 2 to 7 were found to be active. Fraction 7 was selected for it had only two bands in the TLC. Further purification of fraction 7 gave a fraction 1 with a single band (Fig.7).

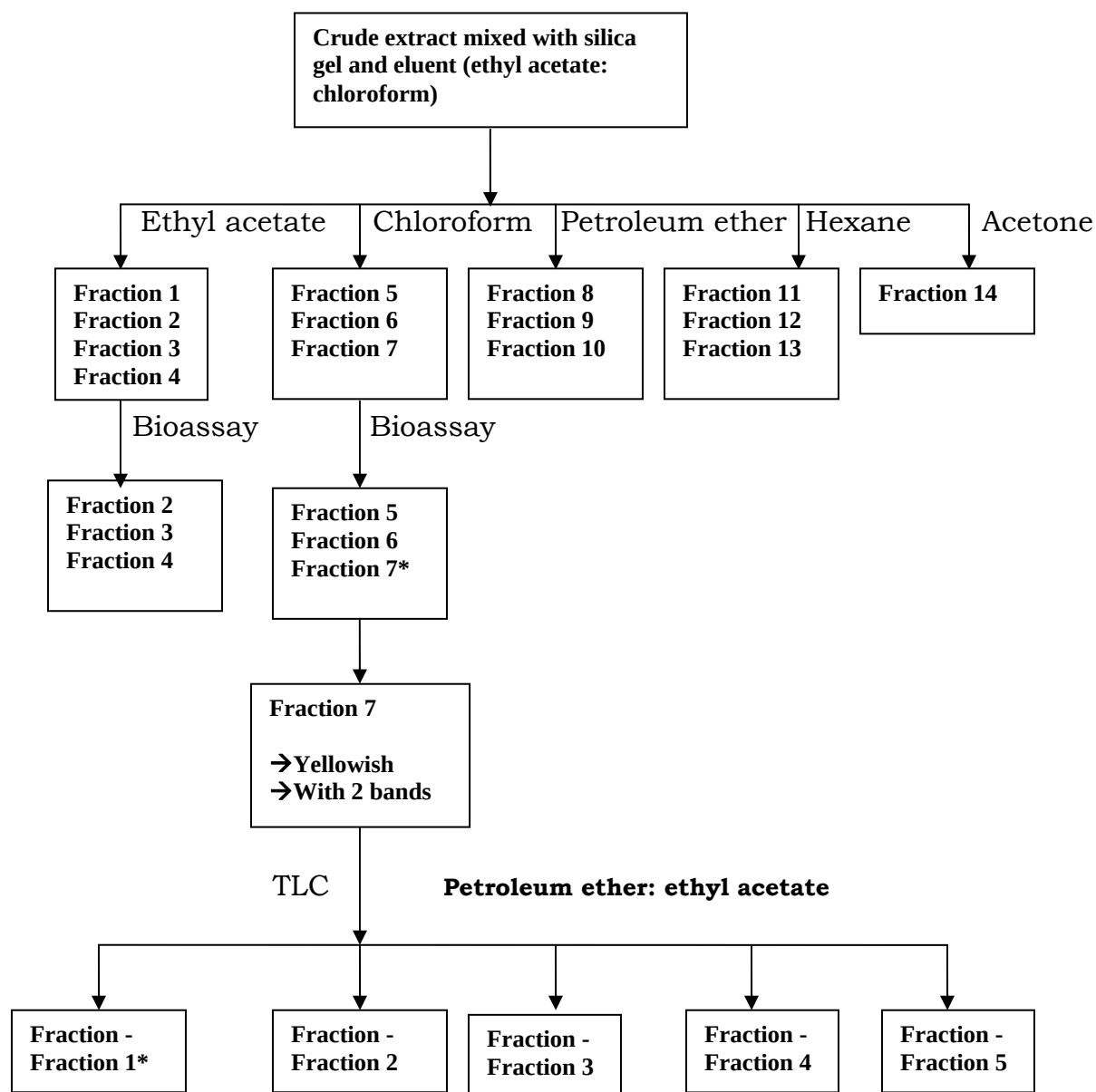


Fig. 7 Fractionation procedures used in the purification process

F-7*; fraction 7 easily distinguished from others in being yellowish

FF-1*; fraction-fraction 1 appeared pale yellow

4.7 Testing Strategies

4.7.1 Test Organism and Culture Condition

The pathogenic yeast, *Candida albicans*, was a clinical isolate (not from culture collection) obtained from a patient at the Bacteriology Laboratory of the Black Lion Hospital. It was tested for its germ tube formation being dipped in to a horse serum for 4 to 6 hrs according to Terleck *et al.* (2006). It was then seeded on sabouraud dextrose agar (SDA) and incubated for 24 hr at 37°C. Afterwards, the yeast culture was harvested, suspended in sabouraud dextrose broth (SDB) and grown overnight on a shaker. The late exponential phase cells were washed with 6.5 ml of phosphate buffered saline (PBS) whose pH adjusted at 7.4 and final yeast suspensions at a concentration of 1×10^6 cells ml^{-1} (0.5 McFarland Standard units) were prepared.

4.7.2 Agar Diffusion Assay (ADA)

The standard agar diffusion assay for sensitivity testing was performed as a screening assay according to Bauer *et al.* (1996). 100 μl aliquots of suspensions of *Candida albicans* were spread on SDA plate. Afterwards, 6 mm diameters of Whatman paper discs were soaked in 10 μl of 10 mg/ml of the crude extract and were placed concentrically on the inoculum. Discs impregnated in 10 μl of 10 μg ketoconazole ml^{-1} were used as positive controls while discs soaked with 10 μl of ethyl acetate were used as negative controls. These SDA plates were incubated for 24 hr at 37°C. Following incubation, naked eye measurements of gross zones of growth inhibition, if any, was evaluated using a finely calibrated transparent ruler. The diameters of growth inhibition of four different areas were measured and the mean diameters were obtained. The experiment was repeated on three separate occasions.

4.7.2.1 Bio Assay 1

After collecting the crude extract, it was tested against *Candida albicans*, *Bacillus sp.*, *Shigella boydii*, *Staphylococcus aureus*, and *Escherichia coli*. In order to prove that the compound was active, bioassay was performed. First, several 6 mm paper disc were placed in three different containers labeled as positive, negative, and crude extract. As a negative control, 20 µl of ethyl acetate was pipetted on three discs. For positive control, 10 µl of 100 mg ketoconazole was pipetted on other 3 discs. Then, 20 µl of crude extract was poured on separate discs.

4.7.2.2 Bio Assay 2

The purpose of this procedure was to show that the test compound could be placed straight onto the agar plate and a zone of inhibition would still form only if the compound was active. After collecting different fractions from the latter column, it was crucial to see which fraction was indeed active against *Candida albicans*.

4.7.3 Antifungal Agent and Stock Solution Preparation

The commercial antifungal agent used was ketoconazole. It was diluted in water and further dissolved in a drop wise ethanol, once it was not water soluble. On the other hand, the purified compound was prepared much the same as ketoconazole before each experiment. The concentration of both antifungal agents was determined and their activity was tested based on the minimal inhibitory concentrations (MIC) of each agent using the broth microdilution method.

4.7.4 Broth Microdilution Susceptibility Testing

The purified compound, which showed higher antifungal activity against *Candida albicans* in the ADA screening test, was selected for MIC determination. For this purpose, a broth microdilution assay was performed according to Polanco *et al.* (1995). Briefly, inocula of 24 hr test cultures were standardized to a turbidity equivalent to 0.5 McFarland standards at 660 nm with a spectrophotometer. The suspensions were further diluted in Rosewell Park Memorial Institute (RPMI) 1640 medium to yield an inoculum concentration of approximately 1×10^6 CFU/ml. The MIC assay was performed in test tubes containing sabouraud dextrose broth (SDB) and each of the *Candida* suspension was exposed to tubes containing concentrations of 10-100 µg/ml of ketoconazole and the extract suspensions. The tubes were incubated at 37°C for 24 hr. At the end of the incubation period, the tubes were examined visually for turbidity. Cloudiness indicates that fungal growth has not been inhibited by the concentration of the antibiotic contained in the medium.

An attempt has also been made to determine the break point concentration. The optical density was determined in each tube using a spectrometer. The inhibitory effect of both antifungal agents was determined by comparing the absorbance of control and treated cells at 660 nm. On the other hand, the turbidity of the suspension was matched against the turbidity standard. The MIC of each compound was defined as the lowest concentration of the substances that had no visible turbidity (Anil *et al.*, 2002). The MIC of ketoconazole and the pure compound was determined for each *Candida* suspension in parallel. Experiments were repeated on three different occasions with duplicate determination on each occasion.

5. Results and Discussion

5.1 Results

5.1.1 Culture Collection of the Microfungi from different habitats

A total of 20 microfungi cultures were collected as shown in Table 2. The isolate (AFC003) was found to produce effective compound and was selectively cultivated on solid state fermentation. Based on hyphal and spore structures, the microfungus was identified as *Syncephalastrum sp.* (AFC003) according to Barnett (1972).

Table 2. Culture Collection of Soil Fungi

Place of soil sample	Habitat (leaf litter)	Candidate ID	Growth Media	Incubation temperature (°C)	Preservation temperature (°C)	Date of preservation (dd/mm/yy)
Menagesha Suba forest	<i>H.abysinnica</i>	AAUCC HAC001	PDA	25°C	4°C	14/11/2008
		AAUCC HAC002	PDA	25°C	4°C	14/11/2008
		AAUCC HAC003	PDA	25°C	4°C	14/11/2008
		AAUCC HAC004	PDA	25°C	4°C	14/11/2008
		AAUCC HAC005	PDA	25°C	4°C	14/11/2008
		AAUCC HAC006	PDA	25°C	4°C	14/11/2008
		AAUCC HAC007	PDA	25°C	4°C	14/11/2008
		AAUCC HAC008	PDA	25°C	4°C	14/11/2008
		AAUCC HAC009	PDA	25°C	4°C	14/11/2008
		AAUCC HAC010	PDA	25°C	4°C	14/11/2008
		AAUCC HAC011	PDA	25°C	4°C	14/11/2008
		AAUCC HAC012	PDA	25°C	4°C	14/11/2008
	<i>A.falcatus</i>	AAUCC AFC001	PDA	25°C	4°C	14/11/2008
		AAUCC AFC002	PDA	25°C	4°C	14/11/2008
		AAUCC AFC003	PDA	25°C	4°C	14/11/2008
	<i>J.procera</i>	AAUCC JPC001	PDA	25°C	4°C	14/11/2008
		AAUCC JPC002	PDA	25°C	4°C	14/11/2008
		AAUCC JPC003	PDA	25°C	4°C	14/11/2008
	<i>O.africana</i>	AAUCC OAC001	PDA	25°C	4°C	14/11/2008
	<i>E.brucei</i>	AAUCC EBC001	PDA	25°C	4°C	14/11/2008

5.1.2 Taxonomy of *Syncephalastrum sp.* (AFC003)

Under the microscope *Syncephalastrum sp.* (AFC003) appears to be abundantly branched, with rapidly grown mycelium; sporangiophores erect, branched, tips enlarged, bearing a head of rod-shaped sporangioles, each producing a row of nearly spherical spores, resembling a chain of merosporangiospores (Fig.8). It is a filamentous fungus that is commonly isolated from soil and animal feces particularly in tropical and subtropical areas.

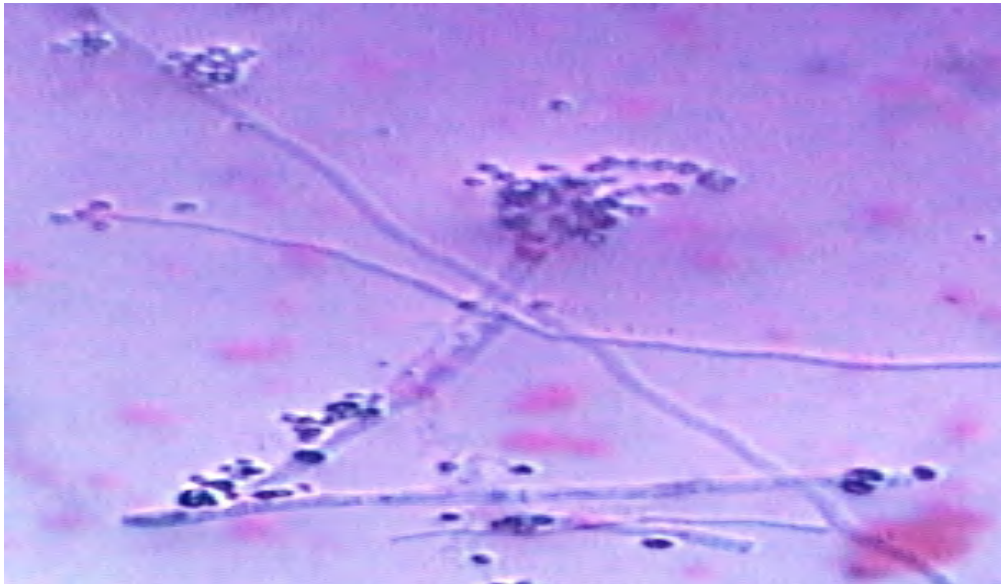


Fig.8 *Syncephalastrum sp.* (AFC003) as it looks under Microphotograph.

5.1.3 Growth of *Syncephalastrum sp.* (AFC003) on SSF

Moist sterile rice of about 50-60% moisture content was found to be optimal for the growth of *Syncephalastrum sp.* (AFC003). It took nearly 15 days to sporulate and about 28 days to colonize the rice almost entirely. It appears to be greenish and powdery just at the time of harvest (Fig.9).



Fig.9 Growth of *Syncephalastrum sp.* (AFC003) on sterile moist rice (SSF)

5.1.4 Extraction and Isolation of the Bioactive Compounds from *Syncephalastrum sp.* (AFC003) Culture

A biomass of 222.7g was extracted with 500ml of ethyl acetate (100%). The gross weight of the crude extract was approximately computed as 2.7g. Afterwards, the ethyl acetate extract was packed into vials and was subject to column chromatography once its activity was confirmed.

5.1.5 Bio Assay

5.1.5.1 Bio Assay for the Crude Extract

The inhibition diameters for the crude extracts were computed against different test organisms as given in Table 3. Accordingly, AFC003 showed

highest activity over the tested organisms such that 16, 16, 15, 14 and 13 mm inhibition diameter against *Candida albicans*, *Staphylococcus aureus*, *Shigella boydii*, *Escherichia coli* and *Bacillus sp.* respectively.

Table 3. Agar Diffusion Assay for the crude extracts

Test organism	Crude extract	Inhibition diameter [mm]		
		Plate1 [x1]	Plate2 [x2]	Average
<i>Candida albicans</i>	HAC001	12	14	13
<i>Candida albicans</i>	HAC003*	0	0	0
<i>Candida albicans</i>	HAC007	10	12	11
<i>Candida albicans</i>	AFC003**	16	16	16
<i>Bacillus sp.</i>	HAC001***	0	0	0
<i>Bacillus sp.</i>	HAC007	15	15	15
<i>Bacillus sp.</i>	AFC003	12	14	13
<i>Shigella boydii</i>	HAC001	0	0	0
<i>Shigella boydii</i>	HAC007	12	15	13.5
<i>Shigella boydii</i>	AFC003	15	15	15
<i>Staphylococcus aureus</i>	HAC001	0	0	0
<i>Staphylococcus aureus</i>	HAC007	10	15	12.5
<i>Staphylococcus aureus</i>	AFC003	16	16	16
<i>Escherichia coli</i>	HAC001	0	0	0
<i>Escherichia coli</i>	HAC007	10	12	11
<i>Escherichia coli</i>	AFC003	12	16	14

* Since HAC003 had no activity against *Candida albicans* it was not tested over other organisms

** Over all *Syncephalastrum sp.* (AFC003) had an outstanding activity against *Candida albicans* and selected for further analysis

*** Similarly, HAC001 was discarded since it had no activity against the tested strain

5.1.5.2 *In vitro* Antibacterial Activities of the Fractions

The diameter of the resultant zones of inhibition of growth around the discs impregnated with 10 µl of each fraction (fraction 1 to 14) was measured after assay as shown in Table 4. The ethyl acetate and chloroform fractions (2-7) gave inhibition diameter of 12, 28, 20, 21, 15, and 26 mm respectively when tested against *Escherichia coli* and *Shigella boydii*. Further purification was attempted for fraction 7 as it had only a few bands on the TLC when visualized under U.V. and this makes identification much easier.

Table 4: bioassay directed separation technique used in this study

Crude extract	Solvent mixture	Eluent Used (10ml)	Conc. applied	Fraction collected	Plate seeded	Organism tested	Media used	Inhibition diameter (mm)
AFC003	Ethyl acetate: chloroform (1:1)	Ethyl acetate	100%	F ₁	#1	<i>E.coli</i>	NAm	NA
		Ethyl acetate	100%	F ₂	#1	<i>E.coli</i>	NAm	12
		Ethyl acetate	100%	F ₃	#1	<i>E.coli</i>	NAm	28
		Ethyl acetate	100%	F ₄	#1	<i>E.coli</i>	NAm	20
		chloroform	100%	F ₅	#2	<i>Shigella</i>	NAm	21
		chloroform	100%	F ₆	#2	<i>Shigella</i>	NAm	15
		chloroform	100%	F ₇	#2	<i>Shigella</i>	NAm	26
		Petroleum ether	100%	F ₈	#3	<i>Salmonella</i>	NAm	NA
		Petroleum ether	100%	F ₉	#3	<i>Salmonella</i>	NAm	NA
		Petroleum ether	100%	F ₁₀	#3	<i>Salmonella</i>	NAm	NA
		Hexane	100%	F ₁₁	#4	<i>Strept.</i>	NAm	NA
		Hexane	100%	F ₁₂	#4	<i>Strept.</i>	NAm	NA
		Hexane	100%	F ₁₃	#4	<i>Strept.</i>	NAm	NA
		Acetone	100%	F ₁₄	#4	<i>Strept.</i>	NAm	NA

NA; no activity

NAm; nutrient agar media

5.1.5.3 Bio Assay Analysis of the Purified Active Fraction- fraction 1

10 μ l of Ketoconazole was used as a positive control, where as 10 μ l of ethyl acetate was used as a negative control. The bioassay results were 14, 9 and 0 mm inhibition diameter against *Candida albicans* was observed respectively for ketoconazole, purified compound, and ethyl acetate (Fig.10c). Accordingly, 35.714 % of inhibition has been shown over the control.

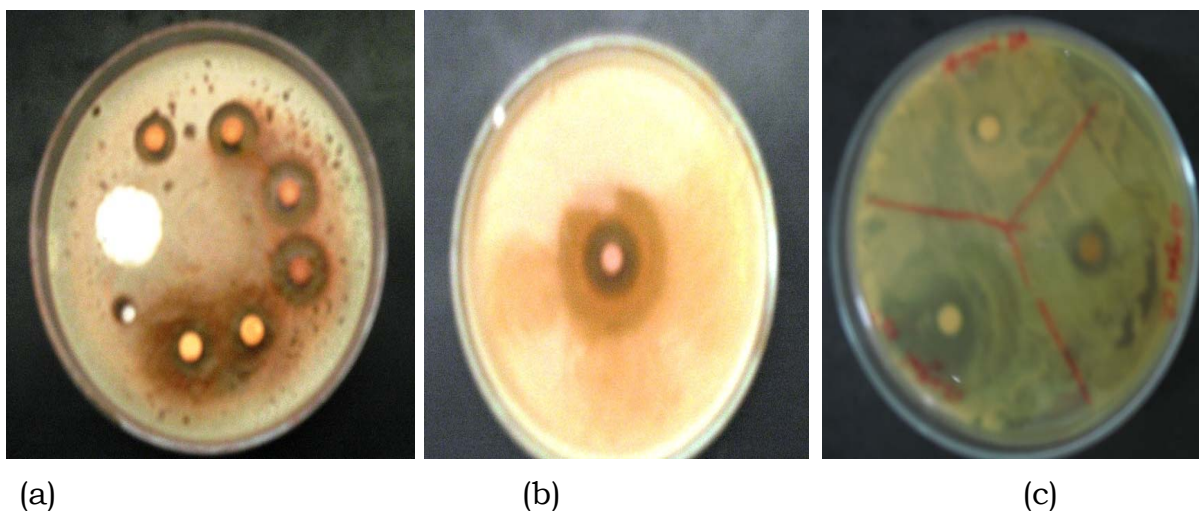


Fig.10 The bioassay image of the crude extract as well as the purified compound. (a) HAC003, AFC003, HAC007 against *Candida albicans* (b) AFC003 against *Candida albicans* (c) The positive control was ketoconazole and the negative control was ethyl acetate when compared with the purified compound.

5.1.6 Thin layer Chromatography for the Crude Extract

After making sure that the crude extract was indeed active against *Candida albicans*, TLC was performed to determine the best suited solvent system. The plate was then placed in a 1:1 solvent mixture of chloroform: ethyl acetate. After many trials, the best ratio for separating the crude extract initially was a 50:50 solution by volume containing chloroform and ethyl acetate solution respectively. After separation, the plates were analyzed under two different wavelengths (254 and 366 nm) and nine bands appear to be compact.

5.1.6.1 Thin Layer Chromatography for the Active Fractions

Fraction 3 to 5 had four bands with R_f value of 0.97, 0.55, 0.53 and 0.37. Fraction 6 had five bands with R_f value of 0.93, 0.85, 0.73, 0.65, and 0.58. However, fraction 7 had only two bands with R_f value of 0.35 and 0.49. The latter fraction was selected due to ease of separation.

5.1.6.2 Thin Layer Chromatography for the Purified Compound

Since fraction 7 showed higher activity against *Candida albicans*, TLC was performed for it. After placing the plate in a 1:1 solution of Petroleum ether: ethyl acetate, the compounds were separated out and two bands were displayed. In turn, fraction 7 gave the most recognizable fraction-fraction1 with a single visible band as shown in figure 11.

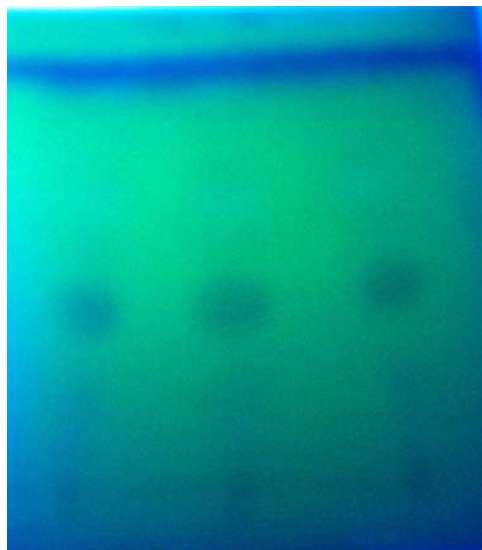


Fig.11 TLC plate of the fraction-fraction 1. The solvent used to develop the plate was a 1:1 solution of chloroform: ethyl acetate. The R_f value for the single visible band was 0.53.

5.1.7. Preparative TLC for the Purified Fraction

Spots were made on the P-TLC plate from the purified compound and a single band was observed when immersed in to a 1:1 solution of chloroform: ethyl acetate (Fig.12).

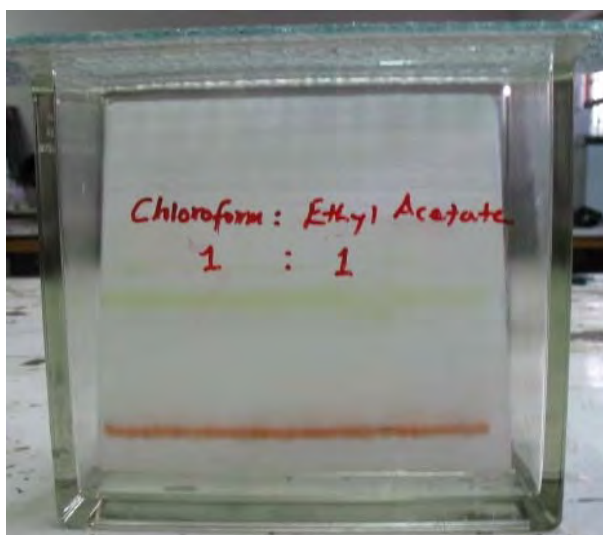


Fig.12 P-TLC for the purified compound

5.1.8 Column Chromatography for the Separation of Fractions from the Crude Extract

Five millimeter of the crude extract was placed in a glass column previously equilibrated and eluted with 3 ml of ethyl acetate and 14 fractions of 5 ml each were collected and their antimicrobial activity was tested. The active fractions were identified and concentrated to be further characterized as given in Table 11. Accordingly, fractions 3 to 7 were only active (Fig.13). As fractions 3 to 5 had nearly the same R_f values, they were pooled together once confirmed by the TLC method.



Fig.13 Active fractions collected from column chromatography

5.1.8.1 Column chromatography for fraction 7

Fraction 7 was distinct from the other fractions in that it was yellowish in color. 50ml of 1:1 petroleum ether: ethyl acetate was used as an eluent and five fractions of about 5 ml were collected. Eventually, fraction-fraction 1 came out to be pale yellow in color while the rest were colorless (Fig.14).

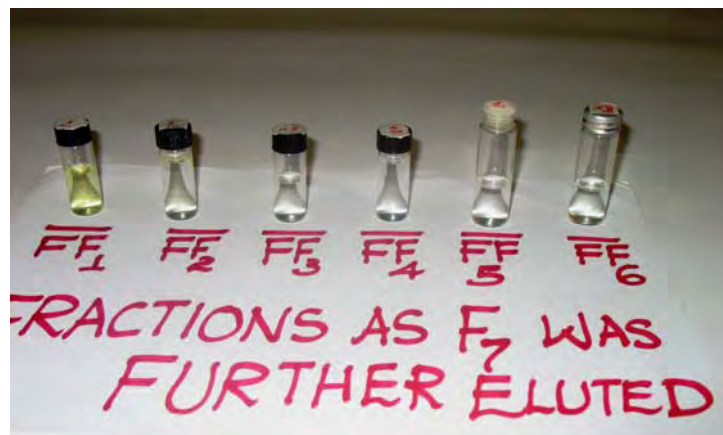


Fig.14 Fractions obtained from fraction 7.

5.1.9 Minimal Inhibitory Concentrations of the purified compound

The broth microdilution method was performed for the assessment of the minimal inhibitory concentrations (MICs). For this, ten test tubes each containing a concentration ranging from 10-100 µg/ml of SDB with purified compound as well as the commercial antibiotics were used. In parallel, ethanol dilutions and SD broth alone were used as microbial growth controls. Each tube was inoculated with 10^6 CFU/ml of the target microorganisms. Afterwards, spectrophotometer reading was taken at 660 nm as given in Table 13. Initially, the McFarland standard had OD of 0.48, where as the blank broth had OD of 0.24. The MIC was defined as the lowest antibiotic concentration that completely prevented visible growth after incubation at 37°C for 24 hr. Hence, MIC was found to be 80-100 µg/ml for the purified compound.

The purified compound showed better result for the concentration 80-100 µg/ml demonstrating an absorbance of 0.19, 0.09, and 0.04. This excel the the absorbance (0.28, 0.20 and 0.11) obtained by the positive control (ketoconazole) within the same concentration range. The McFarland standard (control) had 0.48 absorbance reading through out.

Table 5. Comparison for the sensitivity of *Candida albicans* against ketoconazole and the purified compound using OD measurement at 660 nm.

Concentration range(µg/ml)	10	20	30	40	50	60	70	80	90	100
Ketoconazole (OD ₆₆₀)	0.80	0.74	0.69	0.56	0.53	0.42	0.40	0.28	0.20	0.11
Purified compound(OD ₆₆₀)	0.80	0.76	0.59	0.61	0.46	0.33	0.25	0.19	0.09	0.04
McFarland standard(control)	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48

5.2 Discussion

Twenty isolates from five different indigenous trees were obtained of which twelve of the isolates were obtained from the leaf litter of *Hygenia abyssinica*, three from *Afrocarpus falcatus*, three from *Juniperous procera*, one isolate from *Olea Africana* and one from *Erythrina brucei*. These were preserved and maintained on potato dextrose agar (PDA) at incubation temperature of 25°C and preservation temperature of 4°C. More over, the culture collection was also named to be Addis Ababa University Culture Collection (AAUCC).

Screening of the isolate for antimicrobial activity was made after randomly selecting four fungi and growing them on the rice culture. Following extraction, the crude extracts of HAC001, HAC003, HAC007, and AFC003 were assayed on *Candida albicans*, *Bacillus sp.*, *Shigella boydii*, *Staphylococcus aureus*, and *Escherichia coli*. Among the four fungi, crude extract from AFC003 was over all effective against all the test organisms and hence it was selected for the purification process.

The most effective isolate on *Candida albicans* was taken and characterized on the basis of morphological characteristics and AFC003 was found to be a *Syncephalastrum* species. *Syncephalastrum sp.* (AFC003) was successfully cultured and harvested. Accordingly, *Syncephalastrum sp.* (AFC003) appears to be greenish and powdery on the rice culture. Moreover, the results obtained by the SSF method agreed with the previous reports on higher yields by SSF (Lambert and Meers, 1983; Losane and Ramesh, 1990; Abate, 1999).

The moisture content of the grain was determined in such away that the grain would not be logged or form clump or become too dry. Hence, it was

determined to be optimal at 50-60%, which agreed well with the water content of the substrate could oscillate between 30 and 75% as it was reported by (Cannal and Moo-Young, 1980).

Determination of the best time for the growth of the fungus especially for extraction of the secondary metabolites is very important. Since secondary metabolites are very active after exponential phase is restricted, estimation of this period is crucial. Due to various difficulties in growth determination of fungi growing on solid substrates as well as the lack of well-established procedure, the growth kinetics was not determined in this study.

Solid substrate gives more of the active substance since it is not in water and would not remain bound if it is highly polar and hence the active metabolites are found freely and physically bound in the grain. However, purification of the crude extracts might give different relative yields of pure metabolite (s). The lower yields by SLF have been attributed to the use of water immiscible solvent such as ethyl acetate for extraction (Sanchez-Puelles and Elson, 1997; Abate, 1999).

After trying many different types of solvent mixtures, 1:1 solution of chloroform: ethyl acetate was the best solvent mixture in order to separate the bands as it can be seen in figure 11. There were five different bands separated with 1:1 solution of chloroform: ethyl acetate. The band of interest is at R_f value 0.53, which is proven via various different separation and analyzing techniques performed. It is important to note in figure 10 that the positive control shows a zone of inhibition of 14mm, while the active compound shows 9mm zone of inhibition. It can be assumed that the unknown compound is less potent due to the higher dilution used.

Germ tube test was done according to Terleck *et al.* (2006) and a positive outcome was obtained. He demonstrated the reliability of performance of the

germ tube test and reported high sensitivity and specificity for differentiation of *Candida albicans* from non-albicans.

Syncephalastrum sp. (AFC003) showed higher activity towards *Candida albicans*. In order to show that it was indeed very active, the crude extract of the fungus was collected and tested by using a bioassay procedure. As Table 3 shows, the crude extract showed a 16 mm zone of inhibition. One can see in figure 19 the 16 mm zone of inhibition. This proves that the crude extract was indeed active. After proving the activity, a good solvent system was needed in order to separate the compound and to test each band, which would help narrow down to the final compound that shows activity against *Candida albicans*.

There were marked differences between the activities of the crude extract and those of the pure antifungal drug. Such significant differences were normally present when crude (unpurified) extracts were compared with pure drugs that were already in clinical use (Rex, 2001). Therefore, further purification of the crude extract would be expected to enhance the observed activity. Although the agar diffusion method is commonly employed in preliminary susceptibility testing, it is not always dependable for accurate assessment and comparison. This is because of the high degree of interference inherent in this method arising from drug diffusion problems (Rex, 2001).

The most remarkable achievement of this study was to purify the active compound and to against *Candida albicans*. Accordingly, the MIC (minimal inhibitory concentration) comes out to be 80-100µg/ml. Further investigation of the potency of the compound would help figure out its effectiveness against other organisms and confirm its utility toward animals and humans.

6. Conclusion and Recommendations

6.1 Conclusion

- Soil fungi especially from the Ethiopian habitat could be one potent source for the exploration of novel bioactive compounds.
- *Syncephalastrum sp.* (AFC003) grows well on the sterile rice grain by the solid state fermentation method.
- The best time to harvest the secondary metabolites from *Syncephalastrum sp.* (AFC003) was about a month.
- *Syncephalastrum sp.* (AFC003) extract demonstrated broad spectra of activity
- *Syncephalastrum sp.* (AFC003) extract showed a remarkable antimicrobial activity against *Candida albicans*. These promising results should encourage further investigations of the active metabolite.

6.2 Recommendations

- As time constraint did not allow to tell what the actual organism is, where the purified compound belongs, and what it looks like, further research is needed.
- Further experiments with multiple strains of other organisms warranted to confirm the utility of the bioactive compound from *Syncephalastrum sp.* (AFC003) as an alternative medicine in the war against mycotic diseases especially seen in compromised population groups world wide.
- Optimization of the solid state fermentation method for *Syncephalastrum sp.* (AFC003) would help in improving the scale-up process for industrial production of the active metabolite
- Determination of the growth kinetics of *Syncephalastrum sp.* (AFC003) would enable to estimate the exact period of extraction
- Further investigation of this potential antifungal agent may help in developing new chemical classes of antibiotics.

Appendix

Malt Extract Agar (MEA)

Malt extract 20g
Agar 20g
Distilled water 1000ml

Malt Extract Broth (MEB)

Malt extract agar 6g
Maltose 1.8g
Glucose 6g
Yeast extract..... 1.2g
Distilled water 1000ml

Malt Extract Agar with peptone (MEAP)

Malt extract 20g
Peptone 1g
Glucose 20g
Agar 20g
Distilled water 1000ml

Potato Dextrose Agar (PDA)

Peeled diced potatoes	200g
Glucose	20g
Agar	20g
Distilled water	1000ml

Commercial PDA

PDA	39g
Distilled water	1000ml

Czapek Dox Agar (CDA)

Sucrose	30.0g
NaNO ₃	2.0g
K ₂ HPO ₄	1.0g
MgSO ₄ + 7H ₂ O	0.5g
KCl	0.5g
FeSO ₄ + 7H ₂ O	0.01g
Agar	15.0g
Distilled water	1000ml

Commercial CDA

CDA	48g
Distilled water	1000ml

Sabouraud Dextrose Agar (SDA)

Mycological peptone 10.0g
Glucose 40.0g
Yeast extract 10.0g
Agar 15.0g
Distilled water 1000ml

Sabouraud Dextrose Broth (SDB)

Mycological peptone 10.0g
Glucose 40.0g
Yeast extract 10.0g
Distilled water 1000ml

Commercial Nutrient Agar

NA 20g
Distilled water 1000ml

Nutrient Agar (NA)

Peptic digest of animal tissue 5g
NaCl 5g
Beef extract 1.5g
Yeast extract 1.5g
Agar 15g
Distilled water 1000ml

Nutrient Broth (NB)

Peptic digest of animal tissue	5g
NaCl	5g
Beef extract	1.5g
Yeast extract	1.5g
Distilled water	1000ml

0.5 McFarland Standard

H₂SO₄ (1% v/v)1ml concentrated H₂SO₄ in 99ml distilled water
Barium chloride (1.175% w/v)2.35g BaCl₂ in 200ml distilled water

Phosphate Buffer Saline

K ₂ HPO ₄	0.1 M
KH ₂ PO ₄	0.1 M
NaCl	0.85g
Distilled water	100ml