ANALYSIS OF LOW MOLECULAR WEIGHT COMPOUNDS PRODUCED BY INDIGENOUS WOOD DECAY FUNGI

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DECLARATION

I, Samuel Yaw Aboagye of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, hereby declare that to the best of my knowledge , this thesis contains neither materials which has been accepted for the award of any degree or any material previously published by another author , except where due reference is made in the text of the thesis.

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DEDICATION

To the most influential personality in my life, Floren

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LIST OF ABBREVIATIONS

WDF	Wood Decay Fungi
PDB	Potato Dextrose Broth
BSAB	Broad Spectrum Antibacterial
NSAM	Non Selective Antimicrobial
SG+	Selective Gram Positive
SG-	Selective Gram Negative
SAF	Selective Antifungal
CA	Control Antibiotics
NA	No Activity
SO	Sample Origin
SF	Solvent Front
M	Median
TLC	Thin Layer Chromatography
ATCC	American Type Culture
NMIMR	Noguchi Memorial Institute of Medical Research
KBTH	Korle Bu Teaching Hospital

ABSTRACT

Over the years, natural products have played a major role in the search for novel drugs or drug candidates. Secondary metabolites from nature especially those of fungal origin exhibit unique biological activities and research continue to meet the keen interests which have potential pharmaceutical value. Fungi constitute an important source of secondary metabolites such as penicillin. The present study analyzed the biological activities of a variety of compound mixtures from wood decay fungi.

A total of 54 wood decay fungi (WDF) were collected, with majority obtained from the University of Ghana campus and its surroundings. The WDF were cultured in potato dextrose broth (PDB) for 48 days and the time course analysis of two selected WDF were also performed to determine the profile of specific bioactivities. Cultures were terminated and extracted with ethyl acetate at the end of the 48^{th} day of culture. WDF extracts were analyzed spectrophotometrically at wavelengths between 200 nm and 900 nm which showed high absorption in the UV region of the spectrum. TLC analyses of the fungal extracts were done using EtOAc: CH_3CN: PetEth (7:2:1) as the solvent system and different classes of compounds were detected on TLC plates sprayed with Anisaldehyde reagent. UV detection of compounds on TLC showed unique band pattern for the different WDF. Assays for biological activities of the fungal extracts were performed against *S. aureus ATCC.2, E. coli NMIMR.3, C. albicans KBTH.2* and *A. niger ATCC.2* using disc diffusion assay method.

From the primary screening of antimicrobial activity, a total of 40 WDF extracts were found to exhibit some form antimicrobial activity towards the test organism. Out of total 40 that had an activity, 10 of the WDF extracts were found to have biological activity selectively (SG+) against *Staphylococcus aureus ATCC.2*, and 13 extracts were also found to have a broad spectrum antimicrobial activity (BSAB) against *Staphylococcus aureus ATCC.2* and *Escherichia coli NMIMR.3.* The number of extracts that had selective antifungal (SAF) activity towards *Candida albicans KBTH.2* was found to be 3. The number of WDF that exhibited a non selective antimicrobial (NSAM) activity towards the three test organisms, *S. aureus ATCC.2*, *E. coli* NMIMR.3and *C. albicans* KBTH.2 were recorded to be 11. The time course analysis showed that fungal metabolites are produced as early as the 7th day of culture, however cultures that were harvested between 22nd and the 48th day of culture produced potent bioactive components. . After the secondary screening of 27 WDF, inhibitory activity against only *S. aureus ATCC.2 was* found to be possessed by 9 refermented extracts. Inhibitory activity towards both Gram positive and Gram negative bacteria tested was found in 6 extracts. Four (4) refermented extracts also exhibited inhibition towards *S. aureus ATCC.2* and *C. albicans KBTH.2.* A shift in antimicrobial activity was observed after the secondary screen. Sephadex LH-20 fractions of the selected WDF extracts showed the broad spectrum activities of the individual fractions. All the extracts (A4, E2, E9 and F3) that showed broad spectrum activities against a Gram + and Gram – bacteria had common fraction/s possessing the biological activities. In those extracts (B6 and B7) that inhibited a Gram + bacteria and a fungus, the biological activities were seen in different fractions.

CHAPTER ONE

1.1 INTRODUCTION

The existence of many drugs today originates from the natural environment **(Newman *et al.,* 2000).** Secondary metabolites from nature especially those of fungal origin exhibit unique biological activities and research needs to continue to discover interesting compound which have potential pharmaceutical value. Secondary metabolites produced by microorganisms have continued to rise over the last few decades. **Cragg *et al.,* 1997** reported that 100,000 secondary metabolites from nature were identified, and this reported number has increased to 200,000 metabolites after nearly a decade **(Tulp and Bohlin, 2005).**

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of organisms **(Fraenkel, 1959).** They use synthetic pathways the role of whose end products are not well established but are characterize as low molecular weight compounds (< 1500 Da). These compounds have unusual and varied chemical structures and have show no direct function in growth of the producing organisms **(Stamp, 2003).** Many clinically useful drugs have been obtained through the screening of natural products **(Newman *et al.,* 2000),** it is reported that, on the average two to three antibiotics derived from microorganisms are launched every year **(Clark 1996)**, and over 60% of antitumor and anti-infective agents that have been approved or are in late stages of clinical trials, are of natural products origin **(Cragg *et al.*, 1997)**. The world's top eight selling drugs are either natural products or derived from natural products, including lovastatin, enalapril, simvastatin, pravastatin,

the combination of amoxicillin and calvulanate, cyclosporin, clarithomycin, and captopril **(Quinn, 1999)**.

Microorganisms such as fungi and bacteria produced a few similar secondary metabolites and showed selective activities in various biological systems such as antimicrobial activities, cytotoxic activity and other secondary metabolites also show a wide range of biological activities **(Bernan, *et al.,* 1997)**. Intensive research is indeed needed to find novel secondary metabolites with biological activities from the natural environment.

The exploration of new sources of fungal secondary metabolites from natural environment is needed in order to treat recently discovered diseases **(Muller *et al.,* 2000).** At the moment, less than half of all diseases can efficiently be treated. This is due to the existence of multi-resistant pathogenic strains that developed resistance to most drugs especially antibiotics; example is AIDS and other viral diseases. These are factors that require the search for new therapeutic agents **(Strohl, 1997; Bernan *et al.,* 1997; Muller *et al.,* 2000; Larsen *et al.*, 2005)**. Many current medicines are no longer effective towards the currently emerging diseases and this requires novel medicines. The search for novel secondary metabolites that have biological activity to overcome the growing human health problems of drug resistance in pathogenic microorganisms and the occurrence of new diseases has been rapidly increasing around the world.

Taking into consideration the capability of microorganisms to produce diverse bioactive molecules and the existence of unexplored sections of the microbial diversity spectrum, researching to isolate and screening of microbes of diverse but unique habitats for

discovery of novel metabolite is a vital undertaking. One such unexplored and less studied microorganism is the wood decay fungi, defined as those fungi that have the ability to degrade cellulose and lignin the major component of wood, releasing the nutrients that have been locked up in the wood and making the available for new growth **(Sugimori *et al.,* 1971).**

Fungi are of special interest because they are eukaryotes and their metabolism is more related to humans than that of prokaryotic bacteria. Therefore, a number of metabolites isolated from fungi which found their way into medical applications such as natural products, starting materials for pharmaceuticals or lead structures for the development of pharmaceutical products. Example in Japan, lentinan, a polysaccharide isolated from *Lentinus edodes,* is commercialized for clinical usage due to its anticancer properties **(Soboleva *et al.,* 2006)**. Another important result that can be gained by studies on fungal secondary metabolites is the understanding of biosynthetic pathways, thus giving information about enzymatic reactions and systematic relationships among the various living species **(Sterner, 1995)**. Secondary metabolites present in the fruiting bodies of wood decay fungi, example basidiomycetes, play a vital ecological role; they are weapon of chemical defense and/or competition towards other living species **(Michael and Mizuno, 2000).** Many fungal metabolites have a deterrent role in nature, protecting the fungus against predators and parasites. Many studies have indicated that some compounds with a wide range of biological activities such as antimalarial, antimicrobial, anticoagulant, antiplatelet, antituberculosis, cytotoxic, and antiviral activities have been successfully isolated from marine as well as terrestrial habitat **(Beltran-Garcia *et al.,* 1997).**

The search for novel bioactive compounds with therapeutic potential has become increasingly difficult, since the discovery of unique natural products for the isolation and cultivation relies on effective strategies example different methods of cultivation, different media and incubation time (**Larsen *et al*., 2005).**

1.1.1 Justification

Fungi are important sources of secondary metabolites such as penicillin, cyclosporine, lovastatin etc. Most of the useful compounds obtained from fungi have been found by screening methods using endophytic fungi, epiphytic fungi as well as fungi from the soil environment. **Dreyfuss, 1985** however, described a problem that is often encountered during microbiological screening of fungal isolates for their secondary metabolite content. Surprisingly known metabolites are rediscovered making screening less efficient. This may be caused partially by the using of the same established isolation methods for fungi. With several thousand fungal metabolites now known from these sources, screening such fungi for new bioactive compounds could lead to re-isolation of known metabolites. Therefore selection of wood decay fungi for the isolation might lead to the discovery of novel secondary metabolite because the different species are easily distinguishable and also many different species found locally. Another rational for antimicrobial compounds from basidiomycetes is that humans and animals share common microbial pathogen with fungi, such as *Escherichia coli* and *Staphylococcus aureus* and therefore we can benefit from defense strategies used by fungi against microorganisms **(Zjawiony, 2004).**

1.1.2 Objective of Study

The purpose of carrying out this study was to determine the biological activity of a variety of mixtures of compounds from wood decay fungi.

1.1.2.1 The specific objectives were:

- Isolation of low molecular weight compounds from a variety of fungi found on decaying wood
- Physicochemical analyses of the compounds in the fungal extracts
- Bioassays of the isolated low molecular weight compounds
  - Antimicrobial assay; effects of test compounds on the inhibition of some selected microbes

CHAPTER TWO

LITERATURE REVIEW

2.1 Fungi

The fungal kingdom is enormous with varied shapes and sizes ranging from microscopic yeast to bulky and fleshy mushrooms. They live in diverse habitats and play a vital role in the process of recycle of organic matter **(Laessoe, 1998)**. Traditionally, biologists have defined fungi as eukaryotic, spore producing, achlorophyllus organism with absorptive nutrition. They generally reproduce both sexually and asexually and possess morphologically features filamentous and branched somatic structures known as hypha. Some of these structures are typically surrounded by cell walls, whereas some others have no cell walls **(Alexopolous *et al*., 1996).** The mass of hypha constituting the thallus of a fungus is called the mycelium, the mycelium of some of the higher fungi forms thick strands. The mycelium of a fungus generally begins as a short germ tube emerging from a germinating spore. It has the tendency to grow more or less equally in all directions from a central point, and develop a spherical colony **(Swann and Taylor, 1993).** Also the mycelium of parasitic fungi grows on surfaces inside the host, either spreading between the cells or penetrating into the cells. If the mycelium is intercellular, food is absorbed through the host cell wall or membrane. If the mycelium penetrates into the cells, the hyphal wall comes into direct contact with the host protoplasm.

2.2 Classification of Fungi

The kingdom fungi consist of four phyla: Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota (**Alexopolous *et al*., 1996)**. The Chytridiomycetes are the only group

with motile cells (known as zoospores). The fungi that form zygospores are called Zygomycetes, those that form ascospores are called Ascomycetes and those forming basidiospores are called basidiomycetes **(Hawksworth *et al.*, 1995).** The phyla Basidiomycetes are a large division within the kingdom that includes those species that produce spores in a club-shaped structure called a basidium.

2.3 Wood decay fungi

Every year an enormous amount of wood and wood products are destroyed by decay, rot and decomposition, the process by which tissues of dead organisms break down into simpler forms of matter. Such a breakdown is essential for new growth and development, because it is the basis for recycling limited chemical compounds, as well as freeing up limited physical space in the environment. Fungi are the main organisms responsible for wood decay **(Blanchette, 1984).**

A wide range of fungi occur on wood using various constituents for their metabolism. Decay fungi need oxygen, water and a food source to exist. Wood as a food source is limited to those fungi which are able to utilize the components and in the process break down the wood. Fungi have an external method for breaking down their food by secreting digestive enzymes and other chemicals into the substrate where they are growing **(Hammel *et al.,* 1985).** This enables the fungi to then absorb predigested food products. This external digestion process requires that liquid water be present so the enzymes can be secreted and then the useable food products can absorbed into the fungus. Without this moisture, the fungus cannot be active or grow; it may either become dormant or die **(Higuchi, 1990).**

Wood decay is generally classified into two main groups, white rots and brown rots, based on the wood residue left behind following fungal digestion. Two other types include "dry rot", which is a form of brown rot caused by water-conducting decay fungi, and "soft rot", referring to decay caused by certain Ascomycetes and asexual fungi **(Eriksson *et al.*, 1990).**

The main wood-inhabiting groups of basidiomycetes are commonly known as the polypores. It's estimated that in North America alone, no less than 100 species of polypores cause decay in woody plants and timber, while approximately 75 species are responsible for 90% of the important decays produced in timber and wood products **(Kirk and Moore, 1972).** Most polypores are saprophytic and utilize dead wood as their food source. These fungi commonly appear as hard, tough, corky, leathery or woody structures of various shapes and sizes. They have a fertile surface (where spores are produced), usually made of pores or tubes closely packed together. Polypores are mostly wood inhabiting fungi that are able to utilize components of wood as their primary source of energy for growth and reproduction. When a fruiting body is seen on wood, the mycelium, or main body of the fungus, is usually not so visible, growing within the wood obtaining nutrients from it. (**Otjen and Blanchette, 1986).**

Figure 2.1 *Wood decay fungus from University of Ghana Botanical Gardens. (Aboagye©2009)*

When fungi decay wood, the process involves breaking down complex chemical compounds, primarily cellulose and or lignin. Cellulose is a polysaccharide composed of linear chains of glucose molecules. All plants have this chemical compound as the primary cell wall component Cellulose is the most common organic compound on Earth and makes up roughly 50% of wood **(Kirk and Cowling, 1984).**

Lignin is a complex polymer of phenolic units and relatively resistant to decay. It plays a key role in the carbon cycle as the most abundant aromatic compound in nature, providing a protective matrix in the plant cell wall. This amorphous and insoluble polymer is not susceptible to hydrolytic attack, in contrast to cellulose. Although lignin is a formidable substrate, its degradation by certain fungi was recognized and described nearly 125 years ago. These basidiomycetes are the only organisms capable of efficient depolymerization and mineralization of lignin **(Kirk and Farrell, 1987).**

2.3.1 White Rot Fungi

The fungi causing white rot are represented in all the main groups of the Basidiomycetes and in some Ascomycetes **(Setliff and Eudy, 1980)**. The term white rot has been used to describe forms of wood decay in which the wood assumes a bleached appearance and where lignin, cellulose and hemicellulose are broken down. Two forms of white rot have been reported **(Blanchette, 1984),** selective delignification and simultaneous rot, which both result in chemically and morphologically different wood characteristics. However, one fungus can cause both forms of white rot. During selective delignification more lignin is broken down than hemicellulose and cellulose, this leads to white pocket rot, which is recognized as light patches compared to preferential lignin degradation which leaves patches of pure cellulose. In the course of simultaneous rot the lignin, cellulose and hemicellulose are broken down at approximately the same rate. Lignin serves as a physical and chemical barrier to enzymatic degradation of wood polysaccharides **(Kirk and Farrell, 1987).** Removal of lignin, therefore, will expose cellulose and hemicellulose to degradation by cellulase and xylanase enzymes.

2.3.2 Brown Rot Fungi

Brown rot is a wood decay caused exclusively by fungi of the Basidiomycetes Class. This class consists of many families though the overwhelming majority of the brown rot fungi belong to the Polyporaceae family. Only 6% of all the known wood decay fungi are now known to cause brown rot**.** With brown rot fungi, cellulose is removed but the lignin remains. In advanced stages, the wood appears brown to dark brown in color and is often cracked cubically. The fibrous texture is lost early on in the decay process, due to the removal of cellulose **(Kimura *et al.,* 1990).** Brown rot is more common in conifer

wood (i.e. gymnosperm wood such as pine (Pinus sp.) and fir (Abies sp.)). The brown rot residues left behind after the decay processes are important organic components of forest soils. These brown rot fungi are extremely important in recycling carbon in the ecosystem **(Jurasek, 1964)**. Species of brown rot fungi are relatively few when compared to species of white rot fungi. Geographically, brown rot fungi become low in number in the tropics when compared to temperate regions of the northern hemisphere.

2.4 Fungal biotechnology

Fungi are the most biotechnological useful organisms **(Smith and Berry, 1975; Kurtzman, 1984)**. Fungal biotechnology is not a new phenomenon as mankind has used fungi for their biochemical activities since the beginning of civilization. The use of fungi for bread baking and alcohol production has a long history, and probably the most well-known industrial use of fungi in modern time is the use of yeasts for brewing and for wine and bread making. In the later decades submerged (liquid) cultivation of filamentous fungi for production of commercially important products has increased. These products can be either primary or secondary metabolites produced by fungi. Primary metabolites are products involved in the growth, development and reproduction. The secondary metabolites are usually produced from common metabolic intermediates, but the production is often species- or strain specific.

The production of secondary metabolites is accomplished by special enzymatic pathways in the fungi and usually takes place in the stationary phase when fungi are grown in culture. However, the expression of enzymes for secondary biosynthesis can be altered by e.g. nutritional and genetic factors and secondary metabolites can be produced during growth (**Demain, 1993**).

As previously mentioned, fungi produce many compounds which have been shown to be useful for mankind, ant find uses in industry, medicine, agriculture, and basic science research. The cultivation of filamentous fungi for the production of metabolites is diverse and of great economic importance and there is a great variety of industrially important fungal products such as antibiotics, organic acids, enzymes, foods, and pharmacologically active products.

One of the major fungal biotechnology processes is the production of antibiotics. Penicillin was discovered in **1928 by Fleming Alexander** as a metabolite of *Penicillium chrysogenum*, which inhibited growth of *Staphylococcus*. The mass production of antibiotics began during World War II and since then, industrial-scale processes for production of antibiotics by fungi have continued to increase. Improvements in the fermentation technologies and the productivity of the producer organisms have led to high recovery yields of the penicillins. However, in search for new antibiotics, many of the penicillins produced today are semi-synthetic by chemically modified natural penicillins **(Elander, 2003).**

Besides the pharmacologically active metabolites discussed in a previous section, another example of industrially important products from fungi is the organic acid citric acid, produced by fermentation of *Aspergillus niger* **(Papagianni, 2007**). Citric acid is used as a constituent of soft drinks and other food products, as a preservative and flavor enhancer. Another organic acid, itaconic acid, produced in large scale by *Aspergillus terreus* fermentations, can be incorporated into polymers and is thus having the potential of substituting petrochemical-based monomers (**Willke and Vorlop, 2001).**

2.5 Fungi as the origin of biologically active metabolites

Species of the kingdom fungi constitute a vast reservoir of pharmacologically active substances and are also a useful means of production of several drug products. Some of these are secondary metabolites which have no obvious role for the producing organism, yet they are produced in abundance and comprise a wide variety of compounds. Many of the secondary metabolites have been shown to be beneficial to mankind and have therefore attracted a lot of attention for their commercial significance. One of the most well-known groups of secondary metabolites from fungi is antibiotics, which inhibit the growth of microorganisms and function as fungal defense of territory. These secondary metabolites are also some of the most recognized pharmaceuticals of fungal origin. B-lactam antibiotics include several penicillins and are produced industrially by fermentation of the filamentous micro fungus *Penicillium chrysogenum*. Another antibiotic, griseofulvin, was originally isolated from *Penicillium griseofulvum* (**Oxford *et al.*, 1939**) and is industrially produced from fermentations of the same fungal species. The cholesterol reducing secondary metabolites, the statins, from different fungi have been developed into drugs and lovastatin is produced industrially by cultivation of the microfungus *Aspergillus terreus* (**Greenspan *et al.*, 1985).**

Ergot alkaloids and their derivatives are secondary metabolites found in fungi of the plant parasitic genus *Claviceps*. This class of compounds is produced on a large scale by e.g. *Claviceps purpurea* and has a high variability of chemical structures. Their pharmacological effects pertain to their structural similarities to neurotransmitters such as dopamine and serotonin. Hence, they have effects on neurotransmission and

circulation and therefore a wide field of therapeutic applications including migraine, Parkinsonism and circulatory disturbances *(***Tudzynski *et al.,* 2001).**

2.6 Fermentation Technology

Fermentation is an ancient process and technology used to produce many foods and value added products **(Souza *et al.*, 2004)**. It is a biological process which can happen under aerobic and/or anaerobic (absence of oxygen) conditions. There are two types of fermentation: submerged liquid fermentation (SLF) and solid substrate fermentation (SSF).

2.6.1 Submerged Liquid Fermentation (SLF) and Solid Substrate Fermentation (SSF)

Liquid fermentation is a controlled process which consists of growing cells in liquid broth where both filamentous and non-filamentous fungi can grow. Parameters such as pH, temperature, oxygen and mixing can be controlled in the fermentation chamber. It is known that fungi grown in liquid produce different enzymes and metabolite products compared to solid substrate fermentation. Solid substrate fermentation is the aerobic growth of microorganisms, fungi in this case, on solid substrate under limited water conditions **(Nagel *et al*., 2000).** The solid material may consist of organic material such as cereal grain, wood chippings, compost and cheese **(Rombouts, 1992).**

Solid substrate fermentation (SSF) has been usually exploited for the production of value-added products such as antibiotics, alkaloids, plant growth factors, biofuels,

enzymes, organic acids, aromatic compounds and also for bioremediation of harzardous compounds. **(Pastrana *et al.,* 2005).** Comparative study done on submerged liquid fermentation (SLF) and solid substrate fermentation (SSF) have showed higher yield for SSF system. It is a better option as the low availability in water reduces contamination of bacteria and yeast. It is also a similar environment to natural habitat of fungi. It allows higher levels of aeration, especially in processes demanding intensive oxidative metabolism. Inoculation of spores facilitates uniform dispersion through medium and the substrate is always found to provide sufficient nutrient for growth. Products obtain from SSF is found to have thermo-tolerance and require low levels of energy **(Pastrana *et al*., 2003).**

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". These processes have been used for the production of food, animal feed, and both pharmaceutical and agricultural products **(Cannel and Moo-Young, 1980)**. 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. However, non-traditional substrates which may also be of interest in industrial process development include an abundant supply of agricultural, forest and food-processing wastes (such as wheat bran and soy grits (flakes remaining after extraction of oil) **(Moo-Young *et al*., 1983)**

2.7 Secondary metabolites and their classes

Secondary metabolites have been classified into groups, based on their mode of synthesis in cell **(Ingold, 1973**). These metabolites are derived from acetyl CoA and fatty acids, mevalonic acids, amino acids, sugars, aromatic amino acids, intermediates of tricarboxylic acid cycle and products of several metabolic pathways **(Bu'Lock, 1968).** Two closely related hypotheses have proposed that the specific products of secondary metabolism itself are of selective advantage to the organism. It is considered to provide a mechanism by which excess intermediates and carbohydrates in the medium **(Foster, 1949)** can be metabolized during adverse growth conditions. Such a mechanism would serve to maintain the cell in a functional state during conditions that prevented growth.

The major source of carbon as described in (Figure 2.2) and energy for most heterotrophic organisms is glucose. The breakdown of glucose by the Embden-Meyerhof pathway gives various aromatic amino acids and secondary metabolites **(Turner, 1971)**. The available metabolites can be those derived from glucose, intermediates of shikimic acid pathways, fatty acids, polyketides, terpenes and steroids, intermediates of the tricarboxylic acid cycle, amino acids and miscellaneous secondary metabolites.

Fungal Metabolism and Fungal products

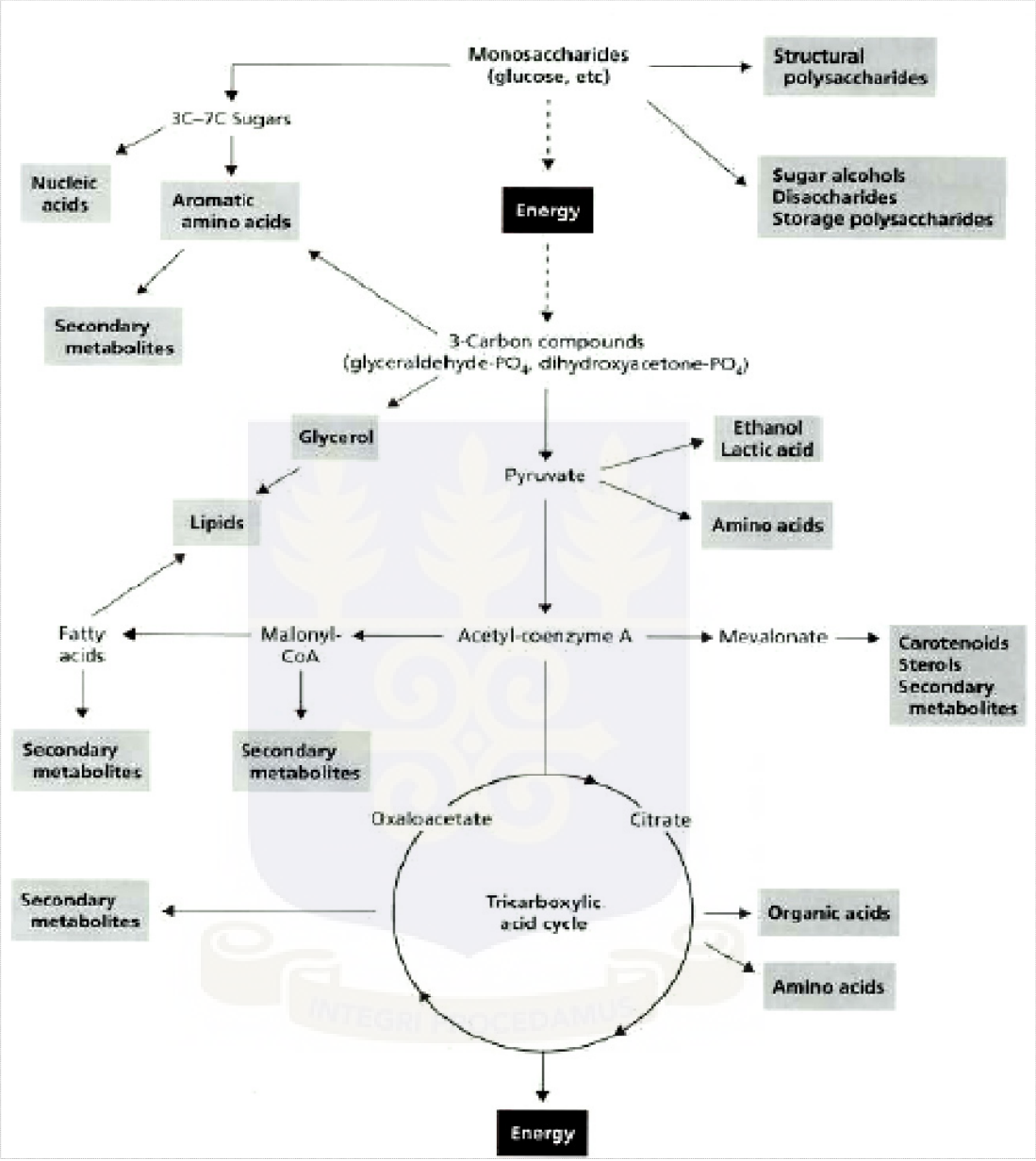
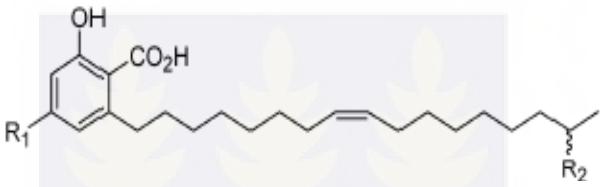


Figure 2.2 Overview of the central metabolic pathways of fungi (Deacon, 2005)

2.7.1 Polyketides

Polyketides are natural products that are formed by the stepwise condensation of acetate (ethanoate) units. Antimicrobial metabolites of polyketide origin are merulinic acids A, B, and C isolated from the fruiting bodies of the polypores *Merulius tremellosus* and *Phlebia radiate* **(Giannetti *et al.*, 1978).** Griseofulvin isolated from *Penicillium griseofulvum* has been used to treat fungal infections of the skin and mycophenolic acid from *P. brevicompactum* used as an immunosuppressive agent.

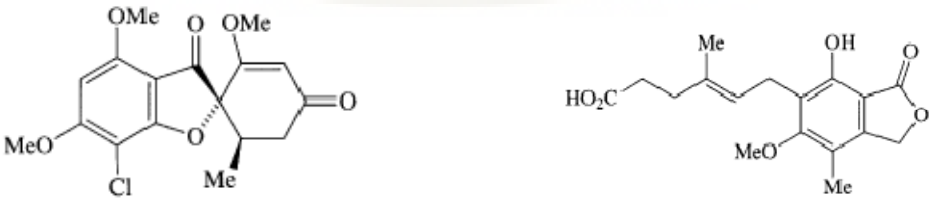


Merulinic acid A (1) R_1= OH, R_2=H

Merulinic acid B (2) R_1= H, R_2= OH

Merulinic acid C (3) R_1=H, R_2=H

Figure 2.3 Structure of Merulinic acids A, B, and C (**Giannetti *et al.*, 1978)**



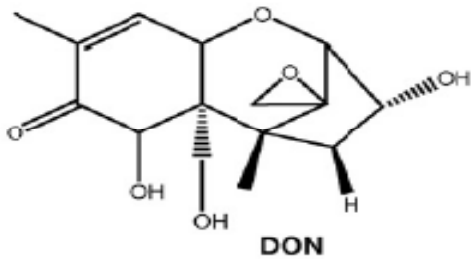
Griseofulvin **Mycophenolic acid**

Figure 2.4 Structures of Griseofulvin **and Mycophenolic acid (Giannetti *et al.*, 1978)**

The merulinic acids showed antimicrobial activity against *Arthrobacter citreus*, *B. subtilis*, *Corynobacterium insidiosum*, *Micrococcus roseus*, and *Sarcina lutea*. *Mycobacterium phlei* was selectively inhibited by merulinic acid B and merulinic acid C, while merulinic acid A was inactive. Similarly, *S. aureus* and *Proteus vulgaris* were inhibited only by merulinic acid B **(Giannetti *et al.*, 1978).**

2.7.2 Terpenoids

The terpenes are compounds that are built up from isoprene units which are products of the mevalonate pathway. Isopentenyl diphosphate and its isomer dimethylallyl diphosphate (DMAPP) are the building blocks (5C isoprene units) for the linear polyprenyl diphosphates, which are precursors of steroids, carotenoids and coenzyme Q in many species (**Tudzynski, *et. al.*, 2001).** The terpenes are classified by the number of these C_5 isoprene units that they contain. The classes are: monoterpenoids C_{10}, sesquiterpenoids, C_{I5} diterpenoids C_{20} , sesterterpenoids, C_{25} triterpenoids, C_{30} and carotenoids, $C_{40.}$ Triterpenes have been found in the fruiting bodies of a number of fungi, particularly from *Basidiomycetes* such as *Polyporus* (polyporenic acids) and *Ganoderma* (ganoderic acids) species **(Keller *et. al.*, 2005).**



Deoxynivalenol (DON)

Figure 2.5 Structure of Deoxynivalenol (Keller *et. al.,* 2005).

2.7.3 Alkaloids

Some of the first natural products to be isolated from medicinal plants were alkaloids. Many alkaloids have neuroactive properties and interact with the receptors at nerve endings. Alkaloids may be grouped according to their plant sources, *e.g.* Aconitum, Amaryllidaceae, Cinchona, Curare, Ergot, Opium, Senecio and Vinca. Another classification is based on the structure of the nitrogen atom containing ring system *(e.g.* piperidine, isoquinoline, indole). This kind of classification reflects their biosynthetic origin from amino acids such as ornithine, lysine, phenylalanine, tyrosine and tryptophan **(Tudzynski, 1999).** Indole alkaloids are derived from the amino acid tryptophan and include poisons such as strychnine. The ergot alkaloids, from the ergot fungus *Claviceps purpureu,* are also indole alkaloids and are amides of lysergic acid.

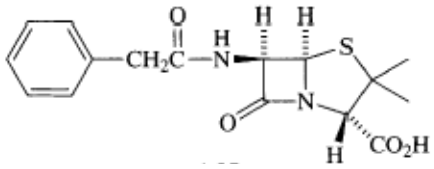
Strychnine **Lysergic acid**

Figure 2.6 Structure of Strychnine and Lysergic acid (Tudzynski, 1999)

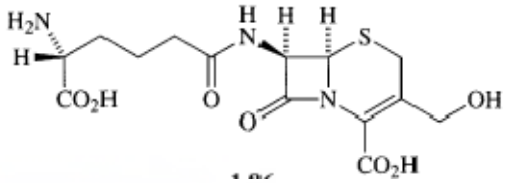
2.7.4 Peptide Antibiotics Derived from Amino Acids

The alkaloids are not the only group of secondary metabolites that are derived from amino acids. Amino acids not only form the building blocks for the large peptides and proteins but also for smaller peptides that are converted into the β-lactam antibiotics such as the penicillins and cephalosporins. The diketopiperazine antifungal agents

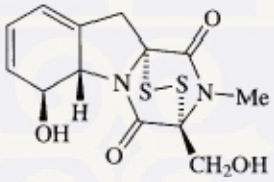
produced by *Trichoderma* and *Gliocladium* species, such as gliotoxin are also derived from amino acids.



Penicillin



Cephalosporin



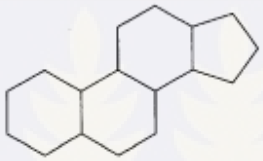
Gliotoxin

Figure 2.7 Structures of Peptide Antibiotics Derived from Amino Acids (Rinehart and Stroshane, 1976).

Amino glycosides are classified into two groups depending on the structure of the aglycone. One class, typified by streptomycin and fortimicin, has the fully substituted aminocyclitol streptamine. The other class has 2-deoxystreptamine as the common aglycone and contains many of the clinically important aminoglycosides like Kanamycin, neomycin, gentamicin and butirosin **(Rinehart and Stroshane, 1976).**

2.7.5 Steroids

The steroids are derived from tetracyclic triterpenes and possess acyclo-penta perhydrophenanthrene backbone **(Kuzuyama and Seto 2003).** Cholesterol is a typical mammalian sterol, whereas ergosterol is found in fungi and stigmasterol in plant oils, cholesterol forms an important constituent of plasma membranes. Another major group of steroids are the steroid hormones, the progestogens *(e.g.* progesterone,**)** and the estrogens *(e.g.* estradiol,**)** are female hormones. **Rossier, (2006)**



Acyclopentaperhydrophenanthrene backbone

Figure 2.8 Structure of Acyclopentaperhydrophenanthrene (Kuzuyama and Seto 2003)

2.8 Fungal Secondary Metabolites

2.8.1 Antimicrobial Metabolites (Antibacterial and Antifungal)

A recent biological evaluation of over 200 mushroom species revealed that more than 75% of screened Basidiomycetes showed strong antimicrobial activity **(Suay and Arenal, 2000).** These activities are associated not only with small molecule secondary metabolites but also with high molecular weight cell wall polysaccharides. The major philosophy of the search for antimicrobial compounds from Basidiomycetes is that humans (and animals) share common microbial pathogens with fungi, such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas areuginosa*, so that we can benefit from defensive strategies used by fungi against microorganisms. Basidiomycetes

have a long history for their medicinal use, example *Fomes fomentarius* was used in the 18th and 19th centuries in hemostatic dressings and bandages **(Roussel *et al.*, 2002).**

There are numerous studies describing antimicrobial properties of secondary metabolites isolated from various basidiomycetes **(Mothana *et al.*, 2000; Nakajima *et al.*, 1976; Giannetti *et al.*, 1978).** Screening of crude extracts *Ganoderma lucidum*, *Ganoderma pfeifferi*, and *Ganoderma resinaceum*, revealed selective activity against *Bacillus subtilis*.

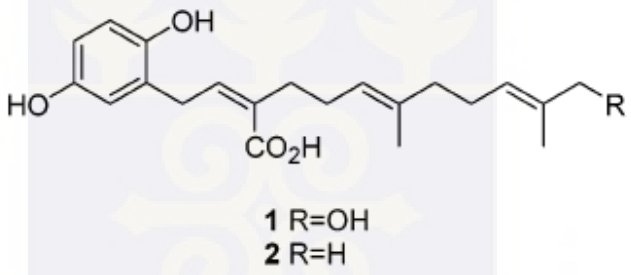
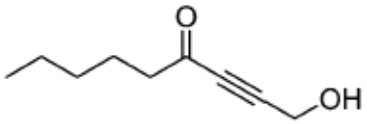


Figure 2.9 Structures of ganomycin A (**1**) and ganomycin B (**2**) (Mothana ***et al.*, 2000**)

For example, two secondary metabolites, ganomycin A (**1**) and ganomycin B (**2**), isolated from *G. pfeifferi* showed moderate growth inhibition of several bacterial strains, particularly Gram-positive strains such as *B. subtilis*, *S. aureus*, and *Micrococcus flavus* **(Mothana *et al.*, 2000).**

The compound 1-hydroxy-2-nonyn-3-one isolated from the fermentation of the *Ischnoderma benzoinum* also exhibit antifungal activity.



1-hydroxy-2-nonyn-3-one

Figure 2.10 Structure of 1-hydroxy-2-nonyn-3-one **(Mothana *et al.*, 2000).**

The fungal species *Favolaschia* produces the metabolite favolon exhibiting greatest antifungal activity against most fungi such as *Mucor miehei*, *Paecilomyces varioti*, and *Penicillium islandicum* **(Anke et *al.*, 1995).**

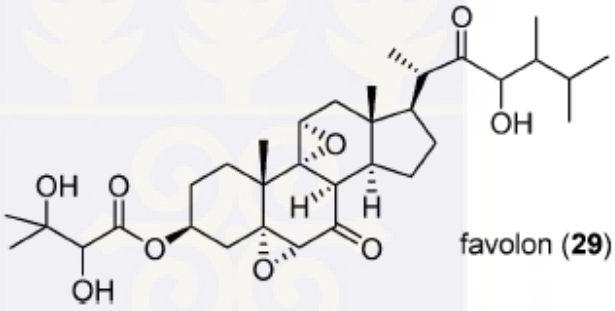
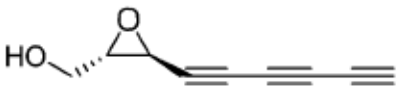


Figure 2.11 Structure of Favolon (**(Anke et *al.*, 1995)**

The antimicrobial compound biformin, isolated from *Trichaptum biforme,* Basidiomycetes is active against a wide variety of bacteria and fungi **(Robbins *et al.*, 1947).**



Biformin

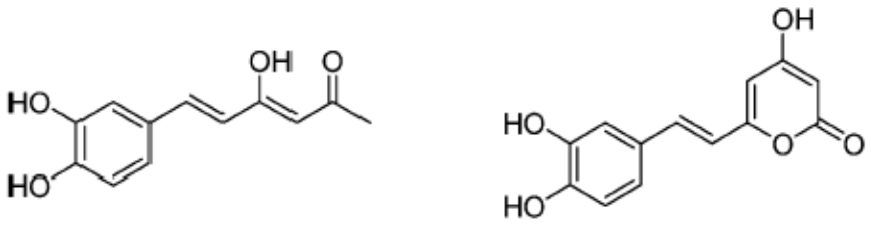
Figure 2.12 Structure of Biformin (Robbins *et al.,* 1947).

2.8.2 Antiviral metabolites

In the review article on mushroom antivirals, Brandt and Piraino, 2000 divided the antiviral compounds from fungi into two major classes. Those acting indirectly as biological response modifiers (usually from polysaccharide fractions) and that act directly as viral inhibitors **(Brandt and Piraino, 2000).**

In Basidiomycetes, several polysaccharide fractions have been found to exhibit inhibitory activity on various viruses. The polysaccharide preparation from *T. versicolor* was found to have an antiviral effect on human immunodeficiency virus (HIV) in vitro **(Tochikura, *et al.*, 1987; 1989).** One of the mechanisms of this effect is due to inhibition of the binding of HIV with lymphocytes. The polysaccharide preparation inhibited reverse transcriptase of avian myeloblastosis virus in vitro **(Hirose *et al.*, 1987).** Also PSK isolated from *T. versicolor* also has been shown to provide protection against exogenous and endogenous infections by the murine cytomegalovirus (MCMV) **(Okada and Minamishima, 1987).**

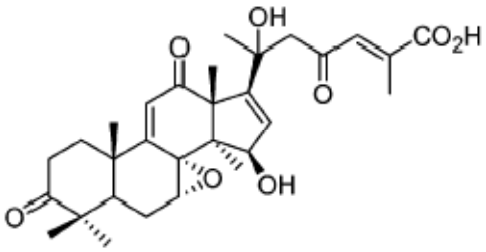
The compounds hispolon and hispidin isolated from the basidiocarps of *Inonatus hispidus* showed considerable antiviral activity against influenza viruses type A and B (**Awadh *et al.*, 2003).**



Hispolon **Hispidin**

Figure 2.13 Structure of Hispolon and Hispidin (Awadh *et al.,* 2003)

Another compound, applanoxidic acid, extracted from *Ganoderma pfeiferri* also showed antiviral activity against influenza virus type A and HSV **(Mothana, *et al.,* 2003).**



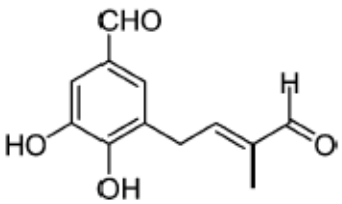
Applanoxidic acid

Figure 2.14 Structure of Applanoxidic acid (Mothana, *et al.,* 2003)

2.8.3 Cytotoxic metabolites

The cytotoxic, antineoplastic, and immunomodulatory activities of extracts from Basidiomycetes are mostly associated with the presence of polysaccharides, although numerous smaller molecular weight cytotoxic metabolites are also known **(Mizuno, 1999; Mizuno *et al.,* 1995).**

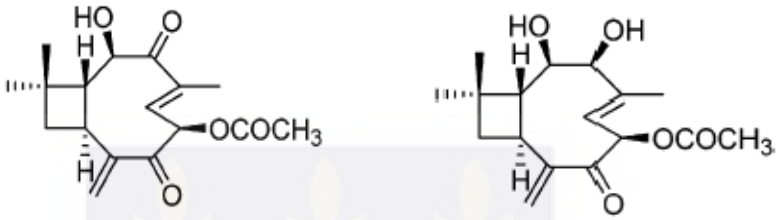
A rare example of a cytotoxic metabolite is montadial A, isolated from *Bondarzewia Montana* **(Sontag *et al.,* 1999).**



Montadial A

Figure 2.15 Structure of Montadial A (Sontag *et al.,* 1999).

Montadial A is cytotoxic against lymphocytic leukemia L1210 cells in mice as well as against promyelocytic human leukemia HL60 cells. Extracts from the basidiocarp of *Panus* species is reported to contain two caryophyllane sesquiterpenes metabolites, naematolon and naematolin **(Lorenzen *et al.*, 1994).**



Naematolon **Naematolin**

Figure 2.16 Structures of Naematolon Naematolin (Lorenzen *et al.,* 1994)

The cytotoxicity of these compounds is probably associated with the presence of an, α, β- unsaturated keto system in their structures. Naematolon possessing two such systems is more cytotoxic than naematolin. Naematolon inhibits the incorporation of thymidine into the DNA of ECA cells. It did not, however, show any significant antitumor activity in vivo with P-388 lymphocytic leukemia, Lewis lung carcinoma, or B-16 melanocarcinoma **(Lorenzen *et al.*, 1994).**

2.9 Regulation of secondary metabolism in fungi

Secondary metabolites (idiolites) are special metabolites usually possessing bizarre chemical structures and although not essential for the producing organism's growth in pure culture, they have survival functions in nature. Secondary metabolites are produced only by some species of a genus. They possess unusual chemical linkages,

such as β-lectam rings, cyclic peptides made of normal and modified amino acids, unsaturated bonds of polyacetylenes and polyenes, and large macrolide rings. Idiolites are produced typically as members of a particular chemical family because of the low specificity of some enzymes involved in secondary metabolism. They include mycotoxins, antibiotics, pigments, and pheromones. An important characteristic of secondary metabolism is that it is usually suppressed by high specific growth rates of the producing cultures.

2.9.1 Intracellular effectors controlling the onset of secondary metabolism

Batch cultures containing nutritionally rich media, high levels of secondary metabolites are usually produced only after most of the cellular growth has already occurred. The growth phase is called the "trophophase" whereas the production phase is termed the "idiophase" **(Bu'Lock *et al.*, 1965).** Expression of the genes coding enzymes for secondary metabolites biosynthesis usually do not occur at high growth rates, suggesting that idiolite synthetases are repressed during growth.

2.9.2 Carbon source regulation

Glucose, usually an excellent carbon source for growth, interferes with the biosynthesis of many secondary metabolites. Polysaccharides, oligosaccharides, and oils are often better carbon sources for production than is glucose. In a medium containing a rapidly used carbon source plus a more slowly utilized carbon source, the former is used first; idiolite production does not occur in this phase. After the favored carbon source is depleted, the second carbon source is used for idiolite biosynthesis.

Carbon source regulation of secondary metabolism exists in most fermentation reactions. The most well known case is the repression of penicillin and cephalosporin production by glucose. In penicillin G formation by Penicillium chrysogenum, glucose represses tripeptide formation from L-a-aminoadipic acid, L-valine and L-cysteine **(Martin and Aharonowitz, 1983).**

Formation of cephalosporin C by *Cephalosporium acremonium* is often delayed until the rapidly utilized sugar (glucose) has been consumed and the slowly utilized sugar (sucrose) begins to be used. In comparisons of individual carbon sources, it was found that readily utilized carbon sources (glucose, glycerol, maltose) yield less antibiotic than do slowly utilized sugars (sucrose, galactose). When different concentrations of glucose are compared, the best antibiotic production occurs at the lowest glucose concentration **(Behmer and Demain, 1983).**

The Cephalosporium fermentation yields two major products, penicillin N and cephalosporin C. Penicillin N is an intermediate in the formation of cephalosporin C but accumulates extracellularly because the enzyme converting it to desacetoxycephalosporin C is a rate-limiting labile enzyme requiring continuous resynthesis throughout the fermentation.

Desacetoxycephalosporin C synthetase (expandase) is the enzyme of the pathway most susceptible to carbon source repression. It does not appear in the mycelium until glucose is consumed and growth has ceased. On the other hand, an earlier enzyme, isopenicillin N synthetase (cyclase) appears during trophophase as does the extracellular intermediate, penicillin N **(Heim *et al.,* 198**

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals and Reagents

Irish potatoes (Mr. Price, Accra Mall - Ghana)

Kanamycin discs and Streptomycin discs (Sigma Aldrich - Germany)

Mycopirox and Cyclopirox olamine (LaGray Chemical Company, Nsawam - Ghana)

P-Anisaldehyde stain (Sigma Aldrich - Germany)

Sephadex LH-20 (GE healthcare – Sweden)

TLC plates (Sigma Aldrich - Germany)

Dextrose (

On Call Plus Glucometer (Acon laboratories - USA)

Nutrient broth, Agar and Sabouraud Dextrose Agar (Liofilchem Diagnostics – Germany)

Solvents used for extraction, column chromatography and thin layer chromatography were of technical quality and were distilled before being used.

Ethyl acetate, methanol, acetonitrile, pet ether, ethanol were purchased from Sigma Aldrich, Germany.

3.2 Strains of Microorganisms

The following test organisms with their designated sources were used for the antimicrobial assay.

S. aureus ATCC.2 (American Type Culture Strain 2)

E. coli NMIMR.3 (Noguchi Memorial Institute of Medical Research Strain 3)

C. albicans KBTH.2 (Korle Bu Teaching Hospital Strain 2)

A. niger ATCC.2 (American Type Culture Strain 2)

3.3 Collection of wood decay fungal (WDF) strains

The inclusion criteria used was that the fungi must be attached to dead wood at the time of collection. After identification of the attached wood decay fungi, the area was cleared and photograph taking while still attached to the decay wood. The detached wood decay fungi were put in tightly sealed containers and brought to the laboratory. All the 54 WDF photographs were matched with the sealed container and then coded into groups using alphabets and numerals such as A1-A9, B1-B9, C1-C9, D1-D9, E1-E9, and F1-F9. The WDF were kept at room temperature until they were ready for culturing.

3.4 Preparation of potato dextrose broth (PDB)

Fresh Irish potatoes were obtained (Mr. Price, Accra Mall), peeled and sliced into pieces. The sliced potatoes were weighed (200g) into 1000 ml flask and 500 ml of distilled water added into conical flask and capped. The content of the flask was autoclaved at 121^0C for 60 min. The cooked potatoes were strained into 1L conical flask and re-autoclaved at 121^oC for 15 min. The volume of the media was topped up to 1L with autoclaved water under aseptic condition. The media was cooled to about 50^0C and 20g of glucose dextrose added. The reduced temperature was to prevent glucose degradation. The PDB medium (200 ml) was put into sterilized 500 ml conical flasks and used for the inoculation of the wood decay fungi (WDF).

3.5 Inoculation of WDF into potato dextrose broth (PDB)

A piece about 5 g of the fruiting bodies of the WDF which were collected and stored in sealed containers were cut under aseptic conditions. The fungal pieces were added to 200 ml PDB medium in a 500 ml conical flask. All the WDF were treated similarly under the same conditions. The fungal cultures were incubated at room temperature (30-34 ^{0}C) for a period of 48 days with daily swirling to allow aeration. Fungal growths of the cultures were terminated with ethyl acetate extraction at the end of the 48th day for extraction of fungal metabolites.

3.5.1 Inoculation of WDF into potato dextrose broth (PDB) for time course strains of WDF.

The time course strains WDF C9 and WDF F7 were inoculated into PDB medium in a similar manner as previously described except that the WDF were cultured in 100 ml of PDB. The flasks were labeled with the appropriate days the cultures will be ready for extraction (Days 3, 7, 9, 14, 18, 22, 27, 32, 40, and 48). The fungal cultures were swirled daily till the 48th day of culture. Cultures were terminated with ethyl acetate extraction at the end of each stated day for the extraction of fungal secondary metabolites.

3.6 Extraction of metabolites from the 54 WDF

The fungal cultures were terminated at the end of their due dates. The extraction solvent used was ethyl acetate. Equal volume of ethyl acetate was added to the final volume of the cultures. Cultures were placed on magnetic stirrer for 24hrs at room temperature (30-34)^{o}C to ensure evenly mixture. The WDF cultures after the 24 hr stirring were poured

into a separating funnel and allowed to settle for 15 minutes for the phases to separate. The aqueous phase was discarded and the ethyl acetate phase kept in sealed flask for evaporation of solvent. The ethyl acetate phase was evaporated to dryness using the rotary evaporator. The fungal secondary metabolites obtained were reconstituted with absolute ethanol and stored at -4^{o}C.

3.6.1 Extraction of metabolites from time course strains

The cultures for WDF C9 and F7 at days 3, 7, 9, 14, 18, 22, 27, 32, 40, and 48 were extracted using ethyl acetate. Equal volumes of the WDF culture and ethyl acetate (10 ml: 10 ml) were added in 50 ml test tubes and capped. The tubes were vigorously shaken for 5 minutes to ensure efficient extraction; the shaking was repeated for another 5 minutes and allowed to settle. The ethyl acetate phases were pipetted into new 50 ml test tubes. Ethyl acetate phase was allowed to evaporate at room temperature and the fungal metabolites were recovered with 20 ul of absolute ethanol into 0.5 ml epindorf tubes and stored at -4 ^{o}C.

3.6.1.1 Glucose clearance determination for time course strains

Glucose clearance determination was conducted for the strains WDF C9 and WDF F7. The aqueous phases that were obtained at days 0, 3, 7, 9, 14, 18, 22, 27, 32, 40, and 48 for the above strains after the extraction of the fungal metabolites were used for the glucose clearance analysis. 100 ul of each WDF culture filtrate was taken into 1.5 ml epindorf tubes and spin in a bench centrifuge at 5000g for 5 minutes. The supernatants were pipetted into 0.5 ml epindorf tubes and used for the glucose clearance test using the On Call Plus Glucometer. The test trip was dipped into each of the supernatant solutions.

The On Call Plus Glucometer gives the reading after 9 seconds count down. The readings from the Glucometer readings were recorded.

3.7 UV-Visible spectroscopy analysis of WDF crude extracts

The crude extracts obtained from the WDF were analyzed spectrophotometrically. Crude metabolites were diluted 1 in 1000 with absolute ethanol and 1000µl measured into a glass cuvette. UV-visible absorbances of the metabolites were read from 200 nm to 900 nm using absolute ethanol as the blank. The chromatographs of the individual profiles were printed.

3.8 Thin layer chromatography (TLC) analysis of 54 WDF

TLC was performed on silica gel, 60 F_{254} aluminum backed plates. Volumes, in ul of the extracts to be examined were spotted unto the TLC plate approximately 1 cm from the bottom of the plate. The spots were dried with a warm current of air and then developed in a saturated glass TLC tank using the appropriate solvent system. Optimization of the solvent system for the extracts was investigated before the final run of the TLC. When the mobile phase had travelled to the required distance, the plates were removed from the TLC tank and allowed to dry. Compounds were viewed under visible and UV light (254 and 365 nm) and then sprayed with Anisaldehyde reagent.

3.9 Preparation of Anisaldehyde staining reagent

The Anisaldehyde reagent was used for unspecific detection of steroids, terpenes, essential oils, bitter principles and saponin drugs. To 135 ml of absolute ethanol was added 5 ml of concentrated sulfuric acid, 1.5 ml of glacial acetic acid and 3.7 ml of *p*-

Anisaldehyde. The solution was stirred vigorously to ensure homogeneity. The TLC plates were then sprayed with this reagent, heated at 100 ^{0}C for 5 minutes and evaluated under visible light.

3.10 Antimicrobial Assay

3.10.1 Preparation of Media for Organism Growth

3.10.1.1 Nutrient Agar (NA) for growth of *S. aureus* and *E.coli*

Nutrient agar prepared according to the manufacturer's instruction.

3.10.1.2 Sabouraud Dextrose Agar (SDA) for growth of *Candida albicans* and *Aspergilus niger*

Sabouraud Dextrose Agar also prepared according to manufacturer's instruction

3.11 Test organisms

- *Staphylococcus aureus ATCC.2*
- *Escherichia coli NMIMR.3*
- *Aspergilus niger KBTH.2*
- *Candida albicans ATCC.2*

3.12 Preparation of WDF extract discs

Empty commercial paper discs were used for the extract disc preparation. The diameter of the discs used was 6 mm. Each disc was placed into a 96 well plates labeled with the corresponding extract names. WDF extract discs were prepared by adding 20 µL of each

WDF extract onto a sterile antibiotic disc in the wells and allowed to dry. After the discs were dried, another 20 ul of the WDF extracts were pipetted onto the paper discs, making up to 40 ul. The discs were allowed to dry and were stored at room temperature until they were ready to be used.

3.13 Turbidity standard for inoculum preparation

For the bacterial suspension, 3×10^7 cell /ml was prepared, compared to that of the McFarland standard. For the filamentous fungi and yeast-like fungi, a suspension containing 1×10^6 spores or cells per ml was prepared by using the Neubauer Counting Chamber.

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard was used.

3.13.1 A $BaSO_4$ 0.5 McFarland standard may be prepared as follows:

1. A 0.5-ml aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2$. $2H_2O$) was added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.
2. The correct density of the turbidity standard was verified by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm was 0.0089 which is within the range (0.008 to 0.10) for the 0.5 McFarland standard
3. The Barium Sulfate suspension was transferred in 6 ml aliquots into screw-cap tubes of the same size as those used in growing the bacterial inoculum.
4. These tubes were tightly sealed and stored in the dark at room temperature.

5. The barium sulfate turbidity standard was vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance.

3.14 Preparation of bacterial and fungal inoculum

- Well-isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 5 ml of nutrient broth medium.
- The broth culture was incubated at 35°C until it achieved the turbidity of the 0.5 McFarland standard
- The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to that of the 0.5 McFarland standard

3.15 Inoculation of Test Plates

- Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level.

- The dried surface of the Nutrient agar plate was inoculated by streaking the swab over the entire sterile agar surface. This was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum.

3.16 Application of Discs to Inoculated Agar Plates

- The already prepared discs from the WDF extracts examples (A1-A9) were dispensed onto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface.
- Commercial antibiotic discs, Streptomycin, Kanamycin and Cyclopirox olamine (product from LaGray Chemical Company) were used as a positive control while self prepared ethanol and ethyl acetate discs were used as negative controls.

3.17 Reading Plates and Interpreting Results

After 18 to 24 hours of incubation, each plate was examined for the inhibition of zones. Inhibition zones were measured with Muchito digital vernier caliper.

3.18 Refermentation of HITS WDF from the primary screen

Wood decay fungi (WDF) that showed activity on not more than two test organisms were refermented as previously described. In this refermentation (secondary screen) large volumes (1L) of PBD medium were used for the culture also for a period of 6 weeks. Preparation of PDB and inoculation of WDF into the PDB was done as previously described.

3.19 Extraction of secondary metabolites from refermented WDF strains

The refermented fungal cultures were ended at the end of the 48th day of culture. Ethyl acetate was the extraction solvent used. In this process, equal volume of ethyl acetate was added to the cultures as described previously. There was a modification of the

extraction procedure. The cultures were put into separating funnel and vigorously shaking for 5 minutes and allowed to stand for 15 minutes to settle. This was repeated three more times to enable all secondary metabolites to be collected in the ethyl acetate phase. The aqueous phase was separated from the ethyl acetate phase and then discarded and ethyl acetate phase kept in sealed flasks for further processes.

3.20 Recovering of refermented WDF extracts from ethyl acetate phase

The ethyl acetate phase was evaporated to dryness using the rotary evaporator. The flasks used for the recovery were weighed (g) before and after recovery to estimate the weight of the extracted secondary metabolites. The volumes of secondary metabolites recovered were also estimated to enable their densities determined. The fungal secondary metabolites obtained from the refermented WDF were constituted in sealed tubes and stored at -4^{o}C.

3.21 TLC analysis of 27 refermented WDF

TLC was performed on silica gel, 60 F_{254} aluminum backed plates. Volumes, in ul of the refermented extracts to be examined were spotted unto the TLC plate approximately 1 cm from the bottom of the plate. The spots were dried with a warm current of air and then developed in a saturated glass TLC tank using the appropriate solvent system. When the mobile phase had travelled to the required distance, the plates were removed from the TLC tank and allowed to dry. Compounds were viewed under visible and UV light (254 and 365 nm) and then sprayed with Anisaldehyde reagent.

3.22 Bioassay of 27 refermented WDF extracts

The 27 WDF extracts that were recovered after the secondary fermentation were bioassay using the disc diffusion method as previously described. Filter paper discs with containing 5mg/ml of fungal extracts were prepared in a similar manner as previously stated. Media preparation for test organisms, turbidity standard for inoculum, fungal and bacterial inoculum preparation, inoculation of test plates, application of disc to inoculated plates and the reading of plates and their interpretations were done similarly as stated above.

3.23 LH-20 chromatography

LH-20 slurry was prepared by weighing 20 g into 80 ml methanol and swirled for uniform mixture to obtain a column volume of 80 ml. A column length of 16 cm was used, and the column was stocked with glass wool at the base. A filter paper disc about the diameter of the column was put on top of the glass wool. The column was loaded by pouring the slurry gently into the column with gently striking of the column with a plastic rod to ensure evenly packing of the slurry. Two filter paper disc about same diameter as the column was put on top of the slurry on which the samples will be put. After packing, the column was filled with the eluting solvent and run to the interphase of the two filter paper disc.

3.24 Separation of WDF extracts on LH-20 column

A 1ml (1.25%) of column volume of WDF extract was carefully pipetted onto the filter paper disc and allowed to slowly flow into the column with very small portions of

solvent by opening the tap on the column gently until it reached the interphase. To ensure good separation of extracts, the flow rate was kept very low, approximately **1** ml/min. When all the extracts had been washed from the paper disc at the top of the column, the rate was increased to obtain the appropriate flow rate.

Samples were collected in conical graduated test tubes. Three fractions were collected as void volumes and set aside. Ten fractions were collected for each extract. At the end of the tenth collection, the column was then washed with the eluting solvent (methanol) to remove any remaining contaminants retained by the column.

3.25 Recovery of LH-20 fractions from WDF extracts (A4, B6, B7, E2, E9, and F3)

The 10 fractions obtained from the above extracts were evaporated using the rotary evaporator, Rota vapor Buchi 111 as previously described. The recovered samples were kept in 1.5 ml epindorf tubes and stored at -4^{0}C until ready for bioassay.

3.26 Bioassay of WDF extracts A4, B6, B7, E2, E9, and F3 fractions

The recovered fractions obtained from the above extracts were bioassay for their antimicrobial and antifungal properties. The filter paper disc containing extract fractions were constituted into 5mg/ml concentration as previously stated. The bioassay procedure was similar as already described.

3.27 TLC analysis of LH-20 WDF extracts fractions

The TLC analysis for the individual fractions was the same as previously described.

CHAPTER FOUR

RESULTS

4.1 Collection of Wood Decay Fungi (WDF)

The wood decay fungi (WDF) were collected mainly from University of Ghana campus and the surrounding areas the period of October 2009 and April, 2010. The selection criteria used was to find the wood decaying fungus attached to a piece of dead wood at the time of collection. Photographs of the wood decay fungi were taken prior to detachment of the fungus from the dead wood. Storage of the individual samples was kept in separate and tightly sealed plastic containers.

In total, 54 WDF were collected , with five of the WDF (B6, B7, B8 B9 and C1) obtained outside the University campus, namely Aburi forest, Cape Coast, Lashibi in Community 19 and 16, and Ridge Hospital, Accra respectively. Fungus B7 was detached from a wooden window frame in Cape Coast whilst that of B8 was obtained at Lashibi in Community 19 from a wooden beam on the roof of a building.

Majority of the wood decay fungi collected from the University campus and it surrounding areas were found in a humid environment but very few of the wood decay fungi were collected from the University community were found in moist places. Three of the five WDF obtained outside the university were found in a very dry environmental conditions (B8, B9 C1), B8 was collected from a dry and dusty environment 2 km from a lagoon and B9 was also obtained from a dry and dusty environment near a lagoon. However the WDF B6, which was collected from the Aburi forest, was from a moist environment. Four of the WDF (F3, F4, F5, and F9) that were obtained around the

university campus possessed fresh basidiocarp at the time of collection; however, within few days in storage, the basidiocarp had dried up leaving dried pieces of fruiting body. Photographs of the WDF that were taken at the various sources were matched with the samples in sealed containers to confirm they are the same, and then coded using alphabets and numerals as follows A1-A9, B1- B9, C1-C9, D1-D9, E1-E9 and F1-F9 making up to 54 . After the coding of the WDF, they were again grouped into a particular cluster based on common identity existing among them. Overall, there are eleven (11) clusters with the corresponding members of each cluster positioned at the base of the representative picture for that cluster. The pictures displayed in Figure 4.1(A and B) are the photographs of the WDF grouped in clusters, with the different WDF belonging to each cluster. It must be emphasized that each cluster is completely different from the other morphologically.

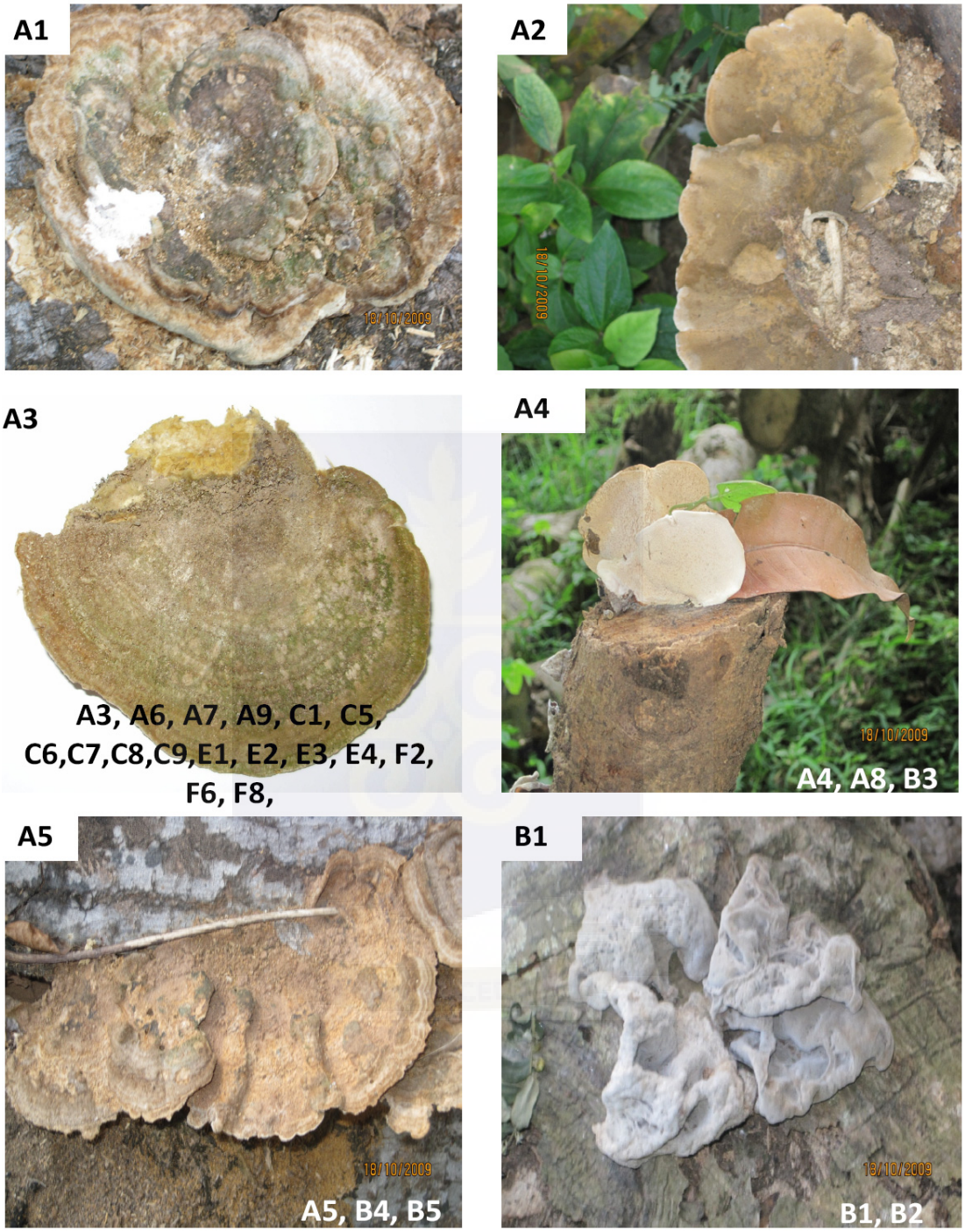


Figure 4.1A. Photographs of Wood Decay Fungi (WDF) taken from their site of collection. *Photographs are grouped into clusters (**A1, A2, A3, A4, A5 and B1**). Members belonging to a single cluster share some common morphological features. Below each cluster are the codes indicating WDF belonging to the same group.*

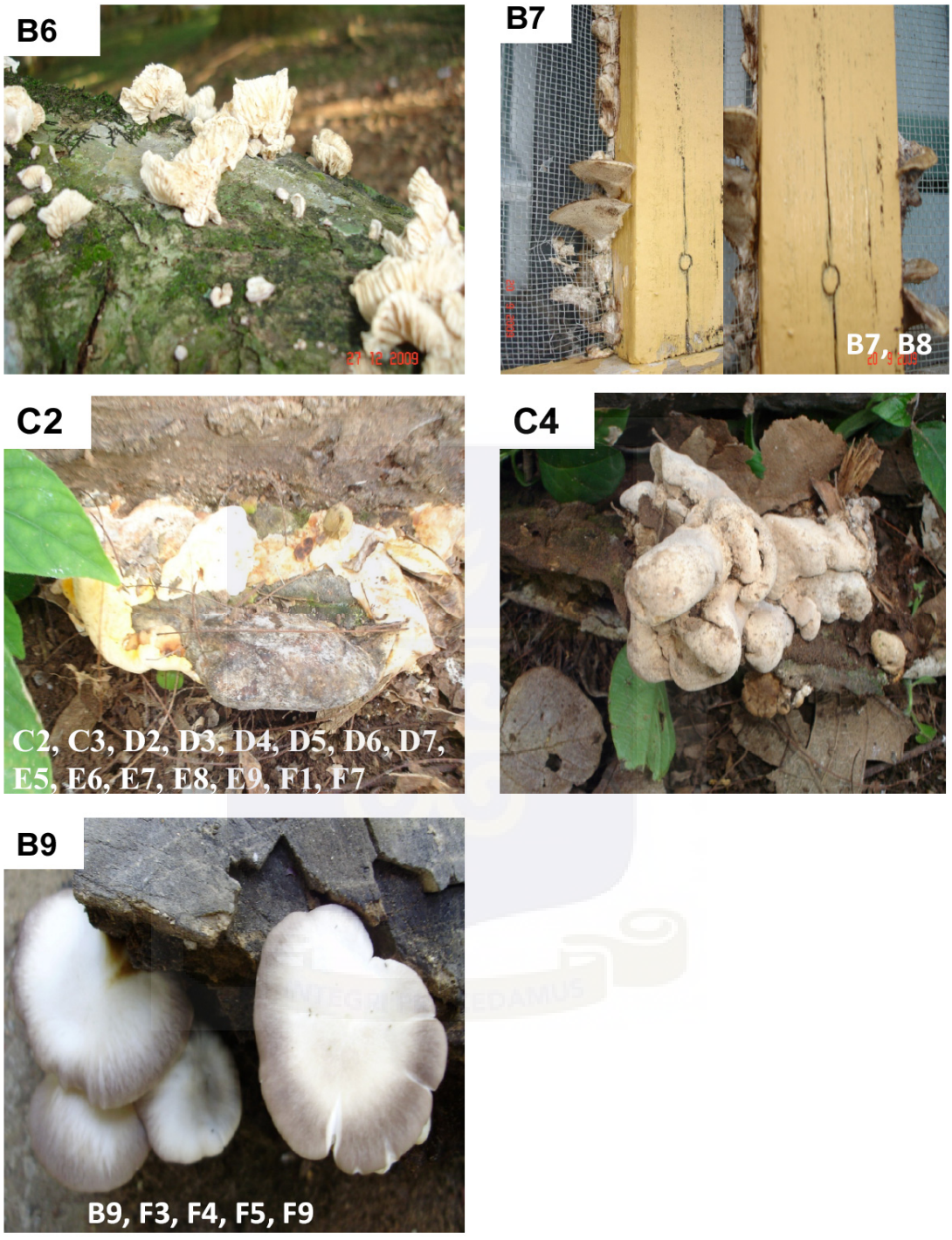


Figure 4.1B. Photographs of Wood Decay Fungi (WDF) taken from their site of collection. *Photographs are grouped into clusters (**B6, B7, C2, C4, and B9**). Members belonging to a single cluster share some common morphological features. Below each cluster are the codes indicating WDF belonging to that group.*

4.2 Culturing of Wood Decay Fungi (WDF) in Potato Dextrose Broth (PDB)

The WDF were inoculated into the PDB medium for 48 days and fungal cultures were kept at room temperature and swirled daily for aeration. At the 3rd day of culture, all the cultures started foaming and bubbling which continued till the 14th day of culture, after which foam and bubbles disappeared. However, the following cultures (A1, A2, A4, B7, E1, E4, E5, F2, F5, F7 and F8) continued bubbling and forming till the 18th day of culture before foams and bubbles disappeared.

Colour changes during the period of culturing were observed for most of the fungal cultures. By the 7th day of culture, the following WDF (A3, A4, A5, A7, A8 and A9) cultures had changed colour from cream to brown, and this brown colouration was maintained throughout the culture period. The WDF B6 by the 5th day of culture had changed colour to light yellow and samples B7 and B8 also changed colour to dark brown by day 7th of culture and these entire colour changes were also maintained till the end of culture. The WDF C1 of culture had changed to pink on the 28th day and this pinkish coloration was maintained till the end of culture.

During the culturing phase, it was also observed that the following WDF, B7, B8 and A5 had developed a thick mass on the surface of the cultures at the following days of culture 14th, 21st and 28th respectively. The thick layer formed remained till the end of the culture period which might be due to a dense stuck of mycelium that were produced during the culturing phase.. Cultures were terminated with ethyl acetate extraction at the end of the 48th day of culture.

4.3 Extraction of secondary metabolites from WDF

After the 48th day of fermentation, fungal cultures were terminated with ethyl acetate extraction and the solvent evaporated to dryness to obtain the crude extracts. The crude fungal extracts were recovered using absolute ethanol and stored in the refrigerator at -4^{0}C until further analysis.

The extracts obtained from the WDF exhibited some colours at the time of recovery. Three major colours were observed for the fungal extracts after evaporation, namely, yellow, brown and red. The yellow coloration was exhibited in the following WDF, A1, A4, A6, A6, B2, B3, B4, B5, B9, C2-C9, D1-D6, D8, D9, E1-E9 and F1-F9. However, the intensity of the yellow colours observed in WDF D5, D8 and F3 was much greater when compared with the other WDF that showed the same colour. Extracts from WDF A2, A3, A5, A8, A9, B1, B6, B7 and B8 also exhibited brownish colours after recovery while WDF C1 was the only one that produced a reddish extract after recovery.

The yield (volumes) of WDF extracts obtained for each sample after recovery is shown in Table4. 1. The ethyl acetate crude extract yields obtained from the 54 WDF cultured in 200 ml PDB for 48days ranges from 0.30ml to 2.0ml. The following WDF C7, E5, F2, F4, and F6 gave the highest yield of 2.0ml each. The lowest crude extract yield was given by the fungus B3 with a volume of 0.25ml, however it must be noted that about 50% of all the WDF extracts recovered gave yields below 1.0 ml as depicted in Table 4.1.

Table 4.1. Volumes of extract from 54 WDF cultured in PDB for 48 days per 200 ml culture.

Code	A	B	C	D	E	F
1	0.65	1.40	0.85	1.00	1.10	1.05
2	0.42	0.85	0.35	0.90	1.60	2.0
3	0.45	0.25	1.05	0.70	1.80	1.80
4	0.43	0.35	0.80	0.92	1.20	2.00
5	0.50	0.40	1.00	0.90	2.00	1.00
6	0.45	0.52	1.60	0.70	1.00	2.00
7	1.10	0.55	2.00	1.00	1.50	1.00
8	0.50	0.55	0.75	1.00	1.50	1.00
9	0.30	0.35	1.10	1.20	1.00	1.00

Wood Decay Fungi (WDF) was cultured in PDB for 48 days in 500ml flasks. Crude extracts yield were calculated from the volume of extracts obtained after deducting recovery solvent volumes from the final volumes.

4.4 Time series analysis of WDF secondary metabolites

In order to determine the time points during culture period at which the fungal secondary metabolites are produced, and also periods at which bioactive fungal metabolites are being generated, a time course study was carried out to investigate these two parameters. The time course analysis was conducted for the WDF C9 and F7 and this was carried out after the culturing of all the 54 WDF. This is because a previous random culture of WDF for this purpose failed and could not give a good TLC profile, therefore the selection of the two samples C9 and F7 after the general culture.

The samples were fermented using the procedure as previously described. The cultures were made to last for 48 days after which it is assumed most if not all, secondary metabolites will be produced. Cultures were terminated with ethyl acetate extraction. The cultures were extracted with ethyl acetate at the following days of culture 3, 7, 9, 14, 18, 22, 24, 32, 40, and 48. The extraction procedure for the time series samples differed slightly from the general 54 WDF extraction. In the time course extraction, cultures were not put on magnetic because of their smaller volumes (10 ml), but rather put in Folin Wu's tube. Equal volume of ethyl acetate was added and shaken vigorously and allowed to settle. The solvent phase kept in new test tubes and stored at room temperature to allow solvent to evaporate, leaving the crude extracts in the test tubes.

Once the solvent was evaporated, the crude extracts obtained from samples C9 and F7 from 3rd day to 48th were subjected to TLC. The TLC profiles generated for the WDF C9 and F7 are shown in the Fig 4.2A (B and C). The TCL profiles for both C9 and F7 detected at UV- 254 nm and 365 nm show the patterns for the components from the fungal extracts at the different time points. At UV-254 nm, extract C9 at day 3 produced a compound that absorbs only at the sample origin and so was for extract at day 22 of

culture. Extracts from cultures at days 7 and 27 also produced UV-254 nm absorbing compound at both the sample origin and the solvent front. Day 9 extract produced a compound that absorbs only at the median region. Days 14 and 18 also produced compounds absorbing at both the sample origin and the median region. Days 32, 40 and 48 produced compounds that absorb at all the three polarity regions, sample origin, median region and the solvent front. However at UV-365 nm, there were no fluorescence for extracts at days 3, 7, 27, and 32; however, day 9 produced a fluorescing compound at both the sample origin and the median region. Days 14, 18, 22, 40 and 48 all produced compound that fluoresces only at the sample origin.

In F9 extracts, days 3 and 14 produced compounds that absorbs only at the sample origin. Days

18, 22, 27, 32, and 40 produced absorbing compounds at both the sample origin and the solvent front. In days 7, 9, and 48, the compounds produced absorbs at all the polarity regions. At UV-365 nm, day 3 produced that fluoresces only at the solvent front. Days 7, 9, 22, and 27, produced no fluorescing compound, but days 14 and 40 produced compounds that fluoresces at all the polarity regions. Day 18 also produced fluorescing compound at both the sample origin and the median region, whilst day 48 produced a fluorescing compound at both the solvent front and the median region. From the TLC profiles of the two WDF, both at UV-254 nm and UV-365 nm, there was no regular pattern of compounds, suggesting that the periods at which a bioactive was produced and so the time of production cannot be determined by TLC. And therefore an assay for bioactivity was necessary to determine the periods of active metabolites production.

In order to determine the periods at which fungal metabolites were generated, antimicrobial activities of the WDF C9 and F7 extracts were tested against *S. aureus*, *E.*

coli and *C. albicans* as shown in Fig. 4.2B. The antimicrobial activity of WDF C9 extract against *S. aureus* started from the 7th day of culture and reached it maximum on the 22nd day of culture then remained uniform till day 48 of culture Fig. 4.2B (C). However the antimicrobial activity for *E. coli* started on the 9th day of culture, reached it maximum on the 32nd day of culture Fig.4.2B (C). The inhibition zones achieved for *S. aureus* were greater than for *E. coli.*

The antimicrobial activity of WDF F9 extract against *S. aureus* started from the 7th day of culture, maintained a steady growth until the 48th day of culture Fig. 4.2B (D). Antimicrobial activity against *C. albicans* was the same from the 7th day of culture and again maintained a steady growth till the 48th day of culture. However, it must be emphasized that the inhibition zones obtained for the *S. aureus* were higher than for *C. albicans* Fig. 4.2B (D).

Glucose clearance test was also performed on the culture filtrates obtained after the extraction of secondary metabolites from WDF C9 and F7. The purpose of this test was to determine if the glucose which was added during the broth preparation is being utilized by the fungi, and if it is, at what point in culture is it cleared. The filtrates from the individual WDF extracts on days 0, 3, 7, 9, 14, 18, 22, 24, 32, 40, and 48 were spin on a bench centrifuge to obtain supernatants.

Initially, the Folin Wu's glucose determination method was being employed for the glucose clearance analysis. However several attempts to use this method failed. The reactions were supposedly interfered by the copper sulphate in the Folin Wu's reagent and the compounds from the fungal extracts inhibiting the generation of the coloured

component in the Folin Wu's reaction. Therefore, On Call Plus Glucometer was finally purchased and used for the glucose clearance analysis.

The glucose clearance test using the On Call Plus Glucometer was carried out by dipping the test trips into the individual supernatant, which were sucked in by capillary action. The On Call Plus Glucometer gives the reading after 9 seconds count down. The results shown in Fig. 4.24 (A) indicate that at day 0 of culture, the amount of glucose read was 550 mmol/L. The amount of glucose reduced sharply by day 3 of culture, which could be attributed to active metabolic processes of the WDF Fig. 4.2A (A).By day 22 of culture, the amount of glucose in the culture medium is almost undetectable, indicating that the amount of the glucose used had been used up and cleared from the culture medium.

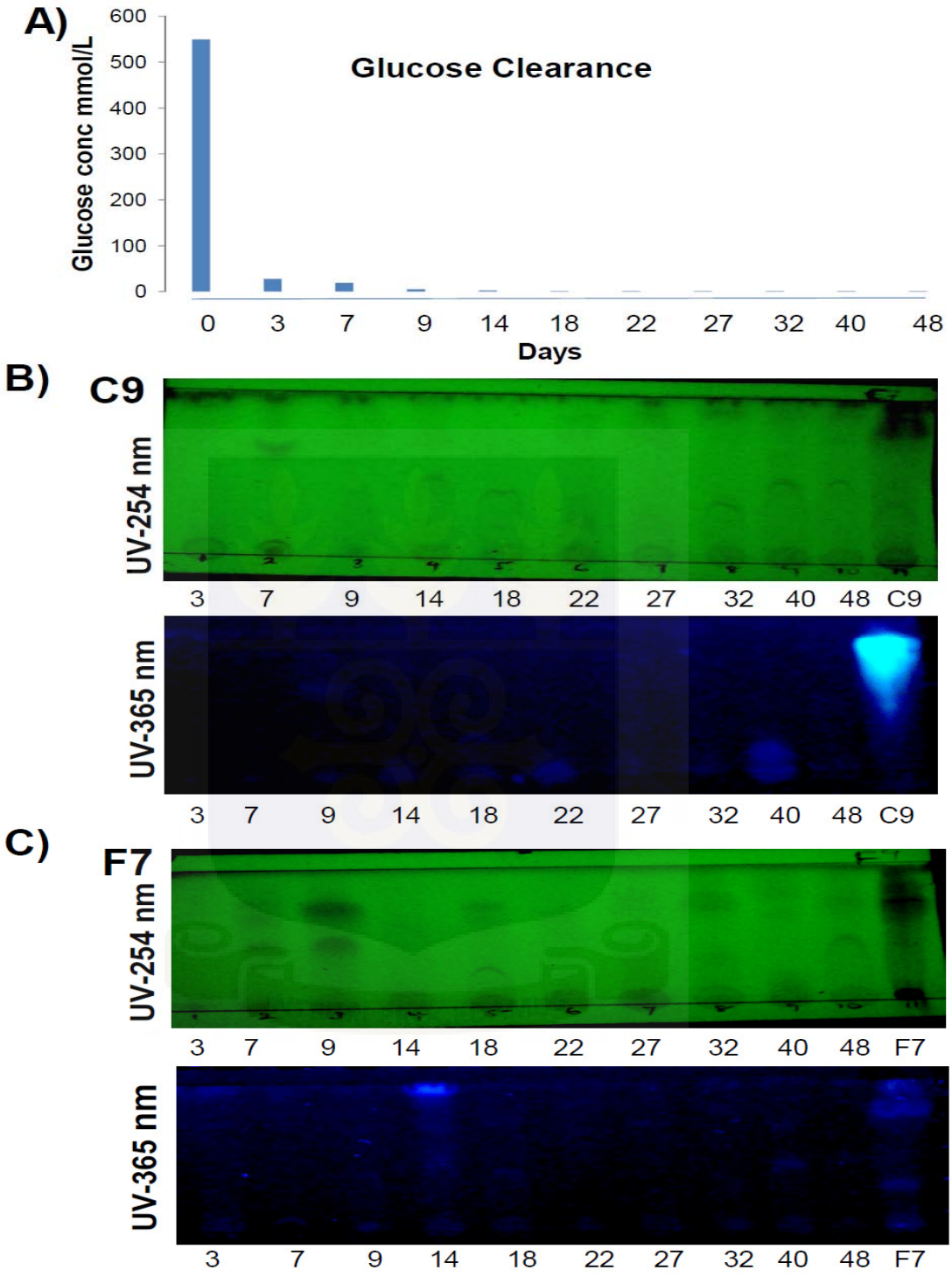


Figure 4.2A. Glucose clearance and TLC analysis of secondary metabolites from WDF C9 and F7. *Glucose clearance profile for time course samples and their TLC profiles (A) Glucose utilization (mmol/L) test for samples at stated days of culture. (B) TLC profile for WDF C9 at wavelengths UV- 254 nm and UV- 365 nm. (C) TLC profile for WDF F7 at wavelengths UV- 254 nm and UV- 365 nm*

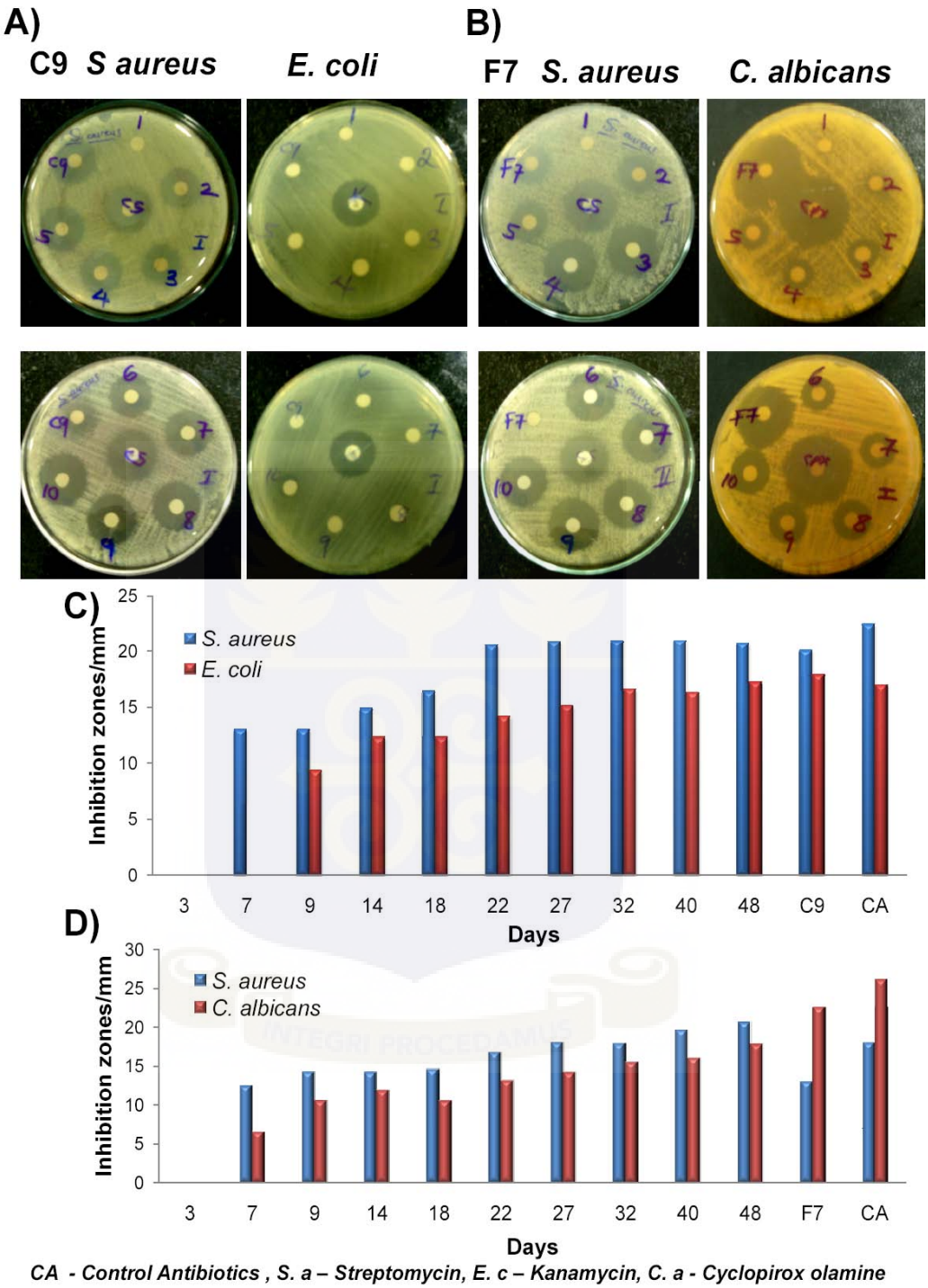


Figure 4.2B.Time series analysis conducted on secondary metabolites from C9 and F7. *Time course antimicrobial activity of WDF C9 and F7 cultured for 48 days on S. aureus, E. coli and C. albicans. (A). Plates showing inhibition zones of C9 extract on S. aureus and E. coli. (B). Plates showing inhibition zones of F7 extract on S. aureus and C. albicans. (C). Time course antimicrobial activity of C9 extract on S. aureus and E. coli. (D). Time course antimicrobial activity of F7 extract against S. aureus and C. albicans.*

4.5 UV/Vis Spectroscopy analysis of extracts from Wood Decay Fungi (WDF)

Compounds extracted from all the 54 WDF cultures were measured spectrophotometrically for their absorbance profile in the UV-Visible spectrum (200 - 900 nm). For each extract, 10 ul was diluted 1: 1000 in absolute ethanol; the UV/ visible absorbance of the extracts were measured in glass cuvette from 200nm to 900nm using absolute ethanol for blanking. UV/Visible spectroscopy profiles were obtained for each extract analyzed unique although some were similar. The extracts with similar profiles were therefore grouped into clusters according to peculiar features showing on each profile. The 4 major clusters were formed as shown in Fig. 4.3. The four major clusters are A1 (2/54), A2 (21/54), A5 (19/54) and C7 (12/54). The A1, A2, A5, and C7 clusters had the following percentage members of 3.7%, 38.9%, 35.2% and 22.2% respectively for all the 54 WDF extracts analyzed. Members of A1 cluster have two major peaks absorbing at wavelengths between 254nm and 367nm. Clusters A2, A5 and C7, have single peaks absorbing at wavelengths 287nm, 288nm and 288nm respectively. Although clusters A5 and C7 had major peaks absorbing at the same wavelength 288nm, however there is a striking difference between the spectrophotometric profiles of these two categories (Figure 4.3), A5, has a smaller absorbing peak at wavelength 400 nm.

There is a greater chance for members belonging to a particular cluster to have similar active components absorbing at such wavelength. In A1 cluster, absorption of compounds start at the origin, this was also observed in A2 cluster (Figure 4.3). In A5 cluster, absorption of the components starts at points above 0.100 whilst that of C7 starts around 0.060. The A5 cluster had an absorbance of 0.334 at wavelength 288 nm and that of C7 cluster was absorbed at 0.120 at wavelength 288 nm. The absorption starts points was also found to be slightly varied among all the extracts analyzed.

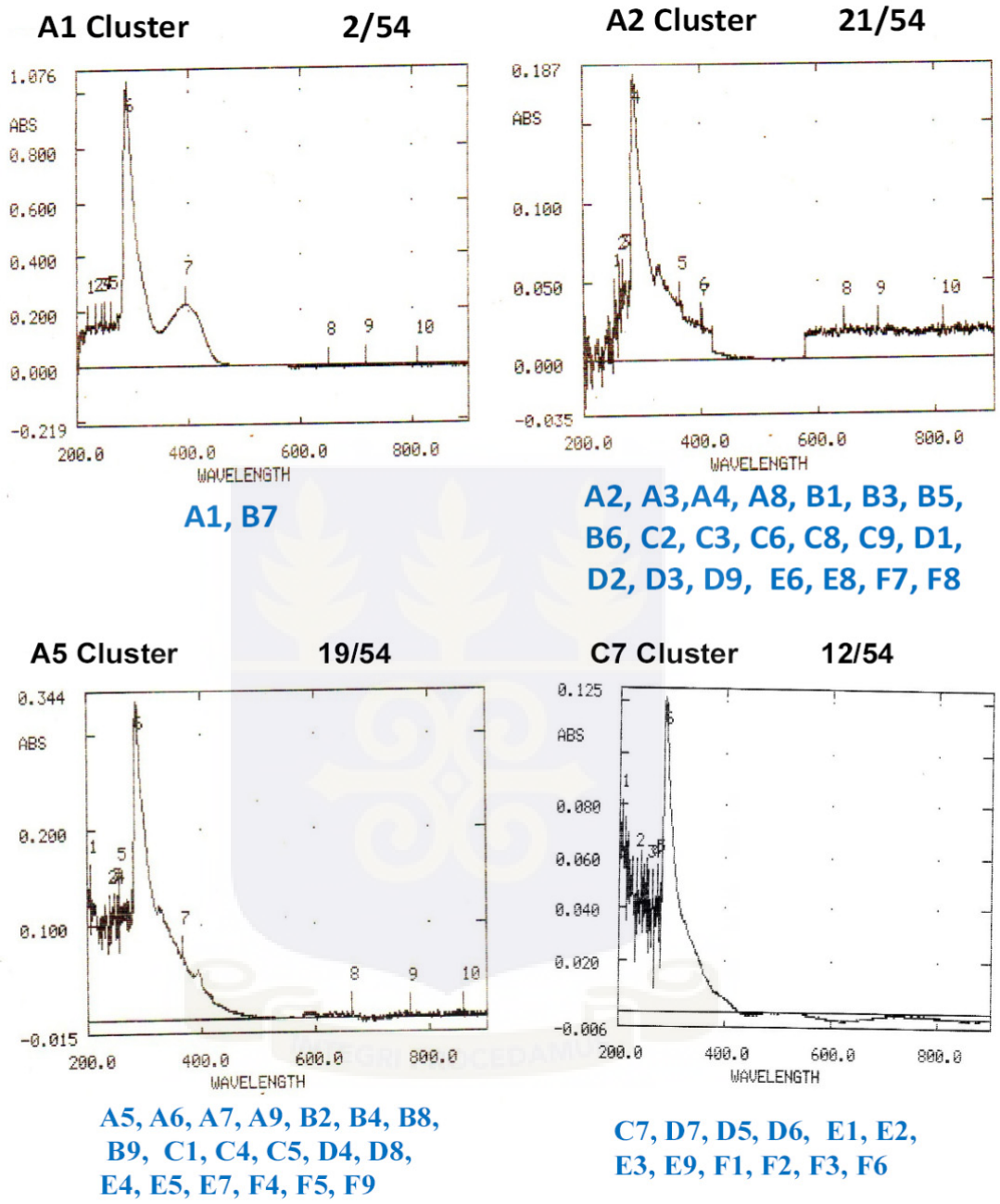


Figure 4.3. Profiles of UV/Vis spectroscopy analysis of secondary metabolites from WDF. *Clusters showing UV-visible absorbance of metabolites extracted from WDF. UV-visible Spectrophotometric scanning of fungal extracts measured at 200 nm to 900 nm. Profiles are grouped into cluster A1, A2, A5 and C7, each cluster showing members with similar spectrophotometric profile.A1 cluster represented by A1 and B7, with two major UV absorbing peaks at wavelengths 254nm and 367nm. A2 cluster represented by A2, A3,A4, A8, B1, B3, B5, B6, C2, C3, C6, C8, C9, D1, D2, D3, D9, E6, E8, F7and F8 with UV absorbing peak at 287nm. A5 cluster represented by A5, A6, A7, A9, B2, B4, B8, B9, C1, C4, C5, D4, D8, E4, E5, E7, F4, F5, and F9 with UV absorbing peak at 288nm. C7 cluster represented by C7, D7, D5, D6, E1, E2, E3, E9, F1, F2, F3, F6 with UV absorbing peak at 288nm. A1, A2, A5 and C7 clusters had percentage members of 3.7%, 38.9%, 35.2% and 22.2% respectively of the total WDF analyzed.*

4.6 Thin layer chromatographic analysis of metabolites from WDF

TLC analysis was performed to determine the number of different components presents in the crude WDF extracts. The analysis of the compounds from the WDF extracts were routinely done by detection on TLC using UV-254 nm (fluorescence shadowing), UV-365 nm (fluorescence) of separated zones. As the number and identity of the WDF extracts component was not known at the time, there was the need for the optimization of a suitable solvent system and the amount of WDF extracts to be spotted on the TLC plates in order to achieve better separation.

TLC was performed as described in the previous section by spotting varying volumes of fungal extracts. The following solvent systems ; EtOAc: PetEth (8:2), EtOAc: PetEth (0.4:9.6), EtOAc: PetEth (9:1), EtOAc: H_2O: PetEth (7:2:1), EtOAc: MeOH (8:2), EtOAc: MeOH: EtOH (8:1:1), EtOAc: MeOH: EtOH: PetEth (7:1:1:1), EtOAc: HCOOH: PetEth (9:0.5:0.5), EtOAc: HCOOH: H_2O: PetEth (8:0.5:0.5:1), EtOAc: $CH_3(CH_2)_3OH$: PetEth (8:0.5:1.5), EtOAc: $CH_3(CH_2)_3OH$: PetEth (7:0.5:2.5), EtOAc: $CH_3(CH_2)_3OH$: PetEth (7:1:2.) and EtOAc: CH_3CN: PetEth (7:2:1) were investigated before a suitable system was obtained.

After a laborious optimization process, EtOAc: CH_3CN: PetEth (7:2:1) was found to be the most suitable solvent system, which yields good separation of the different components of the fungal extracts that were run and therefore was used to run the TLC for all the 54 WDF extracts. After the TCL run, this mobile phase which has a dielectric constant of 116, resulted in the separation of components in the WDF crude extracts at the sample origin and the solvent front. A lot of the components of the extracts was observed to fluoresce at UV-365 nm whiles others were detected at UV-254 nm (Figure 4A i and ii). After TLC plates were sprayed with Anisaldehyde reagent, some fungal

extract components were also observed as coloured spots (Fig. 4.4A i). The intensity of the spot on the TLC plate is a rough measure of the concentration of that component in the mixture. It was clear that the ethyl acetate extracts from the WDF had components present in varying concentrations as different intensities of the components were observed under UV light.

Anisaldehyde reagent is known for the detection of phenols, sugars, terpenes and steroids. Spraying TLC plates with Anisaldehyde reagent revealed several coloured components Fig 4.4. (A (i), B (i), C (i) and D (i)). These colours are associated with specific functional groups. The coloured components observed were yellow, blue, pink, violet, brown and green. From literature, phenolic compounds are detected by the presence of violets spots after spraying with Anisaldehyde reagent, **(Wagner *et al.*, 1984).** Sugars are also detected by the appearance of yellow colours. Carbohydrates (aldohexose, ketohexose, and aldopentose) and uronic acids have also been reported to produce brown spots, yellow spots, green spots and pink spots respectively after Anisaldehyde spray. Terpenes and steroids are found to be associated with blue and green spots respectively. Classes of terpenoids, the triterpenes that can be specifically detected by Anisaldehyde reagent is the cardiac glycosides **(Harbourne 1973, Wagner *et al.*, 1984).** Cardiac glycosides are detected by the appearance of pink zones in visible light after Anisaldehyde spray.

4.6.1 Classes of diverse compounds detected by UV-254 nm based on their relative polarities

In the determination of the relative polarities of the various compound generated by the WDF extracts, the following parameters were used which are compounds at solvent

front (as low polarity), plate median (medium polarity), whilst those at the sample origin (represent the highest polarity), These parameters were used to classify the different extracts according to the appearance of their compounds on the TLC plates.

A total of 54 WDF extracts were analyzed on TLC plates and visualized at UV-254 nm, none of the extracts produced UV254 absorbing compounds at the solvent front (low polarity) or the plate median (medium polarity) only. Again, none of the extracts produced U-V254 absorbing compounds both the solvent front and the median region. However, even (7) WDF extracts were found to produce UV absorbing compounds at the sample origin only. Absorption at the sample origin suggests that such compounds are highly polar and therefore could not move up the mobile phase of the solvent system. Twenty (20) extracts produced compounds that absorb UV at both the sample origin and the solvent front only. At both the sample origin and the plate median, only 6 extracts produced compounds that absorb UV at 254 nm. At the sample origin, plate median, and the solvent front, 21 extracts again produced UV absorbing compounds at UV-254 nm.

The WDF extracts that showed absorption at the sample origin are, D4, E5, E7, E7, E8 and F3, at both the sample origin and the solvent front, the following WDF extracts (A7, B1, B2, B3, B5, B9, C2, C3, C6, C7, C8, D6, E1, E2, E3, E4, E9, F2, F4, and F8) produced UV absorbing compounds. The following WDF extracts (A1, A9, D2, D3, D5 and F1) also showed compounds at both the sample origin and the median region. WDF extracts that produced compounds at all the three polarity regions, sample origin, median region, and the solvent front are, A2, A3, A4, A5, A6, A8, B4, B6, B7, C1, C4, C5, C9, D1, D7, D9, F5, F6, F7 and F9.

4.6.2 Classes of diverse compounds detected by UV-365 nm based on their relative polarities

In the determination of the relative polarities of the various compound generated by the WDF extracts, the following parameters were used which are compounds at solvent front (as low polarity), plate median (medium polarity), whilst those at the sample origin (represent the highest polarity), These parameters were used to classify the different extracts according to the appearance of their compounds on the TLC plates.

At UV-365 nm out of the 54 WDF extracts, twenty (20) extracts were estimated to produce compounds that fluoresce only at the solvent front. None of the extracts were able to produce a fluorescing compound only at the sample origin, however, at the plate median, 4 WDF extracts produced a fluorescing compounds. Again none of the extracts produced compounds that absorb at both the sample origin and the median region. Only 2 extracts produced fluorescing compound at both the sample origin and the solvent front. At both the solvent front and the plate median, 9 extracts produced fluorescing compounds. At the sample origin, plate median, and the solvent front, 14 extracts again produced fluorescing compounds at UV-365 nm.

The following extracts (B2, B5, B9, C1, C2, D4, D5, D6, D7, D8, E1, E4, E6, E7, F1, F2, F4, F6, and F7) produced the fluorescing compounds at only the solvent front. The compounds that fluoresced at only the plate median were produced by the extracts C8, D1, E2, and E3. At both the sample origin and the solvent front, the fluorescing compounds were also produced by extracts A3 and A7. The following extracts, C4, C5, C6, C9, D9, F3, F5, F8, and F9. These extracts, A1, A2, A4, A5, A6, A8, B1, B3, B4,

B6, B7, B8, C7, and D3 also produced fluorescing compounds in all the polarity regions, thus, sample origin, plate median and the solvent front. However extract B7 alone was able to produce a compound that absorbed UV at 365 nm close to the solvent front.

4.6.3 Classes of diverse compounds detected by Anisaldehyde based on their relative polarities

In the determination of the relative polarities of the various compound generated by the WDF extracts, the following parameters were used which are compounds at solvent front (as low polarity), plate median (medium polarity), whilst those at the sample origin (represent the highest polarity), These parameters were used to classify the different extracts according to the appearance of their compounds on the TLC plates.

After the Anisaldehyde reagent spray, 11 WDF extracts were found to produce detectable compounds only at the solvent front. None of the extracts produced a detectable compound only at the sample origin. Only a single extract produced a detectable compound at only the plate median. For both the sample origin and the solvent front, 14 WDF extracts produced detectable compounds. Eight (8) detectable compounds were also produced by the extracts at both the sample origin and the plate median. Only 3 WDF were able to produce detectable compounds at both solvent front and the plate median. In all the polarity regions, sample origin, plate median and the solvent front, 15 WDF extracts produced detectable compounds.

The only solvent front compounds were produced by extracts, B2, C7, D3, D4, D6, E2, F1, F2, F6, and F7. The only plate median compound was produced by extract E8, but

both sample origin and solvent front compounds were produced by extracts, A8, B5, B9, C3, C6, C8, D8, E1, E4, E6, E7, E9, F3, and F4. Again both sample origin and the plate median compounds were produced by extracts, A4, B8, C2, C9, D1, F5, F8, and F9. Both the solvent front and the median region detected compounds were produced by extracts, A9, B1 and E5. All the polarity regions, sample origin, median region and the solvent front, compounds were produced by extracts A1, A2, A3, A5, A6, A7, B4, B6, B7, C1, C4, C5, D5, D7, and E3.

4.6.4 Occurrence and distribution of functional groups

The Anisaldehyde reagent detection revealed five coloured components in all the 54 WDF extracts analyzed; the colours were yellow, blue, green, brown, pink and violet. The number of extracts that showed the above colours was 10, 34, 10, 10, 5, and 8 respectively. After the Anisaldehyde reagent spray (Figure, 4.4A, B, C, D and E), the following extracts (A2, A4, A7, B6, B7, E1, E3, E5, E6, and F2) showed yellow colour after Anisaldehyde reagent spray. The blue coloured components revealed were also shown by these WDF extracts, (A2, A3, A4, A5, A7, A8, A9, B6, B9, C1, C4, C5, C7, C8, C9, D1, D5, D6, D7, D8, D9, E3, E4, E5, E7, E7, E8, E9, F1, F3, F4, F5, F8 and F9). The pink colouration was shown by few extracts such as A1, A3, A5, E3, E6, and F7. An interesting observation made was that four of the pink components were found close to the solvent front except in extract A5 which was found in the median region. The fungal extracts A6, B4, B6, B8, D1, D7, E6 and E7 showed a violet component after Anisaldehyde reagent spray. The following WDF extracts (A4, A8, B1, B5, C9, D1, D7, F5, F7 and F8) also revealed brownish colouration after spraying. Lastly the extracts B1, B3, B5, B7, B8, B9, C1, C4, D5, and E5 also displayed a green coloured component after Anisaldehyde spray as shown in the Fig. 4.4 (A, B, C and D).

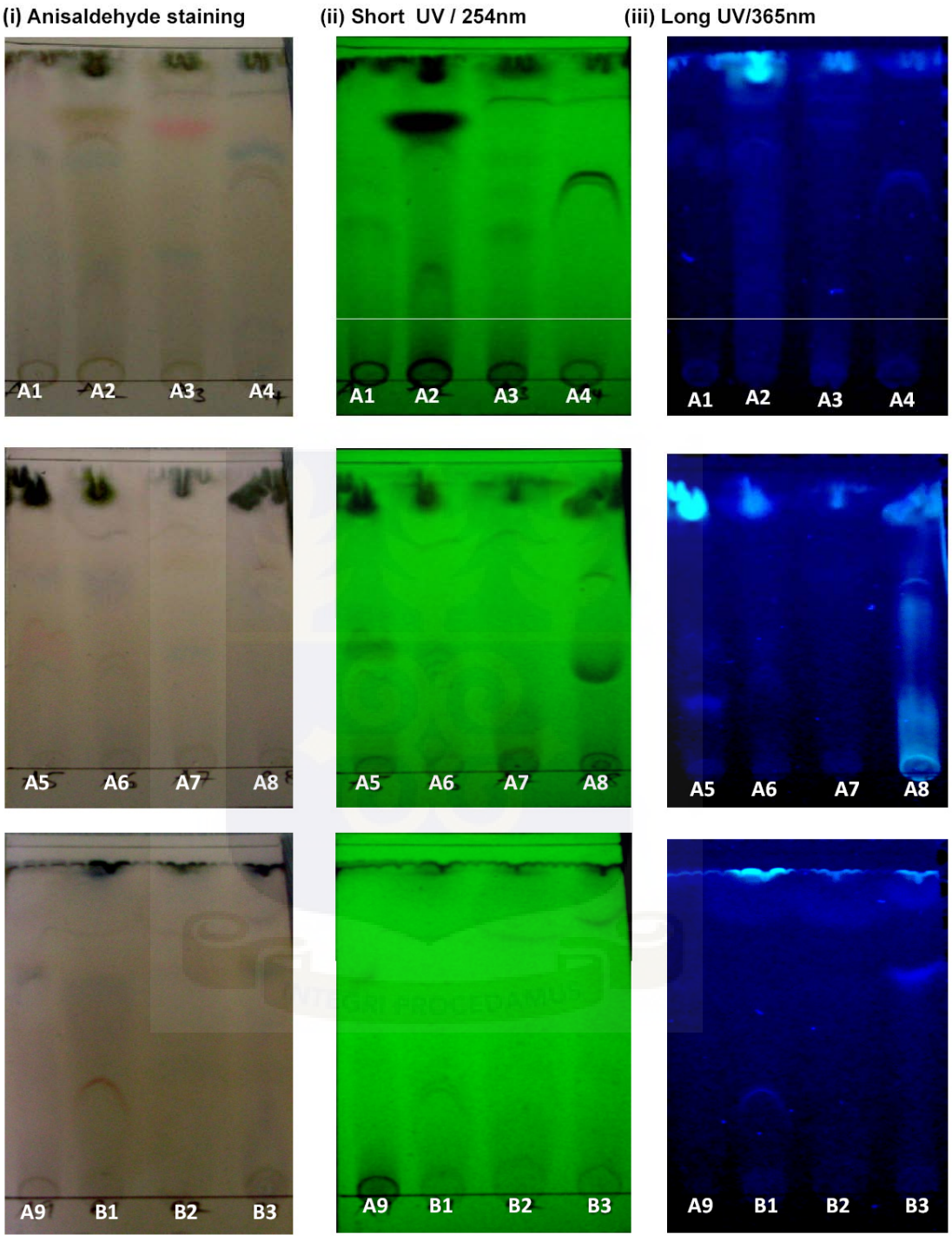


Figure 4.4A.Thin layer chromatographic study of fungal secondary metabolites. *TLC profiles of extracts from WDF (A1-A9) and (B1-B3) developed in* ***EtOAc: CH_3CN: PetEth (7:2:1)*** *solvent system. A 5ul of the fungal extract spotted onto the TLC plate, developed and the compounds visualized under UV and sprayed with Anisaldehyde reagent in visible light.* ***(i).*** *Anisaldehyde reagent,* ***(ii).*** *UV 254nm,* (iii). *UV 365 nm.*

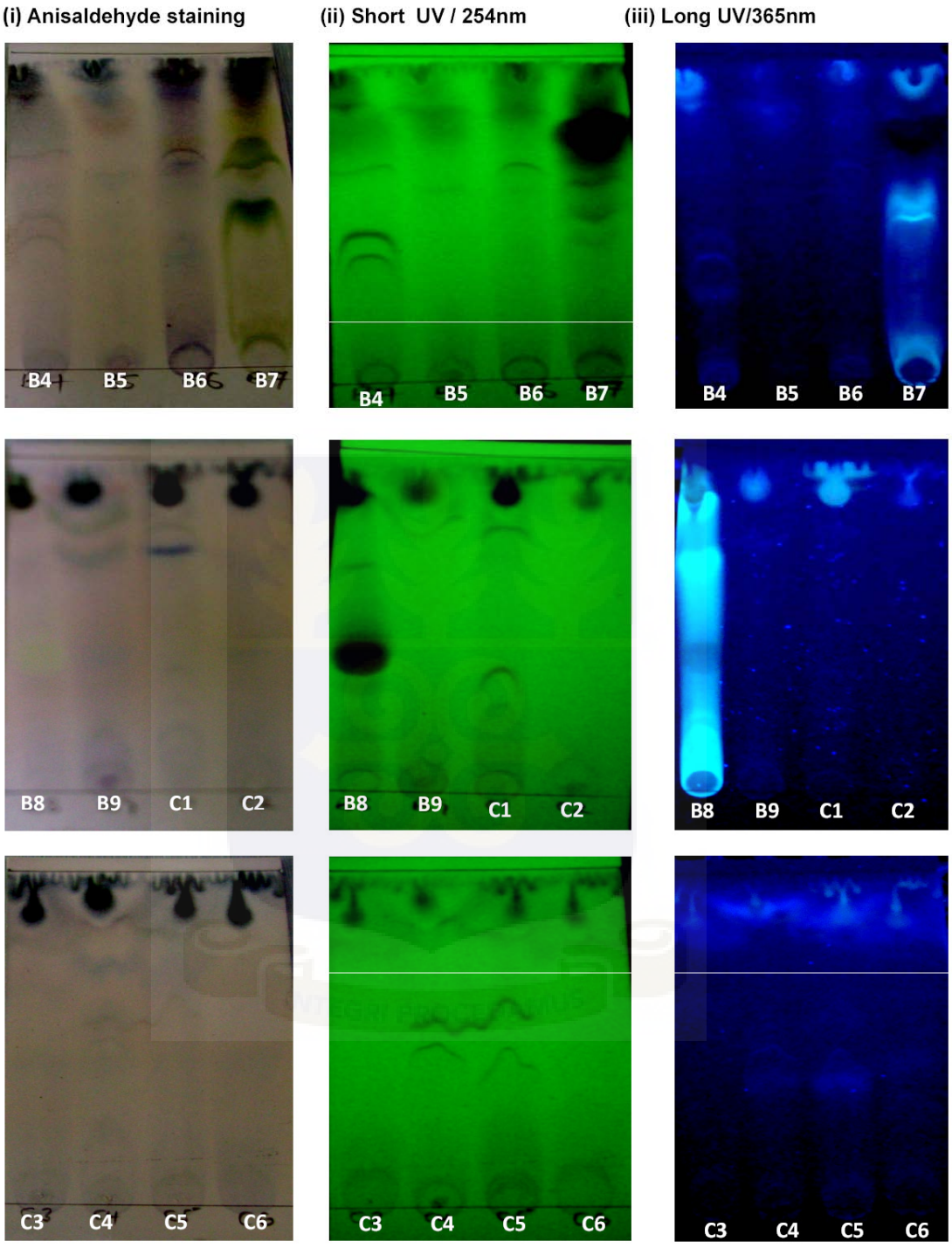


Figure 4. 4B.Thin layer chromatographic study of fungal secondary metabolites. *TLC profiles of extracts from WDF (B4-B9) and (C1-C6) developed in* ***EtOAc: CH_3CN: PetEth (7:2:1)*** *solvent system. A 5ul of the fungal extract spotted onto the TLC plate, developed and the compounds visualized under UV and sprayed with Anisaldehyde reagent in visible light.* ***(i).*** *Anisaldehyde reagent,* ***(ii).*** *UV-254nm,* (iii). *UV-365 nm.*

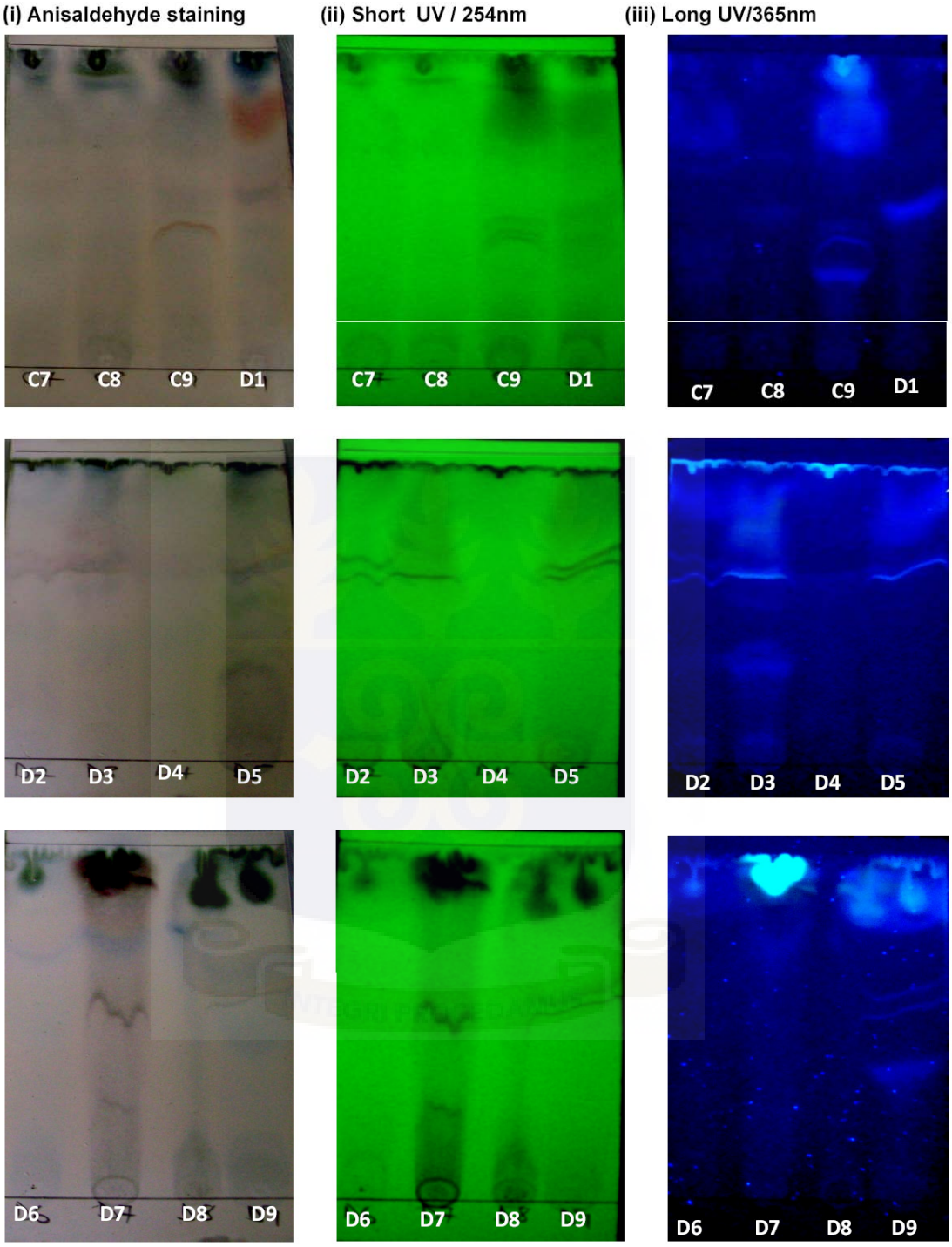


Figure 4.4C.Thin layer chromatographic study of fungal secondary metabolites. *TLC profiles of extracts from WDF (C7-C9) and (D1-D9) developed in* ***EtOAc: CH_3CN: PetEth (7:2:1)*** *solvent system. A 5ul of the fungal extract spotted onto the TLC plate, developed and the compounds visualized under UV and sprayed with Anisaldehyde reagent in visible light.* ***(i).*** *Anisaldehyde reagent,* ***(ii).*** *UV 254nm,* (iii). *UV 365 nm.*

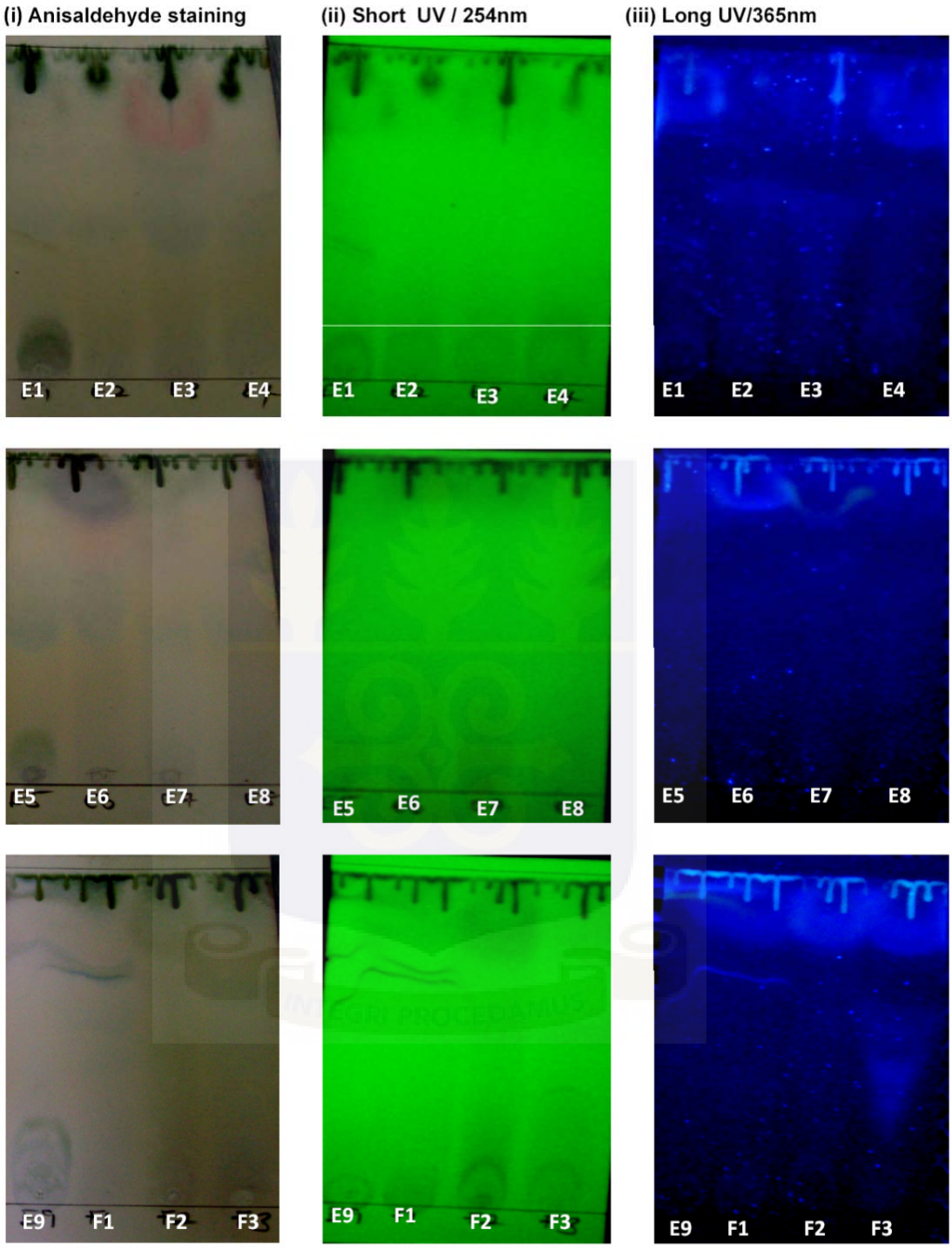


Figure 4.4D. Thin layer chromatographic study of fungal secondary metabolites. *TLC profiles of extracts from WDF (E1-E9) and (F1-F3) developed in* ***EtOAc: CH_3CN: PetEth (7:2:1)*** *solvent system. A 5ul of the fungal extract spotted onto the TLC plate, developed and the compounds visualized under UV and sprayed with Anisaldehyde reagent in visible light.* ***(i).*** *Anisaldehyde reagent,* ***(ii).*** *UV 254nm,* (iii). *UV 365 nm.*

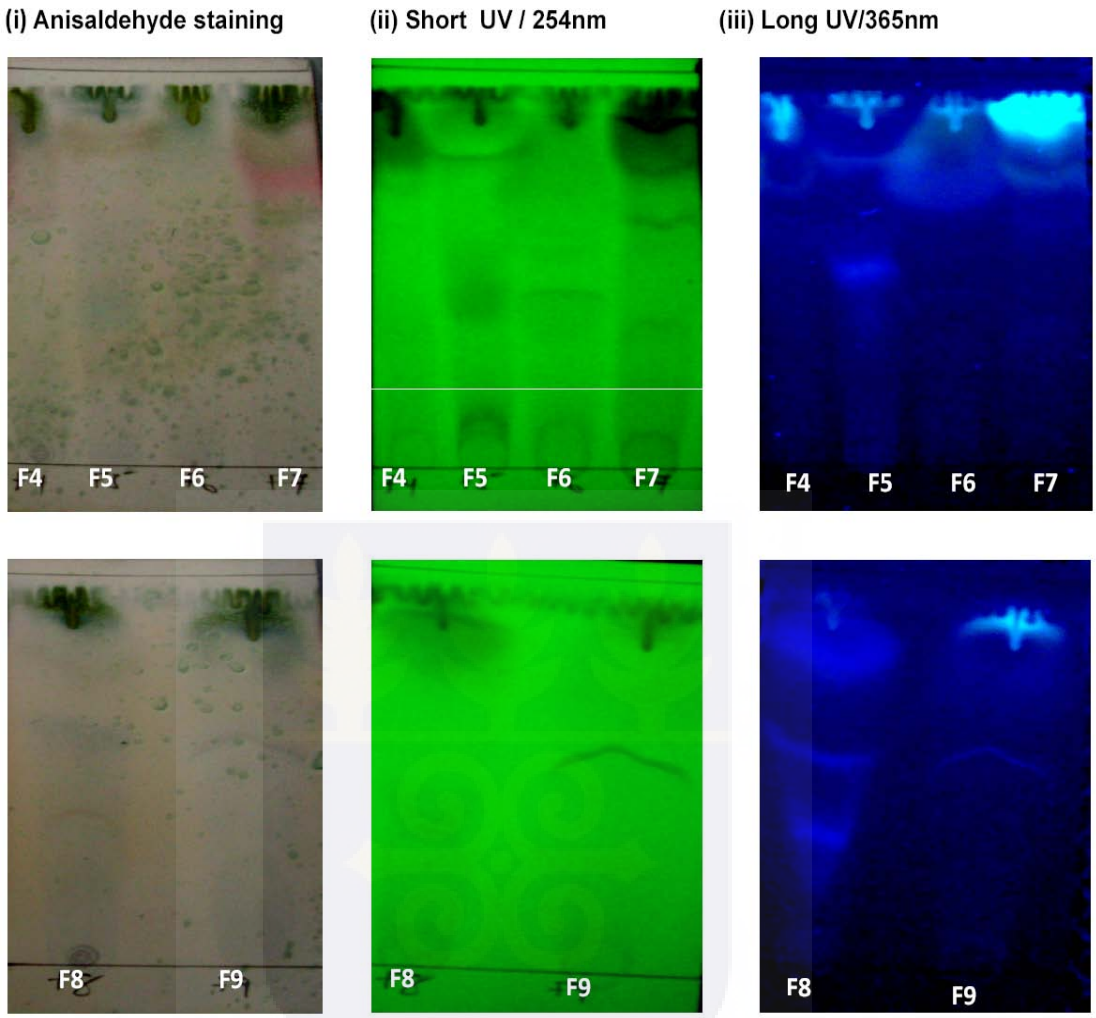


Figure 4.4E.Thin layer chromatographic study of fungal secondary metabolites. *TLC profiles of extracts from WDF (F4-F9) developed in* ***EtOAc: CH_3CN: PetEth (7:2:1)*** *solvent system. A 5ul of the fungal extract spotted onto the TLC plate, developed and the compounds visualized under UV and sprayed with Anisaldehyde reagent in visible light.* ***(i).*** *Anisaldehyde reagent,* ***(ii).*** *UV 254nm,* (iii). *UV 365 nm.*

4.7 Primary screen of WDF for Antimicrobial activity

In order to determine the various antimicrobial activities that are present in each WDF extract, a preliminary antimicrobial screening was performed early in the investigations to determine the specific and the characteristic biological activities of each extracts. The crude extracts from all the 54 WDF were investigated for their respective antibacterial as well as their antifungal activities.

The preliminary screening for antibacterial and antifungal activity was performed using the disc diffusion assay as previously described and was performed for all crude extracts. Crude extracts that were recovered from the WDF were applied to a 6mm paper disc, incubated at the appropriate temperatures and the inhibitory effects measured against a number of Gram positive and Gram negative bacteria and also some selected fungi grown on their respective growth medium. The final amount of WDF extracts that were applied to each disc was 40ul. After 18-24 hours of incubation, the inhibitory effects were assessed on the various test organisms by measuring their zones of inhibition. The extracts were tested against the bacteria *Staphylococcus aureus ATCC.2*, *Escherichia coli NMIMR.3* and the fungi *Candida albicans KBTH.2* and *Aspergilus niger ATCC.2.*

The primary antibacterial testing of the crude organic extracts from the WDF are shown Figure 6A and B. In Figure 4.5 (A, B and C) is also a display of representative plates for *Staphylococcus aureus ATCC.2*, *Escherichia coli NMIMR.3*and *Candida albicans KBTH.2* showing the inhibition zones attained for some of the WDF extracts analyzed. After the primary screening of all the ethyl acetate crude extracts, a total of 40 WDF extracts were found to exhibit some form antimicrobial activity towards the test

organism. Out of total 40 that had an activity, 10 of the WDF extracts were found to have biological activity selectively (SG+) against *Staphylococcus aureus ATCC.2* (Figure 4.5D), and 13 extracts were also found to have a broad spectrum antimicrobial activity (BSAB) against *Staphylococcus aureus ATCC.2* and *Escherichia coli NMIMR.3* Figure 4.5D.

The number of extracts also having a selective antifungal (SAF) activity towards *Candida albicans KBTH.2* was found to be 3 (Figure 4.5D). The number of WDF that exhibited a non selective antimicrobial (NSAM) activity towards the three test organisms, *S. aureus ATCC.2*, *E. coli* NMIMR.3and *C. albicans* KBTH.2 were recorded to be 11 (Figure 4.5D). Moreover, 14 WDF extracts were found to exhibit no biological activity (NA) against any of the test organisms used (Figure 5D). It is clear that majority of the fungal extracts had broad spectrum (BSAB) activity and also selective activity (SG+) for Gram positive bacteria. There was no selective activity (SG-) observed for any of the WDF extracts against the Gram negative bacteria tested (Figure 4.5D). Extract from the WDF D3 (Figure 4.5A), exhibited strong/complete zone of inhibition against *S. aureus ATCC.2.*

Figure 4.6 (A and B), display the various inhibition zones attained for all the 54 WDF extracts analyzed alongside their respective positive control antibiotics. From Figure 4.6A, majority of the WDF extracts showed inhibition towards the Gram positive bacteria *S. aureus ATCC.2.* The zones that were obtained are comparable with the positive control antibiotic, Streptomycin. For example extract E5, caused inhibition towards, *S. aureus ATCC.2, E. coli NMIMR.3* and *C. albicans KBTH.2.* The inhibition

zone that was attained for the *S. aureus ATCC.2* was much higher when compared with the positive control antibiotics.

Very few of the extracts were able to cause inhibition against both the Gram negative bacteria *E. coli NMIMR.3* and the fungi *C. albicans KBTH.2.* Only a single extract in Figure 4.6A was able to achieve inhibition towards the test organism *A. niger ATCC.2.* However, in Figure 4.6B majority of the WDF extracts achieved inhibition towards *S. aureus ATCC.2, E. coli NMIMR.3* and *C. albicans KBTH.2*

Extracts D3 and E6, possessed non selective antimicrobial activity and therefore were able to inhibit all the four test organisms *S. aureus ATCC.2, E. coli NMIMR.3*, *C. albicans KBTH.2* and *A. niger ATCC.2 (*Figure 4.6B). Since a very few of the extracts were able to achieve inhibition towards the test organism *A. niger ATCC.2,* it became necessary for this test organism to be excluded from further analysis.

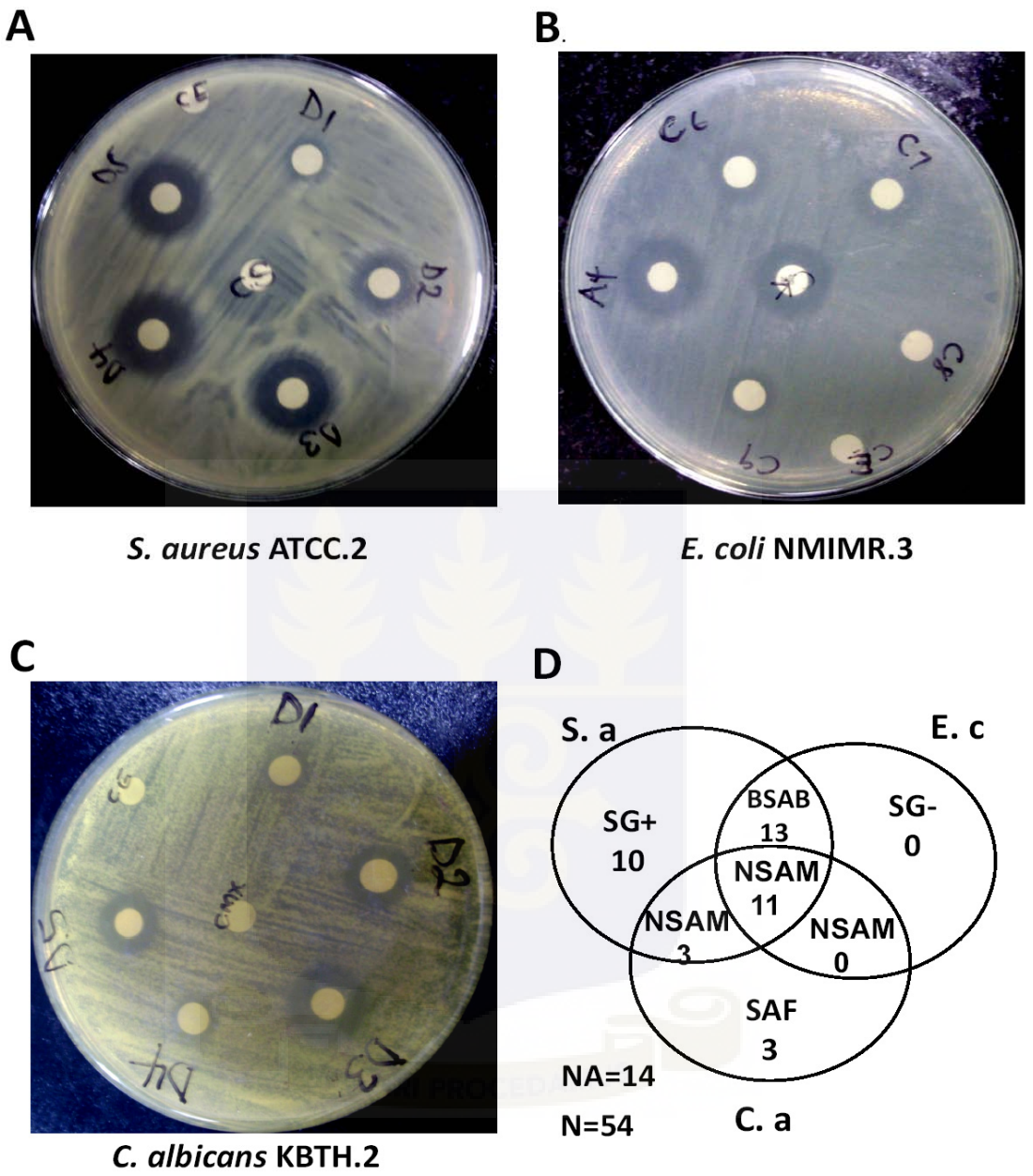


Figure 4.5. Inhibition zones on *S. aureus ATCC.2, E. coli NMIMR.3,* and *C. albicans KBTH.2* from the primary screen of WDF using disc diffusion assay. *Plates showing inhibition zones on S.aureus ATCC.2, E. coli NMIMR.3 and C. albicans KBTH.2.* ***(A).*** *Plate showing inhibition zones on S.aureus ATCC.2 with fungal extracts from samples D1, D2,D3,D4, D5 and the commercial antibiotics Streptomycin.* ***(B).*** *Plates showing inhibition zones on E.coli NMIMR.3 with fungal extracts from samples A4, C7, C8, C9 and the commercial antibiotics Kanamycin.* ***(C)****. Plates showing inhibition zones on C. albicans KBTH.2 with fungal extracts from samples D1, D2,D3,D4, D5 and the commercial antibiotics Mycopirox.* ***(D).*** *A diagram showing the designation of the primary screen for all 54 extracts obtained from the WDF against S.aureus ATCC.2, E. coli NMIMR.3 and C. albicans KBTH.2****. (NA)*** *– Number of extracts that had no activity against any of the test organisms.*

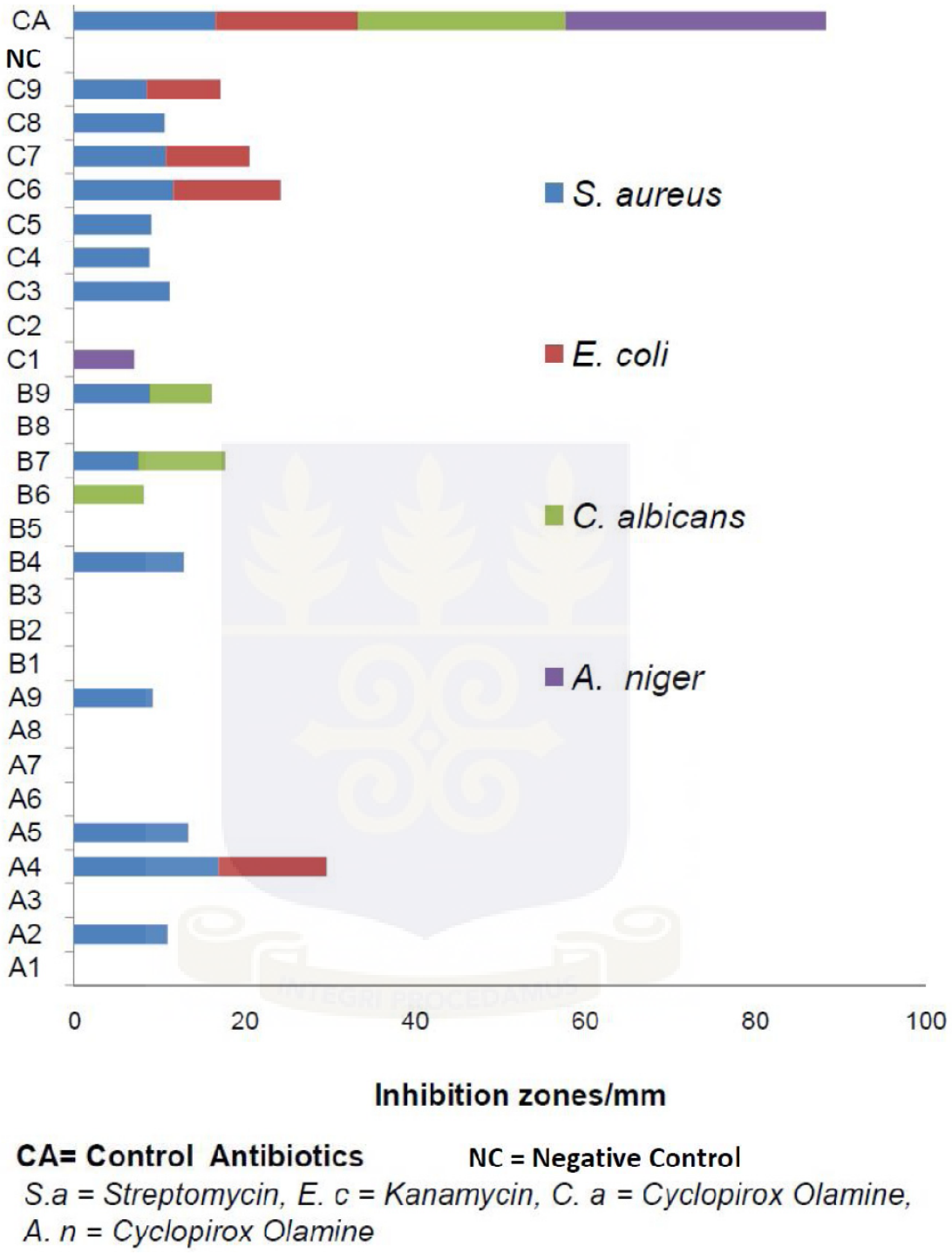


Figure 4.6A.Antimicrobial activities of the WDF extracts against four test microorganisms in the primary screening. *The antimicrobial activity of 27 isolates of WDF (A1-A9, B1-B9 and C1-C9) in the primary screening assay using paper disc agar diffusion method. Positive control: Streptomycin and Kanamycin for antibacterial test, cyclopirox olamine for antifungal test; negative and blank control: ethanol.*

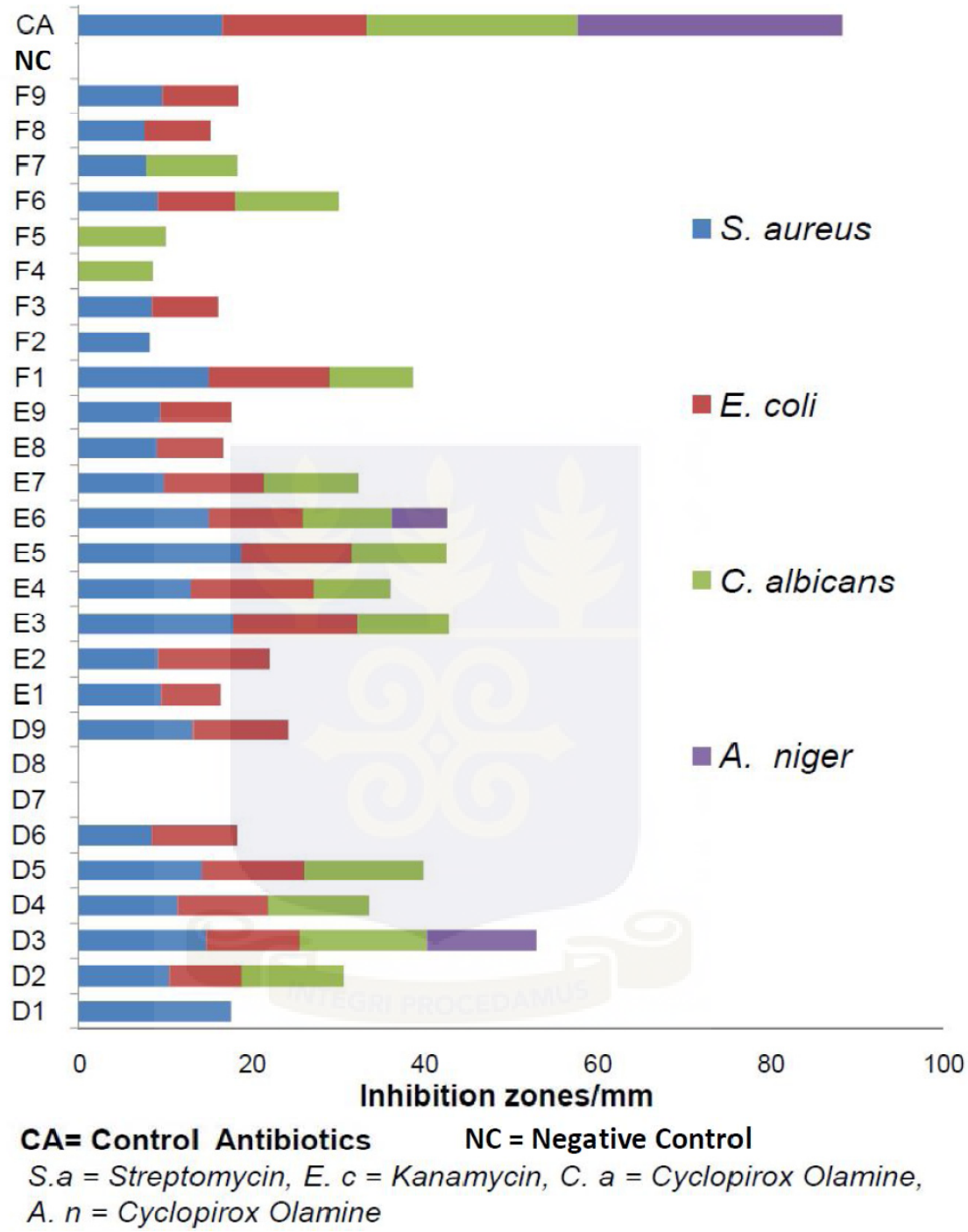


Figure 4.6B. Antimicrobial activities of the WDF extracts against four test microorganisms in the primary screening. *The antimicrobial activity of 27 isolates of WDF (D1-D9, E1-E9 and F1-F9) in the primary screening assay using paper disc agar diffusion method. Positive control: Streptomycin and Kanamycin for antibacterial test, cyclopirox olamine for antifungal test; negative and blank control: ethanol.*

4.8 Refermentation of HITS fungal samples showing promising biological activity (Secondary Screen)

After the preliminary screening, 26 out of the 54 WDF samples that showed biological activity towards a either only a Gram positive or Gram negative bacteria, or both Gram negative and positive, or only towards the fungi C. albicans were selected as HITS. This was the criteria used in selecting the "HIT" samples. The WDF B7, which exhibited non selective antimicrobial (NSAM) activity, although was not considered as HIT, but showed an interesting TLC profile and was included in the HIT samples for further analysis. The WDF B7 refermentation was again to investigate whether the NSAM activity exhibited by it was found in either the same or different fractions.

The 27 HITS were refermented in a 1L culture volume for further investigations. The refermentation process was similar to the general fermentation process except that larger volumes of PDB (1L) were used for the culturing of the WDF. The refermentation lasted for 48 days as the previous fermentations. During the fermentation period, all the cultures by day 3 had started foaming and bubbling profusely. Most of the foams were cleared by the 14th day of culture, whiles the very few that remained disappeared by the 21st day of culture. At the 48th day of culture, the WDF cultures were terminated using ethyl acetate extraction.

The 27 refermented WDF after 48 days of culture were extracted as previously described in the refermentation extraction in the method section. The crude extract yields of fungal extracts were calculated in grams (g) by deducting the weight of empty flask before extraction from weight of flask after extraction. The volumes of crude extracts were also obtained by measuring volumes of extract using micropipette. The densities of extracts

were calculated by dividing the weight of extract by their volumes. The yields of the 27 WDF extracts obtained are shown in Table 4.2 (A and B).

The crude extracts obtained from the 1L culture of refermented hits ranged between 6g and 26g (Table 4.2(A and B). The least extract yield was produced by WDF A2, whilst C6, C7 and D9 gave the highest extract yield. It is worth noting that C7 was among the fungal isolates that produced the highest yield in the primary screen. This was again repeated when C7 was refermented in a 1L PDB culture. Although not all the WDF that produced high yields in the primary screen were refermented, those refermented along with C7, such as F2, and F4, also produced yields that were significantly high, 20g and 18g respectively. Distinctly different colours were again obtained for the refermented WDF extracts as was observed in the primary screen. However it must be emphasized that most of the extracts obtained had yellow colours whiles few others had dark and brownish colorations as those colours observed in the primary screen.

Figure 4.7(A, B and C) summarizes the volumes (ml) and densities (mg/ml) of the wood decay fungal extract obtained during the secondary refermentation. It was observed that extracts C5 and C8 obtained weight 18 g and 20 g respectively, however, the volumes of extract yield were both 20 ml resulting in different densities of 900 mg/ml and 952 mg/ml respectively. This indicates that, all though different compounds might be of the same weight, their densities might not proportionally be equal as shown in this investigation. This was also similar in extracts E8 and F2, both having different weights, but same volumes, thereby given them different density values.

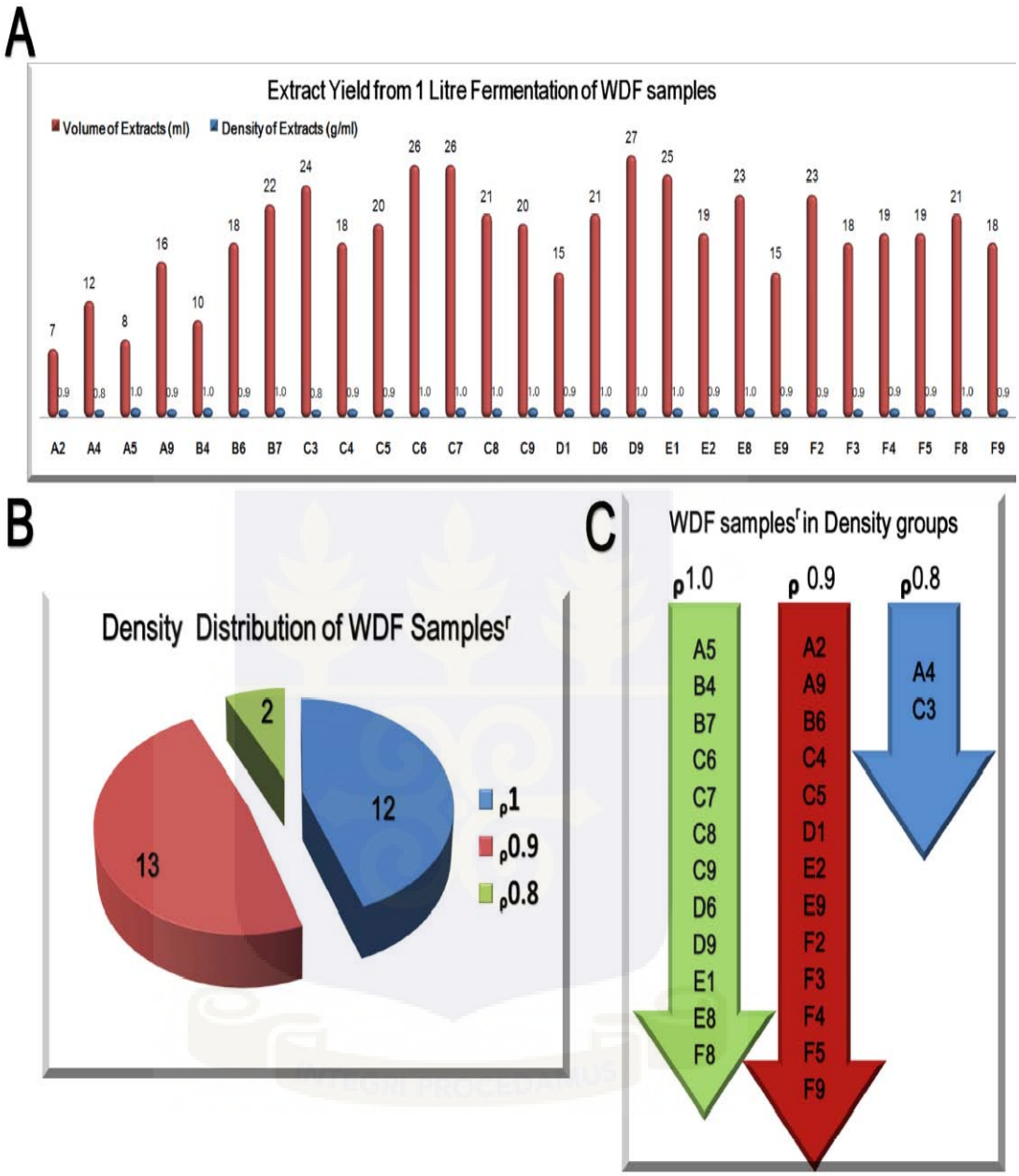


Figure 4.7. Extract volumes from 1L fermentation of (27) WFD samples. *Organic extraction yield of 27 refermented WDF. Wood Decay Fungi (WDF) was cultured in PDB for 42 days in 2000ml flasks. Crude extracts yield were calculated in grams (g) by deducting the weight of empty flask before extraction from weight of flask after extraction. The volumes of crude extracts were also obtained by measuring volumes of extract using micropipette. The densities of extracts were calculated by dividing the weight of extract by their volumes.* ***(A)*** *Shows the volumes of extracts obtained (ml) and the densities of extracts (g/ml).* ***(B)*** *Shows the density distribution of the27 WDF samples.* ***(C)*** *WDF in density groupings*

Table 4.2A. Ethyl acetate extraction volumes (yields) of 14 WDF from 1L fermentation broth

Num.	Code	Weight /g	Volume/ml	Density/mg/ml
1	A2	6	7	857
2	A4	10	12	833
3	A5	8	8	1000
4	A9	14	16	875
5	B4	10	10	1000
6	B6	16	18	888
7	B7	22	22	1000
8	C3	20	24	833
9	C4	16	18	888
10	C5	18	20	900
11	C6	26	26	1000
12	C7	26	26	1000
13	C8	20	21	952
14	C9	20	20	1000

Wood Decay Fungi (WDF) was cultured in PDB for 42 days in 2000ml flasks. Ethyl acetate extract yields were calculated in grams (g) by deducting the weight of empty flask before extraction from weight of flask after extraction. The volumes of crude extracts were also obtained by measuring volumes of extract using micropipette. The densities of extracts were calculated by dividing the weight of extract by their volume

Table 4.2B. Ethyl acetate extraction volumes (yields) of 14 WDF from 1L fermentation broth

Num.	Code	Weight /g	Volume/ml	Density/mg/ml
15	D1	14	15	933
16	D6	20	21	952
17	D9	26	27	962
18	E1	24	25	960
19	E2	18	19	947
20	E8	22	23	956
21	E9	14	15	933
22	F2	20	23	869
23	F3	16	18	888
24	F4	18	19	947
25	F5	18	19	947
26	F8	20	21	952
27	F9	16	18	888

Wood Decay Fungi (WDF) was cultured in PDB for 42 days in 2000ml flasks. Ethyl acetate extract yields were calculated in grams (g) by deducting the weight of empty flask before extraction from weight of flask after extraction. The volumes of crude extracts were also obtained by measuring volumes of extract using micropipette. The densities of extracts were calculated by dividing the weight of extract by their volumes.

4.9 Secondary screening of refermented WDF for antimicrobial activity

After the preliminary investigations of antimicrobial activity of the 54 extracts from WDF using disc diffusion assay, extracts that showed antimicrobial activity (HITS) were refermented in a 1L culture of PDB as previously described. The purpose of the refermentation is to confirm whether the productions of compounds by the specific WDF responsible for inhibition against the various test organisms are reproducible. The HIT extracts that were obtained from the refermented WDF were assessed for their activity on the following test organisms; *Staphylococcus aureus* ATCC.2*, Escherichia coli* NMIMR.3 and *Candida albicans* KBTH.2.

In the secondary screening refermented extracts were constituted into drug concentrations. Paper discs were impregnated with 5mg/ml of refermented extracts for the inhibitory assay. After the secondary screening of the 27 refermented WDF extracts, inhibitory activity against only *S. aureus ATCC.2 was* found to be possessed by 9 refermented extracts. There was no inhibitory activity observed for only the test organisms *E. coli NMIMR.3*and *C. albicans KBTH.2*. Inhibitory activity towards both Gram positive and Gram negative bacteria tested was found in 6 extracts. Four (4) refermented extracts also exhibited inhibition towards *S. aureus ATCC.2* and *C. albicans KBTH.2.* There was no inhibitory activity from 8 extracts towards any of the three test organisms.

Refermentation of HITS samples resulted in a shift of antimicrobial activity. The number of crude extracts that exhibited antibacterial activity against only *S. aureus ATCC.2* was reduced by one. There was no inhibitory activity exhibited towards only the Gram negative bacteria tested. However there was a significant shift in antimicrobial

activity, as the number of extracts that exhibited antimicrobial activity towards both the Gram negative and the Gram positive bacteria were six (6) as opposed to (13) after the primary screen. The shift was as a result of the inability of the extracts to reproduce activity towards the Gram negative bacteria tested. The number of extracts that had non selective antimicrobial activity was increased from 1 in the primary screen to 4 after the secondary screen. Again the 3 WDF extracts that exhibited antifungal activity towards *C. albicans* failed to inhibit the same test organism after the secondary screen. Displayed in Figure 4.8 (A, B and C) are the plates containing 5mg/ml stock of refermented WDF extracts against *S. aureus ATCC.2, E. coli NMIMR.3* and *C. albicans KBTH.2.*

Most of the refermented WDF extracts exhibited no biological activity towards any of the tested organisms, although they showed inhibitory effect towards these same organisms in the primary screen (Fig 4.9). These are the refermented WDF extracts (C3, C8, C9, D6, E1, F4, F5, and F8) that exhibited no biological activity during the secondary screening. Among the extracts C9, D6 and E1 for example, that attained no biological activity during the secondary screening, they showed these inhibition zones 8.68 mm, 8.58 mm and 9.67 mm respectively against *S. aureus ATCC.2* in the primary screen. Extract C9 again, showed much higher inhibition in the time course analysis against both test organisms attaining inhibition zones around 13 mm and 15 mm against *S. aureus ATCC.2* (Figure 4.2B (C). The possible explanation is that, in the primary culturing of the wood decay fungi, a culture volume of 200 ml was used, in the time course analysis a culture volume of 100 ml was also used. However, in the secondary screening a culture volume of 2x500 ml was used, notwithstanding the fact that the same amount of fungal fruiting bodies (5g) was used in all the three different cultures. It is possible that in the 1L fermentation, the amount of active compounds produced were so

little in comparison with the culture volume. This makes sense because in the time course analysis when C9 was cultured in only 100 ml volume culture, greater inhibition zones were attained against the test organisms.

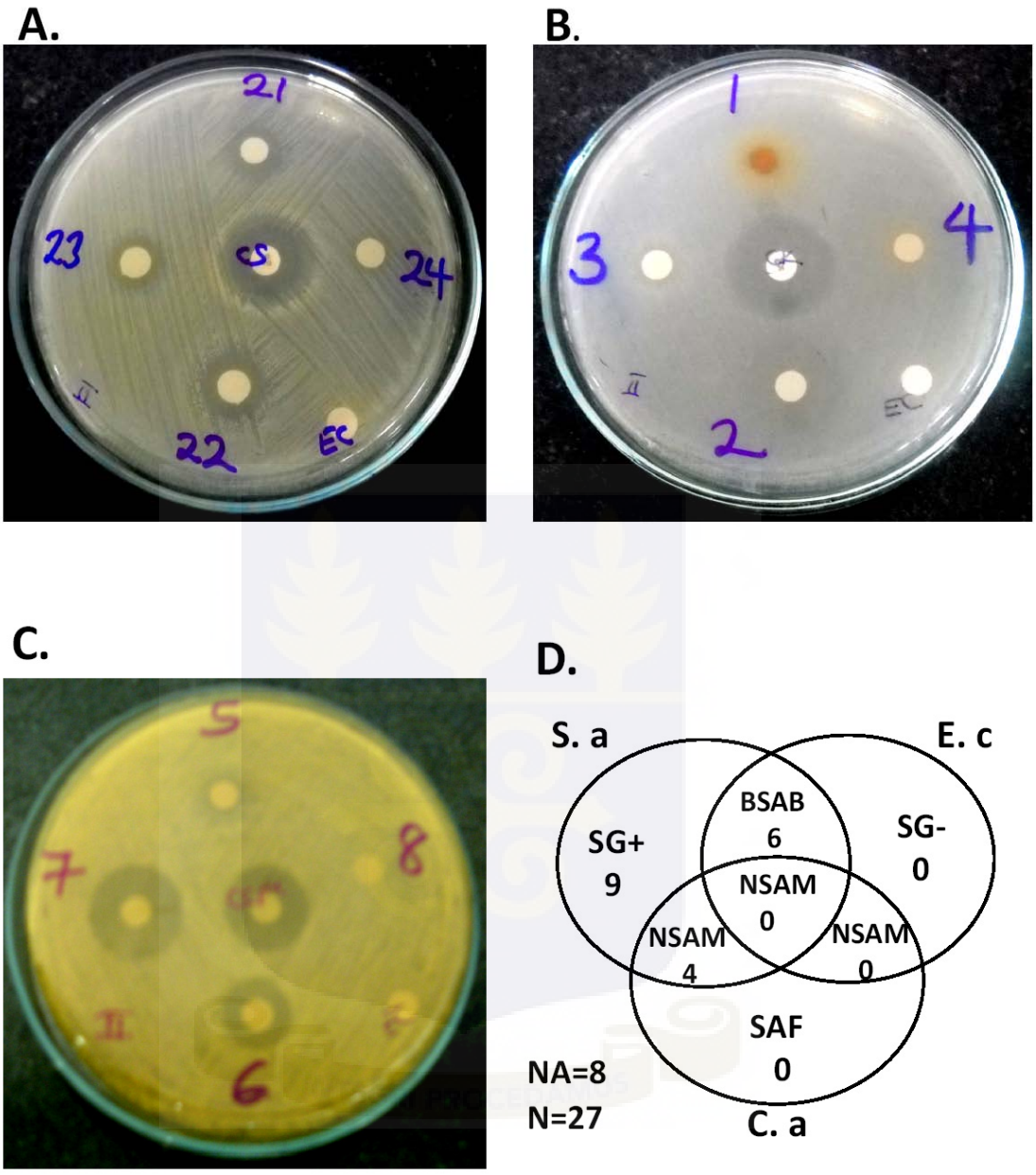


Figure 4.8. Inhibition zones on *S. aureus ATCC.2, E. coli NMIMR.3,* and *C. albicans KBTH.2* from the secondary screen of WDF using disc diffusion assay. *Plates showing inhibition zones on S.aureus ATCC.2, E. coli NMIMR.3 and C. albicans KBTH.2.* ***(A).*** *Plate showing inhibition zones on S.aureus ATCC.2 with fungal extracts from samples 21/E9, 22/F2, 23/F3, 24/F4 and the commercial antibiotics Streptomycin.* ***(B).*** *Plates showing inhibition zones on E.coli NMIMR.3 with fungal extracts from samples 1/A2, 2/A4, 3/A5, 4/A9 and the commercial antibiotics Kanamycin.* ***(C)****. Plates showing inhibition zones on C. albicans KBTH.2 with fungal extracts from samples 5/B4, 6/B6, 7/B7, 8/C3 and the commercial antibiotics Cyclopirox olamine.* ***(D).*** *A diagram showing the designation of the secondary screen for all 27 extracts obtained from the WDF against S.aureus ATCC.2, E. coli NMIMR.3 and C. albicans KBTH.2.* ***(NA)*** *– Number of extracts that had no activity against any of the test organisms*

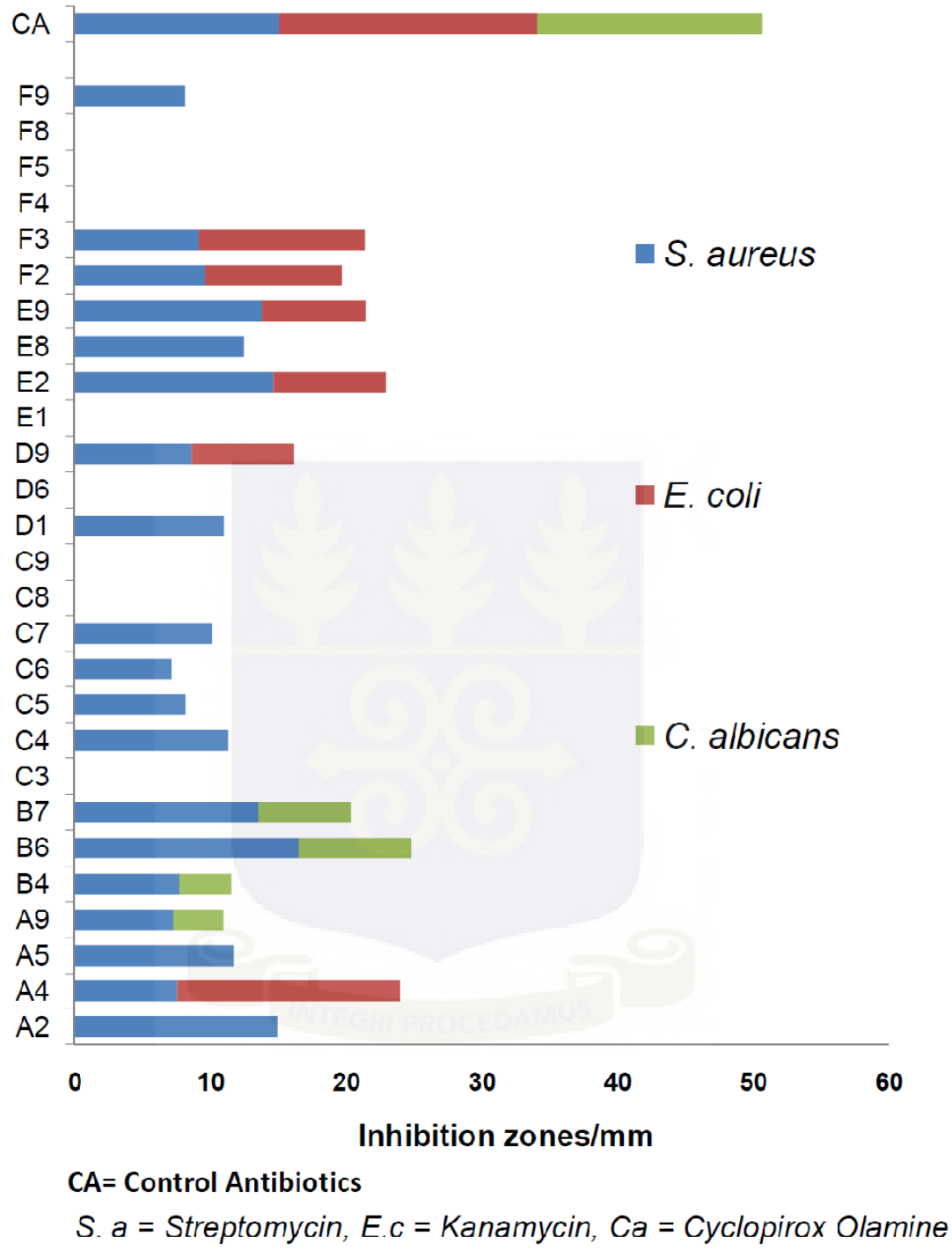


Figure 4.9. Antimicrobial activities of the WDF extracts against four test microorganisms in the secondary screening. *The antimicrobial activity of 27 refermented isolates of WDF (HITS) in the secondary screening assay using paper disc agar diffusion method. Positive control: Streptomycin and Kanamycin for antibacterial test, cyclopirox olamine for antifungal test; negative and blank control: ethanol.*

Table 4.3A Secondary screening of 27 WDF

Num.	Code	S. aureus	E. coli	C. albicans	Inference 2^0	Inference 1^0
1	A2	14.99	-	-	SG+	SG+
2	A4	7.56	16.43	-	BSAB	BSAB
3	A5	11.73	-	-	SG+	SG+
4	A9	7.32	-	7.26	NSAM	SG+
5	B4	7.73	-	7.24	NSAM	SG-
6	B6	16.55	-	12.44	NSAM	SAF
7	B7	13.60	-	14.67	NSAM	NSAM
8	C3	-	-	-	none	SG+
9	C4	11.34	-	-	SG+	SG+
10	C5	8.20	-	-	SG+	SG+
11	C6	7.19	-	-	SG+	BSAB
12	C7	10.18	-	-	SG+	BSAB
13	C8	-	-	-	none	SG+
14	C9	-	-	-	none	BSAB

Key:
CNT = Control
SG+/- = Selective Gram Positive/Negative
BSAB = Broad Spectrum Antibacterial
SAF = Selective Antifungal
NSAM = Non Selective Antimicrobial

Control Antibiotics
S. a = Streptomycin
E. c = Kanamycin
C. a = Cyclopirox Olamine
A. n = Cyclopirox Olamine

Cultures were incubated at 37^OC for 24 hours, except C. albicans KBTH.2 and A. niger ATCC.2 which were incubated at 30^OC for 48 hours. Activity was determined by disc diffusion assay on lawn cultures. A zone of inhibition > 6mm was considered positive, (-), indicates no inhibition zones observed.

Table. 4.3B Secondary screening of 27 WDF

Num.	Code	S. aureus	E. coli	C. albicans	Inference 2^0	Inference 1^0
15	D1	11.01	-	-	SG+	SG+
16	D6	-	-	-	none	BSAB
17	D9	8.62	7.57	-	BSAB	BSAB
18	E1	-	-	-	none	BSAB
19	E2	14.95	8.34	-	BSAB	BSAB
20	E8	12.39		-	SG+	BSAB
21	E9	15.02	7.63	-	BSAB	BSAB
22	F2	8.08	10.12	-	BSAB	SG+
23	F3	8.69	12.19	-	BSAB	BSAB
24	F4	-	-	-	none	SAF
25	F5	-	-	-	none	SAF
26	F8	-	-	-	none	BSAB
27	F9	8.27	-	-	SG+	BSAB
CNT		15.06	19.03	16.53		

Key:
CNT = Control
SG+/- = Selective Gram Positive/Negative
BSAB = Broad Spectrum Antibacterial
SAF = Selective Antifungal
NSAM = Non Selective Antimicrobial

Control Antibiotics
S. a = Streptomycin
E. c = Kanamycin
C. a = Cyclopirox Olamine
A. n = Cyclopirox Olamine

Cultures were incubated at 37^{O}C for 24 hours, except C. albicans KBTH.2 and A. niger ATCC.2 which were incubated at 30^{O}C for 48 hours. Activity was determined by disc diffusion assay on lawn cultures. A zone of inhibition > 6mm was considered positive, (-), indicates no inhibition zones observed.

4.10 LH-20 fractionation for selected WDF extracts

The purpose of the fractionation was to investigate whether the dual or BSAB activity exhibited by the selected extracts are responsible by either the same or different compounds. Fractionation was performed to separate components in the mixture to identify those with antimicrobial activity for extracts A4, B6, B7, E2, E9 and F3. These extracts were selected because they had activity against either two of the test organisms. WDF B7 inclusion for fractionation was due to its NSAM activity. The interest was to investigate the various fractions responsible for such activities. The column volume was 80 ml, height was 16 cm and the void volume was also 36 ml. The fractionation procedure was performed as describe in the method section. The LH-20 Sephadex column was washed with two column volume of ethanol before extracts were added. Crude extracts (1m) applied to the top of the Sephadex LH-20 column and the components eluted using MeOH. During the first step of fractionation process, three fractions were collected as the void volumes, then 10 fractions each consisting of 10ml were collected, evaporated to dryness and the resuspended in the appropriate solvent.

In the process of drying fractions, various colours were observed in the different fractions. In the A4 extracts, fraction 1, 2, 8, 9 and 10 all possessed a colourless liquid, fraction 3 had a milky liquid, and fraction 4 also had pale yellowish liquid with whitish meniscus under vacuum. Fraction 5 had a pale brownish liquid with colourless meniscus under vacuum. Fraction 6 and 7 both had very pale yellow liquid but in fraction 7 there was no colourless meniscus formed under vacuum as there was in fraction 6.

In the B6 fractions recovery, fractions 1, 2, 7, 8, 9, and 10 were all found to have colourless liquid. Fractions 3 and 4 had creamy liquids, but in fraction 4, it had a yellow colour. Fractions 5 and 6 both had pale yellow liquid with fraction 5 having colourless

meniscus under vacuum. In extracts B7, the fractions 1, 2, 8, 9, and 10 that were recovered had colourless liquid. Faction 3 possessed creamy yellow liquid; fraction 4 and 6 had pale yellow liquid, fraction 5 also had pale brownish liquid and fraction 7 had a yellow liquid. In extract E2, all the fractions (1-10) had colourless liquid and this was the same for the fractions from extract E9. In extract from F3, the situation was similar except that the fraction 1 had a cloudy liquid.

Each fraction was subjected to bioactivity evaluation to observe which fractions exhibited antimicrobial activity. Fractions were spotted on TLC plates, developed in an appropriate solvent system, visualized at UV-365 nm and UV-254nm and then sprayed with Anisaldehyde reagent for colour detection.

4.10.1 Antimicrobial activity exhibited by A4 fractions and TLC chromatograph.

The A4 fractions collected at a flow rate of 0.87 ml/min or a drop rate of 1 drop per second. The LH-20 fractions recovered from WDF A4 after drying were reconstituted into an equivalent of 5mg/ml of the A4 crude extract. The disc diffusion assay plates showed the inhibitions of the individual A4 fractions which were tested against *S. aureus ATCC.2* and *E. coli NMIMR.3* Figure 4.10 (A and D). The antimicrobial evaluation of A4 fractions was performed by disc diffusion method. Fig. 4.10 (A and B) display the results of the disc diffusion assay on *S. aureus ATCC.2* and *E. coli NMIMR.3* respectively.

Zones of inhibition were observed for fractions 1, 4, 5, 6, and 7 against *S. aureus ATCC.2.* Fractions 2, 3, 8, 9, and 10 exhibited no activity for *S. aureus ATCC.2*, Fractions 1 and 6 exhibited slight activity. The largest zone was exhibited by fractions 5 and 7, which suggested that there was either a high amount of a single compound or a

number of bioactive compounds in one fraction. Although fraction 4 had antibacterial activity against *S. aureus ATCC.2,* the inhibition zone was less intense when compared to the active fractions 5 and 7. Fractions 2, 3, 4, 5, and 6 exhibited activity on *E. coli NMIMR.3.* Fraction 4 had the least activity against the test organism. The highest zones of inhibition were exhibited by fractions 2, 3, 5, and 6. Fractions 5 and 6 were found to have a broad spectrum activity against both *S. aureus ATCC.2* and *E. coli NMIMR.3,* with fraction 5 having the highest zones in both tested organisms.

Thin layer chromatography (TLC) was performed on A4 fractions using the solvent system ***EtOAc: CH_3CN: PetEth (7:2:1).*** Fractions (10ul) were spotted on TLC plates and developed. Fractions 1, 2, 3, 4, 5, 6 and 7 were observed to absorb fluorescence at UV-254nm. These fractions (1, 2, 3, 4, 5, 6 and 7) produced compounds that were able to UV at the sample origins and the median regions. Fractions 1, 2, 3, 4, 5, 6 and 7 absorb fluorescence at UV-365nm (Fig.4.10D), at the median regions. Only the stock extract fluoresced at the sample origin and the median region. Fewer fractions with faint yellow colours were seen in visible light, however after spraying with Anisaldehyde reagent (Fig. 4.10 D); faint yellow colour disappeared in almost all the fractions.

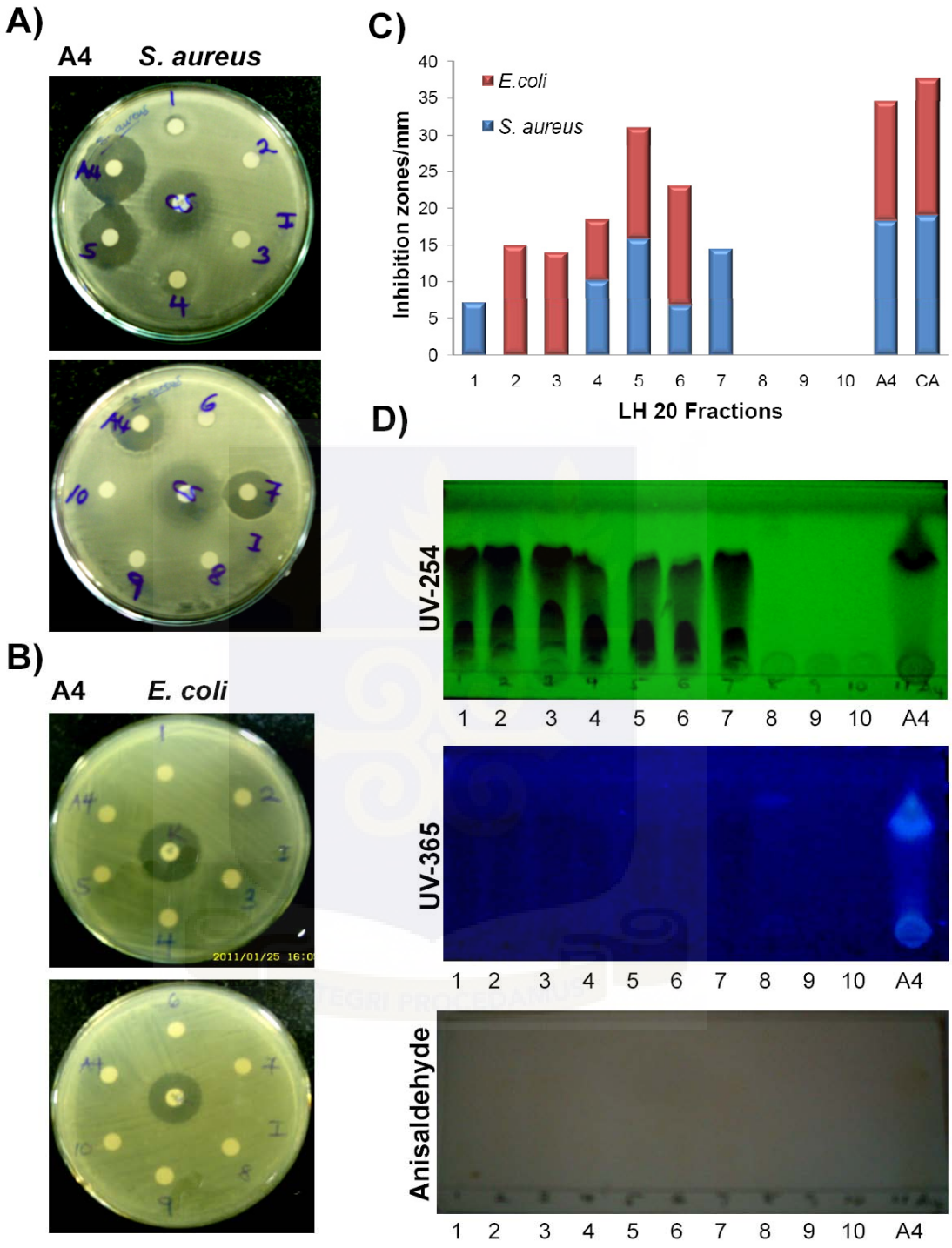


Figure 4.10. ***Analysis of LH-20 fractions of WDF A4 extract. Disc diffusion assay plates for A4 fractions against S. aureus and E. coli (A and B). Inhibition zones of LH- 20 fractions of A4 (C). TLC chromatographs A4 fractions at UV-254, UV-365 and after spraying with Anisaldehyde reagent.***

4.10.2 Antimicrobial activity exhibited by B6 fractions and TLC chromatograph.

The B6 fractions collected at a flow rate of 1.05 ml/minor a drop rate of 1 drop per second. The LH-20 fractions recovered from WDF B6 after drying were reconstituted into an equivalent of 5mg/ml of the B6 crude extract. Fractions from B6 were tested on *S. aureus ATCC.2* and *C. albicans KBTH.2*. Disc diffusion assay method was used to evaluate the antimicrobial activity of the B6 fractions. The results of disc diffusion assay on *S. aureus ATCC.2* and *C. albicans KBTH.2* is displayed respectively Fig 4.11 (A and B). Zones of inhibition were observed for the fractions 4 and 5 for *S. aureus ATCC.2*. Fractions 1, 2, 3, 6, 7, 8, 9 and 10 exhibited no activity for *S. aureus ATCC.2*. Fraction 5 exhibited the highest zone for *S. aureus ATCC.2* Fig 4.11 C. The following fractions 6, 8 and 9 were also found to exhibit activity on *C. albicans KBTH.2* with fraction 6 and 9 almost equal inhibition zones. However, fractions that had activity of *S. aureus ATCC.2* had no activity on *C. albicansKBTH.2* and vice versa, indicating that different fractions different activity on the test organisms.

Thin layer chromatography (TLC) was performed on B6 fractions using the solvent system ***EtOAc: CH_3CN: PetEth (7:2:1).*** Fractions (10ul) were spotted on TLC plates and developed. UV- From (Fig.4.11 D) fractions 4 and 5 were observed to absorb fluorescence at UV-254nm with intensed bands with fraction 4 absorbing at the sample origin and the solvent front and fraction 5 also absorbing at the sample origin, median region and the solvent front. At UV 365 nm, fraction 6, 8 and 9 fluoresce only at the sample origin whiles fraction 7 fluoresces at the solvent front. Fractions 4 and 5 had yellow colours which were detected in visible light, however after spraying with Anisaldehyde reagent (Fig. 4.11 D); faint yellow colour was observed at the solvent front.

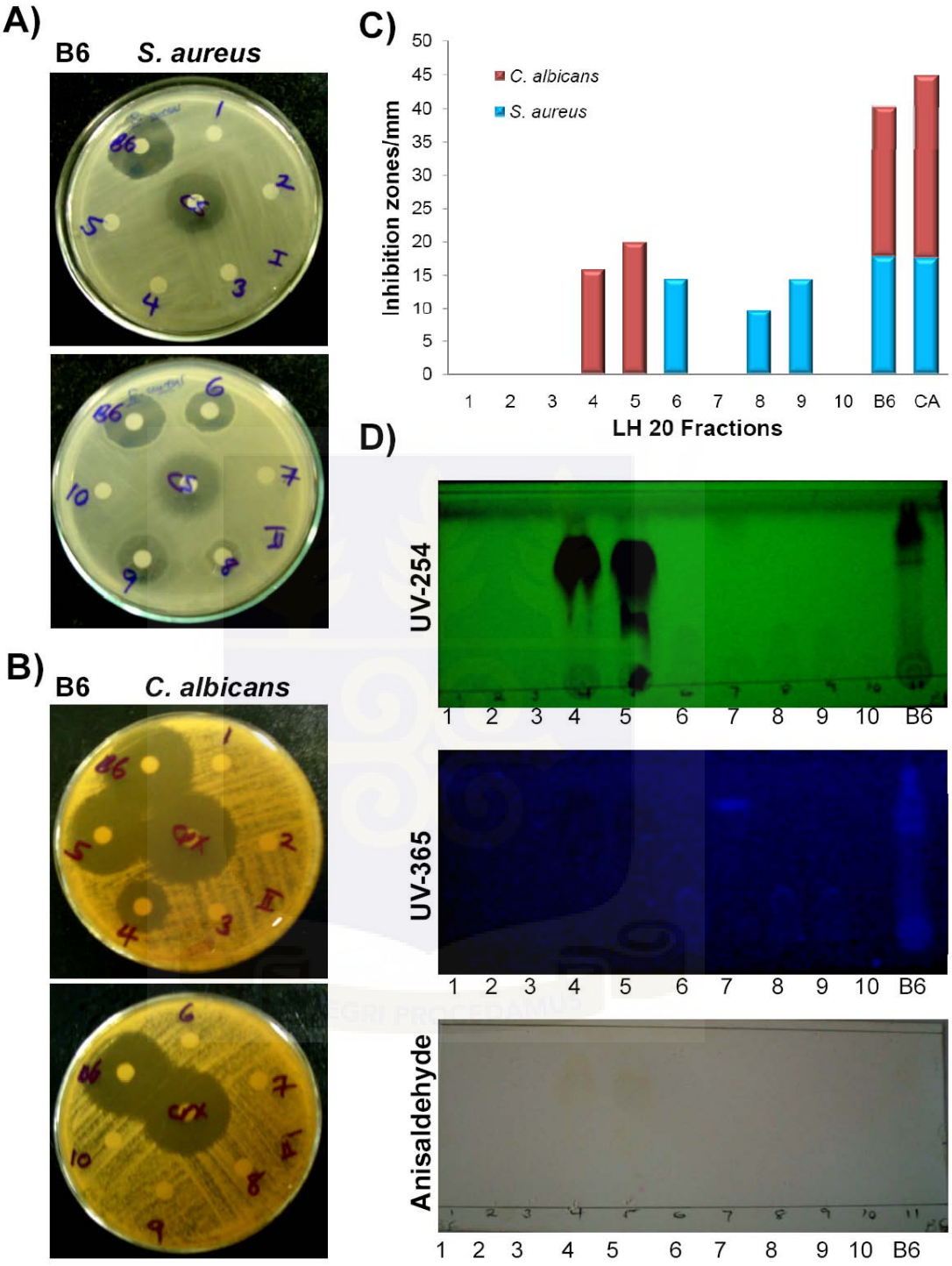


Figure 4.11. Analysis of LH-20 fractions of WDF B6 extract. Disc diffusion assay plates for B6 fractions against S. aureus and C. albicans (A and B) respectively. Inhibition zones of LH- 20 fractions of B6(C). TLC chromatographs B6 fractions at UV-254, UV-365 and after spraying with Anisaldehyde reagent.

4.10.3 Antimicrobial activity exhibited by B7 fractions and TLC chromatograph.

The B7 fractions collected at a flow rate of 0.99 ml/min or a drop rate of 1 drop per second. The LH-20 fractions recovered from WDF B7 after drying were reconstituted into an equivalent of 5mg/ml of the B7 crude extract. Fractions from B6 were tested on *S. aureus ATCC.2* and *C. albicans KBTH.2*. Disc diffusion assay method was used to evaluate the antimicrobial activity of the B7 fractions. The results of disc diffusion assay on *S. aureus ATCC.2* and *C. albicans KBTH.2* are displayed respectively Fig 4.12 (A and B). Zones of inhibition were observed for the fractions 4 and 7 for *S. aureus ATCC.2*. Fractions 1, 2, 3, 5, 6, 8, 9 and 10 exhibited no activity for *S. aureus ATCC.2*. Fraction 4 and 7 exhibited almost the same zone of inhibition for *S. aureus* ATCC.2 (Fig. 4.12 C). Fractions 3, 5 and 6 were found to exhibit antimicrobial activity against C. *albicans KBTH.2.* Fraction 6 exhibited the highest inhibition zone for *C. albicans KBTH.2*. However, fractions that had activity of *S. aureus ATCC.2* had no activity on C. *albicans KBTH.2* and vice versa. Thin layer chromatography (TLC) was performed on B7 fractions using the solvent system ***EtOAc: CH_3CN: PetEth (7:2:1).*** Fractions (10ul) were spotted on TLC plates and developed. Fractions 3, 5, 6, and 7 absorbed fluoresced at UV-254 nm at all the three polarity regions, sample origin, plate median and the solvent front but fraction 8 absorbs at both the plate median and the solvent front with intensed bands (Fig.4.12 D). Fraction 7 alone was observed to fluorescence at UV-254nm at both the sample origin and the solvent front. This same fraction was able to absorb UV at 365 nm at the solvent front. Fractions 3, 5 and 6 had yellow colours which were detected in visible light. These yellow colours were retained after Anisaldehyde spray (Fig. 4.12 D). Fraction 8 although had no activity against any of the test organisms, it was observed to absorb UV at 254 nm and also shadow fluorescence at UV-365 nm.

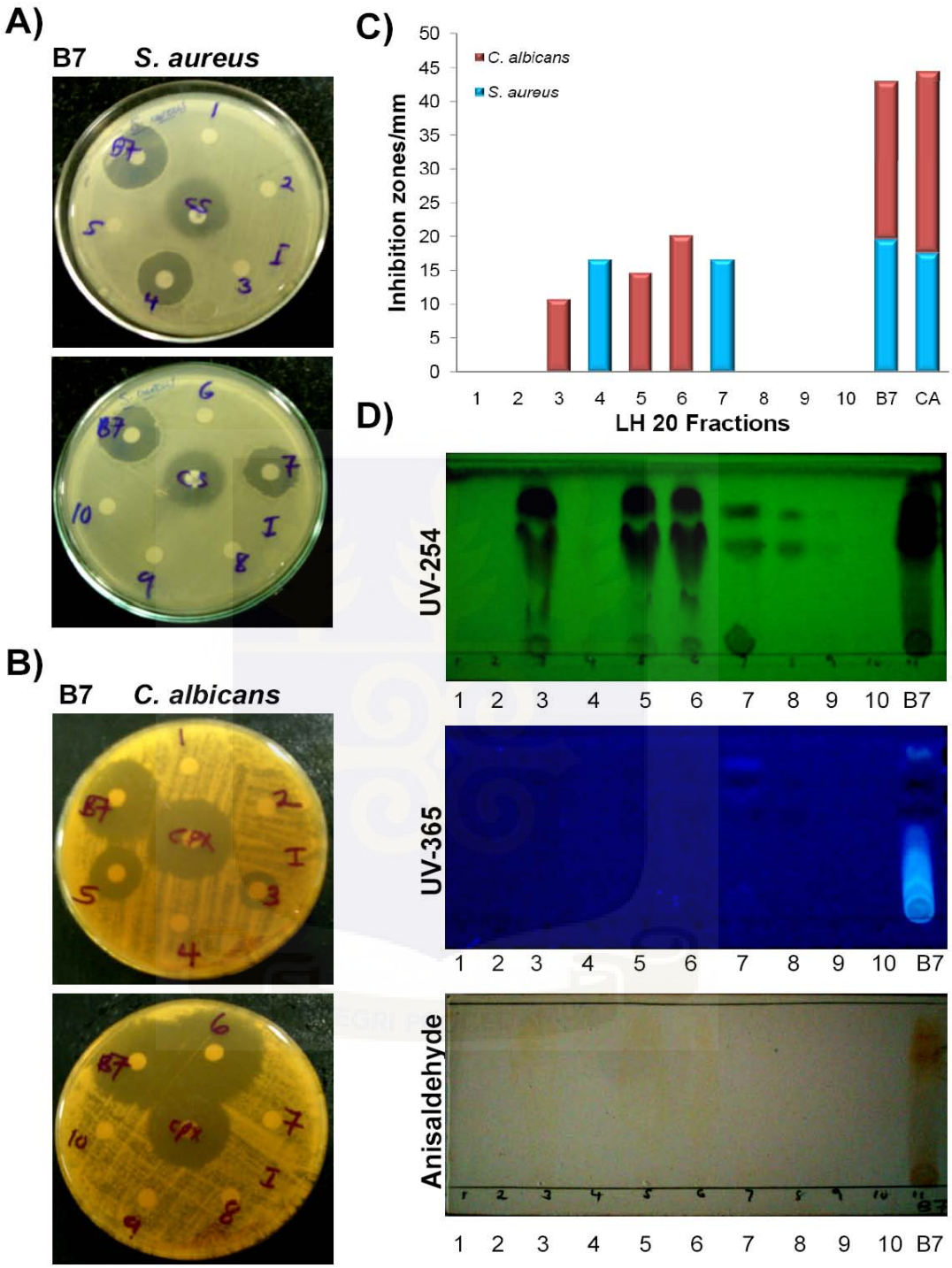


Figure 4.12. ***Analysis of LH-20 fractions of WDF B6 extract. Disc diffusion assay plates for B7 fractions against S. aureus and C. albicans (A and B) respectively. Inhibition zones of LH- 20 fractions of B7(C). TLC chromatographs B6 fractions at UV-254, UV-365 and after spraying with Anisaldehyde reagent.***

4.10.4 Antimicrobial activity exhibited by E2 fractions and TLC chromatograph.

The E2 fractions collected at a flow rate of 1.31 ml/min or a drop rate of 1 drop per second. The LH-20 fractions obtained from WDF E2 after drying were reconstituted into an equivalent of 5mg/ml of the E2 crude extract. The E2 fractions were tested against *S. aureus ATCC.2* and *E. coli NMIMR.3.* The antimicrobial evaluation of E2 fractions was performed by disc diffusion method. Fig. 4.13 (A and B) display the results of the disc diffusion assay on *S. aureus ATCC.*2 and *E. coli NMIMR.3* respectively.

Inhibition zones observed for *S. aureus ATCC.2* were found in fractions 3, 4 and 5. There were no inhibitory activities for fractions 1, 2, 6, 7, 8, 9 and 10 against *S. aureus ATCC.2.* Although fractions 3 and 4 exhibited activity against S. *aureus ATCC.2,* it must be emphasized that they were of minimal inhibitory activity in comparison with fraction 5 which gave the highest inhibition zone. The E2 fractions, 1 and 5 tested on *E. coli NMIMR.3* exhibited inhibitory effect against the test organism. The fractions 2, 3, 4, 6, 7, 8, 9, and 10 were observed to have no inhibitory effect on the test organism. The inhibition zones exhibited by both fractions 1 and 5 were almost equal. Fraction 5 was observed to have a broad spectrum antimicrobial effect since it was able to inhibit both test organisms with the highest zones. Fractions 3 and 4 were found to have inhibitory effect only on *S. aureus ATCC.2* whereas fraction 1 was found to inhibit only *E. coli NMIMR.3.*

Thin layer chromatography (TLC) was performed on E2 fractions using the solvent system ***EtOAc: CH_3CN: PetEth (7:2:1).*** Fractions (10ul) were spotted on TLC plates and developed. Most of the fractions, 3, 4, 5, 6, 7 and 8 absorbed UV-254 nm at the sample

origin (Fig.4.13 D). Fractions 1, 3, 4, and 5 chromatographs at UV-365 nm showed fluorescence at the sample origin. Fractions 7 and 8 again fluoresced at the solvent front. The faint yellow colours seen in visible light in some of the fractions, however, disappeared after spraying with Anisaldehyde reagent (Fig. 4.13 D).

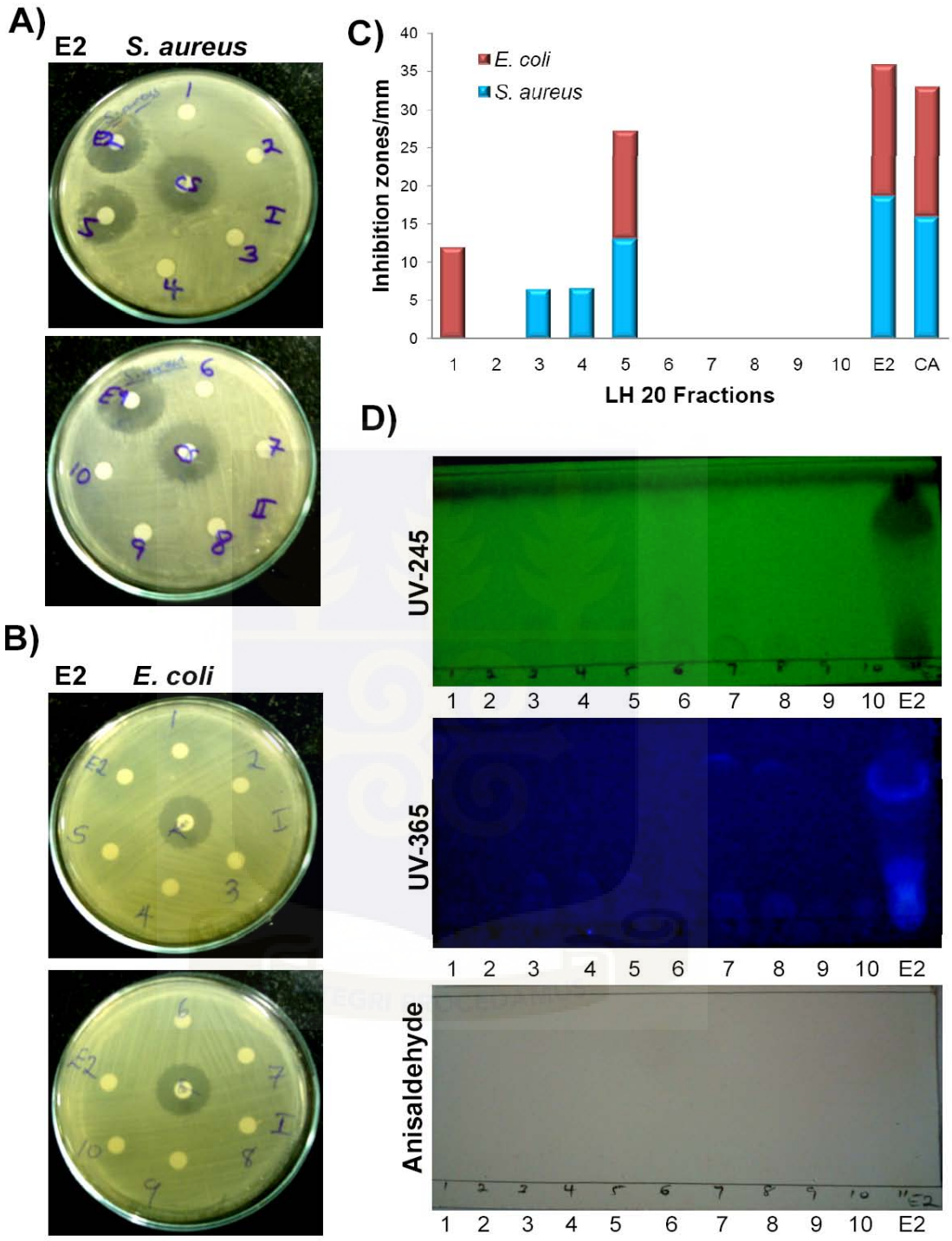


Figure 4.13. ***Analysis of LH-20 fractions of WDF E2 extract. Disc diffusion assay plates for B7 fractions against S. aureus and C. albicans (A and B) respectively. Inhibition zones of LH- 20 fractions of E2(C). TLC chromatographs E2 fractions at UV-254, UV-365 and after spraying with Anisaldehyde reagent.***

4.10.5 Antimicrobial activity exhibited by E9 fractions and TLC chromatograph.

The E9 fractions collected at a flow rate of 1.19 ml/min or a drop rate of 1 drop per second. The LH-20 fractions obtained from WDF E9 after drying were also reconstituted into an equivalent of 5mg/ml of the E9 crude extract. The E9 fractions were tested against *S. aureus ATCC.2* and *E. coli NMIMR.3*. The antimicrobial evaluation of E9 fractions was performed by disc diffusion method.

Fig. 4.14 (A and B) display the results of the disc diffusion assay on *S. aureus ATCC.2* and *E. coli NMIMR.3* respectively. Fractions 4 and 5 were found to have inhibitory effect on both test organisms *S. aureus ATCC.2* and *E. coli NMIMR.3*. The highest inhibition zone was obtained from fraction 4 against *S. aureus ATCC.2*. Fraction 5 even though had activity against S. *aureus ATCC.2;* it recorded the least inhibition zones. Fraction 4 and 5 again exhibited inhibition against *E. coli NMIMR.3*. Both fractions had almost the same inhibition zones against *E. coli NMIMR.3*. The following fractions, 1, 2, 3, 6, 7, 8, 9, and 10 exhibited no inhibitory effect on *S. aureus ATCC.2* and *E. coli NMIMR.3*. Fractions 4 and 5 showed a broad spectrum activity against both test organisms. It can be stated that fraction 4 inhibits both test organisms at equal rates (Fig. 4.14 C.); whiles fraction 5 inhibits *E. coli NMIMR.3* more than *S. aureus ATCC.2*.

Thin layer chromatography (TLC) was performed on E9 fractions using the solvent system ***EtOAc: CH_3CN: PetEth (7:2:1).*** Fractions (10ul) were spotted on TLC plates and developed. Fractions 4, 5, 6 and 7 were observed to absorb UV-254 nm. These fractions absorbed at the sample origin whiles fractions, however 4, 5, and 6 again absorbed at the solvent front. Most of the fractions, 4, 5, 6, and 7 shadowed fluorescence at UV-365nm

(Fig.4.14 D). Fractions 4 and 5 fluoresce at the sample origin and that of 6 and 7 at the solvent front, although fractions 6 and 7 had no inhibitory effect on the test organisms. Fractions 4 and 5 which had faint yellow colours in visible light disappeared after spraying with Anisaldehyde reagent (Fig. 4.14 D).

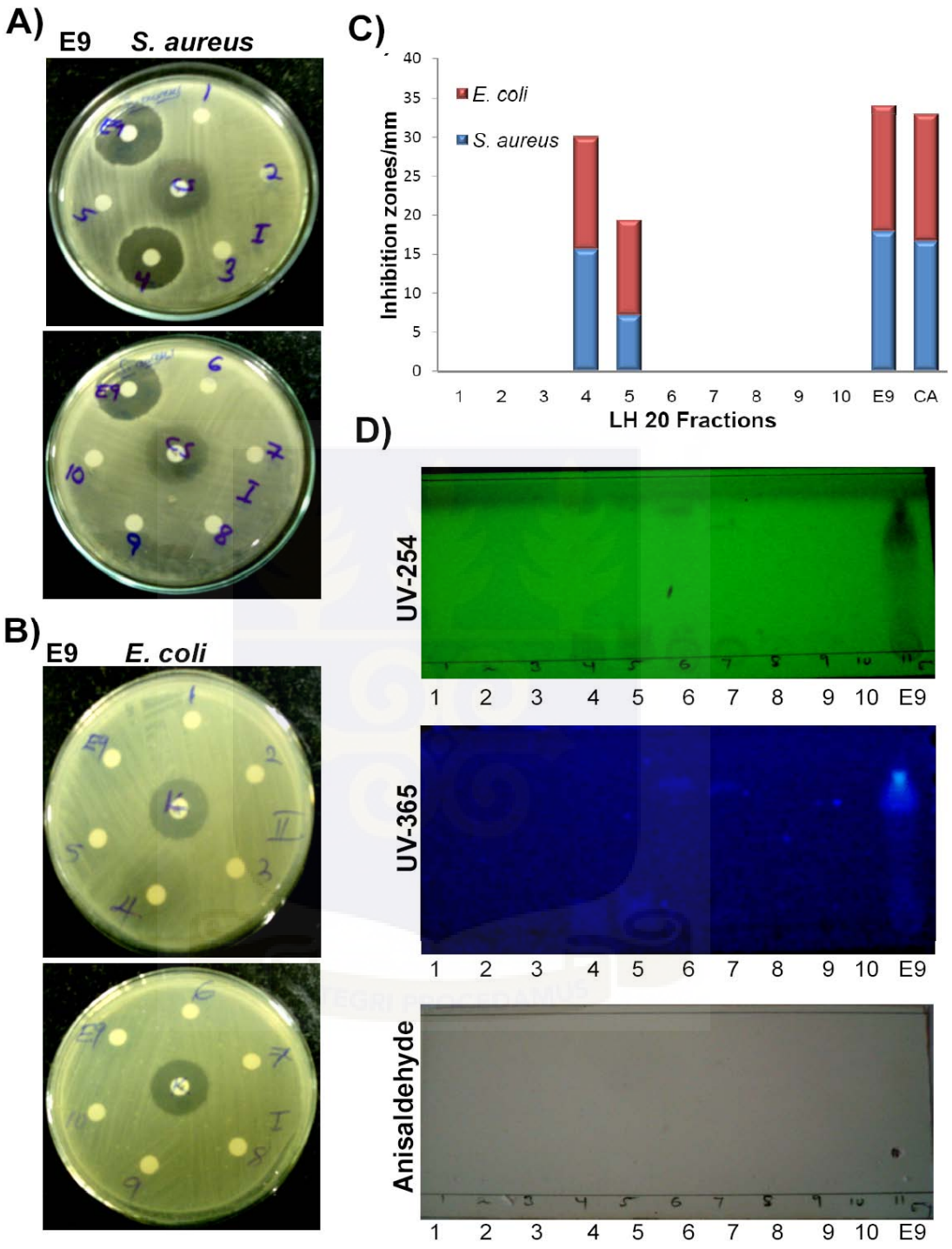


Figure 4.14. ***Analysis of LH-20 fractions of WDF E9 extract. Disc diffusion assay plates for E9 fractions against S. aureus and C. albicans (A and B) respectively. Inhibition zones of LH- 20 fractions of E9(C). TLC chromatographs B6 fractions at UV-254, UV-365 and after spraying with Anisaldehyde reagent.***

4.10.6 Antimicrobial activity exhibited by F3 fractions and TLC chromatograph.

The F3 fractions collected at a flow rate of 1.29 ml/min or a drop rate of 1 drop per second. The LH-20 fractions obtained from WDF F3 after drying were reconstituted into an equivalent of 5mg/ml of the F3 crude extract. The test organisms *S. aureus ATCC.2* and *E. coli NMIMR.3* were tested against the F3 fractions. The evaluation of F3 fractions activity was performed by disc diffusion method. Fig. 4.15 (A and B) display the results of the disc diffusion assay on *S. aureus ATCC.2* and *E. coli NMIMR.3* respectively.

Inhibition zones observed for *S. aureus ATCC.2* were found in fractions 5 and 7. There were no inhibitory activities for fractions 1, 2, 3, 4, 6, 8, 9 and 10 against *S. aureus ATCC.2.* Although fractions 5 exhibited activity against S. *aureus ATCC.2,* it must be emphasized that the effect of fraction 5 on *S. aureus ATCC.2* was minimal in comparison with fraction 7 which gave the highest inhibition zone. The E9 fractions, 4, 7 and 10 exhibited inhibitory effect on the test organism *E. coli NMIMR.3*. The fractions 1, 2, 3, 5, 6, 8, and 9, were observed to have no inhibitory effect on the test organism. The inhibition zones exhibited by fractions 4 and 7 were high when compared with fraction 10. Fraction 7 only was observed to have the broad spectrum antimicrobial effect since it was able to inhibit both test organisms with the highest inhibition zones. Fraction 5 was found to have inhibitory effect only on *S. aureus ATCC.2* whereas fraction 4 and 10 were found to inhibit only *E. coli NMIMR.3.*

Thin layer chromatography (TLC) was performed on F3 fractions using the solvent system ***EtOAc: CH_3CN: PetEth (7:2:1).*** Fractions (10ul) were spotted on TLC plates and developed. Fractions 4, 5, 6, 7, 8, and 9 absorbed fluorescence slightly at UV-254 nm at

the sample origin (Fig.4.15 D). The following fractions (3, 4, 5, 6, and 7) also fluoresce at UV-365 nm at the solvent front. Fractions 4, 5 and 7 which had faint yellow colours in visible light disappeared after spraying with Anisaldehyde reagent (Fig. 4.15 D).

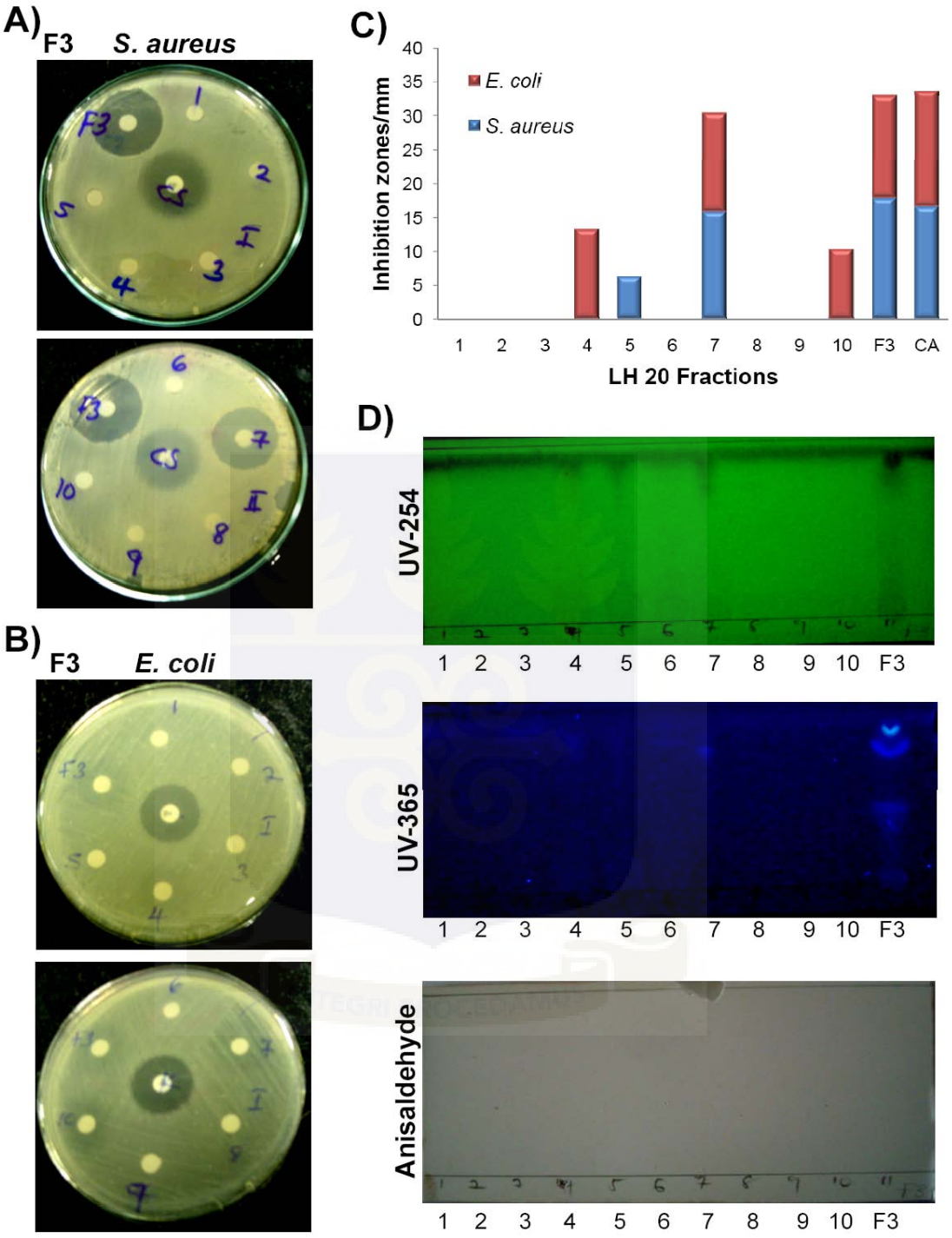


Figure 4.15. ***Analysis of LH-20 fractions of WDF B6 extract. Disc diffusion assay plates for F3 fractions against S. aureus and C. albicans (A and B) respectively. Inhibition zones of LH- 20 fractions of F3(C). TLC chromatographs F3 fractions at UV-254, UV-365 and after spraying with Anisaldehyde reagent.***

CHAPTER FIVE

DISCUSSION

The focus of this project was to investigate the secondary metabolites of wood decay fungi (WDF) with emphasis on biologically active compounds. The fungal isolates were collected from the University of Ghana campus, with exception of four which were obtained outside the University campus. The antimicrobial activity of 54 wood decay fungi (WDF) in liquid culture was studied.

5.1 Temporal profile of active fungal secondary metabolites production`

The time course study was carried out in the study in order to determine the time points during culture at which the fungal secondary metabolites are produced, and also periods at which specific active fungal metabolites are being generated. The onset of bioactive metabolites began as early as the 7th day of culture with a steady rise till the 22nd day of culture at which the highest inhibition was attained against *S. aureus ATCC.2* and *C. albicans KBTH.2* in both WDF extracts. Much of the glucose used in the culture medium by 7th day had been cleared from the culture medium for the growth of the fungi. This point of glucose clearance clearly coincided with the onset of the active fungal metabolites production on the 7th day of culture. This supports the observation made by **B'ulock *et al.*, 1965,** that the consumption and utilization of one or more growth limiting substrates such as glucose, initiate the synthesis of secondary metabolites. Almost undetectable amounts of glucose in the culture medium from the 22nd to the 48th day of culture also coincided with the periods at culture where the highest zones of bioactive compounds were generated. Higher levels of secondary

metabolites are usually produced only after most of the cellular growth has taken place in cultures **(Weinberg, 1970).** According to **Martin and Demain, 1980**, depletion of secondary metabolite precursors, irreversible decay of one or more secondary metabolite synthetase and feedback effect of the metabolite against its production have been found as the probable cause of secondary metabolite synthesis termination. Although the inhibition zones obtained from the 22nd day to the 48th day of culture were almost equal, there is the possibility of attaining higher inhibition zones if the extracts are further diluted to a lower concentration. In order to obtain good yield of the active bioactive compounds and greater inhibition zones, termination of cultures after day 27 is recommended since by that time all the fungal active metabolites are assumed to have been generated.

5.2 Organic extraction of WDF cultures produces varying amounts of fungal extracts

Organic extraction was performed on the cultures of the 54 WDF in the primary screening and the 27 refermented WDF in the secondary screen as previously described, with yields of fungal crude extracts in volumes for the primary screen in Table 1 and density for the secondary screen in Table 2 A and B.

A greater number of extracts (50%) in the primary screen attained crude extract volumes below 1.00 ml. About 11% of the extracts attained the highest volume of 2.0 ml. The amount of crude extracts that are recovered during organic extraction are affected by culture conditions such as volume of culture, aeration, temperature, pH, the number of viable fungal spores present in the fruiting bodies for inoculation and the type of solvent used in the extraction. According to **Boulianne *et al.*, 2000,** the fungal fruiting bodies

contain the spores which are responsible for the production of metabolites. Therefore a culture medium containing fewer viable spores **Buswell and Chang, 1993,** is likely to produce very little or no amount of fungal crude extracts as was observed in the primary screen.

Although the volumes of culture also influences the amounts of crude extracts that are produced, the solvent used in the extraction as well as the extraction procedure also affect the amount or extracts greatly. **Rosecke and Konig, 2000** reported that, although ethyl acetate have been used in the extraction of fungal secondary metabolites, however, they observed differences in the extract volumes when different extraction solvents (ethyl acetate and methanol) were used on different fungal isolates. They observed extract volumes ranging from 1.5ml to 5.6ml from EtOAc extraction, and 2.5ml to 10.3ml from MeOH extraction using the fruiting bodies of different *Ganoderma* isolates. The amounts of crude extract that were obtained in this project had volumes ranging from 0.30ml to 2.00ml which is somehow comparable with 1.5ml to 5.6ml that was reported by **Rosecke and Konig, 2000,** however, the MeOH extracts produced higher volumes of fungal extracts which justifies the argument that the solvent used in this project might have contributed to the lower amounts of fungal crude extracts obtained.

In contrast, the 27 WDF that were cultured in higher volumes (1L) produced greater amounts of fungal extracts, even though about the same amount (5g) of fungal fruiting bodies was used in both cultures. About 48% of the WDF produced amount of extracts greater than 20 ml in this project. The higher amounts of crude extracts produced in the 1L fermentation buttresses the earlier accession that culture volume greatly affect the amounts of crude extracts generated. The higher amounts of crude extracts cannot be categorically attributed to the larger culture volumes. The extraction protocol was

modified slightly after reviewing literature works on organic solvent extraction. The swirling of cultures for 48 hours on magnetic stirrer was reduced to shaking vigorously in a separating funnel for 45 minutes, markedly reducing the shaking time. This modification is also likely to have contributed to the larger amounts of crude extracts produced. The higher volumes obtained in the 1L culture also defeats the earlier observation by **Rosecke and Konig, 2000,** that the solvent type affects the amount of ethyl acetate crude extracts produced as volumes obtained in this project ranges from 7 ml to 27 ml and the MeOH crude extracts by **Rosecke and Konig, 2000,** ranged from 2.5 ml to 10.3 ml, however the culture volumes used by **Rosecke and Konig, 2000,** has not been reported. This indicates that the lower yields in the primary culture might have resulted not because of the extraction solvent, but rather the culture volume, extraction procedure and the number of viable spores that were present in the fruiting bodies used for the culture.

5.3 UV/Vis detects distinct components present in the WDF extract

UV-visible spectra generally show only a few broad absorbance bands. Compared with techniques such as infrared spectroscopy, which produces many narrow bands, UV-visible spectroscopy provides a limited amount of qualitative information. Most absorption by organic compounds results from the presence of π (that is, unsaturated) bonds **(Strong, 1976).**

The presence of an absorbance band at a particular wavelength often is a good indicator of the presence of a chromophore, a molecular group usually containing a π bond. However, the position of the absorbance maximum is not fixed but depends partially on

the molecular environment of the chromophore and on the solvent in which the sample may be dissolved. Other parameters, such as pH and temperature, also may cause changes in both the intensity and the wavelength of the absorbance maxima **(Paul, 1991).**

The UV/vis spectroscopy was to confirm the presence or otherwise of the varied compounds that might have been produced by the 54 WDF extracts. Majority of the WDF extract absorbed UV at lower wavelength, with very few absorbing at medium and higher wavelength. In general, all the spectra obtained for the 54 WDF extracts had had distinct absorption spectra with fewer numbers of bands as reported by **Strong, 1976.** However, some common identity such as, the number of absorbance band and absorbance start point, existed among the spectra. These similarities were the basis for grouping all the 54 WDF spectra into four major clusters. Cluster members absorbing at a particular wavelength have a high propensity of having a similar chromophore as suggested by **(Paul, 1991).**

The A1 cluster for instance has two major peaks with wavelength absorbing at 254nm and 367nm. These two peaks are of different chromophore, an indication that different compounds are absorbing at these peaks. These clusters A2, A5 and C7 all possessed single peaks absorbing at wavelengths 287 nm, 288 nm and 288 nm respectively also indicating that varied compounds are being generated. Although UV-visible spectra do not enable absolute identification of an unknown, they frequently are used to confirm the identity of a substance through comparison of the measured spectrum with a reference spectrum and where spectra are highly similar, derivative spectra may be used **(Burgess and Knowles, 1981).** Cluster A5 and C7 both absorbs at wavelength 288 nm, therefore

the likelihood of possessing the same chromophore is high, however in this project, there was no reference spectrum to be compared with.

The isolation of pure compounds from the 54 WDF crude extracts would enhance the categorization of the individual peaks on the UV/vis spectra. Analytical techniques such as HPLC could be employed to obtain spectra for the pure fungal compounds so as to distinguish the varied compounds that are produced by the WDF extract.

5.4 Chromatographic separation of compounds from the WDF Extracts

TLC analysis was carried out to determine the number of different components present in the crude extracts of the WDF. The bands separation of compounds on the TLC plate were detected under UV absorbing at wavelengths 254 nm and 365 nm, followed by spraying the TLC plates with Anisaldehyde reagent. Anisaldehyde reagent spray has been known for the detection of phenols, sugars, terpenes steroids. All these chemical components are known to exhibit colours upon being sprayed with this reagent. The colours that show are indicative of the functional groups that the extracts are likely to possess.

Phenolic compounds are detected by the presence of violets spots after spraying with Anisaldehyde reagent, **(Wagner *et al.*, 1984)**. Sugars are also detected by the appearance of yellow colours. Carbohydrates (aldohexose, ketohexose, and aldopentose) and uronic acids have also been reported to produce brown spots, yellow spots, green spots and pink spots respectively after Anisaldehyde spray. Terpenes and steroids are found to be associated with blue and green spots respectively. Classes of terpenoids, the triterpenes that can be specifically detected by Anisaldehyde reagent is the cardiac

glycosides **(Harbourne 1973, Wagner *et al.,* 1984)**. Cardiac glycosides are detected by the appearance of pink zones in visible light after Anisaldehyde spray

In the determination of the relative polarities of the various compound generated by the WDF extracts, the following parameters were used which are compounds at solvent front (as low polarity), plate median (medium polarity), whilst those at the sample origin (represent the highest polarity), These parameters were used to classify the different extracts according to the appearance of their compounds on the TLC plates.

The thin layer chromatographs obtained for all the 54 WDF extracts revealed several compounds distributed at different polarity regions such as solvent front, plate median and the sample origin at wavelengths 254 nm, 365 nm and after spraying with Anisaldehyde stain. In the determination of the relative polarities of the various compound generated by the WDF extracts, the following parameters were used which are compounds at solvent front (SF) (as low relative polarity), plate median (M) (medium relative polarity), whilst those at the sample origin (SO) (represent the highest relative polarity), These parameters were used to classify the different extracts according to the appearance of their compounds on the TLC plates. Therefore the relative polarity of the compound ranging from the lowest to the highest are SF< M< SF+M< SO< SO+SF< SO+M< SO+SF+M. At UV-254 nm, majority of the compounds produced by the extracts were found to be distributed at the polarity regions (SO+SF and SO+SF+M). These two regions are relatively highly polar regions suggesting that the compounds that were produced have high polarity. No compound was observed at UV-254 nm for any of the extracts at SF, a region of low polarity as shown in the polarity profile. Fewer numbers of the compounds were produced at the median polarity region.

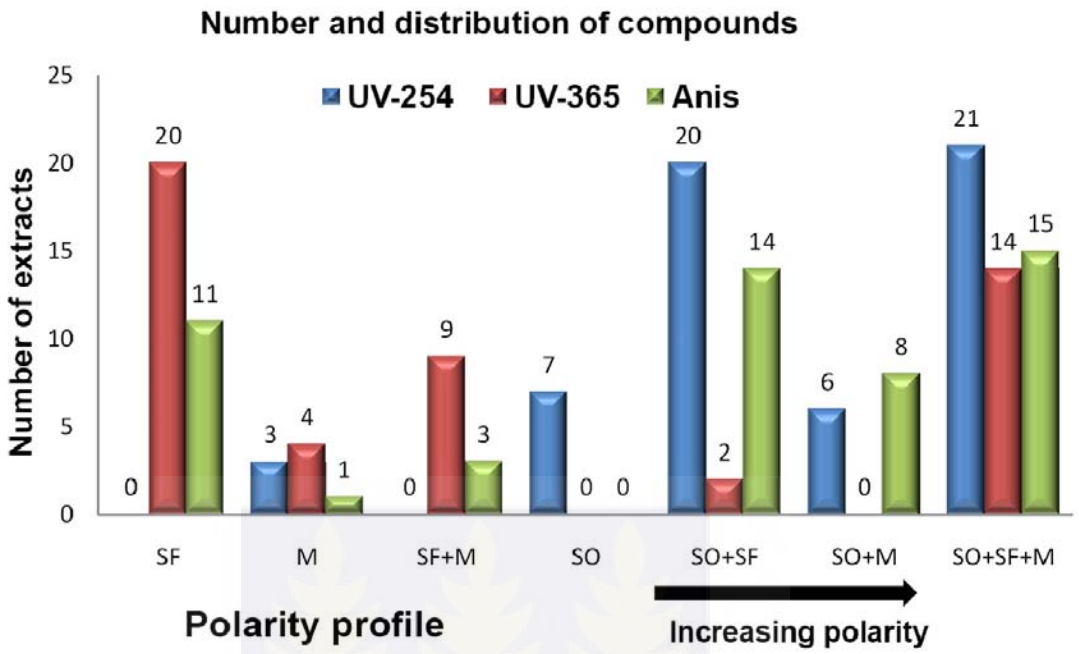


Figure 5.1. *Distribution of compounds by polarity at UV-254 nm, UV-365 nm and after Anisaldehyde reagent spray. SF= Solvent Front, M= Median region, SF+M = Solvent Front and Median region, SO= Sample Origin, SO+SF= Sample Origin and Solvent Front, SO+M= Sample Origin and Median region, SO+SF+M= Sample Origin, Solvent Front and Median region.*

Using the UV-365 nm detection, a higher number of extracts, 20 in all, showed compounds that gave band at the solvent front and 14 extract showed bands at all the polarity regions, SO+SF+M. None of the extracts produced a band fluorescing at the high polarity region, SO, fewer extracts produced band at the median region. The Anisaldehyde gave a widest distribution of the compounds, as almost all the polarity regions revealed bands with the greatest distribution found among these polarity regions, SO+SF+M, SO+SF, and SF.

The polarity profile clearly gives the distribution of the compounds produced by the WDF extracts at all the polarity regions. The results show that different compounds have different polarities at which they can be absorbed.

5.4.1 Occurrence and diversity of functional groups

Table 5.1. Total number of colours and band patterns produced by compounds from WDF extract on TLC

No. of bands	Colour of WDF extract					
	Yellow	Blue	Green	Brown	Pink	Violet
1	10	25	8	9	6	5
2	0	6	1	0	0	2
3	0	0	1	0	0	0
Total bands	10	37	13	9	6	9

Table 5.1.1 Colours shown on TLC by WDF extracts and their possible functional group

Colours of WDF Extracts	Possible Functional Group
Yellow	Sugars
Blue	Terpenes
Green	Steroids
Brown	Aldohexose
Pink	Cardiac glycosides/Uronic acid
Violet	Phenols

Non specific detection of terpenes, steroids, phenols and sugars performed by spraying TLC plate with Anisaldehyde reagent revealed blue, green, violets and yellow coloured components respectively, with majority of the extracts being terpenes. The number of extracts giving a particular colour and the band pattern on TCL plate is shown in Table 4A. Greater number of extracts (31) produced blue colours with 37 being the band number.

Terpenes encompass a number of compounds including volatile mono and sesquiterpenes (C15), to the less volatile diterpenes (C20) to the in volatile triterpenes (Harbourne 1973). Unfortunately there is no sensitive reagent for detecting the classes of terpenoids; therefore differentiation of the terpenoids types is generally impossible using TLC reagent spray **(Harbourne 1973).**

However, Anisaldehyde reagent can somehow distinguish between the triterpenes types, cardiac glycosides and saponins **(Harbourne 1973, Wagner *et al.*, 1984).** Cardiac glycosides are detected by the appearance of pink zones in visible light after Anisaldehyde spray. It can be assumed that the extracts (A3, A5, E3, E6, and F7) that exhibited the pink colouration belong to the triterpenoids with cardiac glycosides as the active components. It can also be inferred that majority of the extracts (57%) have terpenoids as their active compounds as a greater number of them showed blue colouration after Anisaldehyde spray.

From literature there seems to be overlap in the production of coloured components after Anisaldehyde reagent spray which is indicative of their functional groups.

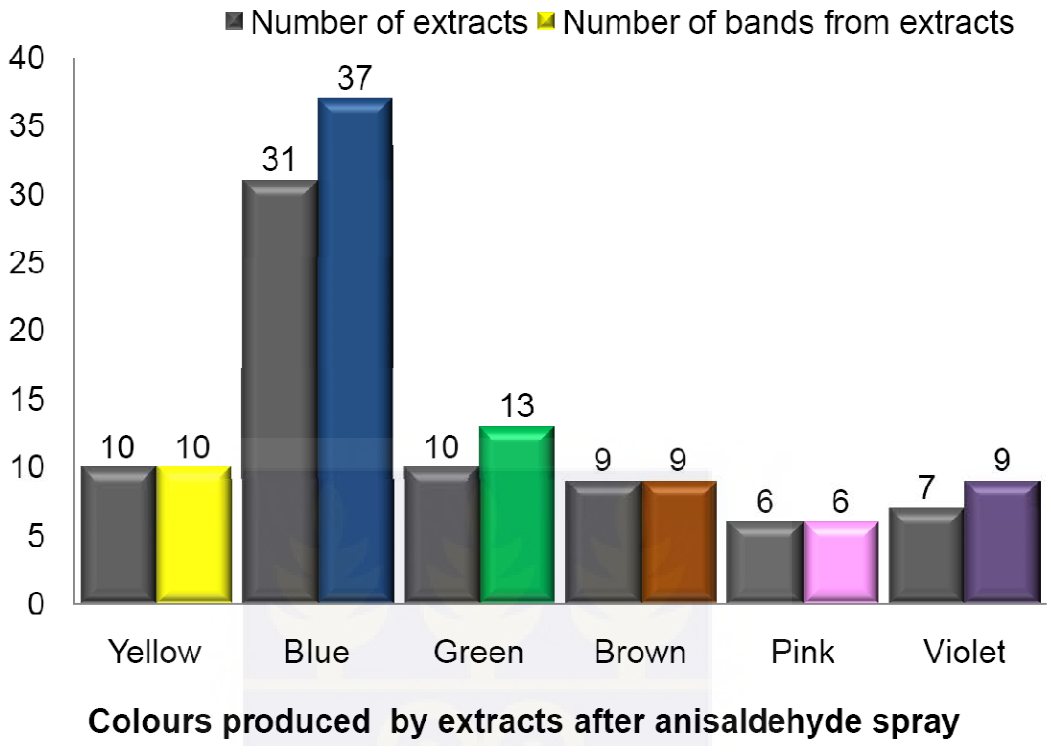


Figure 5.2. *The occurrence and distribution of functional groups after Anisaldehyde reagent spray. The number of colours components produced by the WDF extracts (first bar). The number of coloured bands produced by the WDF extracts (second bar).*

The carbohydrate (aldopentose) gives green colouration after Anisaldehyde reagent spray, nevertheless this green colour is also known to be shown by steroids. Again uronic acids are also found to produce pinkish colouration, as well as a class of the triterpenes cardiac glycosides **(Wagner *et al.*, 1984)**. These overlap in colour detection for the functional groups clearly indicates the non specific detection of the reagent system, therefore the need for a good detection system.

5.5 High number of WDF extracts possessed antimicrobial activity

The extraction process is an important step in the investigation of biologically active compounds. When extracting compounds from fungi, the type of solvent used, the extraction process utilized and the period of culturing of the fungi, all have a marked effect on the type of compounds that can be extracted. Organic extraction using solvents such as chloroform, and ethyl acetate which are non polar and highly polar respectively, different compounds such as flavanoids, alkanoids, phenols, steroids, and terpenoids are commonly extracted **(Cowan, 1999, Lin and Shiao, 1988).**Many compounds extracted from the fruiting bodies of basidiomycetes have been found to exhibit biological activities towards tumor and virus infected cells **(Gao *et al.*, 2003, Oh *et al.*, 2000)**. On review of the literature there appears to be fewer studies on the antimicrobial activity of the organic extracts from basidiomycetes.

The focus on the study was the investigation of wood decay fungi towards their secondary metabolites with emphasis on biologically active compounds, mainly from antimicrobial bioassays. The crude extracts from 54 WDF were evaluated for biological activities using disc diffusion assay against the bacteria *Staphylococcus aureus ATCC.2*, *Escherichia coli NMIMR.3* and the fungi *Candida albicans KBTH.2* and *Aspergilus niger ATCC.2.*

A total of 40 out of the 54 WDF extracts tested were indeed able to produce some antimicrobial low molecular weight substances under the condition of the aerobic liquid fermentation against the organisms tested. This represents a 74% inhibition towards the various test organisms. A report by **Suay and Arenal, 2000,** on antimicrobial evaluation of 200 mushrooms revealed 75% antimicrobial activity. This high success rate was achieved in this project with respect to antimicrobial activity that was attained by the

individual extracts. Variations with respect to antimicrobial activity towards the test organisms were seen in a number of extracts. Majority of the extracts (13) possessed broad spectrum antimicrobial activity (BSAB) towards *S.aureus ATCC.2* and *E. coli NMIMR.3*. This was expected as most of the literature reviewed reported on the dual activity of fungal extracts on both Gram + and Gram – bacteria. A higher number of the extracts assayed again possessed selective antimicrobial activity towards the Gram + organism, *S.aureus ATCC.2,* whilst none of the extract had an activity selectively on the Gram – bacteria. **Mothana *et al.,* 2003,** reported on the organic extracts from Basidiomycetes e.g. *Ganoderma* to have antibacterial only some selected Gram negative bacteria. It is imperative to note that the bioactivity of the organic extract was from only the mycelium and not the liquid cultivated fruiting body medium, as it is in this research. Only 3 extracts were found to have selective antifungal activity on the test organisms, *Candida albicans KBTH.3,* and *A. niger ATCC.2*. The 3 extracts inhibiting *A. niger ATCC.2* are C1, D3, and E6 with inhibition zones of 7.15 mm, 12.64 mm, and 6.39 mm respectively. A confirmation of the antifungal activity against *A. niger ATCC.2* by repeating the disc diffusion assay method for the above extracts failed to reproduce an activity in all the 3 extracts, therefore the test organism *A. niger ATCC.2* was excluded from further bioassays. A number of factors can be associated with the failure of the above 3 extracts: instability of the bioactive components present in these extracts, changes in medium composition and incubation conditions.

A total of 11 extracts were observed to have inhibitory activity on all the three test organisms. These extracts were termed to have a non selective antimicrobial (NSAM) action against the test organisms. Another 3 extracts were also found to have non selective antimicrobial (NSAM) activity against both bacteria and fungi (*S. aureus*

ATCC.2 and *C. albicans KBTH.2*). Activity of compounds (NSAM) for different organisms is of great advantage since there is the likelihood of the activities to be possessed by different compounds.

It is extremely difficult comparing the inhibitory zones obtained in this project with what has been reported in the literature, as the identities of these fungal isolates are not known and therefore equating the inhibition zones obtained in this project with inhibition zones for known fungal isolates might not be valid. However generalized comparisons can be used to evaluate the inhibitory effect of crude extracts. **Yoon *et al.*, (1994),** who investigated the bioactivity of crude extracts from the fruiting bodies of selected Basidiomycetes, found that the extracts also exhibited inhibitory activity towards *S. aureus ATCC.2.* Again **Yoon *et al.*, (1994),** found their crude extracts to have strong activity against both the Gram positive and Gram negative bacteria (S. *aureus* and *E. coli),* which is similar to what has been observed in this project; none of the extracts selectively inhibited *E. coli NMIMR.3* but there were a number of inhibitions for both *S. aureus ATCC.2* and *E. coli NMIMR.3.*

It has been suggested that there can be variations in the bioactive components of the fungal fruiting bodies compared to the mycelium **(Lorenzen and Anke, 1998)** and that during the different stages of growth, there can be structural changes of the bioactive components and these changes could either enhance the activity of the extract or otherwise. Therefore the smaller range of activity exhibited by some of the crude extracts in this investigation can be attributed to the different bioactive compounds generated by the fruiting bodies of the fungi during the culture period.

Surprisingly, eight of the WDF extracts (A4, D1, D3, D5, E3, E5, E5, and F1) produced antimicrobial activities that were potent enough to be compared with the positive control

streptomycin against *S. aureus ATCC.2.* The antimicrobial activities exhibited by the extracts, A4, E2, E3, E4 and F1, towards *E. coli NMIMR.3* were potent enough to also be compared with the positive control Kanamycin.

On close inspection of some of the inhibition zones, there were definite areas where bacterial growths were seen. One possibility of this observation is that the bacteria had time to grow within such regions after the compounds were rendered inactive. It would then appear the extract continued to move through the agar medium and killed the bacteria after growth had occurred. The use of diameter measure of inhibition zones as a reliable measure of antibacterial activity is debated, as the size of the inhibition zone is affected by the rate of diffusion of the compound as well as the moisture content of the agar plate.

5.6 High numbers of refermented WDF maintained their bioactive compounds

The purpose of the refermentation was to confirm whether the bioactive compounds produced by specific WDF that caused various inhibitions against the test organisms are reproducible. Out of the total 27 WDF extracts assayed, 19 (70%) of the extracts were able to reproduce their bioactive principles also represent a high success rate. Although a higher success rate has been attained, there was a marked shift in antimicrobial activities after secondary screening. In the primary screening, 13 WDF extracts were recorded to possess BSAB, however, after the secondary screen; there has been a significant reduction in the numbers to 6, about 50% reduction. Amazingly the 3 WDF extracts that selectively inhibited the test organism *C. albicans* in the primary screen lost their inhibitory effect in the secondary screen.

There was a switch in bioactivity of the extracts after the secondary screen, out of the 27 WDF that were recultured in PDB, only one possessed a non selective antimicrobial (NSAM) activity, however in the secondary screen the number had increased to 3 (Fig. 5.3). The possible reasons for the re-designation in bioactivity could be that different compounds are produced under different culturing conditions, and although culture conditions are assumed to be the same, however certain parameters such as temperature, aeration etc, which cannot be adequately and accurately monitored and controlled, could affect the production of compounds resulting in switch in bioactivity.

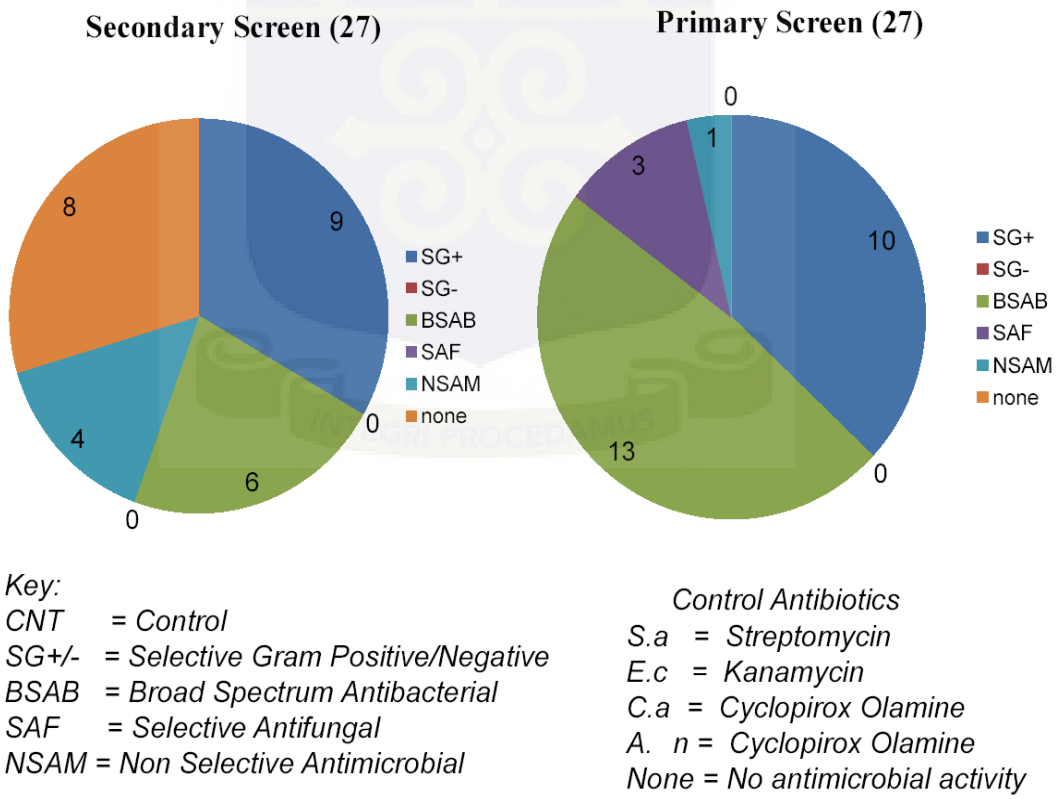


Figure 5.3. Changes in designation of antimicrobial activities during secondary screening. *Redesignation of antimicrobial activity after refermentation of HITS samples from primary screen. Shift of antimicrobial activity pattern after refermentation.*

All the WDF that were selected for the secondary screen attained some level of biological activity during the primary screen, therefore their involvement in the secondary screen. There was loss of antimicrobial activity for 8 of the WDF extracts after the secondary screening. This could be that the equivalent 5mg/ml disc concentration of WDF extract probably did not contain enough in these eight extracts to cause appreciable inhibitions against the various test organisms. Again it is also possible that the bioactive principle could not be produced in the refermentation period, or there extraction procedure employed might have affected the active principle. The results obtained so far clearly show that most of the compounds responsible for various antimicrobial actions are reproducible whilst a few others are not. However, the inability of some extracts to exhibit the activity they possessed during the primary screen in the secondary screen could be attributed to a greater number of factors.

The extracts following 8 WDF (C3, C8, C9, D6, E1, F4, F5, and F8) that failed in achieving biological activities toward their target organisms in the secondary screen. A typical example is the WDF extract C9, which was used in the time course analysis. During the primary screening, average inhibition zones of 8.68 mm and 8.59 mm were attained against *S. aureus ATCC.2* and *E. coli NMIMR.3* respectively. However in the time course analysis as early as day 9 of culture, extract C9 had attained an average inhibition zones of 13.01 mm and 9.35 mm towards *S. aureus ATCC.2* and *E. coli NMIMR.3* respectively. And by the 48th day of culture of this same WDF extract, an average inhibition zone of 20.76 mm and 17.24 mm were attained. It is possible that the concentration of the active compound produced in the secondary screen was so little due to the larger volumes of culture resulting in the failure to produce inhibition zones in the

secondary screen considering the fact that the time series cultures were 100 ml only as compared to 200 ml for primary and 1000 ml in the secondary screen.

Another possible explanation could be due to the variations in the extraction procedures as stated in the method section, looking at the fact that the extraction protocol used slightly differs among the primary screen, time course screen and the secondary screen. It is also possible that the number of fungal spores that were present in the fruiting bodies used for the secondary screen was so little that, secondary metabolites with substantial activity could not be generated resulting in their inabilities to attain any inhibition in the secondary screen **(Buswell and Chang, 1993).**

5.7 Different WDF LH-20 fractions exert different antimicrobial activities

Sephadex LH-20 fractionation was performed on 6 different WDF extracts that showed broad spectrum antimicrobial (BSAB) activity in the secondary screen. The purpose was to determine the individual fractions responsible for the various inhibitory activities that were observed and also investigate whether the activities are achieved by the same or different compounds. The selected WDF extracts are A4, B6, B7, E2, E9 and F3. Extracts A4, E2, E9 and F3. Extracts B6, B7 were selected for fractionation due to their NSAM activities towards test bacteria and fungi. The LH-20 fractionation of the WDF extracts (A4, B6, B7, E2, E9 and F3) yielded 10 fractions each, which were evaporated to small volume. Bioactivity evaluation was performed on each fraction using disc diffusion method as already described. A summary of the biological activities of all the fractions is shown in Table 5.2.

Table 5.2 Biological activities of the 10 fractions of all 6 WDF extracts

Fractions	A4		B6		B7		E2		E9		F3	
	S.a	E.c	S.a	C.a	S.a	C.a	S.a	E.c	Sa	E.c	S.a	E.c
1	SG+	-	-	-	-	-	-	SG-	-	-	-	-
2	-	SG-	-	-	-	-	-	-	-	-	-	-
3	-	SG-	-	-	-	SAF	SG+	-	-	-	-	-
4	**BSAB**		-	SAF	SG+	-	SG+	-	**BSAB**		-	SG-
5	**BSAB**		-	SAF	-	SAF	**BSAB**		**BSAB**		SG+	-
6	**BSAB**		SG+	-	-	SAF	-	-	-	-	-	-
7	SG+	-	-	-	SG+	-	-	-	-	-	**BSAB**	
8	-	-	SG+	-	-	-	-	-	-	-	-	-
9	-	-	SG+	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	SG-

The various activities of the 10 fractions from WDF A4, B6, B7, E2, E9, and F3. Selective and dual activities are shown by some of the fractions against the different test organisms. SG+ = Selective Gram +, SG- = Selective Gram -, SAF= Selective Antifungal. S.a = S. aureus, E.c = E. coli and C.a = Candida albicans

Fractions from extract A4 were tested on *S. aureus ATCC.2* and *E. coli NMIMR.3* for their broad spectrum activity. Seven (7) fractions were evaluated to posses the bioactive components against the test organisms. Inhibitions against S. aureus ATCC.2 were found to be exhibited by fractions 1, 4, 5, 6, and 7 whilst fractions 2, 3, 4, 5, and 6 were also found to inhibit *E. coli NMIMR.3.* There is an overlap of antimicrobial components in the fractions 4, 5, and 6 and this can be attributed to the broad spectrum antimicrobial activity against *S. aureus ATCC.2* and *E. coli NMIMR.3.* However, the largest inhibition zone was exhibited by fraction 5 towards the two test organisms, suggesting a

concentrated amount of the active compounds present in fraction 5. There is the possibility of these 3 extracts having the same active compounds looking at the fact that they are eluted close to each other. TLC detection at UV-254 nm also confirms that these 3 fractions are likely to be of the same compound.

Another observation was that fractions 1 and 7 were found to possess activity against *S. aureus ATCC.2* and fractions 2 and 3 against *E. coli NMIMR.3*. However fractions 1 and 7 were eluted very far from each other, which indicate they might be produced by different compounds. Fractions 2 and 3 are also eluted close to each other as well as fractions 4, 5 and 6, which mean they are likely to be of the same compound; however the broad spectrum activity was seen in fractions 4, 5 and 6. The number of fractions that exhibited antimicrobial activity against the test organisms corresponds to the bands showing on the TLC plates at UV- 245 nm. Also confirming that these fractions were responsible for the inhibitory effect achieved towards the test organisms.

Fractions from extract B6 were assessed for their activity towards *S. aureus ATCC.2* and *C. albicans KBTH.2*. Against the test organism *C. albicans KBTH.2* the fractions 4 and 5 were found to posses the bioactivities, with fractions 5 exhibiting the greatest activity when compared with the positive control Cyclopirox olamine. The elution of these fractions was so close to each other inferring that they might be of one and the same active compound. Inhibition to *S. aureus ATCC.2* was found in fractions 6, 8 and 9, with the greatest inhibition found in fractions 6 and 9 when compared with the control antibiotics. Surprisingly fraction 7 which was eluted between fractions 6 and 8 exhibited no antibacterial activity towards *S. aureus ATCC.2* which makes it possible for fraction 6 to be of different active component from fraction 8 and 9 which can be assumed to have the same active compounds. It can be inferred that the bioactive

activities observed are from different compounds. The TLC profile at UV-254 confirms this by showing intensed bands for fractions 4 and 5. The band pattern is somehow related also suggesting that the active components in both fractions might be the same.

Fractions of extracts from B6 shows a similar pattern as B6, where different compounds are supposedly responsible for the biological activities observed. Fractions 3, 5 and 6 were active against *C. albicans KBTH.2* with the greatest activity exhibited by fraction 6 in comparison with the positive control cyclopirox olamine. It is possible for fraction 3 to be of different active component from that of fractions 5 and 6 which were eluted close to each other, therefore the likelihood of exhibiting the same active compound or be of the same compound but modified during the course of production. Antimicrobial activity was seen in fractions 4 and 7 against *S. aureus ATCC.2* with both fractions having almost equal inhibition zones when compared with the positive control antibiotics. Fractions 4 and 7 can be inferred to be of different active components with regards to their positions during elution. Although fraction 3 was eluted not close to fractions 5 and 6, their TLC profile at UV-254 nm reveals similar band pattern which seems to contradict the earlier assertion that fractions 3 might be of different active components from fractions 5 and 6. At UV-254 nm fraction 4 did not show a good visible band pattern, however, fraction 8 revealed a visible band pattern at UV- 254 nm, which was somehow related to the band pattern shown by fraction 8, although it exhibited no activity towards any of the test organisms. It is possible that the active component of fraction 4 is not a UV absorbing component, thereby failing to show at the stated UV-254 nm.

In the E2 fractions, it was observed that fractions 1 and 5 exhibited strong antimicrobial activity against *E. coli NMIMR.3,* giving an average inhibition zones that are potent

enough to be compared with the positive control antibiotics, Kanamycin. Again fractions 1 and 5 possessed activity towards the same organism, it can be inferred that they are of different active compounds looking at the distance between their elution points. Biological activity was observed in fractions 3, 4, and 5 towards *S. aureus ATCC.2* with the greatest inhibition zone attained by fraction 5 which is also potent enough to be compared with the positive control antibiotics. Since fraction 3, 4, and 5 were eluted close to each other, it is likely they are of the same active compound.

The broad spectrum activity of extract E2 can be seen to be exerted by fraction 5, which had the highest inhibition zones towards the two test organisms. Surprisingly their TLC profiles at UV-254 nm could not reveal the bands responsible for these activities that were seen, which can be assumed they are not UV absorbing. However at UV-365 nm spots can be found very close to the baseline. Although fractions 7 and 8 showed bands at UV-365 nm, they exhibited no antibacterial activity towards the test organisms.

Fractions 4 and 5 from E9 extract also exhibited antimicrobial activity against the two test organisms. Fraction 4 attained the greatest inhibitory effect on the two test organisms and since the elution of fraction 4 and 5 were close to each other it can be inferred they are of the same active compound. The inhibition achieved by these two fractions explains the broad spectrum activity that was observed in E9 extract on *S. aureus ATCC.2* and *E. coli NMIMR.3* in the primary and secondary screening.

In the extract F3, fraction 4 and 7 caused inhibition towards *E. coli NMIMR.3,* with both fractions exerting almost equal potency. However they were eluted distantly apart, which probably suggest they are of different active compounds. Inhibition towards *S. aureus ATCC.2* was caused by fractions 5 and 7, with the highest inhibition zones attained by fraction 7, which was potent enough to be compared with the positive control

antibiotics. Again it can be inferred that fractions 5 and 7 are of different active compounds looking at their elution points. The broad spectrum activity that was observed in the F3 extracts in both the primary and secondary screen was attained by fraction 7, which was gave the highest inhibition zones towards the two test organisms. However, their TLC profiles at UV-254 nm and UV-365 nm could not reveal the active components. The probable explanation could be due to the fact that the active components are not UV absorbable.

It would be interesting to know the effect those fractions that possess antimicrobial activity towards a particular organism, but however are eluted distantly apart from each other. Will their effect add up to the activities or subtract from their activities.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

A total of 54 wood decay fungi (WDF) were collected from dead wood were cultured, extracted and analyzed in this study using these analytical techniques TLC and UV/VIS spectrometry. The two extraction procedures employed in the study affected the yields of fungal extracts that were generated. Lower yields were observed in the cultures that were kept on magnetic stirrer for 48 hrs before extraction as compared to cultures that were vigorously shaken in separating funnel for 45 minutes before extraction.

Although number of fungal spores in the fruiting bodies used for inoculation after yield of extract (Boulianne *et al.,* 2000) and the type of extraction solvent used (Rosecke and Konig, 2000) have also been found to affect the yield of extracts. It was observed in this study that culture volume could also affect the yield of fungal extracts as the fungal strains that were cultured in 1L PDB resulted in high yields of fungal secondary metabolites when compared with 200 ml cultures in PDB notwithstanding the fact that about same (5g) fruiting bodies was used in all the culture.

The time course analysis from this study showed that active fungal metabolites are produced as early as the 7th day of culture, however greater activity is observed when culture are terminated between the 22nd and 48th day of culture when the fungi have completed their growth. This is consistent with what **Weinberg, 1970 reported that** higher levels of secondary metabolites are usually produced only after most of the cellular growth has taken place, since consumption and utilization of one or more

growth limiting substrates have been demonstrated to initiate the synthesis of secondary metabolites **(B'ulock *et al.*, 1965).**

The UV/VIS spectroscopy results obtained from the analyzed fungal extracts clearly indicate that varied fungal extracts were produced looking at the unique spectrophotometric profiles that were generated for all the 54 WDF extracts. However, it must be emphasized that some of the extract profiles share some common identity. Therefore the need for further analysis such as the use of HPLC, LC-MS etc to be able to distinguish the individual spectra that were generated.

The TLC analysis of the fungal extracts showed the absorption and fluorescence of compounds at 254 nm and 365 nm wavelengths, with the compounds showing varied relative polarities. Anisaldehyde reagent spray revealed diverse coloured components such as blue, green, yellow, brown, violet and pink. These colours are associated with specific functional groups such as terpenes, steroids, sugars etc. Majority of the extracts possessed blue colour indicative of possessing terpenes functional group. The overlap of colours with respect to functional group as was observed in the carbohydrate (aldopentose) and steroids as well as uronic acids and the cardiac glycosides requires the need for a detection system that are able to specify the functional group of a particular colour. Again different detection reagents can be varied, so as to associate a given colour to a specific functional group; nevertheless this task might be time consuming.

The primary screening of the 54 fungal extracts for biological activity against the test organisms revealed 40 extracts having various activities towards the test organisms. Out of the total 40 that had an activity, 10 of the WDF extracts were found to have biological activity selectively (SG+) against *Staphylococcus aureus ATCC.2*, and 13 extracts were

also found to have a broad spectrum antimicrobial activity (BSAB) against *Staphylococcus aureus ATCC.2* and *Escherichia coli NMIMR.3*. The number of extracts also having a selective antifungal (SAF) activity towards *Candida albicans KBTH.2* was found to be 3. A non selective antimicrobial (NSAM) activity towards the three test organisms, *S. aureus ATCC.2*, *E. coli* NMIMR.3and *C. albicans* KBTH.2 were recorded to be 11. Moreover, 14 WDF extracts were found to exhibit no biological activity (NA) against any of the test organisms used.

During the secondary screen for the reproducibility of the active components that were generated in the primary screen, a marked shift of antimicrobial activity was observed after the secondary screen as most of the extracts lost their inhibitory activities in the secondary screen. The number of crude extracts that exhibited antibacterial activity against only *S. aureus ATCC.2* was reduced by one in the secondary screen. There was no inhibitory activity exhibited towards only the Gram negative bacteria tested. The number of extracts that exhibited antimicrobial activity towards both the Gram negative and the Gram positive bacteria were six (6) as opposed to (13) after the primary screen. The number of extracts that had non selective antimicrobial activity was increased from 1 in the primary screen to 4 after the secondary screen whilst the 3 WDF extracts that exhibited antifungal activity towards *C. albicans* failed to inhibit the same test organism after the secondary screen.

The possible explanation is that, in the primary culturing smaller volumes of PDB (200) however, in the secondary screening a culture volume of 2x500 ml PDB was used, notwithstanding the fact that the same amount of fungal fruiting bodies (5g) was used in

all the three different cultures. It is possible that in the 1L fermentation, the amount of active compounds produced were so little in comparison with the culture volume.

The purpose of the Sephadex LH-20 column chromatography was to investigate whether the dual or BSAB activity exhibited by the selected extracts are responsible by either the same or different compounds. All the extracts (A4, E2, E9 and F3) that showed broad spectrum activities against a (Gram + and Gram –) bacteria had common fraction/s possessing the biological activities. In those extracts (B6 and B7) that inhibited a Gram + bacteria and a fungus, the biological activities were seen in different fractions. Example in A4 extract, the fractions 4, 5 and 6 all had activity against both bacteria, however the highest activity was seen in fraction 5. For the B7 extract, fractions 4 and 7 had activity against the Gram + bacteria and fractions 3, 5 and 6 had activity against the fungi, *C. albicans KBTH.2.*

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