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COLLEGE OF BASIC AND APPLIED SCIENCES

ISOLATION AND CHARACTERIZATION OF COMPOUNDS FROM WOOD DECAY FUNGI (WDF) WITH ACTIVITY AGAINST GRAM-POSITIVE BACTERIA

BY

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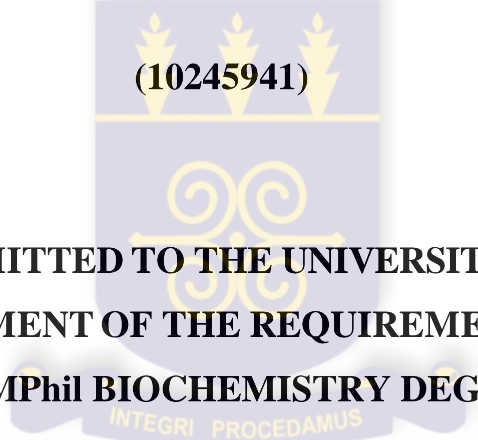

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MPhil BIOCHEMISTRY DEGREE

DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY, UNIVERSITY OF GHANA

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DECLARATION

I certify that this thesis is the result of research done by me, Gloria Baaba Arkaifie in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, under the supervision of Dr. Patrick Kobina Arthur and kind advice and input from Professor Dorothy Yeboah-Manu at Noguchi Memorial Institute for Medical Research. All references have been duly cited

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DEDICATION

To God Almighty, all Glory to His name

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I sincerely want to express my heartfelt gratitude to my main supervisor, Dr. Patrick Kobina Arthur. He has been very instrumental in my training and this project would not have materialized had it not been for him. He gave himself up to see this project come to fruition, His time, critical evaluation, rich biochemistry experience and financial support is invaluable and I appreciate him greatly.

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LIST OF ABBREVIATIONS

MSSA	Methicillin susceptible *Staphylococcus aureus*
MRSA	Methicillin resistant *Staphylococcus aureus*
IHDAC	Inhibitors of histone deacetylases
WDF	Wood decay fungi
MHA	Mueller Hinton Agar
YPDA	Yeast extract, peptone, dextrose agar
YPMD	Yeast extract, peptone, malt extract, dextrose
YPMD +PB	Yeast extract, peptone, malt extract, dextrose and potato broth
YPMD+SE	Yeast extract, peptone, malt extract, dextrose supplemented with soil extract
YPMD+SE+PB	Yeast extract, peptone, malt extract, dextrose and potato broth supplemented with soil extract
YPMD+SE+RB	Yeast extract, peptone, malt extract, dextrose and rice broth supplemented with soil extract
PDB	Potato dextrose broth
PDB+SE	Potato dextrose broth supplemented with soil extract

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ABSTRACT

For decades, fungi have been a source of anti-infective substances, especially after the discovery of penicillin. However, in recent years, the efforts of pharmaceutical companies have dwindled due to the redundancy in the discovery of anti-infective compounds. Antibiotic resistance is also on the increase worldwide, and this poses a serious clinical threat. This study explored metabolites from indigenous Ghanaian Wood Decay Fungi (WDF) by screening a library of 180 WDF. Extracts obtained from these fungi were tested against Gram-positive organisms, Methicillin-sensitive *Staphylococcus aureus* (MSSA) and Methicillin-Resistant *Staphylococcus aureus* (MRSA). Out of 180 WDF screened, 48% had antibacterial activity against both susceptible and resistant strains of *S. aureus*, with 23.45% showing selective activity against only the resistant strains of *S. aureus.* Techniques to optimize the fermentation of WDF were explored by varying the closing mechanism of fermentation vessels using filter paper capping, foam capping, loose capping and tight capping. Filter paper-covered vessels were the most suitable for the production of extracts with consistent activity. Six different media were tested for maximum production of bioactive compounds. Yeast, Peptone, Malt extract, and Dextrose (YPMD) broth aided the production of consistent antibacterial activity among the six media tested. Inhibitors of histone deacetylases (iHDACs) were used to modify broth in which two fungi were cultured. iHDACs increased the antibacterial activity of the two fungal extracts. Using a phenotypic array assay that employed synergy between standard antibiotics and phenotype modulating compounds, fungal isolates taken through activity-guided fractionation revealed five potential compounds with antibacterial activity. This study is a proof of concept that Wood Decay Fungi (WDF) have the potential of producing secondary metabolites with anti-Gram positive activity. The study also established an assay that provides a predictive power of WDF with potential novel antibacterial

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compounds, as well as techniques for optimizing fermentation of WDF to produce antibacterial compounds.

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CHAPTER ONE

1.1 INTRODUCTION

"Miracle drugs" was the term used to describe antibiotics half a century ago, when the discovery of penicillin saved the lives of millions of people during World War II (Penesyan *et al.,*2015). This paved the way for the discovery of more antibiotic classes like the sulfonamides, macrolides, and β-lactams. Based on the success of antibiotics, that period of antibiotic research was described as the "golden age" (Singh *et al.,* 2006).

Presently, bacterial infections are among the biggest global public health concerns. Primarily, over use and misuse of antibiotics has induced selection pressure, and resulted in the development of resistance in bacterial populations. The short generation time of bacteria, means mutations occur more frequently in bacteria than in eukaryotes. Rapid mutation and the mechanism of horizontal transfer of resistant genes have also contributed to the current surge in antibiotic resistance (Walsh & Fischbach 2010).

Of great concern is the increasing occurrence of infections caused by Gram-positive organisms. This is, in part, thought to be due to the slow evolution and selection of Gram-positive resistant bacteria when scientists focused on development of drugs active against Gram-negative bacteria in the 1970s and 1980s (Baquero, 1997). *Staphylococcus aureus* infections are one of such threats, with multidrug resistant strains occurring at a very fast rate.

The World Health Organization (WHO), in its 2014 global report on surveillance of antimicrobial resistance, stated that "A post-antibiotic era, in which common infections and minor injuries can kill, far from being an apocalyptic fantasy, is instead a very real possibility for the 21st Century" (World Health Organization, 2014). This makes research to find new and better antibiotics imperative. Natural products have been a thriving source of potential drug

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leads, and are the forerunners to antibiotic discovery (Butler *et al.,* 2004). Most antibiotics are either obtained from natural products of microorganisms, semi-synthetically produced from natural products, or chemically synthesized using the structure of natural products as a template (Demain, 2009). This is mainly because natural products possess a unique structural diversity in contrast to standard combinatorial chemistry, and this affords great opportunities for discovering novel low molecular weight lead compound. Plunging into natural products for the discovery of new antibiotics is thus a viable prospect, especially since less than 10% of the world's biodiversity has been explored for potential biological activity (Cragg *et al.,* 2005).

Almost all kinds of living things have the ability to produce secondary metabolites with antibiotic properties. This ability is however not evenly distributed among different species (Berdy, 2005). In general, it is clear that unicellular bacteria, eukaryotic fungi, and filamentous actinomyces are the most common and most versatile producers of secondary metabolites with antibiotic properties (Watve *et al.,* 2001). Fungi prove to be a particularly good source of natural products (Dais *et al.,* 2012). They are abundant and diverse, with one previous estimate suggesting 1.5 million fungal species (Hawksworth, 1991). Recent estimates of the number of land plants suggest 400,000 species (Paton *et al.,* 2008; Joppa *et al.,* 2010). A different report also suggested that the ratio of fungi to land plants is 10.6:1 (O' Brien *et al.,* 2005), although higher ratios have also been predicted using data from high-throughput sequencing of clone libraries (Blackwell, 2011). Fungal genomes are rich in biosynthetic gene clusters encoding genes for small molecule production (Miao & Davies, 2010). Genome sequencing of fungi has revealed that the number of genes involved in secondary metabolism is higher than the number of characterized secondary metabolites from fungi (Zutz *et al.,* 2014).

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Epigenetic manipulation mechanisms are currently used to activate "hidden" gene clusters. One major epigenetic signal of chromatin regulation is acetylation of histones, which results mainly in activation of gene transcription. Recent studies have shown that various low molecular weight chemicals are able to inhibit histone deacetylases (HDACs), and consequently activate gene transcription (Zutz *et al.,* 2014). Hence, the fungi world appears to be one of the largest reservoirs of bioactive metabolites (Berdy, 2005).

Fermentation is the bridge between a microorganism and an active pharmaceutical ingredient. For maximum product isolation from fermentation, vital elements of fermentation development should be considered, namely strain selection and optimization; media and process development; and scaling up to maximize productivity. Downstream processes include tools for extracting, concentrating and purifying the product from a dilute fermentation broth. Therefore, innovative screening and detection techniques which require a multi-disciplinary approach covering microbiology, organic chemistry, biochemistry, and molecular biology is essential to identify novel antibiotics.

1.2 Problem Statement

Staphylococcus aureus has been one of the main causes of nosocomial infections worldwide (Voss & Doebbeling, 1995). In Ghana, the threat of *S. aureus* became very apparent when the Korle-Bu Teaching Hospital Children's Ward was temporarily closed down in 2012 due to an outbreak of Methicillin-Resistant *Staphlococcus aureus (MRSA)* (www.myjoyonline.com, 2012). New antibiotic interventions are, therefore, needed to combat this threat. Natural products from fungi are a potential source of new antibiotics, but are understudied in Ghana.

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Furthermore, methods for production of bioactive compounds from fungi are sub-optimal since they are species-specific.

1.3 Hypothesis

Wood decay fungi sampled from Ghana produce secondary metabolites that are active against Gram-positive bacteria.

1.4 Objective of Study

To isolate and characterize compounds from WDF with bioactivity against Gram-positive bacteria by employing a synergistic activity screening approach with compounds of known bioactivity.

1.4.1 Specific objectives

1. To screen and isolate WDF extracts with anti-Gram-positive activity.
2. To develop media formulation and fermentation techniques for optimum production of anti-Gram-positive compounds from WDF.
3. To perform activity based fractionation and characterization of WDF extracts
.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Antibiotic Resistance

Antibiotics are a chemically varied group of small organic molecules of microbial origin that inhibit the growth or metabolic activities of other microorganisms at low concentrations (Thomashow *et al.,* 1995). The "golden age" of antibiotic research was used to describe the new era of treatment of infectious diseases, with the discovery of penicillin being the torchbearer for that period (Singh *et al.,* 2006). Other antimicrobials were later discovered, including streptomycin, chloramphenicol, and tetracycline; the threat of many common bacterial diseases for the first time was no more. Victims of World War II especially benefited from the first antibiotics (Lerner, 2004). The success of antibiotics resulted in the term "miracle drugs".

Presently, the situation is different. The increasing use of antibiotics has led to the development of resistance by pathogenic bacteria to the effects of antibiotics (Barriere, 2015). Thus, despite their initial effectiveness, most antibiotics now have a limited lifespan, and cause the development of pathogen variants with intrinsic or acquired resistance mechanisms. (Alanis, 2005). Antimicrobial resistance currently threatens the effective prevention and treatment of a range of infections that are increasingly becoming dangerous. Slowly, it has become a serious threat to global public health and requires immediate attention (WHO, 2014).

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2.2 Gram-positive Bacterial Infections

Gram-positive bacterial infections have become a great concern due to the increasing rate of infections caused by antibiotic-resistant strains. Baquero suggests that the increase in Gram-positive infections is partly due to scientists in the 1970s and 1980s focusing on the development of drugs active against Gram-negative pathogens, thereby allowing the slow evolution and selection of resistant Gram-positive bacteria (Baquero, 1997). There are three major mechanisms by which antibacterials may become ineffective. First is by modifying or destroying the antibiotic, a classic example being the production of lactamases and aminoglycoside-inactivating enzymes. The second mechanism is by blocking access to the target through alteration of permeability or efflux. The third mechanism is by alteration of the target site (Tenover, 2006).

In Gram-positive bacteria, these mechanisms are mediated by either chromosomes or plasmids (Baquero, 1996). Methicillin resistance in *S. aureus*, for example, is due to the incorporation of an additional chromosomal DNA, *mec*, into the genome. This *mec* DNA encodes *mecA* which is the structural gene for Penicillin binding protein 2A (PBP 2a). PBP2a has a lower affinity for beta-lactam antibiotics than normal PBPs, and thus prevents lactams from interfering with cell wall synthesis, creating resistance (Schalbeg 1994).

Contrary to this, *Entero-coccus faecium* develops resistance to vancomycin by acquiring new DNA on plasmids or chromosomes (Schalbeg 1994). This prevents vancomycin from disturbing cell wall synthesis.

Due to the threat posed by Gram-positive organisms such as pneumococci which are resistant to penicillin and macrolides; beta-lactam- and aminoglycoside-resistant streptococci; vancomycin- and teicoplanin-resistant enterococci; and MRSA (Baquero, 1997), it is urgent to find new effective antibiotics.

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2.2.1 *Staphylococcus aureus* Infections

Most *S. aureus* strains are producers of lactamases, and hence are resistant to penicillin (Cormican, 1996; Thornsberry, 1995). MRSA is a major nosocomial pathogen causing infections in hospitals globally. Infection with MRSA makes up 28% of surgical wound infection, 28% of skin infections, and 12% of all bacteremia (Baquero, 1997).

2.3 Natural products in drug discovery

Natural products, which are mostly secondary metabolites of microorganisms, have proved to be the most successful source of potential drug leads (Mishra *et al.,* 2011). Even though there has been a decline in the interest of pharmaceutical companies in natural products drug discovery, natural products continue to be the store of unique structural diversity in comparison to standard combinatorial chemistry. This presents opportunities for discovering novel low molecular weight compounds (Mishra *et al.,* 2011). More natural compound-leads are available to be discovered since less than 10% of the world's biodiversity has been explored for potential biological activity (Cragg, 2005).

The medicinal value of natural products is well documented. As early as 2600 B.C, there were records on oils from *Cupressus sempervirens* (Cypress) and *Commiphora* species (myrrh) which are still used today to treat coughs, colds and inflammation. They were made on clay tablets in cuneiform from Mesopotamia (Cragg, 2005). The Egyptians also have a pharmaceutical record called the Ebers Papyrus (2900 B.C.) which contains over 700 plant-based drugs including gargles, pills, infusions, and ointments (Cragg, 2005). The plant, *Alhagi maurorum* Medik (Camels thorn) produces a sweet, gummy material, called mama, from its

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stems and leaves during hot days. This material has been documented to aid in the treatment of anorexia, constipation, dermatosis and obesity (Duke *et al.,* 2008).

2.3.1 Natural Products from Fungi

Secondary metabolites with novel biological activity, derived from fungal sources have yielded some of the most important natural products for the pharmaceutical industry (Cragg, 2005). Fungi, like mushrooms, serve as food, are useful in preparation of alcoholic beverages (yeasts), and have been used in traditional medicine. With current advances in microbiology, the use of fungi has been extended to enzymes, antibiotics and other pharmacologically active products (Lorenzen *et al.,* 1996).

2.3.2 Wood decay Fungi

Based on the fungus to plant ratio of 6:1, work done by Hawksworth estimated total species of fungi to be 1.5 million (Hawksworth, 1991). More recent work suggests that the number of fungal species is expected to outnumber land plants by 10.6: 1 (O' Brien *et al.,* 2005). The ratio is predicted to go higher using data from high-throughput sequencing of clone libraries, although specific ecosystems will differ (Blackwell, 2011).

Wood decay fungi are classified into three main groups, namely white rot, which consists of basidiomycetes and ascomycetes; brown rot, which consists of basidiomycetes; and soft rot, which is made up of basidiomycetes, ascomycetes and deuteromycetes (Schwarze, 2007).
Basidiomycota (basidiomycetes) and Ascomycota make up the majority of fungal species identified (Figure 2.1), and they are a major source of pharmacologically active substances.

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Investigations on the potential of basidiomycetes as sources of antibiotics were carried out in 1941, when extracts of fruiting bodies and mycelia cultures from over 2000 species were examined (Florey *et al.,* 1949). There are about 30,000 species of basidiomycetes, (Zjawiony, 2004). Approximately 75% of tested polypore fungi have shown strong antimicrobial activities. This may serve as a rich source to rely on for development of novel antibiotics. Many compounds from fungal origin have displayed some form of biological activity. Examples are antiviral, anti-inflammatory, immune-stimulating and anticancer activities (Zjawiony, 2004). Edmund Kornfeld first isolated vancomycin (Figure 2.2), a glycopeptide antibiotic, in 1953 (Dias, 2012). It was found to be produced in cultures of *Amycolatopsis orientalis*, which is active against a wide range of gram-positive organisms including *Staphylococci* and *Streptococci* and against gram-negative bacteria, mycobacteria and fungi. Vancomycin is one of the second-line drugs for patients resistant to penicillin.

Another antibiotic of the macrolide class, erythromycin (Figure 2.2), was isolated from *Saccharopolyspora erythraea.* It has broad-spectrum antibacterial activity against gram-positive cocci and bacilli. It is also used to treat upper and lower respiratory tract infections of mild to moderate intensity (Butler, 2004).

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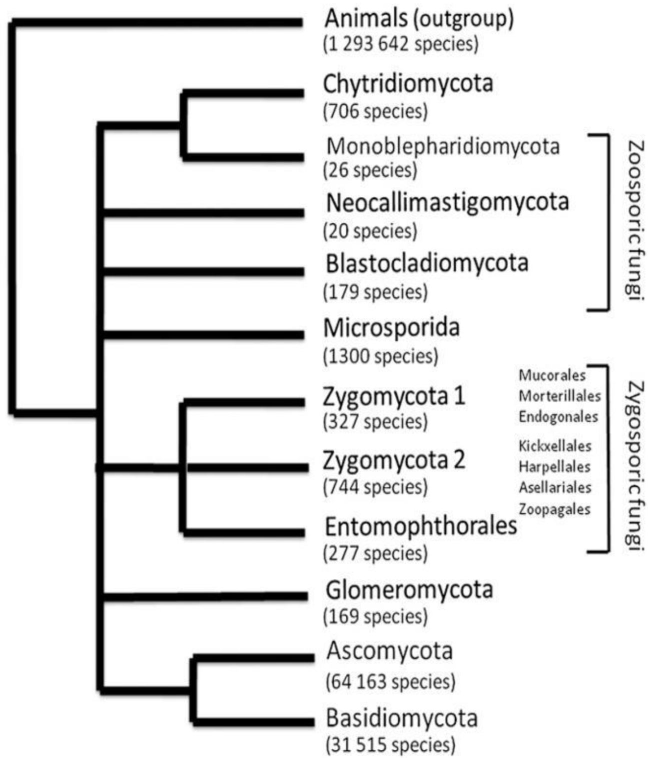

Figure 2-1: Phyla of fungi and approximate species in each group (Kirk *et al.,* 2008)

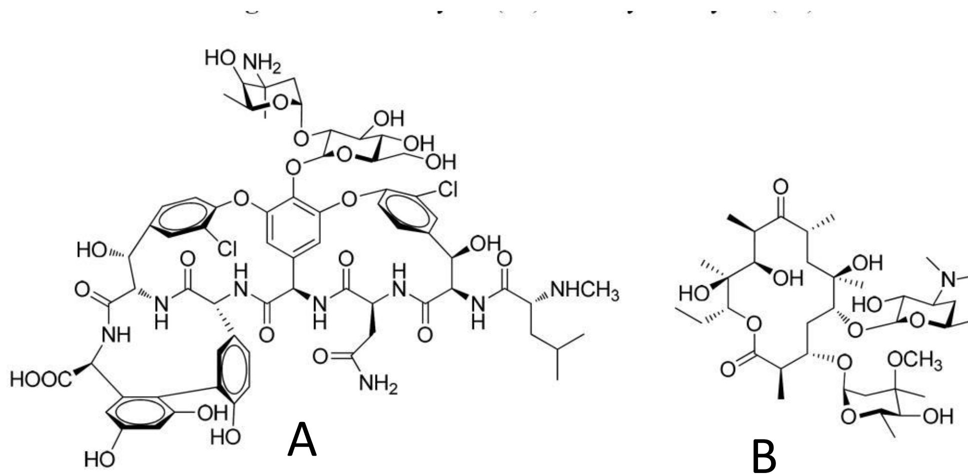

Figure 2-2: Chemical structure of (A) *Vancomycin* and (B) *Erythromycin* (Dias *et al*, 2012)

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2.4 Optimization of Fermentation Conditions

The basis for the production of most pharmaceutical products including antibiotics and vaccines is microbial fermentation. The processes which make up the natural synthesis of these molecules involve specific multi-step routes which can be manipulated for the production of varying compounds. Microorganisms may either be genetically or metabolically manipulated for the production of desired compounds. Strain selection and optimization, media development, optimization of physical factors, and scaling up to maximize productivity are important elements of the fermentation process (Darah *et al.,* 2011; Jain *et al.,* 2011).

Nutritional and environmental factors have been shown to significantly affect the quantity and diversity of secondary metabolite production (Fiedurek *et al.,* 1996; Bode *et al.,* 2002; Miao *et al.,* 2006; Bills *et al.,* 2008; Xu *et al.,* 2008; Mohanty & Prakash, 2009; Kossuga *et al.,* 2012; Shang *et al.,* 2012). Therefore, optimizing these factors in the fermentation of a microbial culture may have improved effects on metabolite profile.

2.5 Employing epigenetics to explore silent gene clusters of secondary metabolism in Fungi

Epigenetics is the use of mechanisms other than the DNA sequence to change gene expression. The epigenome affects gene expression under varying conditions. Molecules such as acetyl and methyl groups can be added to histones due to the many positively charged lysine moieties which act as attachment sites on the histones (Grunstein, 1997). Electrostatic interactions which exist between the positively charged histone and the negatively charged DNA aid in binding DNA to histones. When histones are acetylated, its positive charge density decreases and this affects how tightly bound the DNA is to the histone. The chromatin structure is thus relaxed, and

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subsequently transcription is increased (Grunstein, 1997). De acetylation of histones has a reverse effect, and thus prevents transcription.

Epigenetic manipulation is thus being exploited in the transcription of silent gene clusters. Inhibitors of histone deacetylation can be used to allow for transcription of otherwise silent genes. Some research groups have already demonstrated the potential of this technique in the isolation of novel compounds (Williams, 2008; Asai, 2012; Fisch, 2009; Beau, 2012). In fungi, production of antimicrobial compounds is during secondary metabolism. Sequencing whole fungal genomes has revealed that the number of genes implicated in secondary metabolism is more than the number of fungal secondary metabolites that have been characterized (Winter *et al.,* 2011; Walsh *et al.,* 2010; Keller *et al.,* 2005). Recent studies have shown the potential of various low molecular mass chemicals to inhibit histone deacetylases (iHDACs) in fungi (Cole, 2008). It was also demonstrated that, in fungi, specific chemicals influence the secondary metabolite profile (Cichewicz, 2010; Zutz *et al.,* 2013).

2.6 Phenotypic Screening for Bioactive compounds

Two approaches can be taken towards drug discovery, namely target-based approach, and cell-based or phenotypic screens. Each approach has its advantages and disadvantages (Cong *et al.,* 2012). The target-based approach has advantages, which include the ability to apply molecular and chemical knowledge to investigate a specific molecular hypothesis and ability to perform High throughput screening against the target. However, the main disadvantage is that the molecular hypothesis or target may not be relevant in the disease setting, and this places a major emphasis on target validation. Contrary to this approach, the phenotypic strategy draws its strength from the fact that it optimizes hits with multi-parametric cell-based activity. The advantage of a phenotypic screen is the possibility to find new compounds that inhibit new targets or pathways. It also has

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the potential to find pro-drugs and other complex mechanisms of action. The significance of this approach is emphasized by the fact that all currently used antibiotics were discovered using cell-based phenotypic screening (Manjunatha *et al.,* 2014).

2.7 Separation of crude extract to obtain pure bioactive compounds

Bioassay guided fractionation involves following the activity of fractions obtained from a separation step and progressing in the separation attempts based on only fractions that show the desired activity (Weller, 2012). The agar diffusion method has been the classical test for antibacterial activity.

A number of separation techniques are available for the isolation of pure compounds. Size exclusion chromatography, reverse phase chromatography and a more optimum High performance liquid chromatography.

2.8 Gel filtration/Size exclusion chromatography

This type of separation is based on differences in the size and shape of the molecules being separated. The exclusion limit of a size exclusion packing indicates the molecular weight, larger molecules which have less access to the pore volume are first to elute and the smallest molecules elute last.

Sephadex LH-20 is size exclusion liquid chromatography media made for separating natural products such as steroids, terpenoids, lipids and low molecular weight peptides (up to 35 amino acid residues). Components of a sample are divided between the stationary and mobile phases depending on the solvent used. Sephadex LH-20 has unique chromatographic selectivity due to the dual hydrophilic and lipophilic nature of the matrix, its elution behavior can also be easily

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predicted based on its chemical structure and it has been seen to have excellent batch to batch reproducibility.

2.9 Reverse phase chromatography

This is the opposite of normal phase chromatography. It involves the adsorption of hydrophobic molecules onto a hydrophobic solid support in a polar mobile phase. As the mobile phase is decreased by adding more organic solvent, the hydrophobic interaction between the solute and solid support reduces and this results in de-sorption. The more hydrophobic molecules stay longer on the solid support and requires higher concentration of organic solvent to elute it.

2.10 High performance liquid chromatography (HPLC)

High performance liquid chromatography is an improved form of column chromatography. In this method, high pressure is used to force the solvent through the column instead of allowing it to drip through the column under gravity. This makes separation faster and more efficient.

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals and reagents

Growth media for microorganisms: Mueller Hinton Agar, Nutrient broth, Yeast extract, malt extract, peptone, dextrose and agar were all obtained from Becton, Dickson and company (BD, USA).

Standard antibiotics: Ampicillin, Amoxicillin, Vancomycin, Isoniazid, Ethambutol, Pyrazinamide, Moxifloxacin, Rifampicin, Linezolid, Tetracycline, Chloramphenicol, Erythromycin, Streptomycin, Cycloserine, and 5-Fluorouracil were all obtained from Sigma Aldrich, Germany.

Inhibitors of histone deacetylases: Valproic acid, Trichostatin A, Sodium butyrate, Sodium -4-phenyl butyrate, Suberoylanilide Hydroxamic Acid (SAHA), and 2-hexyl-4-pentynoic acid were obtained from Sigma Aldrich, Germany.

Solvents: Ethyl acetate, methanol, acetonitrile, petroleum ether, ethanol were all purchased from Lab Aid, Ghana. All the solvents were of technical grade.

3.2 Bacterial Strains used in the study

Antimicrobial activity of fungal cultures was tested against Gram-positive pathogens, namely Methicillin-Susceptible *Staphylococcus aureus* (MSSA GGP 200), and two strains of Methicillin-Resistant *Staphylococcus aureus*, namely MRSA GGP E120 and MRSA Noguchi. The bacterial strains used were all kind gifts from the Bacteriology Department of Noguchi Memorial Institute for Medical Research (NMIMR). Initial bacteria cultivation was performed

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on Mueller Hinton Agar (MHA) at 37ºC for 12 hours. A loop-full of the isolate was inoculated into 50ml nutrient broth and incubated for 12 hours with shaking at 37ºC. To obtain cells at mid- log growth phase, the 12-hour cultures were used to inoculate new 50ml nutrient broth to obtain an OD_{600} of 0.01. Afterwards, the set-up was kept in culture for an extra 12 hours. The OD_{600} of the culture was adjusted to 0.3 before being used for the antibacterial assay.

3.3 Collection of Wood decay Fungi (WDF)

Previous students from our laboratory have collected WDF from the Greater Accra, Volta, Central, Ashanti, Eastern and Brong Ahafo Regions of Ghana. Fungi attached to decaying wood were picked and transported in tightly sealed containers to the laboratory. Prior to their use, the fungi were stored at room temperature in a dry place. The fungi were given codes based on their morphological characteristics. These codes were a continuation of the already existing WDF library. A total of 180 WDF were collected. Fungi were maintained on Yeast Peptone Dextrose Agar (YPDA) slants and stored at 4ºC. Slants were inoculated with mycelia and incubated at room temperature for 5 days before storage.

3.4 Liquid media cultivation of Wood Decay Fungi (WDF)

Liquid media of eight different types representing a broad range of nutrient sources were used in this study. Each WDF was initially cultured on Yeast peptone dextrose agar (YPDA) for four days. Mycelia discs obtained from YPDA were used to inoculate culture broths in high-density polyethylene fermentation vessels. Culture broths used in this study include PDB, (200g boiled **potato**, 20g **dextrose**, 1L H_2O); PDB+ 2X SE, (200g boiled **potato**, 20g **dextrose**, 120ml **soil extract**, 1L H_2O); YPMD, (5g **yeast extract**, 5g **peptone**, 5g **malt extract**, 30g **dextrose**,

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1L H_2O); YPMD+PB, (5g **yeast extract**, 5g **peptone**, 5g **malt extract**, 30g **dextrose**, 200g boiled **potato**, 1L H_2O); YPMD+2X SE, (5g **yeast extract**, 5g **peptone**, 5g **malt extract**, 30g **dextrose**, 120ml **soil extract**, 1L H_2O); YPMD+2X SE+PB, (5g **yeast extract**, 5g **peptone**, 5g **malt extract**, 30g **dextrose**, 200g **potato**,120ml **soil extract**, 1L H_2O); YPMD+SE+RB; (5g **yeast extract**, 5g **peptone**, 5g **malt extract**, 30g **dextrose**, 200g boiled **rice**,120ml **soil extract**, 1L H_2O).

Broth media was autoclaved at 121°C for 15 minutes and allowed to cool before inoculating with WDF. Broth cultures were incubated for four weeks at room temperature with frequent swirling. Each culture was grown in duplicate.

3.5 Solid media cultivation

Two forms of solid media were used in this project. YPMD+2x SE +Potato, (5g **yeast extract**, 5g **peptone**, 5g **malt extract**, 30g **dextrose**, 60ml soil extract, 200g potato, 140ml H_2O) and YPMD+2x SE + Rice, (5g **yeast extract**, 5g **peptone**, 5g **malt extract**, 30g **dextrose**, 60ml **soil extract**, 200g **rice**, 140ml H_2O). Media was autoclaved and allowed to set. Pieces of mycelia from mycelia disc grown on YPDA were used to inoculate solid media and allowed to grow for four weeks. Each culture was grown in duplicate.

3.6 Preparation of soil extract

Fresh garden soil was mixed with distilled water in the ratio 1:2 by their respective volumes. The mixture was autoclaved and filtered twice through cotton wool. The filtrate was allowed to stand for 48hours and autoclaved again. 1x concentration was constituted by adding 60ml of soil extract to 1L of broth.

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3.7 Extraction of fungal cultures

To extract liquid cultures, equal volume of ethyl acetate was added to each fungal broth culture. Ethyl acetate and broth cultures were shaken thoroughly and allowed to stand for 24 hours. The solvent and extract mixture was pipetted off the broth and concentrated with a rotary evaporator. Crude extracts were reconstituted in methanol and pipetted into vials.

To extract solid cultures, a spatula was used to scrap off the solid culture and transferred into flasks. Water and ethyl acetate (1:3) was added to the culture to help dissolve the solid culture and extract metabolites. Extracts were concentrated with a rotary evaporator and reconstituted in methanol.

3.8 Disc diffusion assay

In order to assess the antibacterial activity of WDF extracts, the disc diffusion method was used. Round 5mm filter paper discs were cut out and sterilized by autoclaving at 121°C for 15 minutes. WDF extracts were pipetted unto the discs and allowed to air-dry. Discs were placed on the surface of Mueller Hinton agar plates spread with test microorganisms. Plates were incubated for 12 hours at 37°C. The zones of inhibition generated by each extract was measured. Each WDF extract was assayed in duplicates

3.9 Investigating the use of submerged mycelia from previous fungal cultures as inoculum for successive cultures on bioactive metabolite production

The wood decay fungi (WDF) B7 NEW BM and B7 OLD PM were used in this study. To obtain the starting inoculum for the study, fungi were initially inoculated from stored YPDA slants unto YPDA on a petri dish. Mycelia discs from the petri dish were then used as the starting inoculum, the discs were transferred into sterile YPMD+ SE+PB broth in fermentation vessels made of high-density polyethylene. These were called Step 1 cultures. Cultures were made in

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duplicates, and incubated at room temperature for four weeks. Mycelia (1g) from Step 1 cultures were used to inoculate fresh YPMD+ SE+PB broth to make Step 2 cultures. These were also incubated for four weeks. Mycelia (1g) from Step 2 cultures were used as inoculum for Step 3 cultures. Step 4 cultures were made in a similar way using mycelia from Step 3 cultures. Crude extracts were obtained from each culture step by solvent extraction using ethyl acetate. Rotatory evaporator was used to concentrate the extracts and they were reconstituted in methanol. The antibacterial activity of the crude extracts were tested using the disc diffusion assay.

3.10 Exploring Different Fermentation Vessel Closing Mechanisms for Optimum Production of Fungal Bioactive Secondary Metabolites

To study the effect of different fermentation vessel closing mechanisms on antibacterial metabolite production of fungi, WDF, B7 OLD PM and B7 NEW BM were selected. Fermentation vessels that were either tightly covered, loosely covered, plugged with foam, or covered with filter paper were used in this study. To make tightly covered fermentation vessels, the screw cover was screwed tightly unto the vessel leaving no allowance for entry or exit of air. Loosely covered vessels had the screw cover lightly placed on the vessel and screwed once with the ability to move the cover up and down without necessarily exposing the contents of the vessel. Foam covered vessels were plugged with a piece of foam such that the opening of the vessel was air tight. For filter paper covered vessels, whatman paper number 5 was cut out into a circular discs. The discs were the size of the opening of the fermentation vessel. The filter paper was placed on the opening of the vessel and taped tightly at the edges to hold it in place. Mycelia discs of fungi cultured on YPDA were used to inoculate 200ml YPMD+SE+PB in the fermentation vessels. Control broth cultures were set up with same fermentation vessel closing mechanisms but with no fungal

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inoculum. Cultures were incubated at room temperature for four weeks and extracted with ethyl acetate. Crude extracts were assayed for antibacterial activity using the disc diffusion assay.

3.11 Comparative Evaluation of Solid versus Broth (submerged) State fermentation on Fungal Bioactive Secondary Metabolite Production

YPMD+SE was used as the base media. Three media types were made for both solid and broth media. Agar (10g/L) was added to the YPMD+SE to make solid media. Broth media had no agar. The second and third media consisted of potato and rice. 50ml and 200ml water was added to make solid and broth media respectively.

3.12 Effect of media composition on the consistent production of fungal bioactive secondary metabolites

In this study, WDF, B7 NEW BM and D7 PM 2pb were used. To study the effect of different media constituting different nutrient sources, six different broth media were set up, namely PDB, PDB+ 2X SE, YPMD, YPMD+PB, YPMD+2X SE, and YPMD+2X SE+PB (media composition in section 3.4). Mycelia discs cultured on YPDA were used to inoculate 500ml cultures of each broth type. For each media type, four replicates were made. Cultures were incubated at room temperature and extracted with ethyl acetate upon maturity. Crude extracts obtained from each culture were assayed for antibacterial activity using the disc diffusion assay

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3.13 Using Inhibitors of Histone Deacetylases (iHDACs) to explore Silent Gene Clusters and improve the production level of Fungal Secondary Metabolites

Epigenetic manipulation of fungi is increasingly being used to activate silent gene clusters of secondary metabolism in fungi. (Zutz *et al.,* 2013). Small molecular weight chemicals which inhibit histone deacetylases (iHDACs) have been studied to aid in this process of activating secondary metabolite gene clusters in fungi (Cole, 2008). To test if these chemicals enhance production of antibacterial secondary metabolites from our fungal library, stock solutions were prepared by dissolving chemicals in DMSO. (Chemicals are listed in section 3.1). The final concentration of all the chemicals used to treat YPDA plates and YPMD+SE+PB was 10µM except for Trichostatin A (0.5µM) and Sodium butyrate (100µM) and Sodium-4-phenyl butyrate (100µM). Stock solutions were stored at -20°C.

To prepare the fungal cultures, YPMD+SE+PB was used as the media. WDF used for this study were B7 OLD PM and B7 NEW BM. Control broth cultures were set up using fungal mycelia discs grown on untreated YPDA. This was used to inoculate 500ml of untreated YPMD+SE +PB. For broth cultures treated with the (iHDACs), three different treatments were done. First, mycelia discs cultured on iHDAC-treated YPDA plates were used to inoculate untreated YPMD+SE+PB (TP, UB). Second, mycelia discs cultured on untreated YPDA plates were used to inoculate iHDAC-treated YPMD+SE+PB (UP, TB). Third, mycelia discs cultured on iHDAC-treated YPDA plates were used to inoculate iHDAC-treated YPMD+SE+PB (TP, TB). Cultures were setup in duplicates. Extraction with ethyl acetate was done after four weeks of incubation at room temperature. Crude extracts were assayed for antibacterial activity using the disc diffusion method

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3.14 Preparation of fungal cultures

YPMD+SE+Potato broth was used as the media for this study. WDF used for this study were B7 OLD PM and B7 NEW BM. Control broth cultures were set up using fungal mycelia discs grown on YPDA untreated with any of the chemicals. This was used to inoculate 500ml of YPMD+SE +PB untreated with the chemicals. For broth cultures treated with the chromatin modifiers, three different treatments were done. First, mycelia discs cultured on chemical-treated YPDA plates were used to inoculate untreated YPMD+SE+PB (TP, UB). Second, mycelia discs cultured on untreated YPDA plates were used to inoculate chemical-treated YPMD+SE+PB (UP, TB). Third, mycelia discs cultured on chemical-treated YPDA plates were used to inoculate chemical-treated YPMD+SE+PB (TP, TB). Cultures were setup in duplicates. Extraction with ethyl acetate was done after four weeks of incubation at room temperature. Crude extracts were assayed for antibacterial activity using the disc diffusion method.

3.15 Phenotypic screening of Wood Decay Fungi

Phenotypic array screening was done with standard antibiotics and phenotype modulating compounds. Mueller Hinton Agar was modified with standard antibiotics with known mechanisms of action. The standard antibiotics used were ampicillin (0.05µg/ml), tetracycline (0.5µg/ml), chloramphenicol (5µg/ml), clavulanic acid (50µg/ml), aspirin (50µg/ml). The phenotype modulating compounds used were N-acetyl glucosamine (2.2mg/ml) and arginine (0.85mg/ml). The modified plates were used to assay the antibacterial activity of standard antibiotics and WDF extracts using the disc diffusion method. The sensitivity pattern of the antibiotics and WDF extracts across all the modified conditioned were determined by measuring the zone of inhibition.

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3.16 Thin layer chromatography (TLC)

To qualitatively observe the different components of the crude extract, pre-coated silica gel TLC strips were used in the TLC analysis. 5µl of crude fungal extract was spotted on a thin line drawn 5mm from the bottom of the plate. The spot was allowed to air-dry and developed in a glass tank filled to 3mm and saturated with the developing solvent (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1). The plates were air-dried after developing with solvent. The resulting bands were viewed under short wavelength (254nm) and long wavelength (365nm) of UV light. The plates were then sprayed with anisaldehyde reagent, developed in an oven (70^oC), and viewed under long wavelength UV light.

3.17 Size Exclusion Column Chromatography Using LH-20

In order to separate the various components of the crude extract based on their sizes, LH 20 Column was washed with 80ml of 0.1M NaOH, this was followed by washing with 80ml distilled water, and then 80ml methanol. The crude fungal extract (1ml) was loaded onto the column and allowed to run down the length of the column. 10ml fractions were collected until all the crude extract was exhausted. The disc diffusion assay was used to test the antibacterial activity of the fractions obtained.

3.18 Reverse Phase (C-18) Chromatography

To do further separation in order to get a pure compound, the active fractions obtained from the LH 20 fractionation were pooled together. Pooled fractions were concentrated using a rotary evaporator. The extract obtained was reconstituted in 10ml of 5% Acetonitrile. C18 columns were equilibrated with 20ml of 5% acetonitrile. The sample in 10ml of 5% acetonitrile was loaded unto the column.

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The fraction collected was loaded again onto the column and eluted with 5% acetonitrile. This was the 5% acetonitrile fraction. Eleven more fractions were collected after eluting with 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80% and 90% Acetonitrile. The elution volume for each fraction was 20ml. The antibacterial activity of each fraction was tested using the disc diffusion assay.

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CHAPTER FOUR

RESULTS

4.1 Screening of WDF collection #2 of Wood Decay Fungi (WDF) Library

Our laboratory has a library of 369 Wood Decay Fungi collected between October 2009 and July 2014. Majority of the sampled fungi were from Accra. However, some were obtained from the Volta, Central, Ashanti, Eastern and Brong Ahafo Regions of Ghana. Upon collection from the field, the fungi were packaged in airtight plastic containers and stored at room temperature.

Previous students of the laboratory have screened part of the WDF library employing the disc diffusion assay. WDF were first cultured in PDB. The metabolites produced from the mature culture were extracted using ethyl acetate. A total of 189 out of the 369 fungi were screened against MRSA. An activity of 68% was obtained from the screen. Out of this number, 20 were selected for further analysis in this project. The remaining 180 WDF were screened in this project.

The new collection of 180 WDF were cultured in Yeast, Peptone, Malt extract, and Dextrose (YPMD) broth and extracted with ethyl acetate. The extracts were tested for antibacterial activity against MSSA GGP 200 and two strains of MRSA using the disc diffusion method. Out of the 180 WDF extracts screened, 81 (48%) had antibacterial activity for at least one of the three strains of *S. aureus* (Figure 4.1). From the 81 WDF extracts which showed activity, 48 (59.2%) exhibited antibacterial activity against all the three strains of *S. aureus*. MSSA GGP 200 was sensitive to 63 of the extracts, with WDF AM4 recording the highest zone of inhibition (28.5mm). Two strains of MRSA were tested, namely MRSA GGP E120 and

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MRSA Noguchi. Out of the extracts, 19 had selective antibacterial activity against the resistant strains (Figure 4.2).

Extract from WDF AG6 had the highest zone of inhibition against MRSA GGP E120 (17.5mm). Extract from WDF A04 recorded the highest zone of inhibition (23mm) against MRSA Noguchi. (Figure 4.1 B)

Thirty-nine (39) of the WDF extracts that had activity against MSSA GGP 200 had zones of inhibition ranging from 10mm to 29mm (Figure 4.3 A). For the two resistant strains, MRSA GGPE120 and MRSA Noguchi, 22 out of 67 and 25 of 60 extracts respectively had zones of inhibition 10mm and above. (Figure 4.3 B and C).

Having confirmed that WDF produce metabolites with antibacterial properties, media formulation, fermentation, and techniques for optimum production of antibacterial metabolites from WDF were studied

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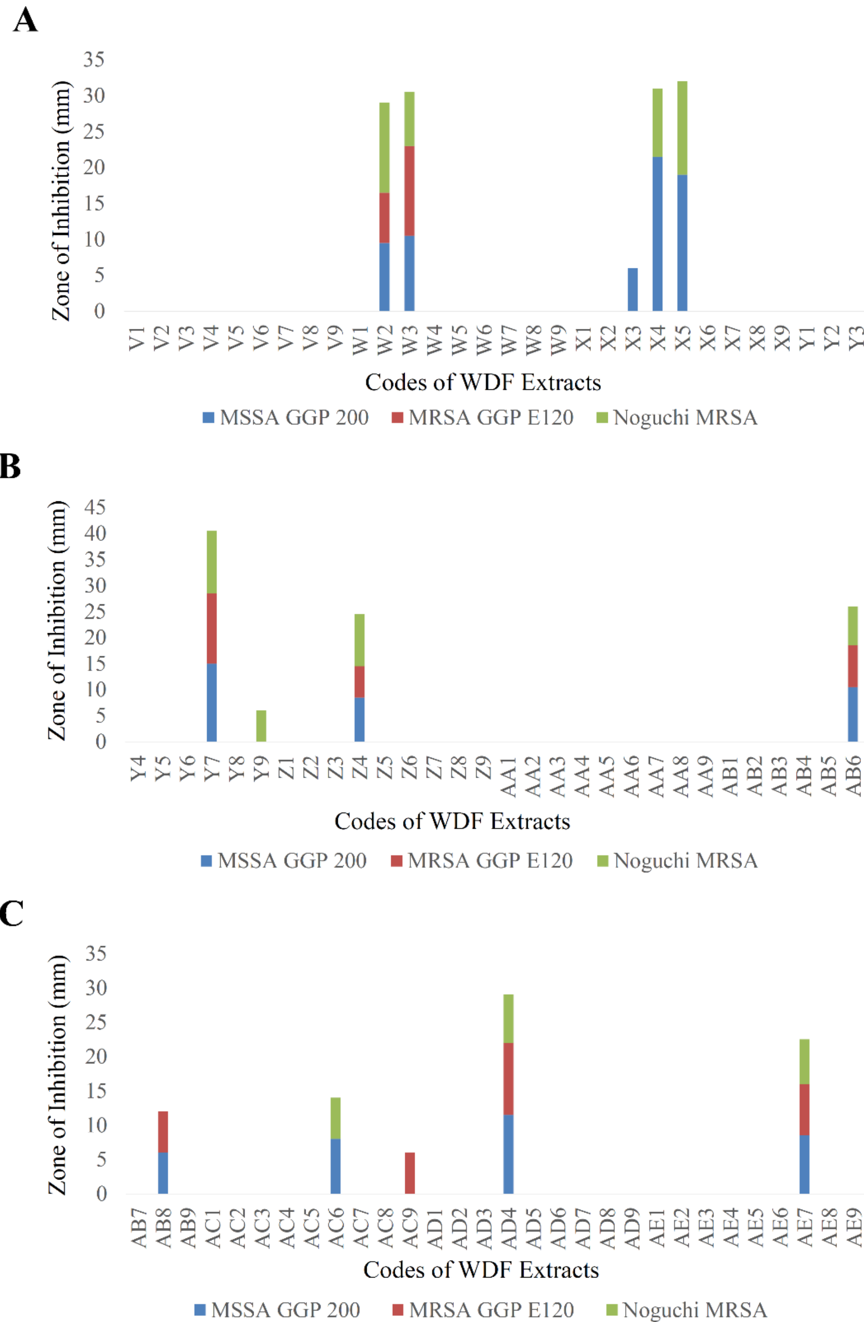

Figure 4-1A: Antibacterial activity of crude extracts of Wood Decay Fungi (WDF) against MSSA GGP 200, MRSA GGP E120 and MRSA Noguchi. WDF were cultured in Yeast, Peptone, Malt extract, Dextrose (YPMD) broth for four weeks and extracted with ethyl acetate. Antibacterial activity of extracts were assayed using the disc diffusion assay. (A), (B), (C) are groups of 30 WDF. Zones of Inhibition generated by each fungal extract was a readout for its antibacterial activity. V1-AE9 are codes for individual WDF. MSSA (Methicillin-Susceptible *Staphylococcus aureus*); MRSA (Methicillin-Resistant *Staphylococcus aureus*); MRSA GGP E120 and MRSA Noguchi are two strains of MRSA, both kind gifts from Noguchi Memorial Institute for Medical Research (NMIMR).

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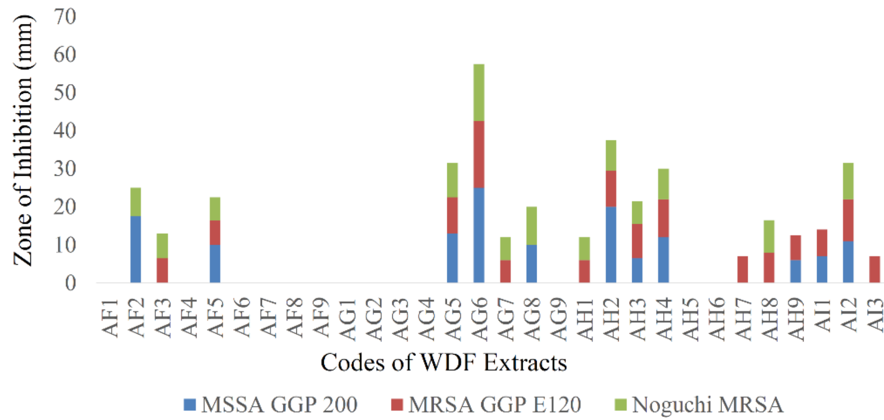

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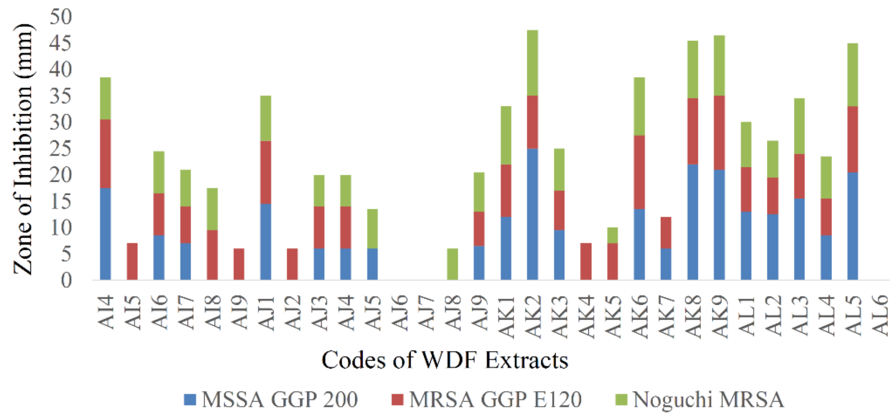

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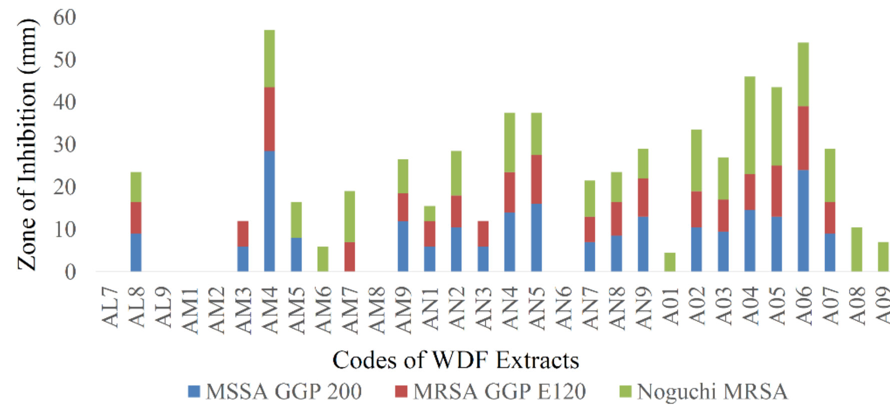

Figure 4-1B: Antibacterial activity of crude extracts of Wood Decay Fungi (WDF) against MSSA GGP 200, MRSA GGP E120 and MRSA Noguchi. WDF were cultured in Yeast Peptone Malt extract Dextrose (YPMD) broth for four weeks and extracted with ethyl acetate. Antibacterial activity of extracts were assayed using the disc diffusion assay. (D), (E), (F) –groups of 30 WDF. Zones of Inhibition generated by each fungal extract was a read out for its antibacterial activity. AF1-A09 are codes of individual WDF. MSSA-Methicillin Susceptible *Staphylococcus aureus*, MRSA-Methicillin Resistant *Staphylococcus aureus.* MRSA GGP E120 and MRSA Noguchi are two strains of MRSA, both kind gifts from Noguchi Memorial Institute for Biomedical Research (NMIMR)

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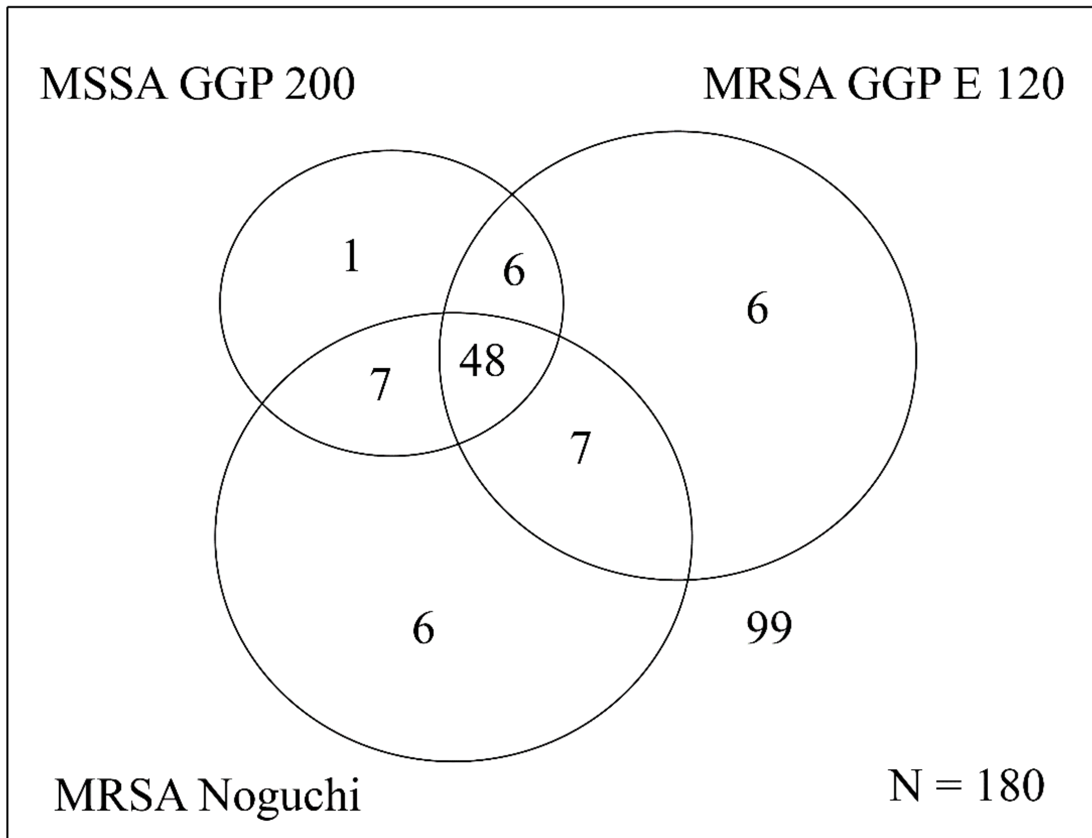

Figure 4-2: Distribution of 180 screened crude extracts of Wood Decay Fungi (WDF) with antibacterial activity against MSSA GGP 200, MRSA GGP E120, and MRSA Noguchi. WDF were cultured in Yeast, Peptone, Malt extract, Dextrose (YPMD) broth for four weeks and extracted with ethyl acetate. Antibacterial activity of extracts were assayed using the disc diffusion assay. The zone of inhibition generated by each fungal extract was a readout for its antibacterial activity. MSSA (Methicillin-susceptible Staphylococcus aureus); MRSA (Methicillin-resistant Staphylococcus aureus). MRSA GGP E120 and MRSA Noguchi are two strains of MRSA, both kind gifts from Noguchi Memorial Institute for Medical Research (NMIMR). N-Total number of WDF screened.

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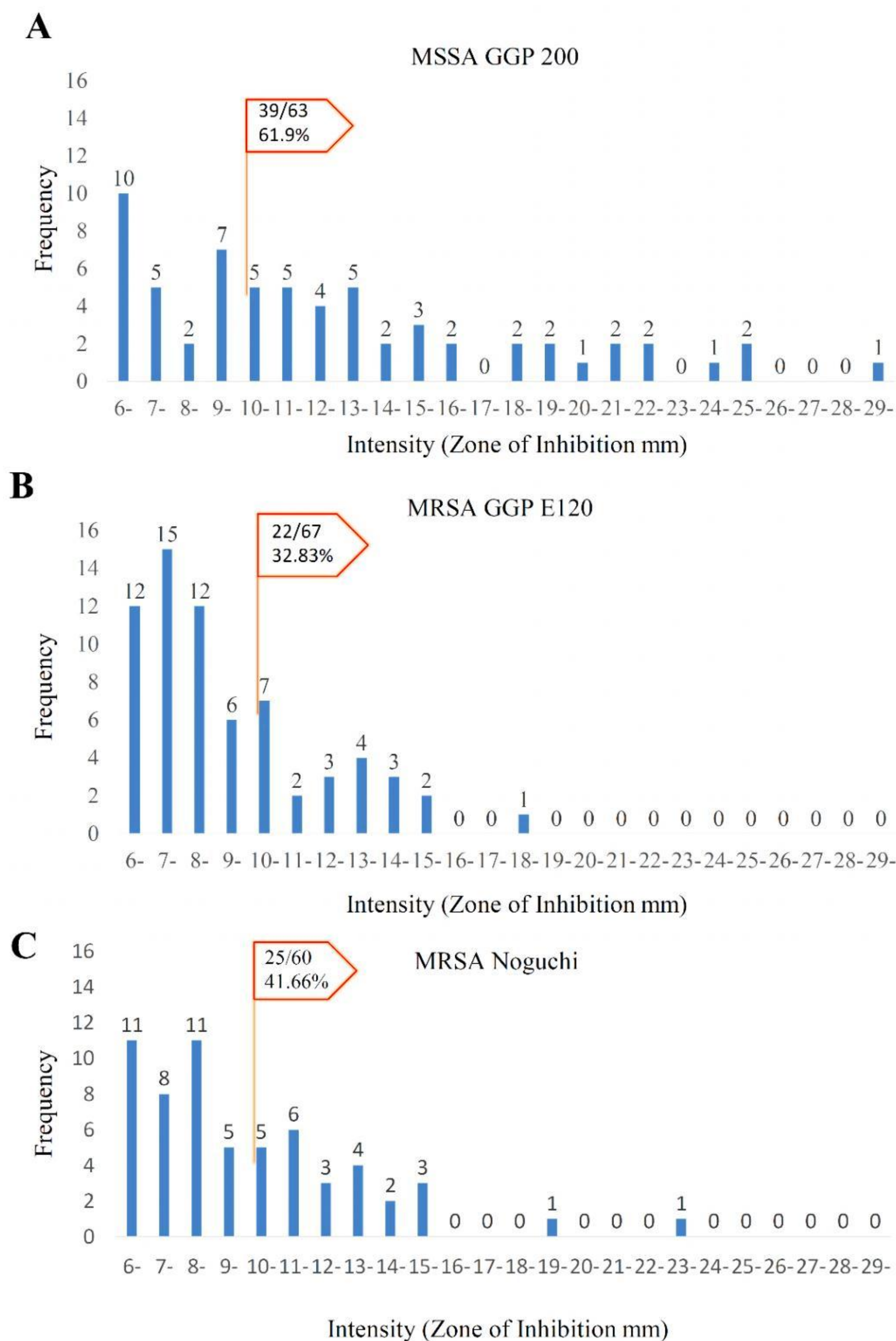

Figure 4-3: Intensity and frequency of antibacterial activity of Wood Decay Fungi (WDF) against (A) MSSA GGP 200, (B) MRSA GGP E120 and (C) MRSA Noguchi. The disc diffusion assay was used to assess the antibacterial activity of crude extracts of WDF. The size of the zone of inhibition generated by each fungal extract was used as a measure of the intensity of its antibacterial activity. Red flag demarcates number of WDF among total number of WDF with antibacterial activity having zones of inhibition ≥10mm and their percentages. MSSA (Methicillin-Susceptible *Staphylococcus aureus*); MRSA (Methicillin-Resistant *Staphylococcus aureus*). MRSA GGP E120 and MRSA Noguchi are two strains of MRSA, both kind gifts from Noguchi Memorial Institute for Medical Research (NMIMR).

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4.2 Investigating the use of submerged mycelia from previous fungal cultures as inoculum for successive cultures on bioactive metabolite production

The scarcity of the fungal fruiting body presents a challenge to fungal culture in the laboratory. To address this, a protocol was developed (Adadey, 2014) that uses mycelia obtained from growing fruiting bodies on plates (referred to as Plate Mycelia, PM), and mycelia obtained from growing fruiting body in broth (referred to as Broth Mycelia). Both inoculums generally proved successful. However, to overcome the challenges associated with long-term storage of PM and BM isolates on slants at 4°C, submerged mycelia from previous fungal cultures were used as inoculums for subsequent fungal cultures. Metabolites produced were extracted and tested for antibacterial activity. **Y**east, **P**eptone, **M**alt Extract, **D**extrose and **S**oil **E**xtract (YPMD+SE) broth was used to culture two fungi (B7 OLD PM and B7 NEW BM). Mycelia discs of the two fungi were grown on **Y**east **P**eptone **D**extrose **A**gar (YPDA) and used as inoculum for Step 1 cultures. After four weeks of culturing, mycelia were harvested from Step 1 cultures, and used to inoculate fresh YPMD+SE broth for Step 2 cultures. This was repeated until Step 4 cultures were also four weeks old.

The antibacterial activity of the metabolites obtained at each step was assayed by the disc diffusion method. Generally, there was decreasing antibacterial activity as the culturing steps increased. The activity of B7 OLD PM (Figure 4.4) against MSSA decreased from an average zone of inhibition of 9mm in Step 1 to 6mm at Steps 2 and 3, and finally disappeared at the end of Step 4. The activity was generally lost against MRSA except for extracts from Step 3, which had an average zone of inhibition of 6.5mm. Similar activity patterns were observed for Extracts from B7 NEW BM (Figure 4.5). After Step 3, the activity against MSSA was lost. No activity was observed against MRSA.

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Thin layer chromatography (TLC) was used to qualitatively examine the metabolite profile of the extracts obtained (Figure 4.6). For B7 OLD PM, there were similar band patterns for metabolites from all the steps (confirming that the same WDF isolate is being propagated) except for Step 3, which had a more prominent fluorescing band close to the origin, which was fainter in the other steps. The band profiles of extracts from B7 NEW BM were more varied. Steps 1 and 2 had bands at the mid-section, which appeared darker than the fluorescing bands observed close to the solvent front in the extracts for Steps 3 and 4. The duplicates for extracts from Step 2 produced interesting band patterns. Step 2A showed band patterns similar to bands from Step 1 while Step 2B showed band patterns similar to bands observed in Steps 3 and 4.

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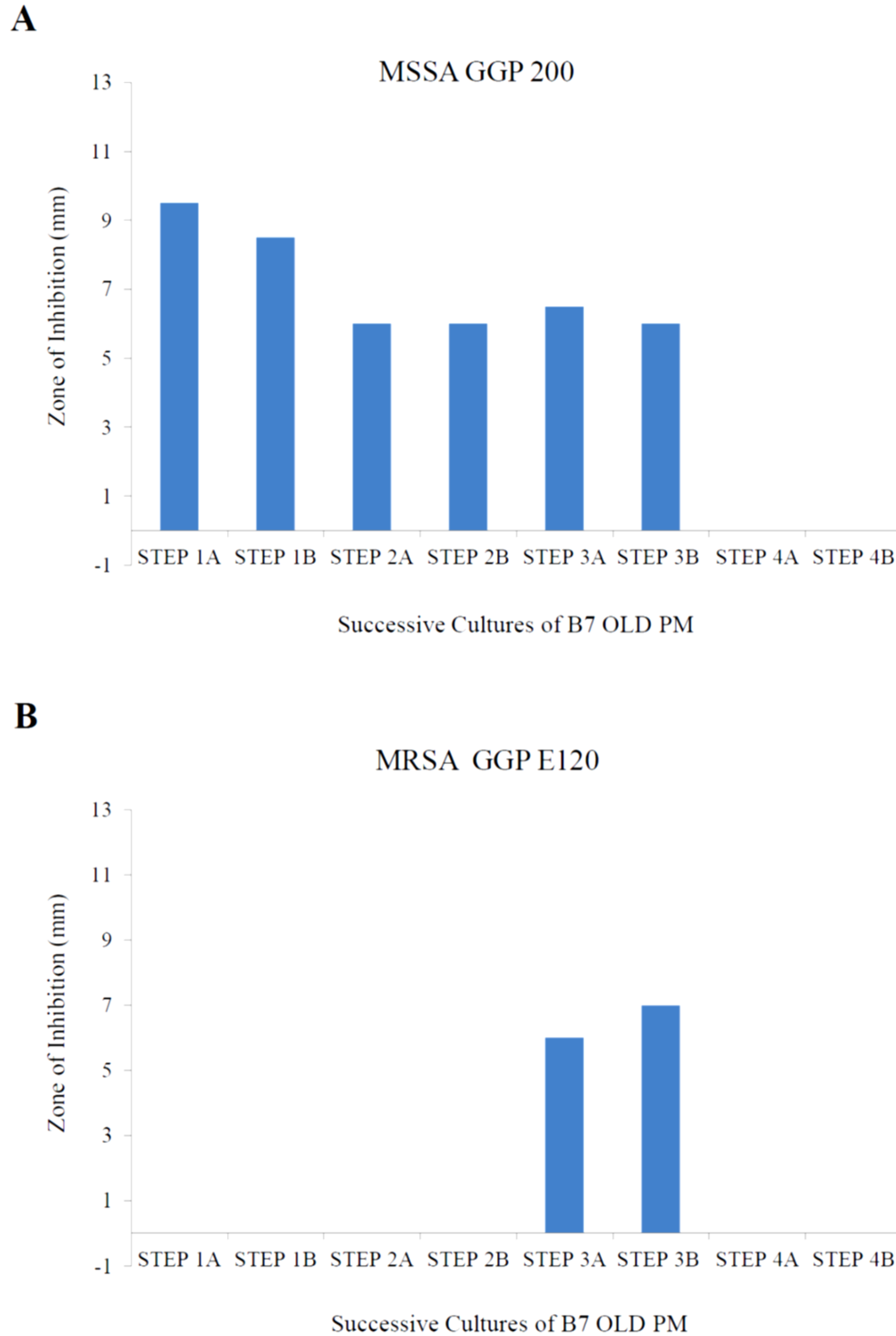

Figure 4-4: Antibacterial activity of crude extracts of the fungi (B7 OLD PM) against (A) MSSA GGP 200 and (B) MRSA GGP E120. Step 1 to Step 4 are successive cultures of B7 OLD PM obtained by passaging mycelia inoculum from previous steps. B7 OLD PM was inoculated onto Yeast, peptone, dextrose, agar (YPDA) plates and incubated for four days. Mycelia discs obtained were used to inoculate Yeast, peptone, malt extract, dextrose (YPMD) broth to make Step 1 cultures. Mycelia from Step1 cultures were harvested after four weeks and used to inoculate Step 2 cultures. This was repeated for Steps 3 and 4 cultures. A and B are duplicates of cultures at each step. Ethyl acetate extracts from each step was assayed for antibacterial activity, which was indicated by the zone of inhibition.

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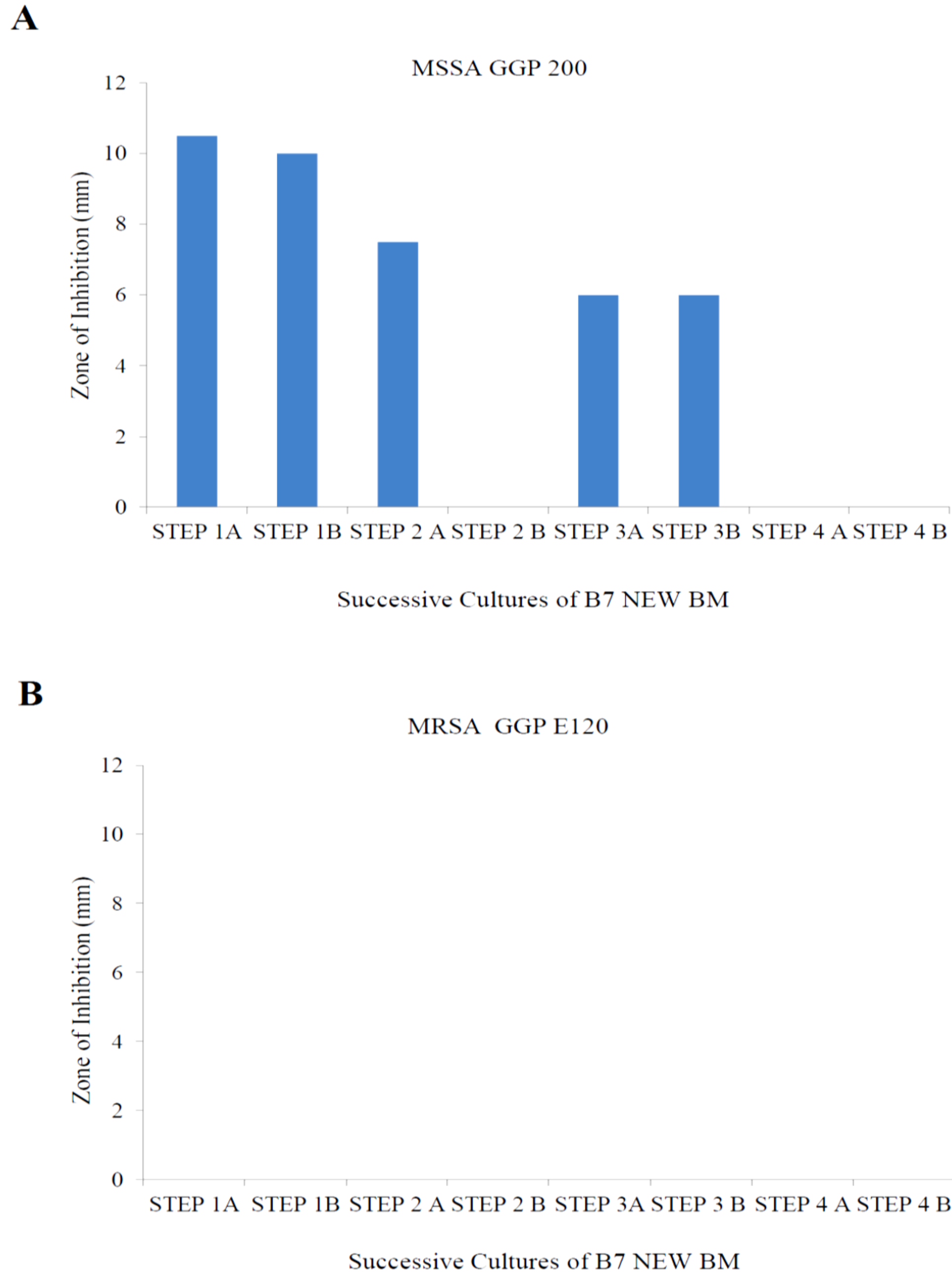

Figure 4-5: Antibacterial activity of crude extracts of the fungi (B7 NEW BM) against (A) MSSA GGP 200 and (B) MRSA GGP E120. Step 1 to step 4 are successive cultures of B7 NEW BM obtained by passaging mycelia inoculum from previous steps. B7 NEW BM was inoculated onto Yeast, peptone, dextrose, agar (YPDA) plates and incubated for four days, mycelia discs obtained were used to inoculate Yeast, peptone, malt extract, dextrose (YPMD) broth to make step1 cultures, mycelia from step1 cultures were harvested after four weeks and used to inoculate step 2 cultures. This was repeated for step 3 and 4 cultures. A and B are duplicates of cultures at each step. Ethyl acetate extracts from each step was assayed for antibacterial activity, which was indicated by the zone of inhibition

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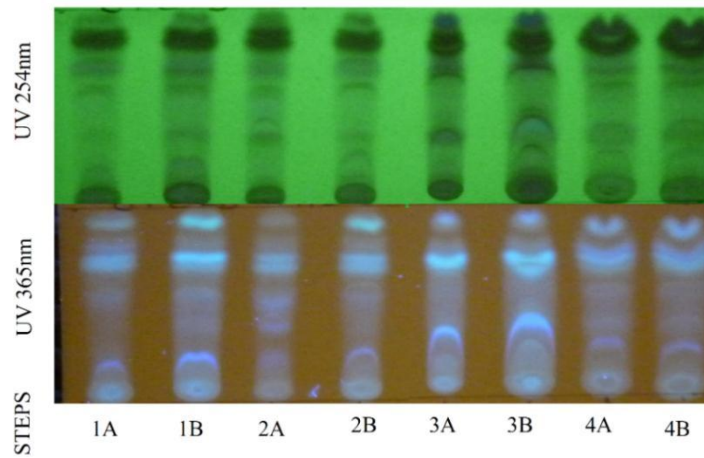

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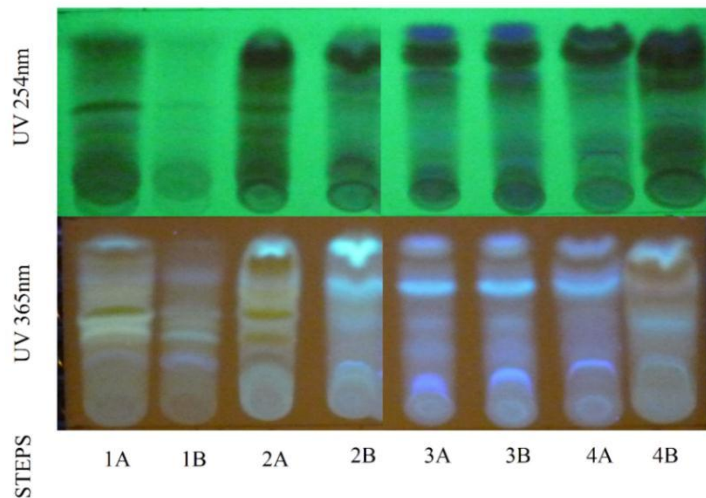

Figure 4-6: TLC profiles of Wood Decay Fungi (WDF) from successive culture steps; (A) B7 OLD PM and (B) B7 NEW BM. Step 1 to step 4 are successive cultures of B7 OLD PM and B7 NEW BM obtained by passaging mycelia inoculum from previous steps. Fungi were inoculated onto Yeast peptone dextrose agar (YPDA) plates and incubated for four days, mycelia discs obtained were used to inoculate Yeast, peptone, malt extract (YPMD) broth to make step1 cultures, mycelia from step1 cultures were harvested after four weeks and used to inoculate step 2 cultures. This was repeated for step 3 and 4 cultures. A and B are duplicates cultures at each step. TLC plates were spotted with 5µl of crude extract, developed with (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1), and viewed under UV 254nm and 365nm.

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4.3 Exploring Different Fermentation Vessel Closing Mechanisms for Optimum Production of Fungal Bioactive Secondary Metabolites

Aeration is a critical factor in culturing fungi for optimum metabolite production. One way this factor can be regulated is in the engineering of fermentation vessels. Previous work done in our laboratory varied the aeration to culture ratio, using 100ml culture in 500ml vessels (4:5 aeration to culture ratio) and 400ml culture in 500ml fermentation vessels (1:5 aeration to culture ratio) (Adadey, 2014). The 4:5 aeration to culture ratio showed consistent bioactivity, but 1:5 aeration ratio gave more yield. However, consistency and yield are important factors in the successful production of fungal secondary metabolites. Thus, the aeration to culture ratio was kept constant at 2:5. The effect of aeration due to fermentation vessel closure mechanisms on the production of bioactive metabolites was investigated. The closure mechanisms examined included loosely capped vessels; tightly capped vessels; vessels capped with foam; and vessels capped with filter paper.

The biological activities of the extracts were assayed against MSSA and MRSA (Figures 4.7 and 4.8). When tested against MSSA, B7 OLD PM extracts from foam-capped vessels had the highest zone of inhibition (11mm), but this was not replicated in its duplicate (8mm). Tightly capped vessels produced the next highest zone of inhibition of 8.5mm and 6mm. The filter paper capped vessels showed consistent activity; this was nevertheless the lowest (6.5mm and 6mm). When tested against MRSA however, the antibacterial activity was lost except for extracts obtained from tightly capped, loosely capped and filter paper capped vessels. These antibacterial activities were low (6mm), and were not observed in their respective duplicate broths. Extracts obtained from B7 NEW BM had lower antibacterial activity compared to B7 OLD PM. B7 NEW BM extracts from loosely capped vessels had the highest zone of inhibition

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(9mm) against MSSA GGP 200 which was same as that of the loosely capped control. This activity was however lost when assayed against MRSA GGP E12O. B7 NEW BM Tightly capped and foam capped vessels on the other hand had lower zones of inhibition against MSSA GGP 200 compared to loosely capped vessels, but their activity was maintained against MRSA GGP E120.

TLC profiles were obtained for extracts of B7 OLD PM and B7 NEW BM (Figure 4.9) cultured in differently capped vessels. In comparison to the control vessels, which were set up with broth only and no fungal inoculum, extracts from B7 OLD PM cultured in vessels with loose capping; tight capping; and filter paper capping had similar band patterns. The band pattern for the cultures from foam-capped vessels had an extra band prominently displayed in the mid-section. In all the set-ups, the control samples produced band patterns that were different from the test samples. For B7 NEW BM, band patterns of extracts obtained from loosely capped and foamed capped vessels were different from that observed in tightly capped and filter paper capped vessels. All the extracts however had different band patterns compared to the controls.

Based on the consistent production of antibacterial compounds by filter paper capped vessels of B7 OLD PM against MSSA GGP 200, filter paper capped vessels were selected as the best bottle closing mechanism to ferment WDF.

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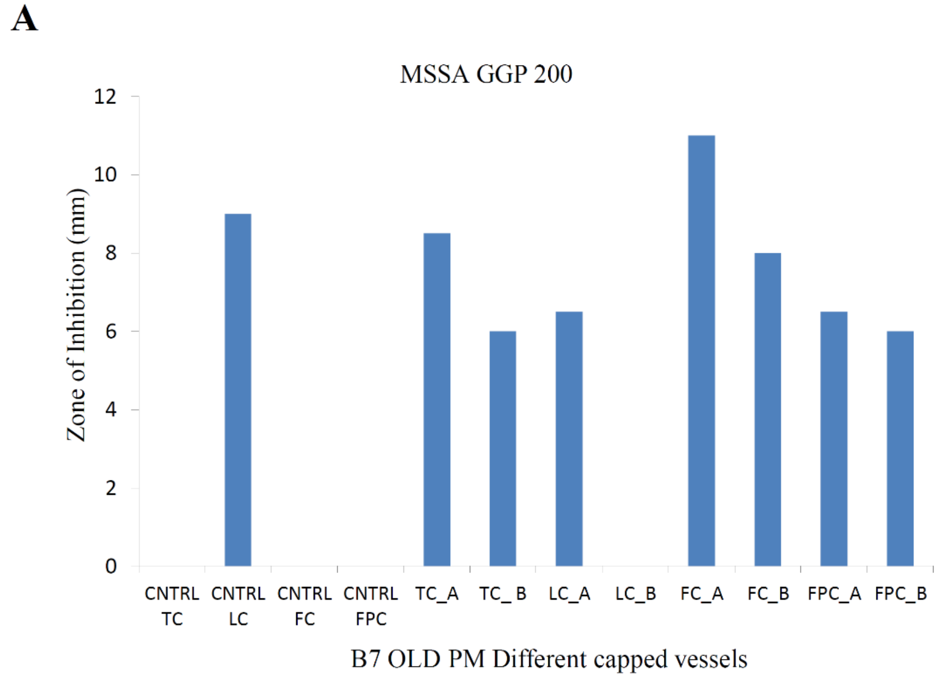

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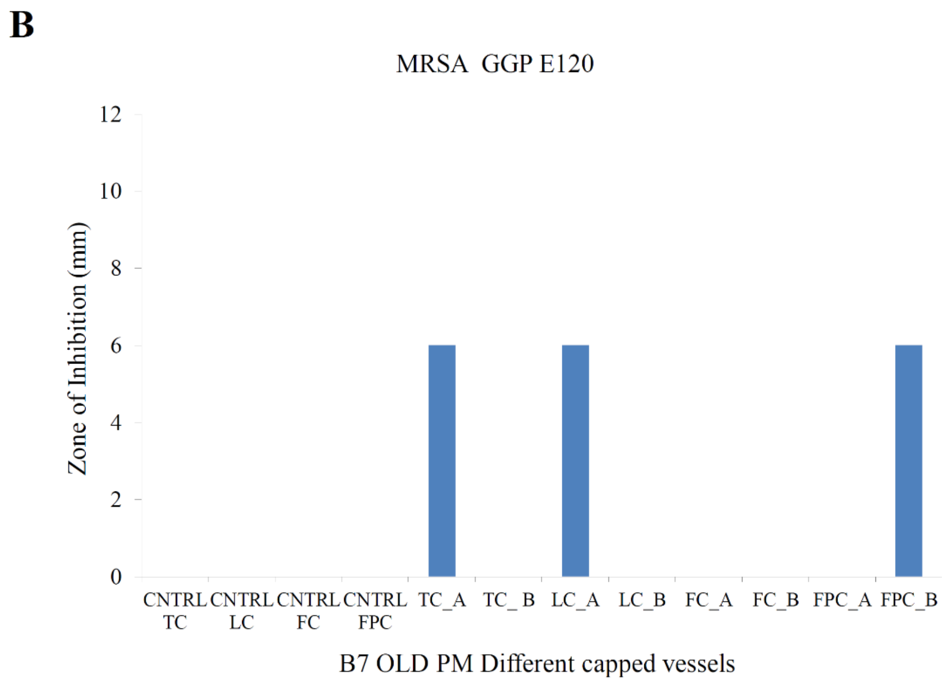

Figure 4-7: Antibacterial activity of crude extracts of Wood Decay Fungi (WDF); B7 OLD PM cultured with different fermentation vessel closing mechanisms against (A) MSSA GGP 200 and (B) MRSA GGP E120. B7 NEW BM was inoculated on Yeast peptone dextrose agar (YPDA) and mycelia discs obtained were used to inoculated Yeast, peptone, malt extract, dextrose (YPMD) broth in fermentation vessels which were tightly covered, (TC), loosely covered (LC), covered with foam (FC) and covered with filter paper (FPC). A and B are culture duplicates. Control cultures were set up with each closing mechanism but with no fungal inoculum. Ethyl acetate extracts from each vessel was assayed for antibacterial activity using the disc diffusion assay.

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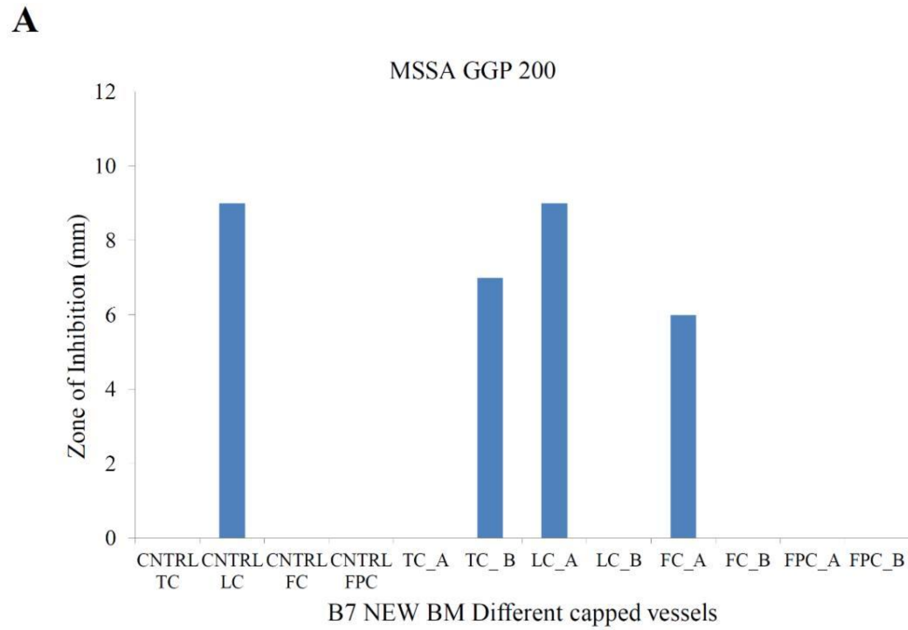

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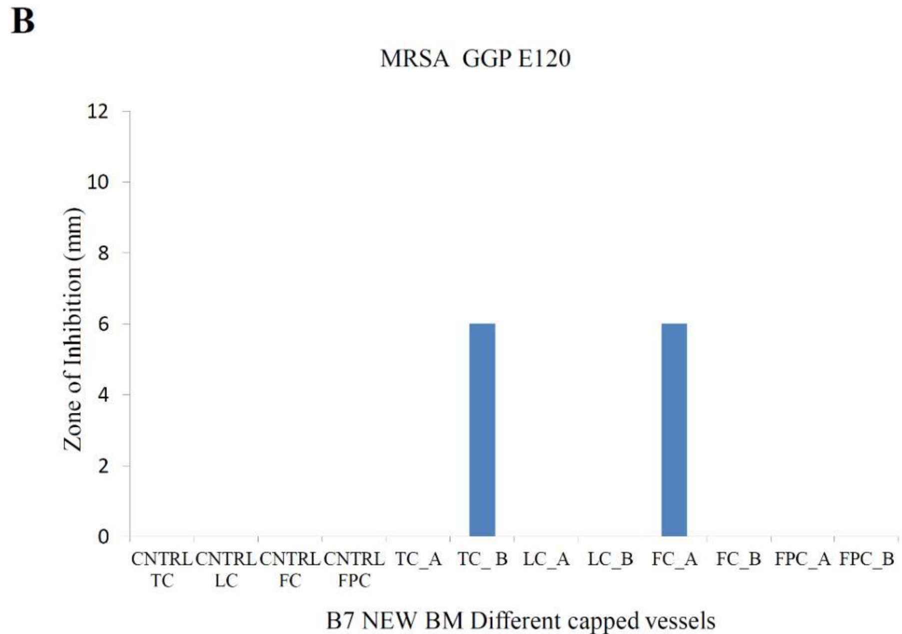

Figure 4-8: Antibacterial activity of crude extracts of Wood Decay Fungi (WDF); B7 NEW BM cultured with different fermentation vessel closing mechanisms against (A) MSSA GGP 200 and (B) MRSA GGP E120. B7 NEW BM was inoculated on Yeast peptone dextrose agar (YPDA) and mycelia discs obtained were used to inoculated Yeast, peptone, malt extract, dextrose (YPMD) broth in fermentation vessels which were tightly covered, (TC), loosely covered (LC), covered with foam (FC) and covered with filter paper (FPC). A and B are culture duplicates. Control cultures were set up with each closing mechanism but with no fungal inoculum. Ethyl acetate extracts from each vessel was assayed for antibacterial activity using the disc diffusion assay.

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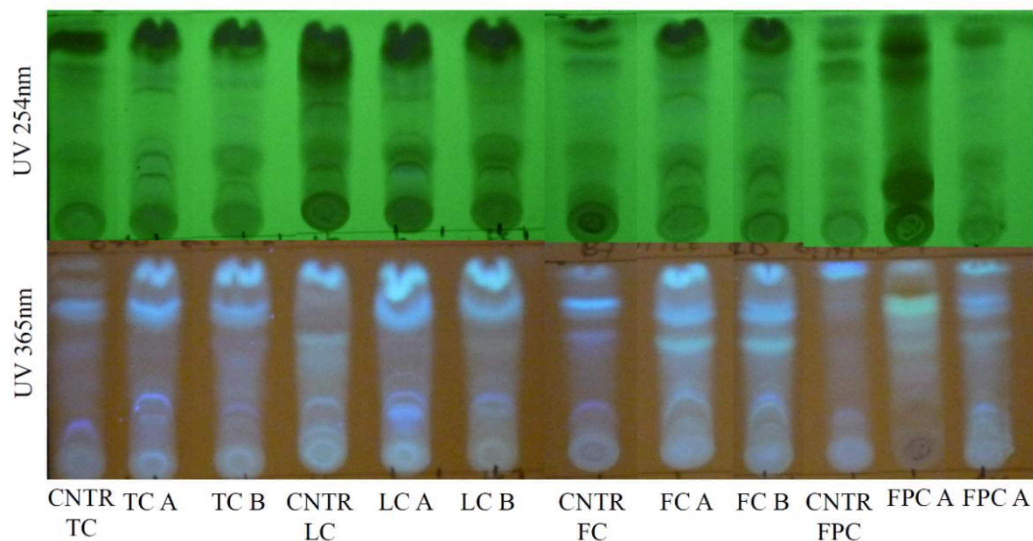

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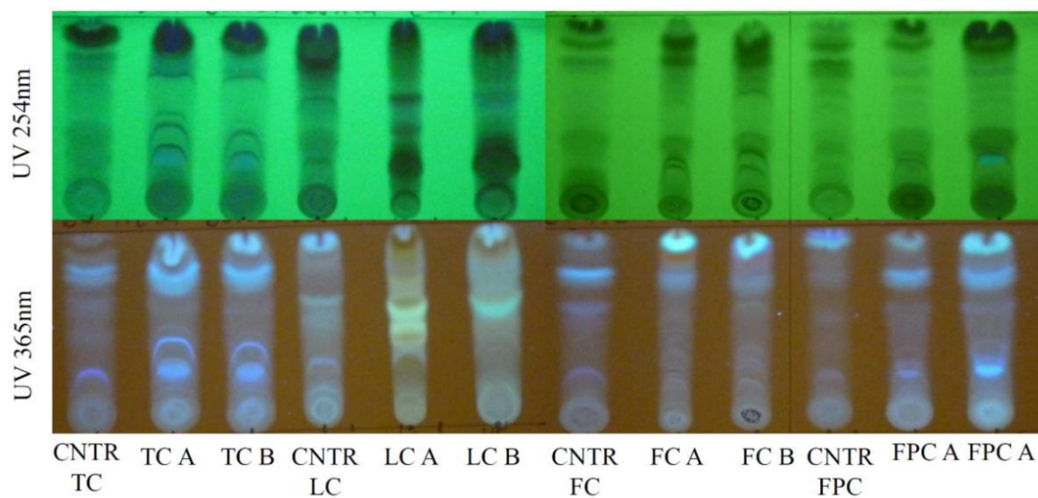

Figure 4-9: TLC profiles of extracts of Wood Decay Fungi (WDF); (A) B7 NEW BM and (B) B7 NEW BM cultured with different fermentation vessel closing mechanisms. WDF was inoculated on Yeast peptone dextrose agar (YPDA) and mycelia discs obtained were used to inoculated Yeast, peptone, malt extract, dextrose (YPMD) broth in fermentation vessels which were tightly covered, (TC), loosely covered (LC), covered with foam (FC) and covered with filter paper (FPC). A and B are culture duplicates. Control cultures were set up with each closing mechanism but with no fungal inoculum. TLC plates on which 5µl of crude extract were spot, were developed with developing solvent (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1) and viewed under UV 254nm and 365nm.

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4.4 Comparative Evaluation of Solid versus Broth (submerged) State fermentation on Fungal Bioactive Secondary Metabolite Production

Media conditions are essential in the production of fungal secondary metabolites. In the past, the laboratory has been using submerged cultures to grow fungi as the standard practice. However, due to challenges encountered in achieving consistency in duplicate cultures, and in progressing to large-scale fermentation, there was the need to compare solid-state fermentation to submerged broth fermentation, and analyze production of fungal metabolites. YPMD+SE was used as the basal media. Three categories were made. Agar was added to the YPMD+SE to make solid media, and withheld in the broth media. Potato was used in the second category. 50ml and 200ml water was added to make solid and broth media respectively. In the third category, potato was replaced with rice. B7 OLD PM and B7 NEW BM were used in this experiment. The cultures were grown for four weeks and extracted with ethyl acetate.

The antibacterial activity assay generally put submerged liquid-state cultures ahead of solid-state cultures in the production of antibacterial compounds. The anti-MSSA activity of the extracts of B7 NEW BM was generally higher than B7 OLD PM (Figures 4.10; 4.11), thus, it was used as a read out. All the broth cultures exhibited antibacterial activity, however, with the solid-state cultures, extracts from solid YPMD+SE had no antibacterial activity. The broth cultures for YPMD+SE and YPMD+SE+Rice had the highest average zone of inhibition of 11.5mm. In the potato modified media however, the solid media had a higher zone of inhibition (9.5mm) compared to the broth media (6mm). Against MRSA GGP E120, the zone of inhibition of broth cultures of YPMD+SE and YPMD+SE+Rice reduced from an average of 11.5mm to 6.5mm and 7mm respectively. Potato modified YPMD+SE solid media also had activity of 6mm whilst its broth counterpart lost its activity.

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For B7 OLD PM against MSSA GGP 200, an average zone of inhibition of 6.5mm was observed across all the media conditions, both solid and liquid (Figure 4.10). The exception was with YPMD+SE, which had the solid media showing activity while the broth media was inactive. Solid and broth media of both Potato and Rice showed similar activity. This observed activity was lost against MRSA GGP E120.

Qualitative assessment of metabolite profiles was done using TLC (Figure 4.12). B7 OLD PM extracts from solid-state fermentation had brighter bands compared to extracts from broth media although similar band patterns were observed in both solid and broth media. For extracts from B7 NEW BM, highly fluorescent bands were observed for YPMD+SE and YPMD+SE +Rice broth media, which was not detected in the solid media. YPMD+SE+Potato broth however had band patterns similar to the solid media even though in the duplicate broth, the second band (from the solvent front) appeared to lag behind its counterpart in the solid media.

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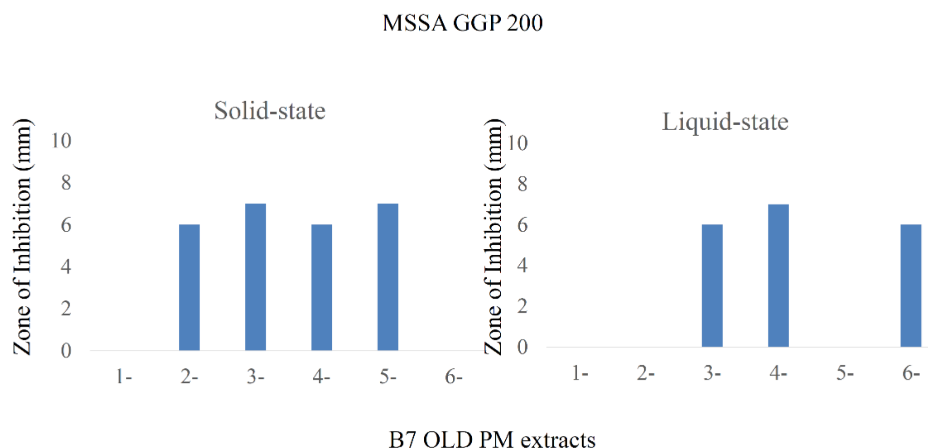

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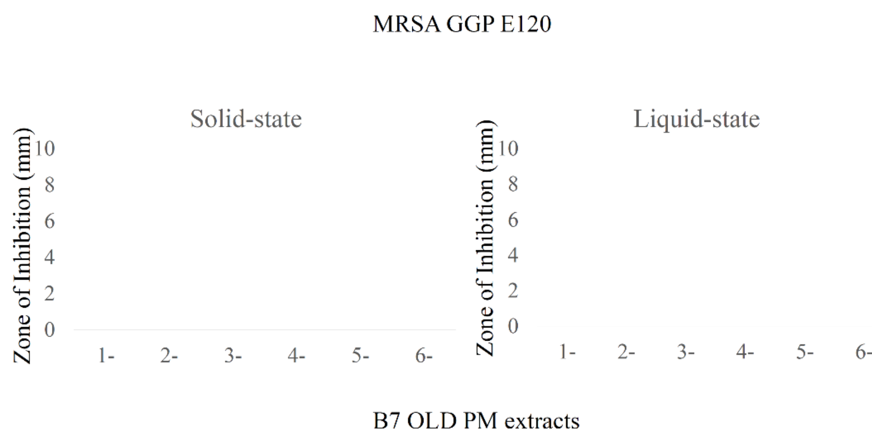

SOLID-STATE
1. YPMD+SE A
2. YPMD+SE B
3. YPMD+SE +POTATO A
4. YPMD+SE +POTATO B
5. YPMD+SE+RICE A
6. YPMD+SE+RICE B

LIQUID-STATE
1.YPMD+SE BROTH A
2.YPMD+SE+BROTH B
3.YPMD+SE+POTATO BROTH A
4.YPMD+SE+POTATO BROTH B
5.YPMD+SE+RICE BROTH A
6.YPMD+SE+RICE BROTH B

Figure 4-10: **Antibacterial activity of extracts of B7 OLD PM cultured on solid and liquid media against (A) MSSA GGP 200 and (B) MRSA GGP E120.** B7 OLD PM was inoculated on Yeast peptone dextrose agar (YPDA) and mycelia discs obtained were used to inoculate solid and liquid cultures of YPMD+SE, YPMD+SE+Potato and YPMD+SE+Rice. YPMD+SE- Yeast, peptone, malt extract, dextrose +soil extract. Agar was added and withheld from YPMD+SE to make solid and broth media respectively. 50ml and 200ml water was added to YPMD+SE+Potato and Rice, to make solid and broth media respectively. The cultures were incubated for four weeks. Ethyl acetate extracts were assayed for antibacterial activity using the disc diffusion assay.

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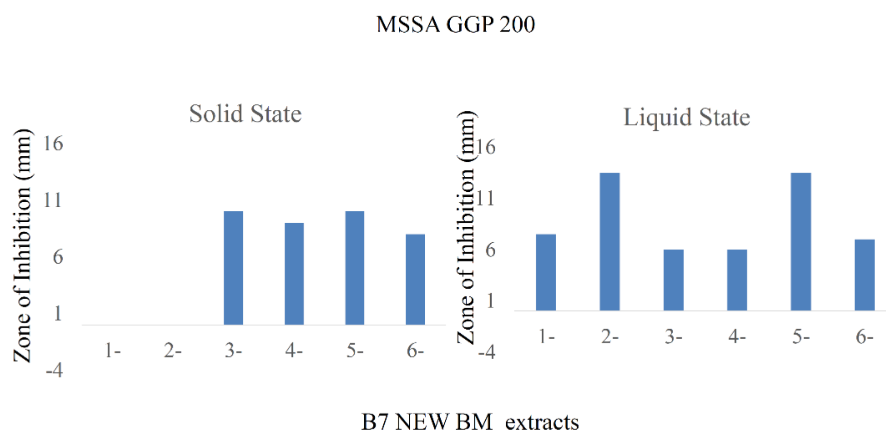

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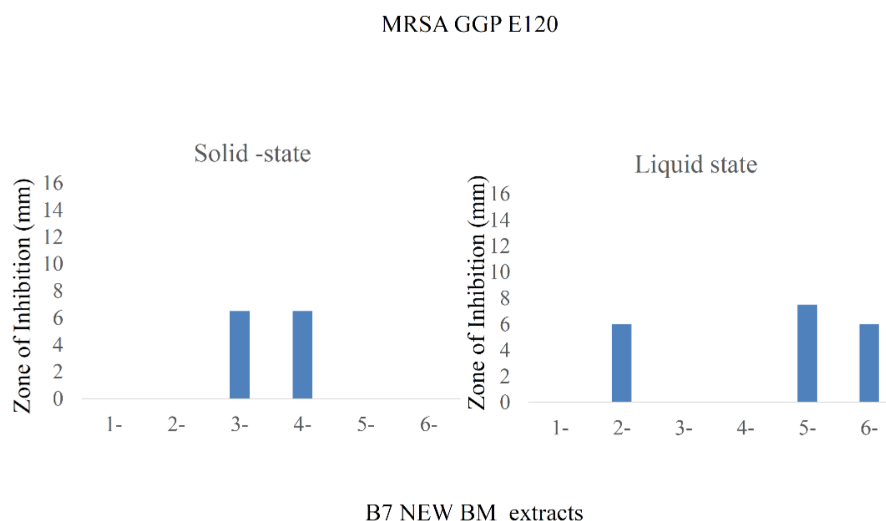

SOLID-STATE
1. YPMD+SE A
2. YPMD+SE B
3. YPMD+SE +POTATO A
4. YPMD+SE +POTATO B
5. YPMD+SE+RICE A
6. YPMD+SE+RICE B

LIQUID-STATE
1. YPMD+SE BROTH A
2. YPMD+SE+BROTH B
3. YPMD+SE+POTATO BROTH A
4. YPMD+SE+POTATO BROTH B
5. YPMD+SE+RICE BROTH A
6. YPMD+SE+RICE BROTH B

Figure 4-11: Antibacterial activity of extracts of B7 NEW BM cultured on solid and liquid media against (A) MSSA GGP 200 and (B) MRSA GGP E120. B7 NEW BM was inoculated on Yeast peptone dextrose agar (YPDA) and mycelia discs obtained were used to inoculate solid and liquid cultures of YPMD+SE, YPMD+SE+Potato and YPMD+SE+Rice. YPMD+SE- Yeast, peptone, malt extract, dextrose +soil extract. Agar was added and withheld from YPMD+SE to make solid and broth media respectively. 50ml and 200ml water was added to YPMD+SE+Potato and Rice, to make solid and broth media respectively. The cultures were incubated for four weeks. Ethyl acetate extracts were assayed for antibacterial activity using the disc diffusion assay.

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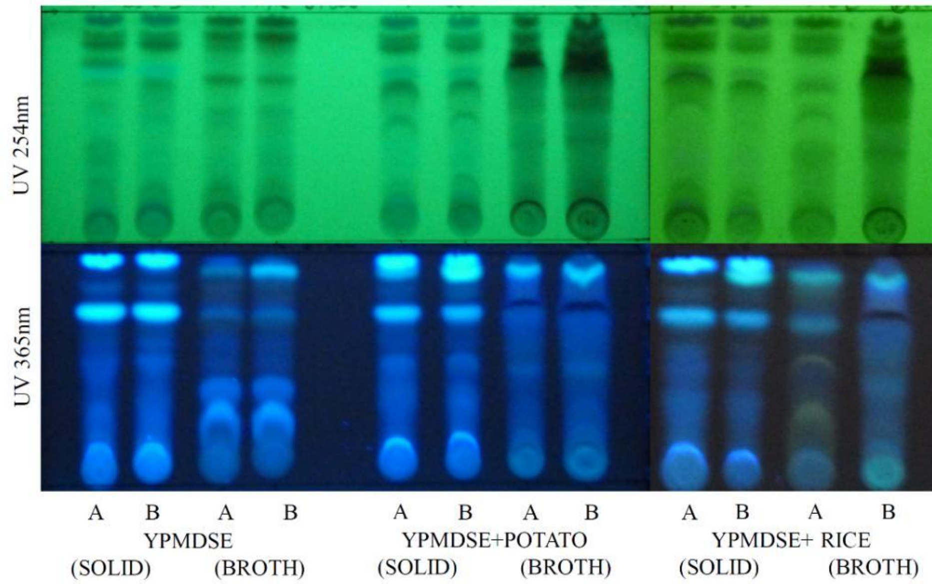

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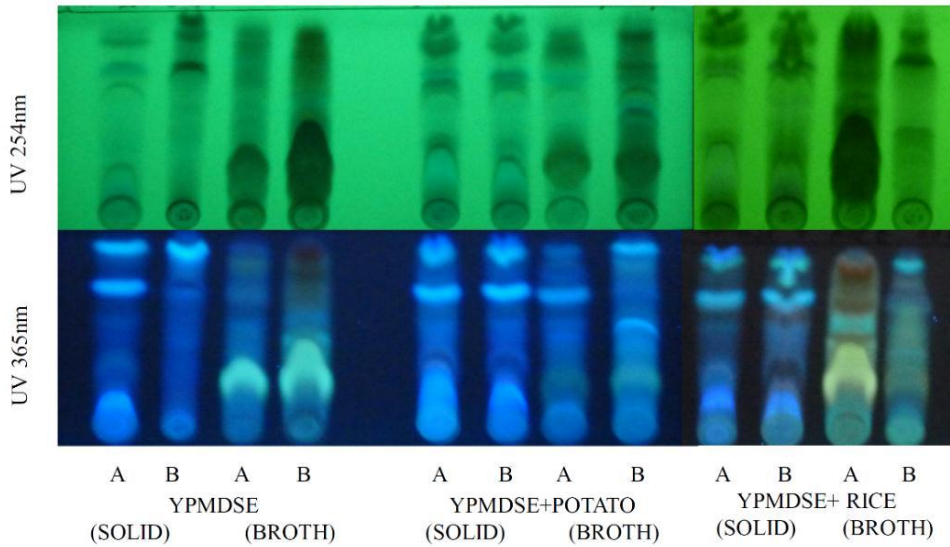

Figure 4-12: TLC profile of extracts of Wood Decay Fungi (WDF); (A) B7 OLD PM and (B) B7 NEW BM cultured on solid and broth media. WDF was inoculated on Yeast peptone dextrose agar (YPDA) and mycelia discs obtained were used to inoculated solid and broth media of YPMD+SE, YPMD+SE+Potato and YPMD+SE+RICE. TLC plates on which 5µl of crude extract were spot, were developed with (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1) and viewed under UV 254nm and 365nm.

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4.5 Effect of media composition on the consistent production of fungal bioactive secondary metabolites

To reduce the inconsistencies in activity profile of fungi cultured in Potato Dextrose Broth (PDB), previous work in our laboratory investigated mineral supplementation in PDB and its effect on metabolite production (Adadey, 2014). Soil extract (SE) moderately increased activity, but the batch-to-batch inconsistencies were still present. Nutrition manipulation is known to have an impact on metabolite production (Fiedurek *et al.,* 1996; Bode *et al.,* 2002; Miao *et al.,* 2006; Bills *et al.,* 2008; Xu *et al.,* 2008; Mohanty & Prakash, 2009; Kossuga *et al.,* 2012; Shang *et al.,* 2012). Thus, it was vital to test the hypothesis that the formulation of rich media consisting of essential proteins, carbohydrates, and minerals salts would aid the consistent production of fungal secondary metabolites.

To test this hypothesis, six different media (PDB; PDB+2X Soil Extract; PDB+2X SE; Yeast, Peptone, Malt extract, Dextrose; YPMD, YPMD+PB, YPMD+2XSE, YPMD+PB+2X SE) were used. Four replicates of each media were made. Wood Decay Fungi B7 NEW BM and D7 PM 2pb were used as inoculum. Antibacterial activity of the extracts against MSSA GGP 200 and MRSA GGP E120 was tested. Extracts of B7 NEW BM cultured in PDB and YPMD showed the highest zone of inhibition with highest activity of PDB being 20.5mm and that for YPMD was 21.5mm against MSSA (Figure 4.13). The other media lagged behind these two with their highest activity being 13mm. The same pattern was observed when the extracts were tested against MRSA GGP E120. Only extracts from PDB and YPMD maintained high zones of inhibition of 11.5mm and 10.5mm respectively. The others had basal activity of 6mm. The third and fourth replicate of PDB+ 2X SE, and the first replicate of YPMD +PB+ 2X SE even lost their activity completely.

Extracts from D7 PM 2pb appeared to be most productive in YPMD broth as it showed its highest activity of 21 mm against MSSA GGP 200 (Figure 4.15).

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The activity decreased to 12.3mm against MRSA GGP E120. This was closely followed by extracts obtained from PDB+2X SE broth, which had the first replicate showing a zone of 17mm. Interestingly, even though this high activity was not replicated in all four broths, it was maintained against MRSA GGP E120 (Figure 4.15).

TLC profiles of extracts obtained from the six media inoculated with B7 NEW BM showed similar band patterns across all six media and their replicates, except for the first two replicates of PDB and YPMD and all the replicates of PDB+SE which did not seem to separate well and appeared as a smear (Figure 4.14). For extracts of D7 PM 2pb, bands viewed under short UV wavelength showed green bands close to the origin for YPMD+PB, which were not observed in the other media types (Figure 4.16). Although bands observed in extracts cultured in PDB were faint, the second replicate of YPMD seemed to taper on reaching the solvent front. The same band pattern was observed for all the media types. YPMD is therefore the media of choice for obtaining batch-to-batch consistency in WDF cultures.

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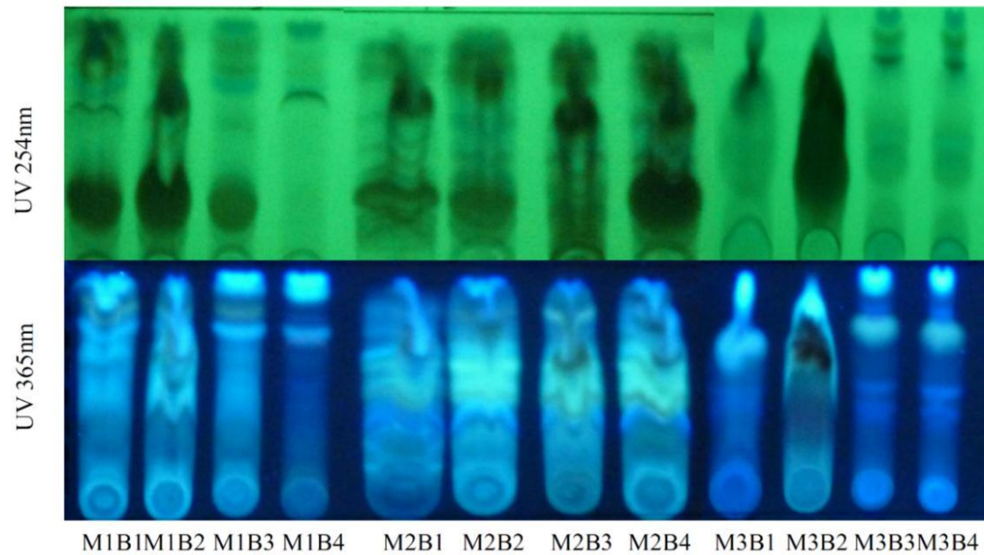

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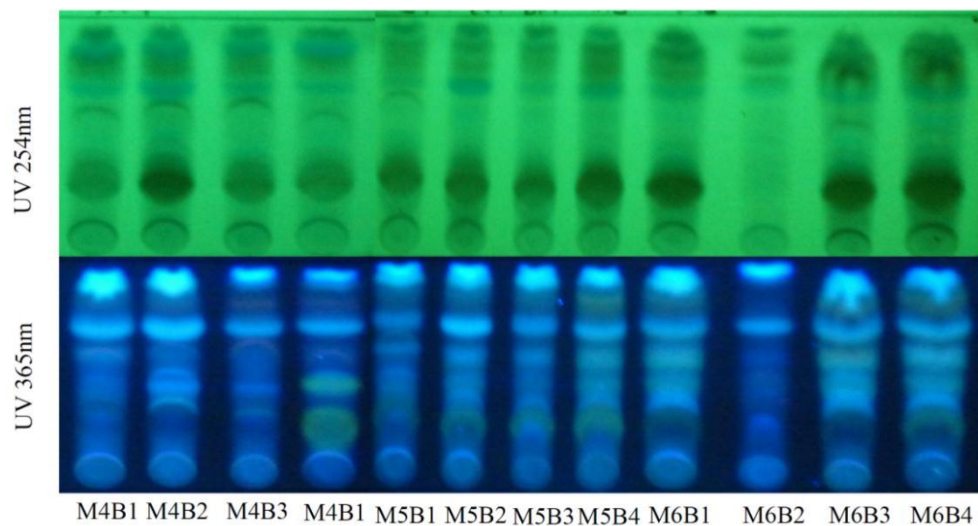

Figure 4-14: TLC profile of extracts of Wood Decay Fungi (WDF); B7 NEW BM cultured in six different broth media. B7 NEW BM was inoculated unto Yeast extract, peptone, dextrose agar (YPDA) and mycelia discs obtained were used to inoculate four replicates (B1-B4) of six different media (M1-M6). M1-Potato dextrose broth (PDB), M2-Potato dextrose broth +Soil extract (PDB+2X SE), M3-Yeast extract, peptone, malt extract, dextrose (YPMD) broth, M4-Yeast extract, peptone, malt extract, dextrose +Potato broth (YPMD+PB), M5- Yeast extract, peptone, malt extract, dextrose +Soil extract (YPMD+2X SE) M6- Yeast extract, peptone, malt extract, dextrose +Soil extract+Potato Broth (YPMD+SE+PB). TLC plates on which 5µl of crude extract were spot, were developed with developing solvent (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1) and viewed under UV 254nm and 365nm

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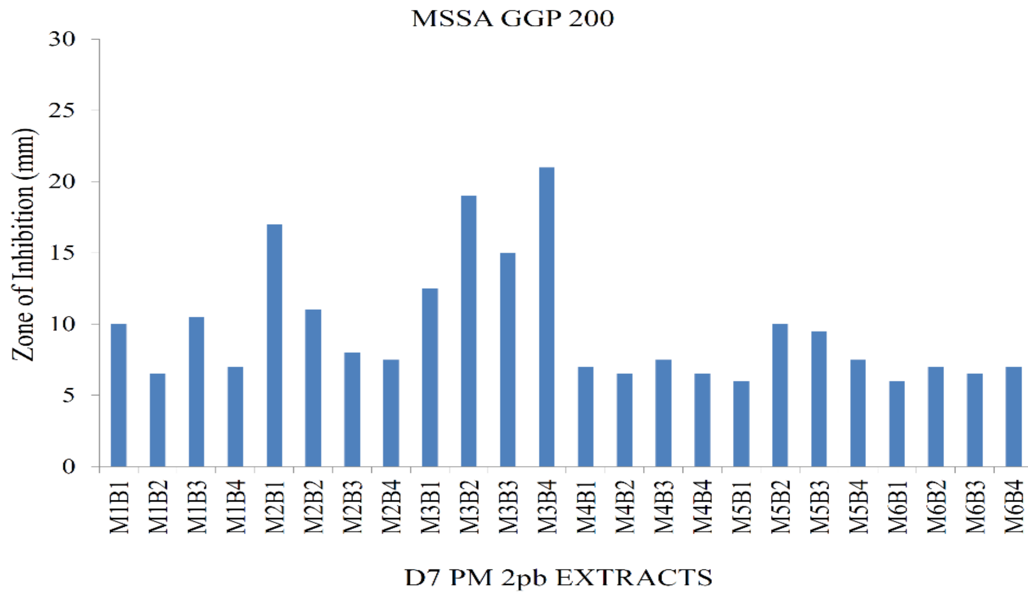

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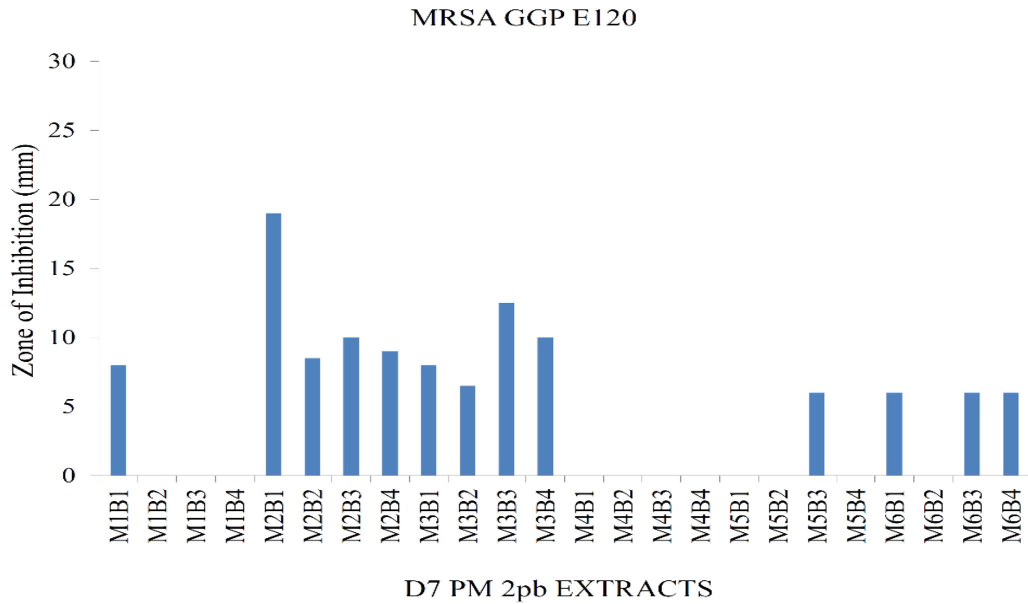

Figure 4-15: Antibacterial activity of extracts of Wood Decay Fungi (WDF); D7 PM 2pb cultured in six different broth media. D7 PM 2pb was inoculated unto Yeast extract, peptone, dextrose agar (YPDA) and mycelia discs obtained were used to inoculate four replicates (B1-B4) of six different media (M1-M6). M1-Potato dextrose broth (PDB), M2- Potato dextrose broth +Soil extract (PDB+2X SE), M3-Yeast extract, peptone, malt extract, dextrose (YPMD) broth, M4-Yeast extract, peptone, malt extract, dextrose +Potato broth (YPMD+PB), M5- Yeast extract, peptone, malt extract, dextrose +Soil extract (YPMD+2X SE) M6- Yeast extract, peptone, malt extract, dextrose +Soil extract+Potato Broth (YPMD+SE+PB). Ethyl acetate extracts of six broth were assayed for antibacterial activity using the disc diffusion assay.

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4.6 Using Inhibitors of Histone De-acetylases to explore Silent Gene Clusters and improve the level production of Fungal Secondary Metabolism

Fungi are known to have gene clusters of secondary metabolism that are usually silent under laboratory conditions. Inhibitors of histone de-acetylases (iHDAC) have been explored in accessing these metabolites in the laboratory. To investigate this, six histone deacetylase inhibitors were tested. Fungi were cultured on plates containing iHDAC-treated YPDA media (Figure 4.17). Two wood decay fungi were used for the experiment. B7 OLD PM and B7 NEW BM. The first significant readout obtained from this treatment was the change in the colour of the fungi colonies on agar plates. B7 OLD and B7 NEW were both green on unmodified YPDA. However, upon modification with iHDAC, B7 OLD PM lost its green colour and turned white. A similar occurrence was observed for B7 NEW BM, except for Trichostatin A-treated plates, which still maintained a tint of green.

Three treatment modalities were used in culturing fungi in the presence of the chromatin modifiers (Figures 4.18 — 4.21). First, untreated broth was inoculated with iHDAC-treated fungal mycelia discs. Next, iHDAC-treated broth was inoculated with untreated mycelia discs. Lastly, iHDAC-treated broth was treated with treated mycelia discs. TLC and disc diffusion assay were employed to analyze the qualitative composition and bioactivity of the extracts obtained respectively. The pure compounds were spotted and run alongside extracts from treated broth cultures. Apart from sodium butyrate which did not show any band on the TLC, the other compounds eluted and showed single bands, with SAHA being the most prominent when viewed under UV short wavelength (Figure 4.19). Generally, all the bands observed for extracts of treated B7 OLD PM under UV long and short wavelength were similar to the bands observed in the control, which had no treatment (Figure 4.19). However, in comparison to bands observed in the control extract, SAHA-, valproic acid-, and 2-Hexyl-4-Pentanoic acid-treated broth cultures

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showed very intense fluorescing bands close to the origin when viewed under UV long wavelength (365nm) (Figure4.19). The activity of these extracts against MSSA GGP 200 revealed that these three treated conditions cause an increase in the zone of inhibition observed relative to the untreated control extracts. Extracts from broth A of SAHA treated broth and untreated Plate inoculum particularly recorded a 10 mm increase in the zone of inhibition from 15mm in the control to 25mm (Figure 4.18). Against MRSA GGP E120, the zone of inhibition of the extract from the control untreated broth decreased to 13mm whilst the SAHA treated extract increased to 30.5mm.

The most significant pattern observed for the effect of the small chemicals on the antibacterial activity of B7 NEW BM was observed in extracts from trichostatin A treated broth (Figure 4.20). The zone of inhibition it exhibited against the resistant strain of S. aureus was increased by 6mm compared to extracts from the untreated broth. Generally, the TLC band patterns for untreated and treated extracts of all the chemicals used were similar (Figure 4.21). The antibacterial activities of the treated extracts did not vary much, except for extracts from valproic acid-treated broth and untreated plate inoculums, which, even though had only slightly higher zones of inhibition against MSSA GGP 200 compared to extracts from the control untreated broth, did not reduce in activity against MRSA GGP E120 as the control did (Figure 4.20). Duplicate broth of Sodium-4-phenyl butyrate-treated plate inoculums and broth recorded the highest zone of inhibition (20.5mm) against MSSA GGP 200, which was 6mm more than that observed in the control untreated broth extracts.

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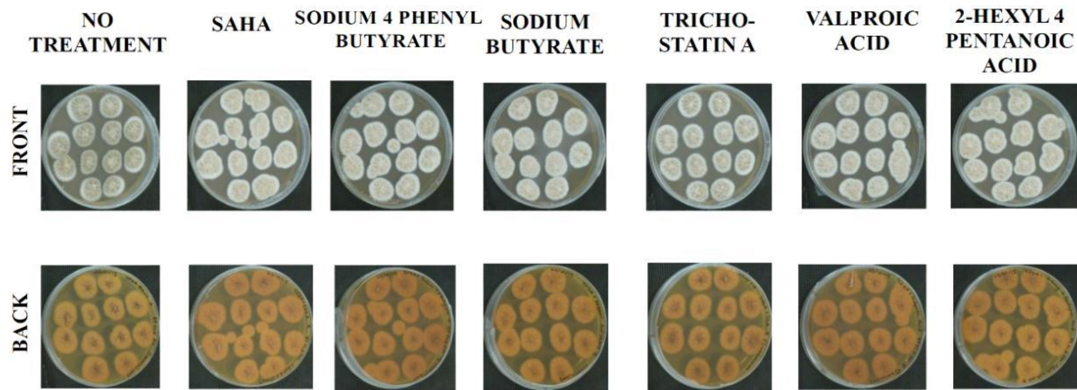
A

B7 OLD PM



B

B7 NEW BM

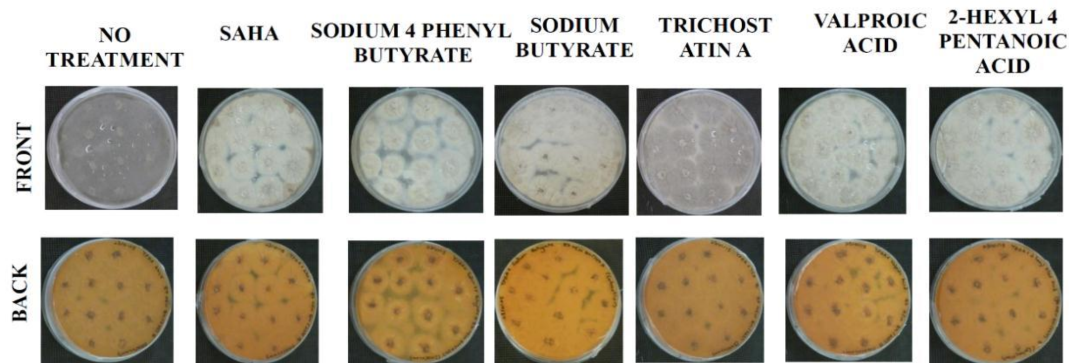


Figure 4.17: Colonies of Wood Decay Fungi (WDF); B7 OLD PM (A) and B7 NEW BM (B) cultured with and without inhibitors of chromatin modifiers. The two WDF were inoculated unto Yeast extract, peptone, dextrose agar (YPDA) modified with SAHA, Sodium-4-phenyl butyrate, Sodium butyrate, Trichostatin, Valproic acid and 2-hexyl-4 pentynoic acid and incubated at room temperature for four days. Control plates were unmodified YPDA plates. Front and back pictures of the growth of fungi from the front and back of YPDA plates

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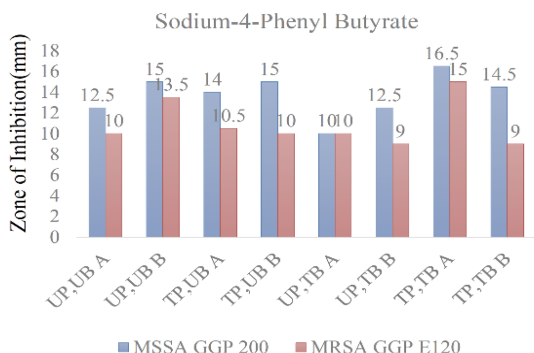

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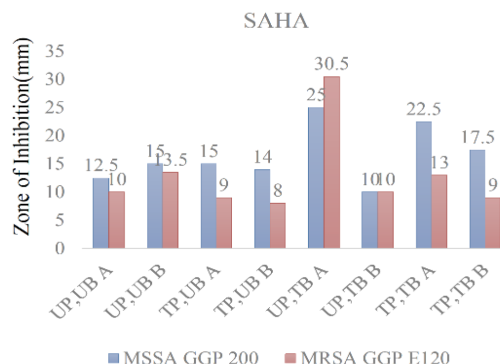

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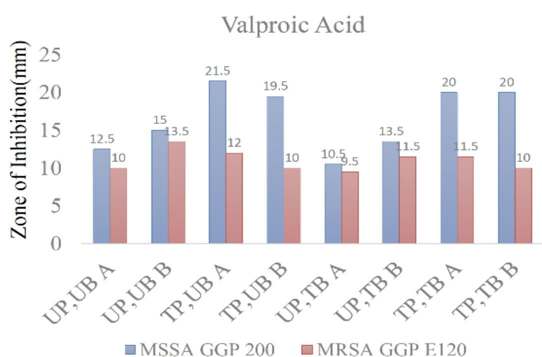

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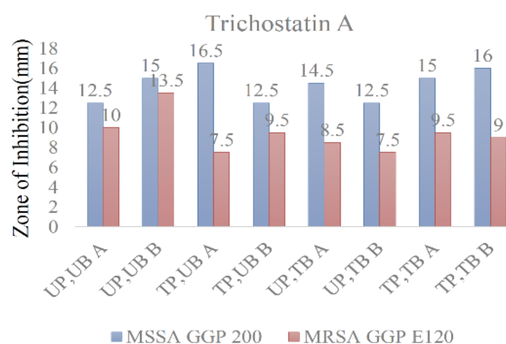

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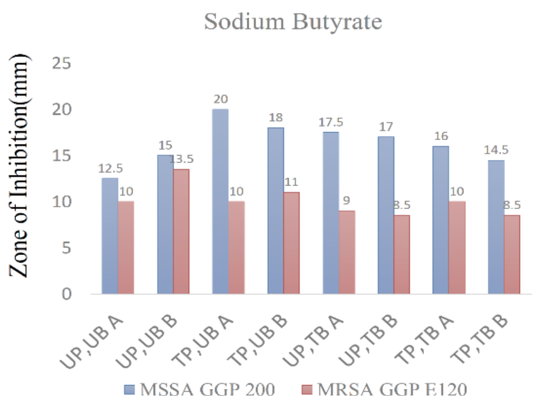

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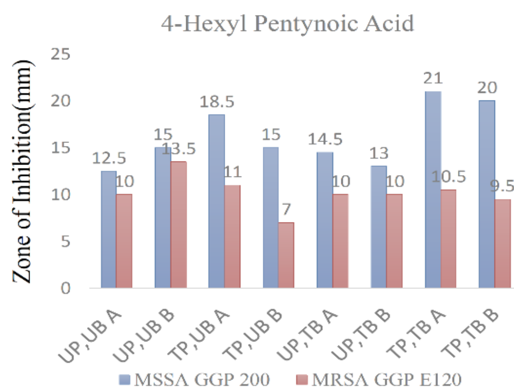

Figure 4-18: Antibacterial activities of extracts of Wood Decay Fungi (WDF); B7 OLD PM treated with inhibitors of histone deacetylases. Three treatments were done; mycelia disc of B7 OLD PM grown on **treated** YPDA plates were inoculated into **untreated** Yeast extract, peptone, malt extract and dextrose (YPMD) broth (TP, UB), next, mycelia disc on **untreated** YPDA plates were inoculated into treated YPMD broth (UP, TB), lastly, mycelia discs grown on **treated** YPDA plates were inoculated into **treated** YPMD broth (TP, TB). Control broth was made up of mycelia discs grown on **untreated** YPDA plates inoculated into **untreated** YPMD broth (UP, UB). Cultures were incubated for four weeks and extracted with ethyl acetate. Antibacterial activity of the extracts were assayed using the disc diffusion assay

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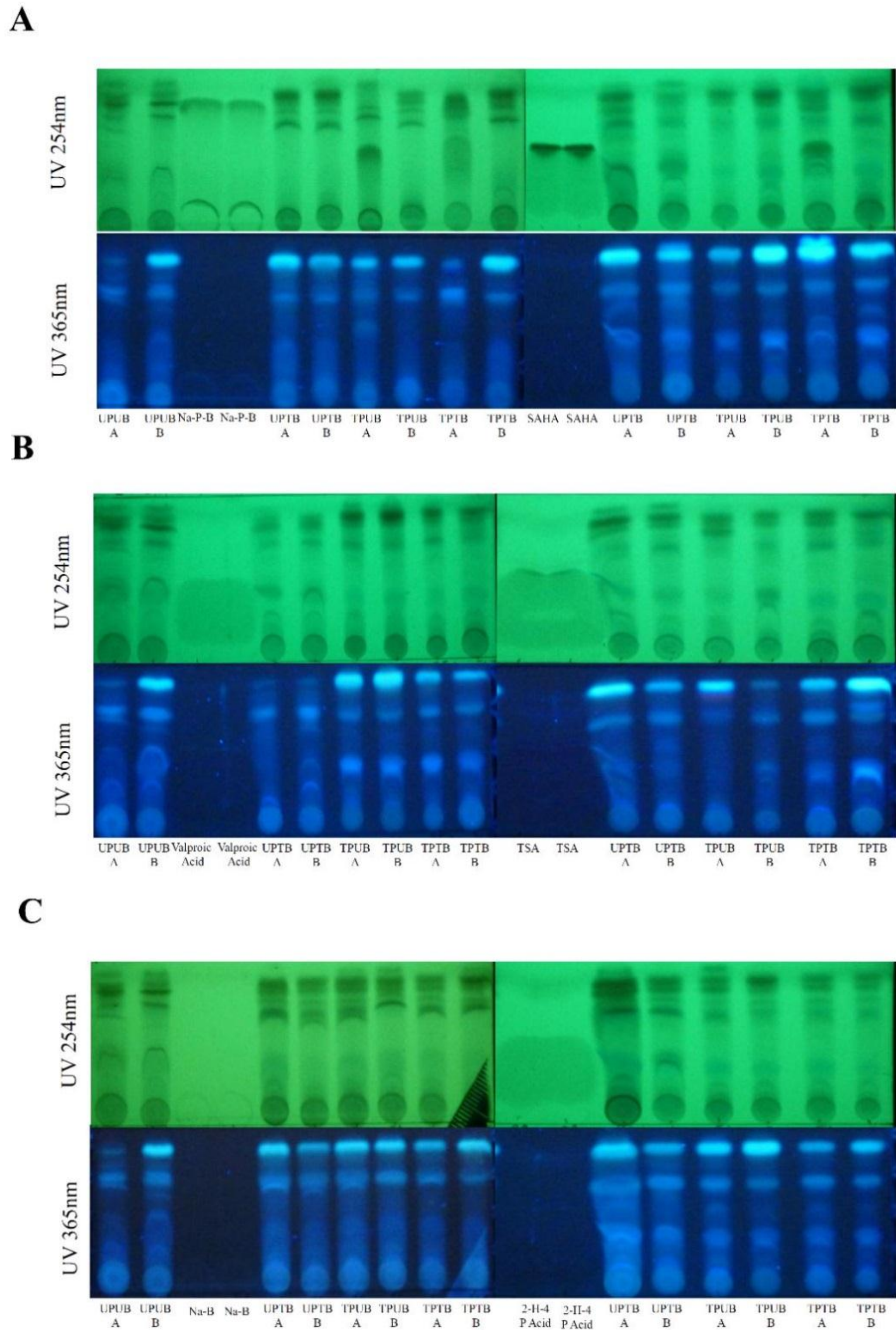

Figure 4-19: TLC profile of extracts of Wood Decay Fungi (WDF); B7 OLD PM treated with inhibitors of histone deacetylases. Three treatments were done; mycelia disc of B7 OLD PM grown on **treated** YPDA plates were inoculated into **untreated** Yeast extract, peptone, malt extract and dextrose (YPMD) broth (TP,UB), next, mycelia disc on **untreated** YPDA plates were inoculated into treated YPMD broth (UP,TB), lastly, mycelia discs grown on **treated** YPDA plates were inoculated into **treated** YPMD broth (TP,TB). Control broth was made up of mycelia discs grown on **untreated** YPDA plates inoculated into **untreated** YPMD broth (UP, UB). Cultures were incubated for four weeks and extracted with ethyl acetate. TLC plates on which 5µl of crude extract were spot, were developed with (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1) and viewed under UV 254nm and 365nm.

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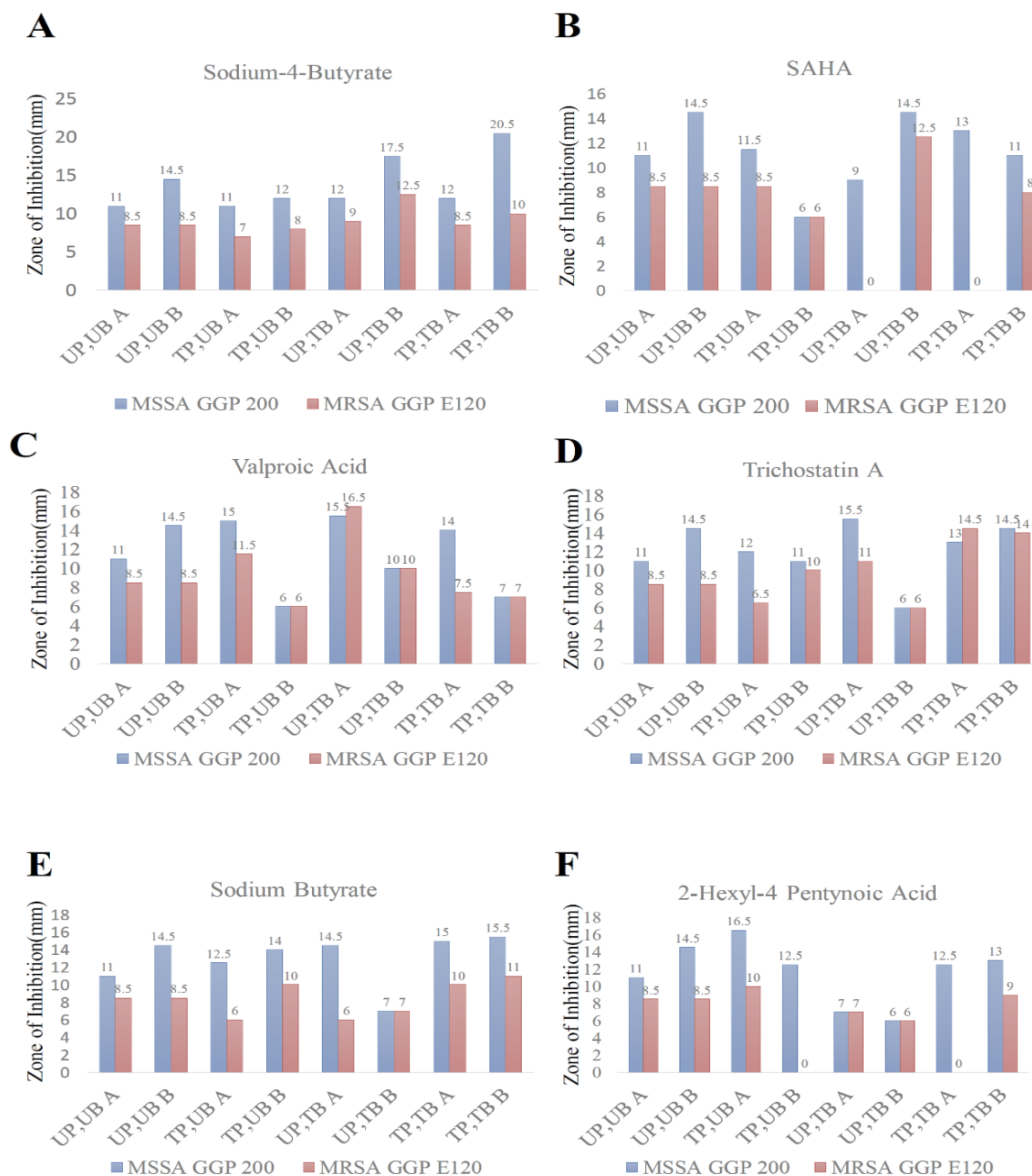

Figure 4-20: Antibacterial activities of extracts of Wood Decay Fungi (WDF); B7 NEW BM treated with inhibitors of histone deacetylases. Three treatments were done; mycelia disc of B7 NEW BM grown on **treated** YPDA plates were inoculated into **untreated** Yeast extract, peptone, malt extract and dextrose (YPMD) broth (TP, UB), next, mycelia disc on **untreated** YPDA plates were inoculated into treated YPMD broth (UP, TB), lastly, mycelia discs grown on **treated** YPDA plates were inoculated into **treated** YPMD broth (TP, TB). Control broth was made up of mycelia discs grown on **untreated** YPDA plates inoculated into **untreated** YPMD broth (UP, UB). Cultures were incubated for four weeks and extracted with ethyl acetate. Antibacterial activity of the extracts were assayed using the disc diffusion assay

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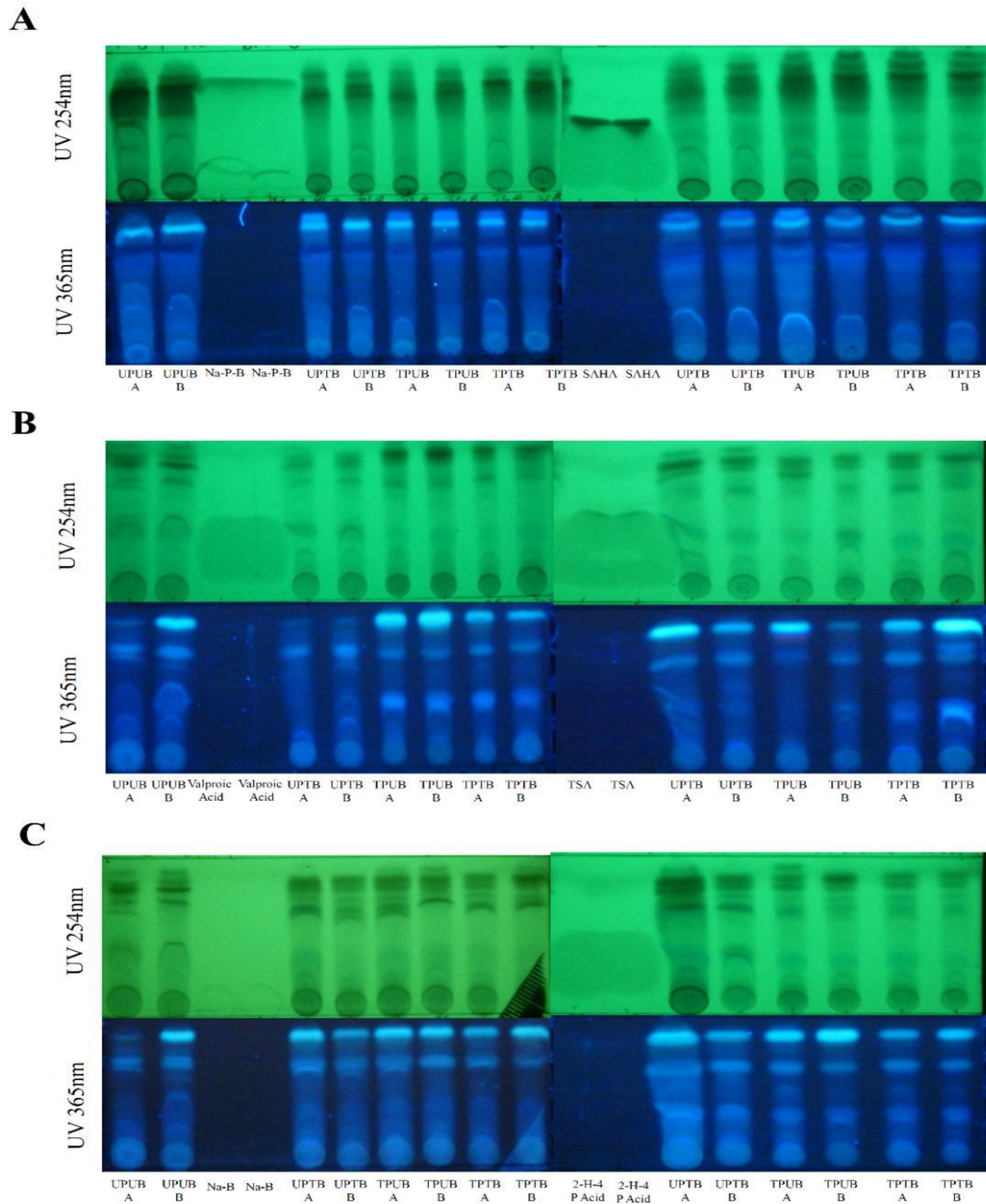

Figure 4-21: TLC profiles of extracts of Wood Decay Fungi (WDF); B7 NEW BM treated with inhibitors of histone deacetylases. Three treatments were done; mycelia disc of B7 NEW BM grown on **treated** YPDA plates were inoculated into **untreated** Yeast extract, peptone, malt extract and dextrose (YPMD) broth (TP,UB), next, mycelia disc on **untreated** YPDA plates were inoculated into treated YPMD broth (UP,TB), lastly, mycelia discs grown on **treated** YPDA plates were inoculated into **treated** YPMD broth (TP,TB). Control broth was made up of mycelia discs grown on **untreated** YPDA plates inoculated into **untreated** YPMD broth (UP,UB). Cultures were incubated for four weeks and extracted with ethyl acetate. TLC plates on which 5µl of crude extract were spot, were developed with (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1) and viewed under UV 254nm and 365nm.

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4.7 Validation of Bioactivity of WDF using Synergy with Standard Antibiotics and Phenotype modulating Compounds

The primary screen of 189 WDF returned 20 WDF with high activity against Gram-positive organisms (*S. aureus*). These were grown on a large scale to get higher yield of extract for further analysis. Different chemicals (Ampicillin, Clavulanic Acid, Chloramphenicol, Tetracycline, Streptomycin, N-acetyl glucosamine and Arginine) with known mechanisms of action were used to probe the cell system in an attempt to choose promising extracts to carry on for product isolation. Standard antibiotics used as controls and the twenty WDF that had high activity from the primary screen were subjected to a synergy assay with these chemicals incorporated into the growth media (Mueller Hinton Agar, MHA).

Distinct activity patterns were observed in *S. aureus* grown on media modified with each of the compounds (Figures 4.22-4.25). Chloramphenicol particularly seemed to have an effect on the activity of almost all the antibiotics. Against MSSA GGP 200, the activity of ampicillin (34mm) on unmodified MHA increased to 45mm in the presence of Chloramphenicol. Ethambutol and Cycloserine, which had no activity on unmodified MHA, showed zones of 15mm and 19 mm respectively in the presence of Chloramphenicol. (Figure 4.22 A)

While chloramphenicol increased the activity of the above-mentioned antibiotics, it also seemed to make some other antibiotics less active. Vancomycin and Streptomycin both had a reduction in their zones of activity from 21.5mm and 25 mm to 15 and 8mm respectively. Arginine, another phenotype-modulating compound, appeared to suppress the activity of Linezolid, as it was observed to have totally lost its activity from 31mm observed on the control MHA plate (Figure 4.22 A).

Chloramphenicol was also active against MRSA GGP E120. Tetracycline and Erythromycin, which initially had zones of inhibition of 12.5 and 12mm on MHA only plates,

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increased to 18.5mm and 18mm respectively in the presence of chloramphenicol. N-acetyl glucosamine increased the sensitivity of Moxifloxacin, increasing its zone of inhibition from 22 mm to 30.5 mm (Figure 4.22 B).

The extracts were analyzed for activity against MSSA GGP 200 and MRSA GGP E120. One extract that stood out in its activity across all the differently modified media was B7 NEW 2BB e5, which had a strikingly high activity of 32 mm against MSSA (Figure 4.23), and 24mm against MRSA (Figure 4.24). The chemicals employed barely affected its (B7 NEW 2BB e5) activity against MSSA. Chloramphenicol and arginine seemed to boost it up by increasing its zone of inhibition to 30 and 29.5mm respectively when its sensitivity reduced against MRSA from the 24 mm on the control plate (Figure 4.24)

Other notable extracts were B8 2pb e2, which had a zone of inhibition of 17 mm on control-unmodified plates. Its activity was increased to 21.5mm in the presence of chloramphenicol however; ampicillin, clavulanic acid and tetracycline reduced this zone to 14.5, 13.5 and 13.5 respectively (Figure 4.23). Its sensitivity against MRSA (12.5mm), however, was improved by chloramphenicol, arginine and ampicillin to 19mm, 18mm and 18mm respectively (Figure 4.24).

Another extract the D7 2pb e4, whose initial zone of inhibition against MSSA GGP 200 on control plates was 21 mm, increased to 26.5 mm in the presence of Streptomycin. Interestingly, chloramphenicol modification caused a drastic reduction in its sensitivity from 21mm to 7.5mm (Figure 4.23). However, this extract was unable to inhibit MRSA GGP E120.

Ampicillin, Chloramphenicol and N-acetyl glucosamine modifications also boosted the activity of D7 2bb_A e1 against MSSA GGP 200 from 9mm to 20mm, 17mm and 14.5mm respectively (Figure 4.23). Based on the activity pattern observed for the extracts against MSSA GGP 200 and MRSA GGP E120, 8 extracts were selected to do further work on.

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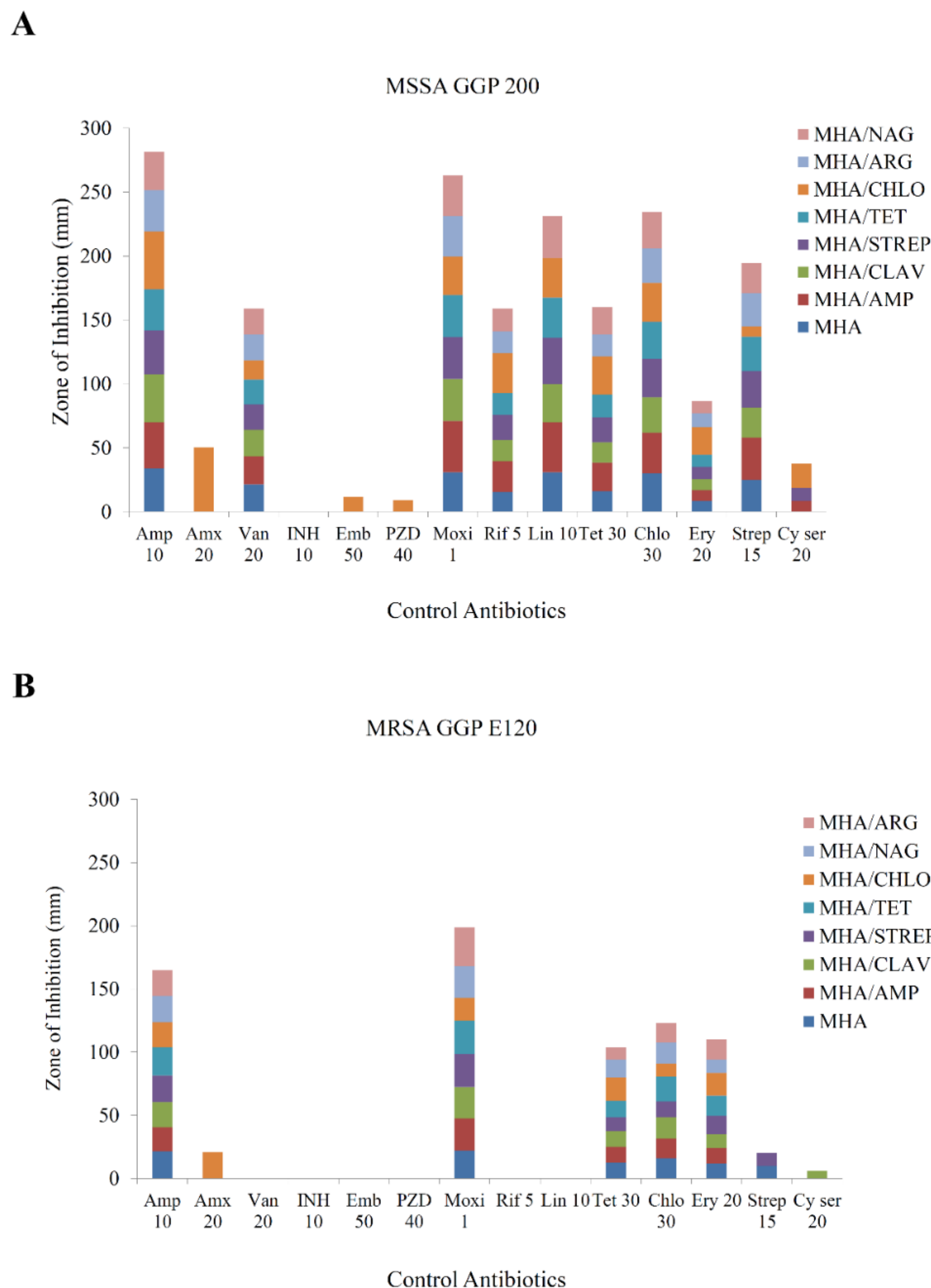

Figure 4-22: Chemical phenotypic screen of 14 standard antibiotics against (A)- MSSA GGP 200 and (B)- MRSA GGP E120. The Disc diffusion method was used to assay the antibacterial activity of standard antibiotics on Mueller hinton agar (MHA), modufied with ampicillin(AMP-0.05µg/ml), clavulanic acid (CLAV-50µgml) tetracycline (TET- 0.5µg/ml), chloramphenicol (CHLO-5µg/ml), N-acetyl glucosamine (NAG-2.2mg/ml), arginine (ARG-0.85mg/ml).Amp 10- Ampicillin 10µg/disc, Amx 20- Amoxicillin 20µg/disc, Van 20-Vancomycin 20µg/disc, INH 10- Isoniazid 10µg/disc, Emb 50- Ethambutol 50µg/disc, PZD 40- Pyrazinamide 40µg/disc, Moxi 1- Moxifloxacillin 1µg/disc, Rif 5-Rifampicin 5µg/disc, Lin 10-Lineolid 10µg/disc, Tet 30- Tetracycline 30µg/disc, Chlo 30- Chloramphenicol 30µg/disc, Ery 20- Erythromycin 20µg/disc, Strep 15- Streptomycin 15µg/disc, Cyser 20- Cycloserine20µg/disc.

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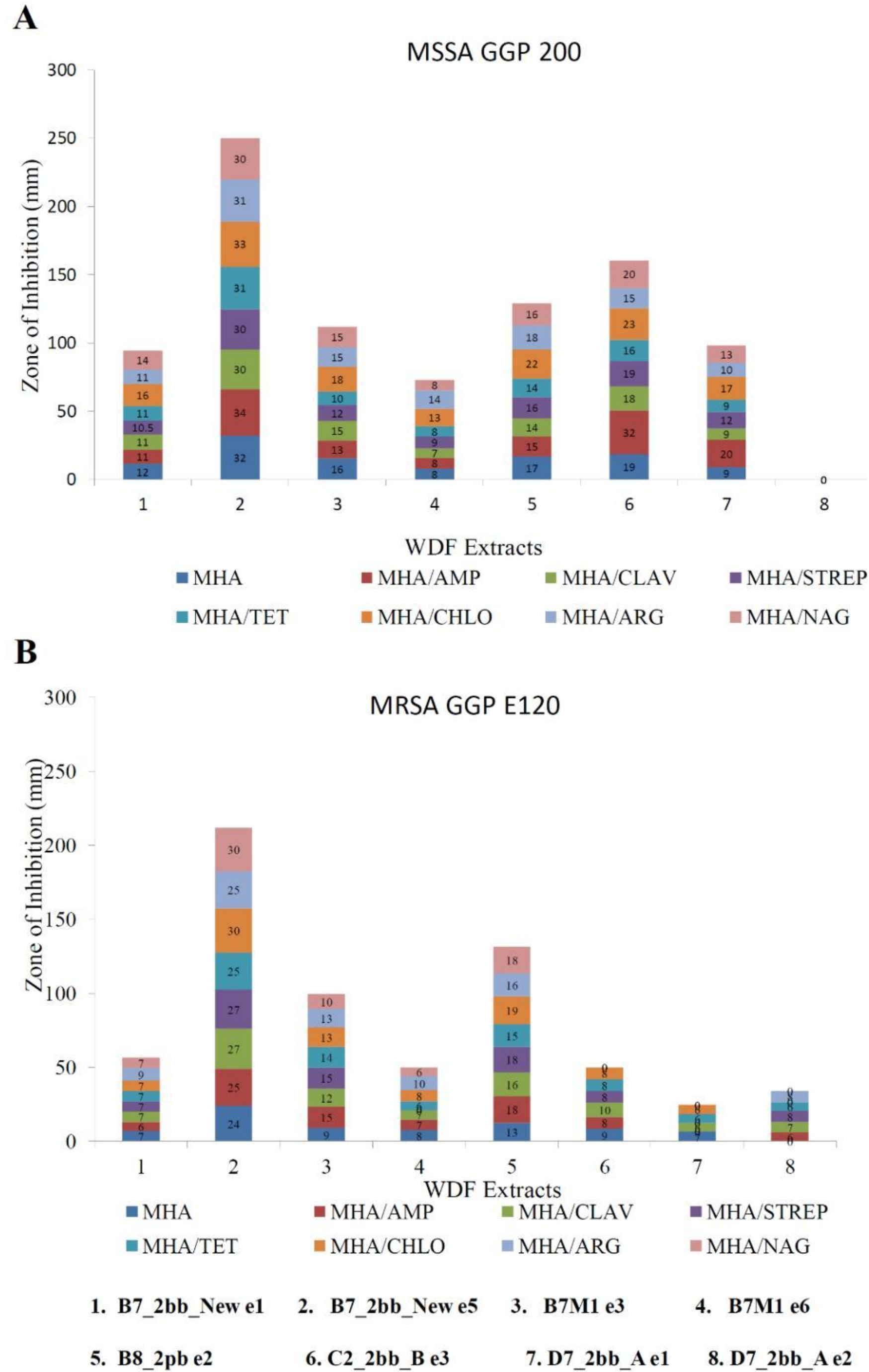

Figure 4-25: Chemical phenotypic screen of top 8 Wood decay fungi (WDF) extracts against (A) MSSA GGP 200 and (B) MRSA GGP E120. The Disc diffusion method was used to assay the antibacterial activity of WDF extracts on Mueller hinton agar (MHA), modified with ampicillin(AMP-0.05μg/ml), clavulanic acid (CLAV-50μg/ml) tetracycline (TET- 0.5μg/ml), chloramphenicol (CHLO-5μg/ml), N-acetyl glucosamine (NAG-2.2mg/ml), arginine (ARG-0.85mg/ml).

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4.8 Effect of Increasing Concentration of Zinc (Zn), Iron (Fe) and Calcium (Ca) on the antibacterial activity of standard antibiotics

Ions are essential for the signaling and metabolic processes of the cell, influencing pathogenicity of microorganisms. The effects of different concentrations of Zn, Fe and Ca on the antibacterial property of standard antibiotics were analyzed. Increasing concentrations of Zn was observed to slightly reduce the zone of inhibition of Moxifloxacillin against both sensitive and resistant strains of *S. aureus*. The same effect was also observed for Streptomycin as its zone of inhibition against MSSA reduced with increasing concentration of zinc. The inverse was observed in the case of Rifampicin and Chloramphenicol against MSSA. In the presence of high amounts of zinc, their zone of inhibition increased (Figure 4.26 A).

Overall, it was observed that increasing concentration of Fe decreased the activity of the standard antibiotics. With the exception of Rifampicin, which recorded an increase in its zone of inhibition against MRSA with increasing concentration of Fe; and Chloramphenicol, which was not significantly affected by Fe, all the other antibiotics recorded some degree of reduction in their zones of inhibition as the concentration of Fe increased for both MSSA and MRSA (Figure 4.26 C and D).

Calcium was seen to increase the zone of inhibition of Ampicillin against MSSA, however on the other antibiotics, an inhibitory effect was observed. Chloramphenicol and Tetracycline did not record any changes in their zone of inhibition (Figure 4.26 E).

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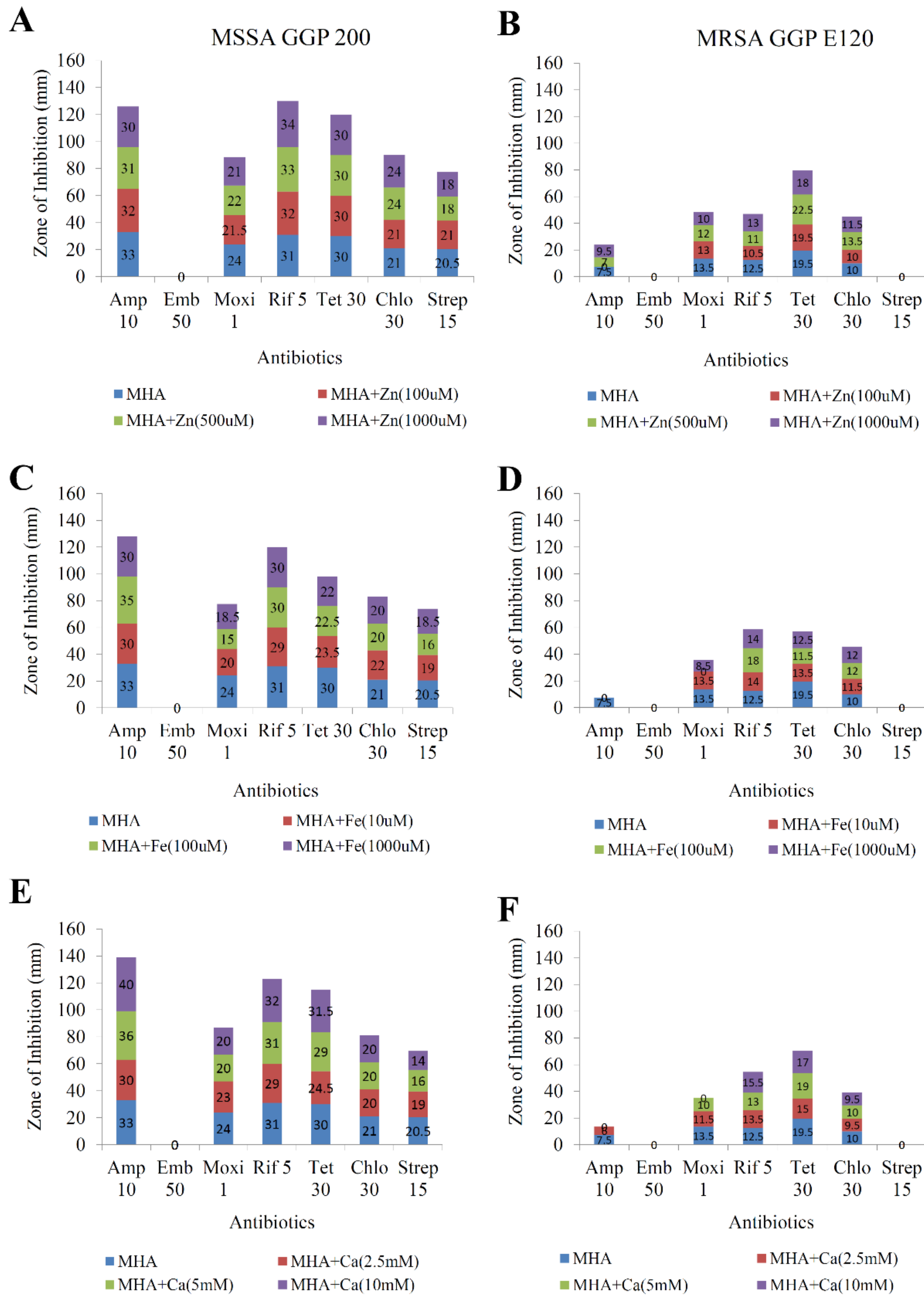

Figure 4-26: Antibacterial activity of 7 standard antibiotics in the presence of increasing concentrations of Zinc (Zn), Iron (Fe) and Calcium (Ca). The disc diffusion method was used to assay the antibacterial activity of Ampicillin 10μg/disc (Amp 10), Ethambutol 50μg/disc (Emb 50), Moxifloxacillin 1μg/disc (Moxi 1), Rifampicin 5μg/disc (Rif 5), Tetracycline 30μg/disc (Tet 30), Chloramphenicol 30μg/disc (Chlo 30) and Streptomycin 15μg/disc (Strep 15). The assay was performed on Mueller Hinton Agar (MHA) modified with Zinc (100,500,1000) μM, Fe(10,100,1000) μM and Ca(2.5,5,10)mM.

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4.9 Effect of DCCD and DNP on antibacterial activity of standard antibiotics

DCCD and DNP were added to the growth media of MSSA and MRSA and the antibacterial activity of standard antibiotics were tested (Figure 4.27). Rifampicin and Linezolid were the most affected. DNP increased the zones of inhibition against MSSA by 3 points in both antibiotics. DCCD had an inverse effect by reducing the zones generated by Rifampicin and Linezolid. Erythromycin was also observed to have a reduction in its zone of inhibition by both DCCD and DNP. In general however, most of the antibiotics were not affected by DCCD and DNP.

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4.10 Bioassay Guided Fractionation of B7 NEW BM 2bb

The first extract analyzed further was B7 NEW BM 2bb. The activity observed earlier was from the crude extract obtained from culturing the fungi. Fractionation of the crude extract was performed as part of the purification scheme to identify the particular compound (or compounds) generating the observed activity. Size exclusion chromatography using LH-20 was done with 1g of extract was fractionated using methanol as the elution solvent. Eleven fractions were collected. TLC was used to qualitatively determine the components of the fractions. Their bioactivity was tested against MSSA GGP 200 and MRSA GGP E120 (Figures 4.29 — 4.30). Fractions 6, 7, 8 and 9 showed bioactivity against both MSSA GGP 200 and MRSA GGP E120. Fraction 7 had the highest zone of activity of 30mm. This was similar to the activity observed for the crude (29.5mm and 28.5mm against MSSA and MRSA respectively). Interestingly, this activity was maintained for both sensitive and resistant strains of *S. aureus*. Fractions 8, 6 and 9 followed with decreasing activity respectively in both strains of *S. aureus* (Figure 4.29 A and B) some correlation was observed between the fractions that showed intense bands on the TLC plate and those that had high bioactivity. Fractions 6 and 7 had two thick bands close to the solvent front. Similar bands were observed for fraction 8 although these bands appeared faint. Fraction 9, which had the least activity among the fractions, only shared one faint of these bands (Figure 4.29 C).

The fractions with bioactivity (Fraction 6,7,8 and 9) were pooled together and further separated by reverse phase C18 column chromatography and eluted with water acetonitrile gradient (5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%). Fractions of each gradient phase were collected and tested for bioactivity using the disc diffusion assay. TLC analyzed the band profiles of the fractions and their bioactivity assayed by disc diffusion. From

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the TLC pattern, the 5% fraction was the only fraction that had prominent bands. The pattern was observed for this fraction was very similar to that observed for the pooled fractions. Faint bands were also observed for fractions 10%, 15%, 20% and 60% (Figure 4.30 C). The bioactivity of the fractions supported the observation on the TLC plates as MSSA GGP 200 was only sensitive to the 5% fraction, with a zone of inhibition of 30mm higher than the pooled fraction (21.5mm) (Figure 4.30 A). In addition to the activity observed in the 5% (ACN) fraction, the activity observed against MRSA GGP E120 exposed some basal activity in the 10%, 15%, and 50% (ACN) fractions (Figure 4.30 B). The 5% fraction was analyzed using HPLC. The respective fractions obtained were tested for their bioactivity. No bioactivity was recorded for these fractions due to low concentration of the active compound however, bands were observed for the input and the first fraction (Figure 4.31)

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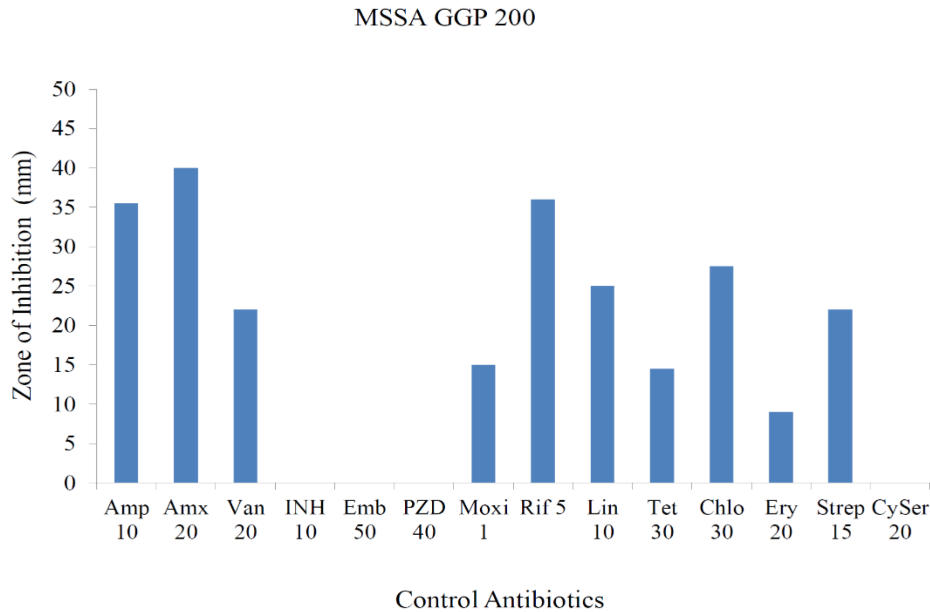

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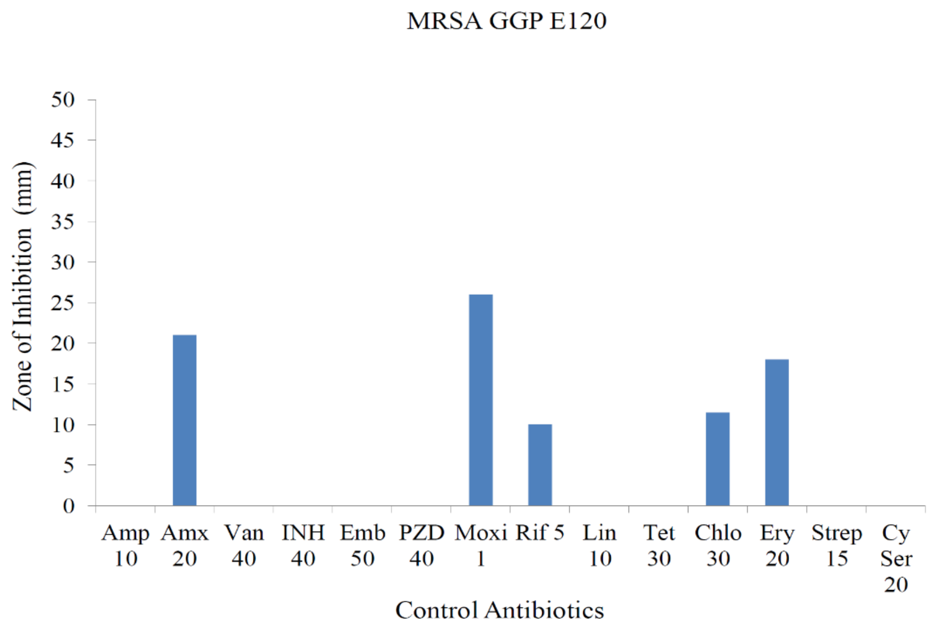

Figure 4-28: Antibacterial activity of 14 standard antibiotics against (A)- MSSA GGP 200 and (B)- MRSA GGP E120. The Disc diffusion method was used to assay the antibacterial activity of standard antibiotics on Mueller hinton agar (MHA), Amp 10- Ampicillin 10μg/disc, Amx 20- Amoxicillin 20μg/disc, Van 20-Vancomycin 20μg/disc, INH 10- Isoniazid 10μg/disc, Emb 50- Ethambutol 50μg/disc, PZD 40- Pyrazinamide 40μg/disc, Moxi 1- Moxifloxacillin 1μg/disc, Rif 5-Rifampicin 5μg/disc, Lin 10-Lineolid 10μg/disc, Tet 30- Tetracycline 30μg/disc, Chlo 30- Chloramphenicol 30μg/disc, Ery 20- Erythromycin 20μg/disc, Strep 15- Streptomycin 15μg/disc, Cyser 20- Cycloserine 20μg/disc.

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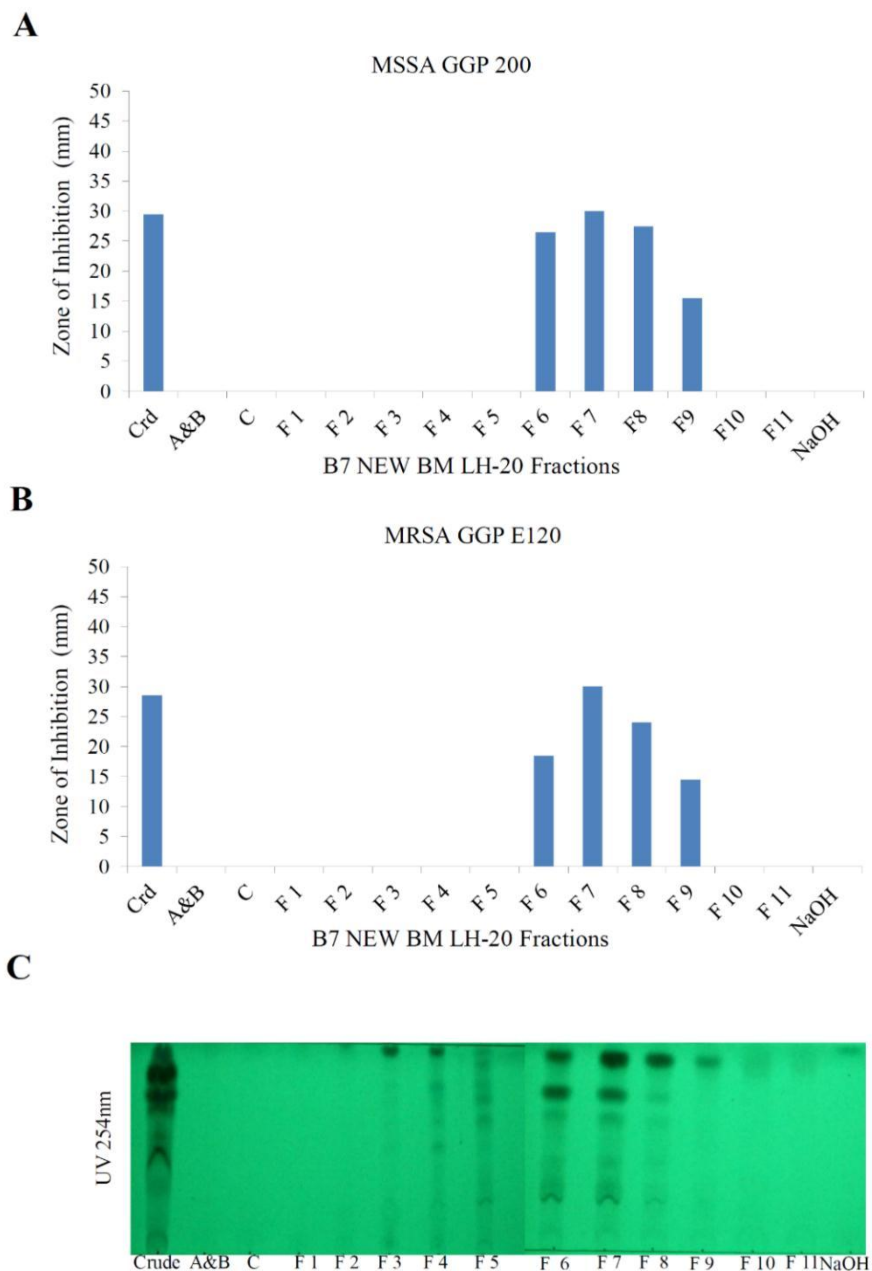

Figure 4-29: Antibacterial activity crude B7 NEW BM extract and corresponding LH-20 fractions of fractions against MSSA GGP 200 (A) and MRSA GGP E120 (B). (C) TLC profile of crude B7 NEW BM and corresponding LH-20 fractions. Crude extract of B7 NEW BM was taken through size exclusion chromatography using sephadex LH-20 as stationary phase and methanol as the solvent phase. A, B and C are fractions of methanol run through the column before elution of extract. F1-F11 – 11 fractions of 10ml each collected during fractionation. NaOH- sodium hydroxide used to wash column after fractionation of extract. Disc diffusion method was used to assay the antibacterial activity of 20ul of each fraction and zones of inhibition obtained after 12hours of incubation at 37°C was recorded.5μl of crude extract and fractions were spot on silica gel TLC plates and developed with (Ethyl acetate: petroleum ether: acetonitrile 7:2:1). TLC plate was dried and viewed under UV 254nm.

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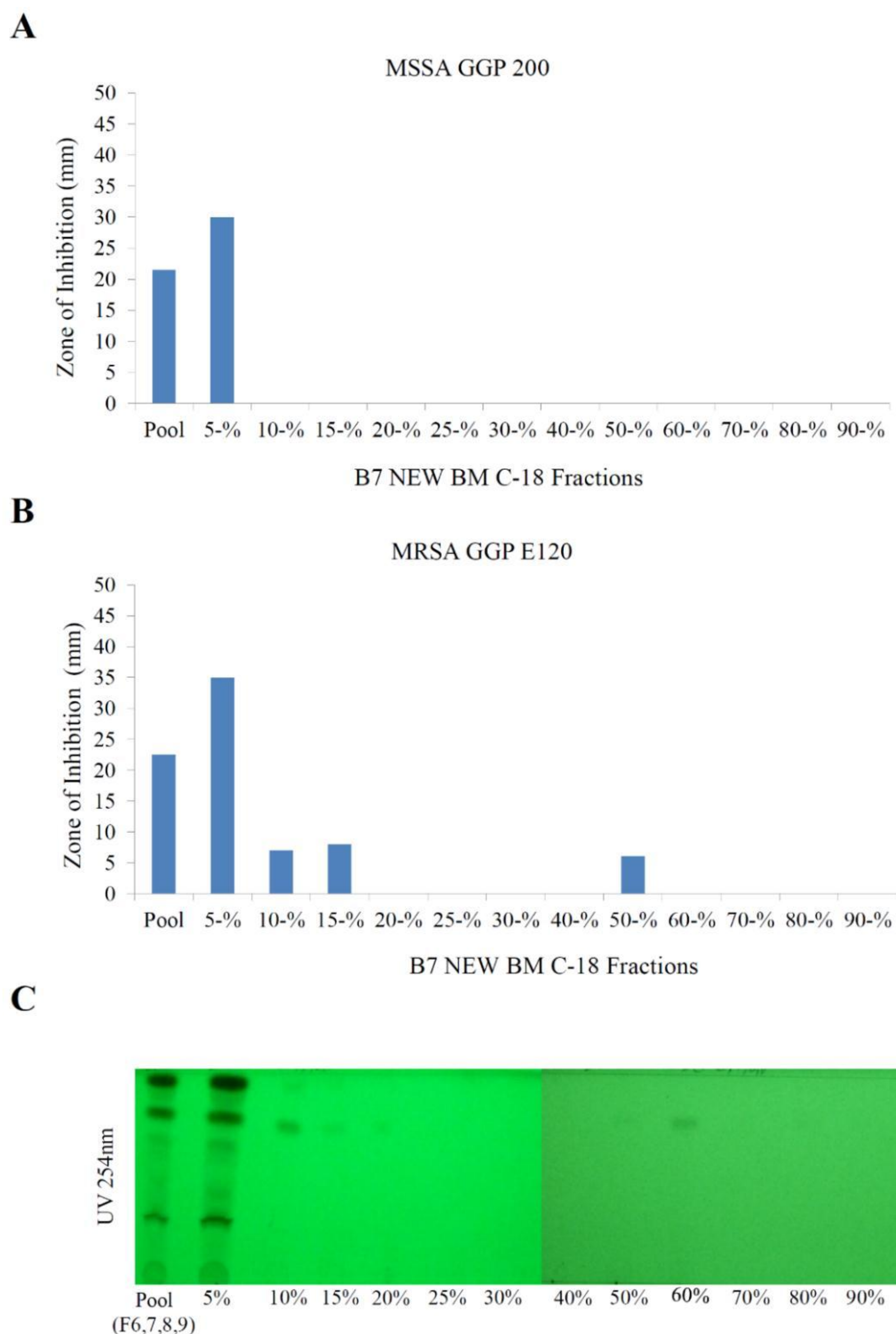

Figure 4-30: Antibacterial activity of LH-20 pooled fractions (6, 7, 8, and 9) of B7 NEW BM extract and corresponding C-18 fractions against MSSA GGP 200 (A) and MRSA GGP E120 (B). (C) TLC profile of LH-20 pooled fractions (6, 7, 8, and 9) and corresponding C-18 fractions. Pooled fractions of B7 NEW BM was concentrated and taken through reverse phase chromatography using C-18 as stationary phase and water- acetonitrile gradient as the solvent phase. 5%-90% are increasing percentages of acetonitrile used in the elution process. The disc diffusion method was used to assay the antibacterial activity of 20ul of each fraction and zones of inhibition obtained after 12hours of incubation at 37°C was recorded.5μl of fractions were spot on silica gel TLC plates and developed with (Ethyl acetate: petroleum ether: acetonitrile 7:2:1). TLC plate was dried and viewed under UV 254nm.

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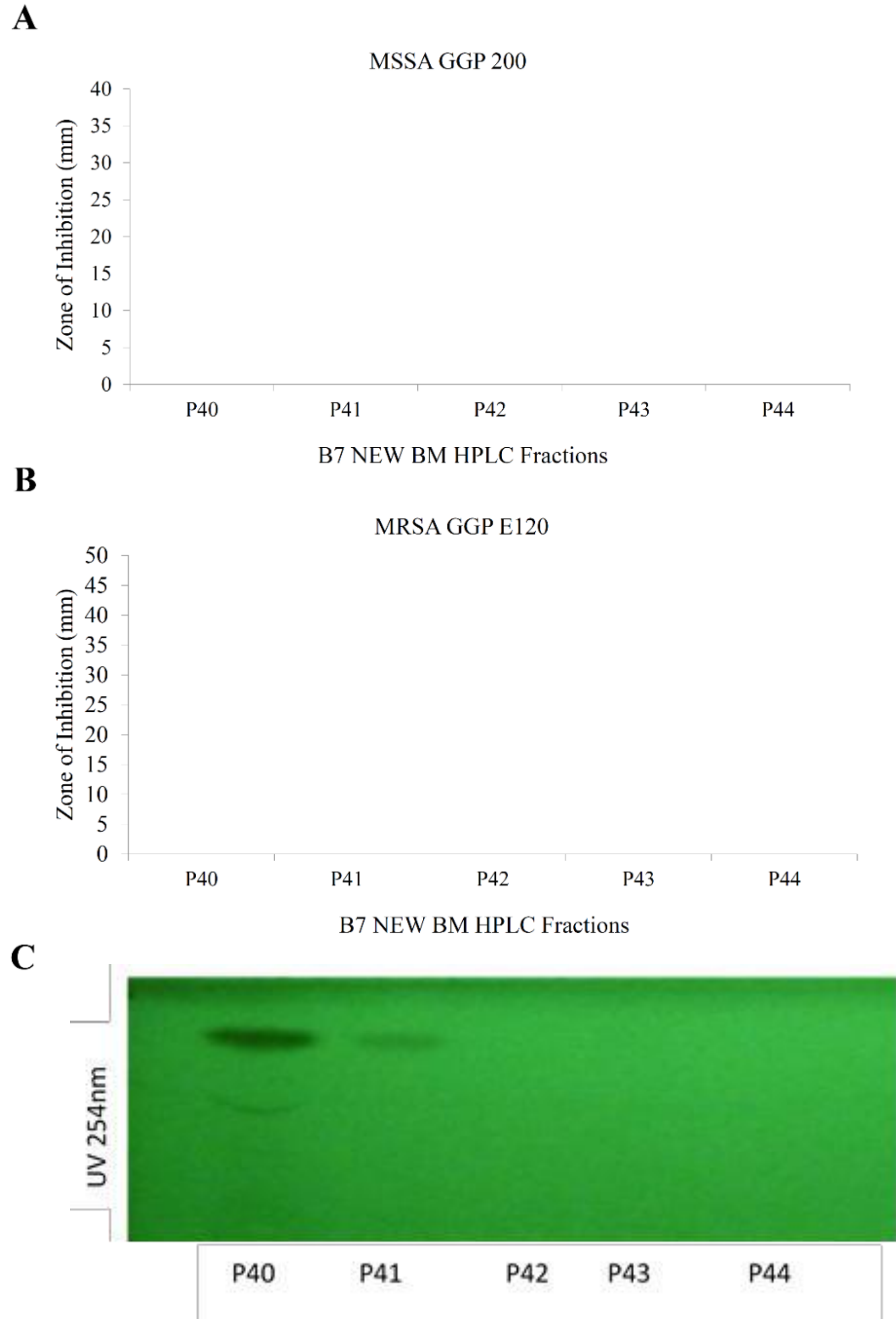

Figure 4-31: Antibacterial activity of HPLC fractions of B7 NEW BM active fractions from C-18 fractionation against MSSA GGP 200 (A) and MRSA GGP E120 (B). (C) TLC profiles of HPLC fractions of B7 NEW BM. Fractions from the C-18 fractionation with antibacterial activity were taken through HPLC. The resultant fractions were assayed for antibacterial activity using the disc diffusion method.5μl of fractions were spot on silica gel TLC plates and developed with (Ethy acetate:petroleum ether:acetonitrile 7:2:1). TLC plate was dried and viewed under UV 254nm. TLC- Thin layer chromatography.

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4.11 Bioassay guided fractionation of D7 PM 2pb

D7 PM 2pb was also fractionated employing size exclusion chromatography (LH-20), reverse phase(C-18) and HPLC (Figures 4.33—4.35). The fractions obtained from each separation step were tested for antibacterial activity by the disc diffusion method and active fractions separated further.

LH-20 fractionation generated 11 fractions. Bands were observed close to the solvent front for fractions 3 to 8. However, the band patterns observed in fractions 5 to 8 were similar, although it seemed to decrease at fraction 8. Fractions 3 and 4 also seemed to share similar band patterns with fraction 4 having a thicker band than fraction 3 (Figure 4.33C). This was mirrored in the activity assay. Against MSSA, apart from the activity of the crude extract (14mm), fraction 6 had the highest zone of inhibition of 15mm closely followed by fractions 7, 2 and 5. (Figure 4.33 A). Against MRSA however, only the crude extract generated a zone of activity (6mm) (Figure 4.33 B). For further separation, fractions 3 and 4 were pooled together and fractions 5 to 8 were also pooled together and taken through reverse phase C-18 chromatography. Eleven fractions were generated for each pooled combination.

TLC band profiles for the C-18 fractions of Pool (3, 4) revealed an intense band, close to the solvent front in the 5% ACN fraction similar to the band observed in the pooled fractions (Figure 4.34 C). 40% and 50% ACN fractions also had a similar band even though with a lower intensity. Similar faint bands were also observed in the 15% to 30% ACN fractions. When these fractions were tested for antibacterial activity against MSSA GGP 200, the 5% and 50% ACN fractions were the ones with activity with the 50% fraction having a zone of inhibition same as the pooled fractions. The fractions however did not have any activity against MRSA GGP E120 (Figure 4.34 A and B).

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Fractions 5,6,7,8 pooled from the LH-20 fractionation was also further separated using reverse phase C-18 chromatography. This time, fraction 10% to 25% were observed to share band patterns similar to the pooled fractions. Fractions 15% and 20% however had the highest intensity and these were the only fractions, which had antibacterial activity against MSSA (Figure 4.35). The fractions had no activity against MRSA. The active fractions from the two pools were further separated using HPLC. In both cases however, only the input showed zones of inhibition against MSSA GGP 200, with the sub-fractions being below detection limit (Figure 4.36 and 4.37).

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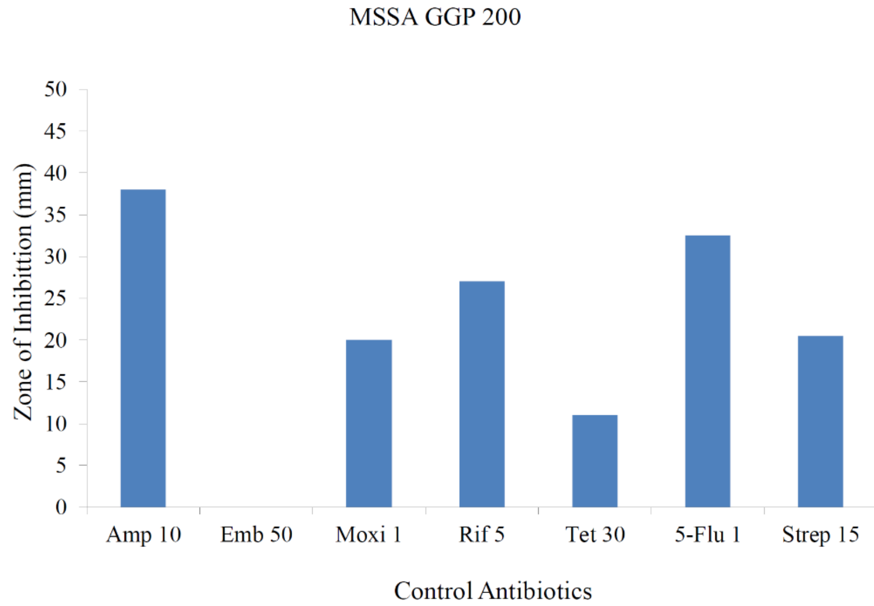

B

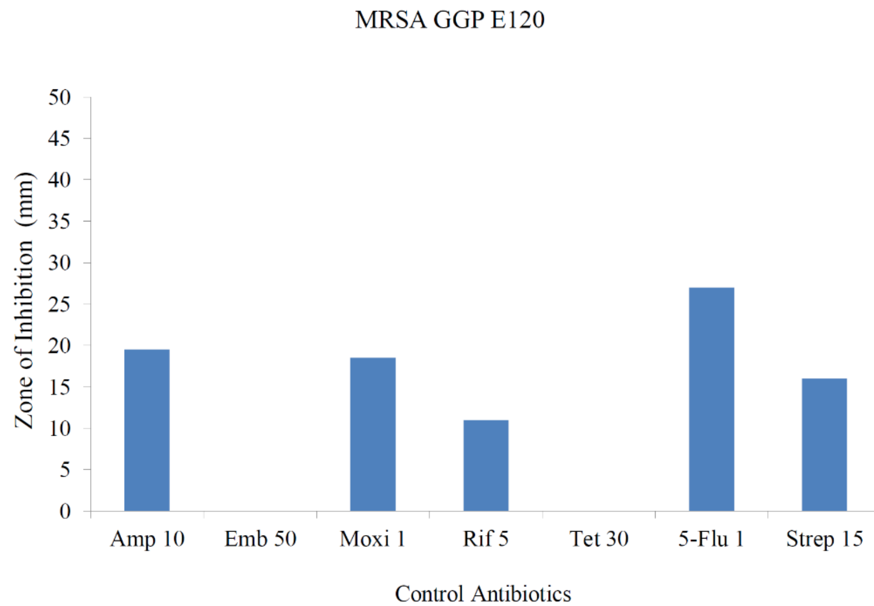

Figure 4-32: Antibacterial activity of 7 standard antibiotics against (A)- MSSA GGP 200 and (B)- MRSA GGP E120. The Disc diffusion method was used to assay the antibacterial activity of standard antibiotics on Mueller hinton agar (MHA), Amp 10- Ampicillin 10μg/disc, Emb 50- Ethambutol 50μg/disc, Moxi 1- Moxifloxacillin 1μg/disc, Rif 5-Rifampicin 5μg/disc,Tet 30- Tetracycline 30μg/disc, 5-Flu 1,5-fluorouracil 1μg/disc, Strep 15- Streptomycin 15μg/disc.

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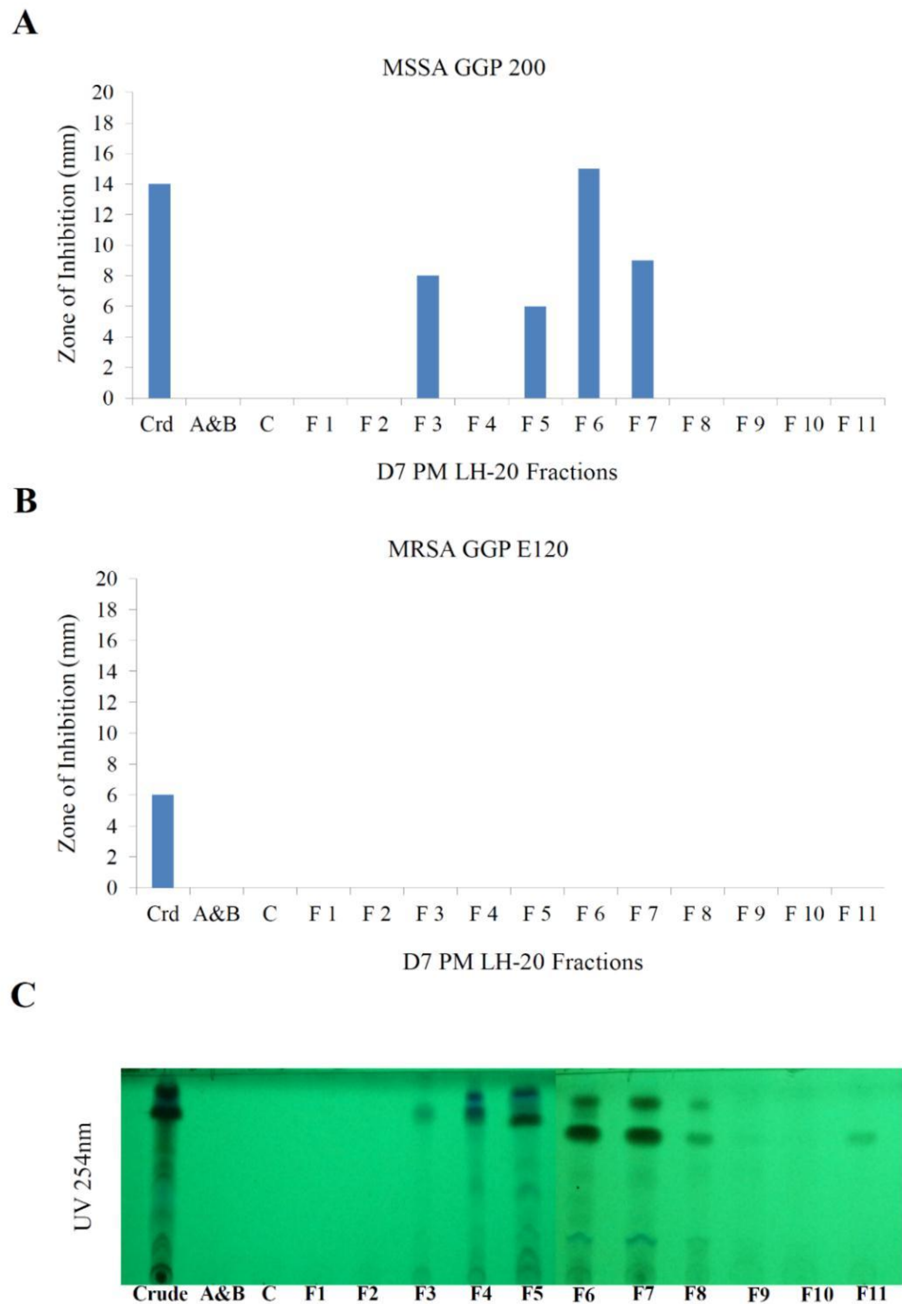

Figure 4-33: Antibacterial activity crude D7 PM 2pb extract and corresponding LH-20 fractions against MSSA GGP 200 (A) and MRSA GGP E120 (B). TLC profile of crude D7 PM 2pb and corresponding LH-20 fractions. Crude extract of D7 PM 2pb was taken through size exclusion chromatography using sephadex LH-20 as stationary phase and methanol as the solvent phase. A,B and C are fractions of methanol run through the column before elution of extract. F1-F11 – 11 fractions of 10ml each collected during fractionation. NaOH- sodium hydroxide used to wash column after fractionation of extract. Disc diffusion method was used to assay the antibacterial activity of 20ul of each fraction and zones of inhibition obtained after 12hours of incubation at 37°C was recorded.5μl of crude extract and fractions were spot on silica gel TLC plates and developed with (Ethy acetate:petroleum ether:acetonitrile 7:2:1). TLC plate was dried and viewed under UV 254nm.

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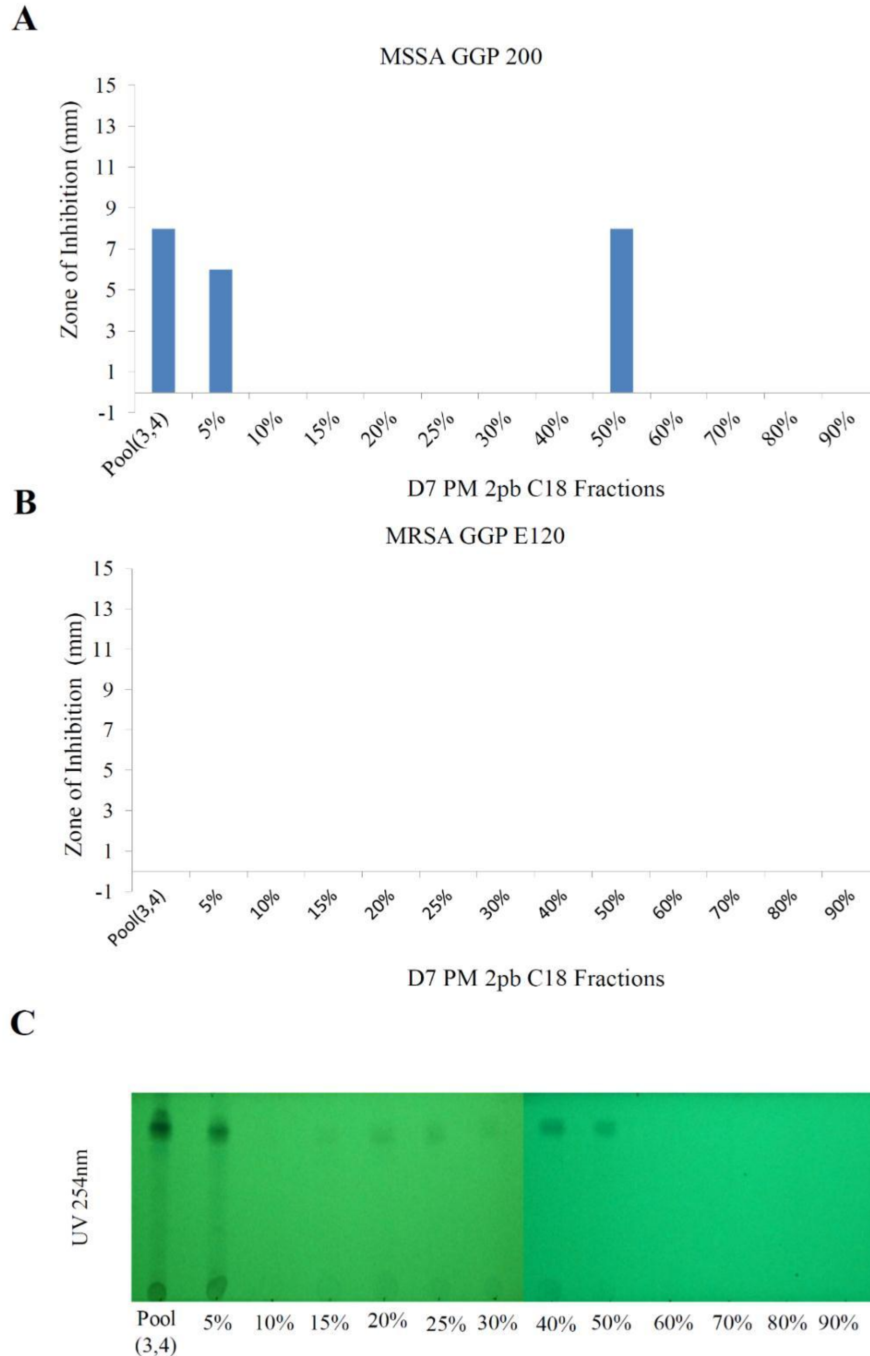

Figure 4-34: Antibacterial activity of LH-20 pooled fractions (3, 4) of D7 PM 2pb extract and corresponding C-18 fractions against MSSA GGP 200 (A) and MRSA GGP E120 (B). TLC profile of LH-20 pooled fractions (3, 4) and corresponding C-18 fractions. Pooled fractions of D7 PM 2pb were concentrated and taken through reverse phase chromatography using C-18 as stationary phase and water- acetonitrile gradient as the solvent phase. 5%-90% are inreasing percentages of acetonitrile used in the elution process. The disc diffusion method was used to assay the antibacterial activity of 20ul of each fraction and zones of inhibition obtained after 12hours of incubation at 37°C was recorded.5μl of fractions were spot on silica gel TLC plates and developed with (Ethy acetate:petroleumether:acetonitrile 7:2:1). TLC plate was dried and viewed under UV 254nm.

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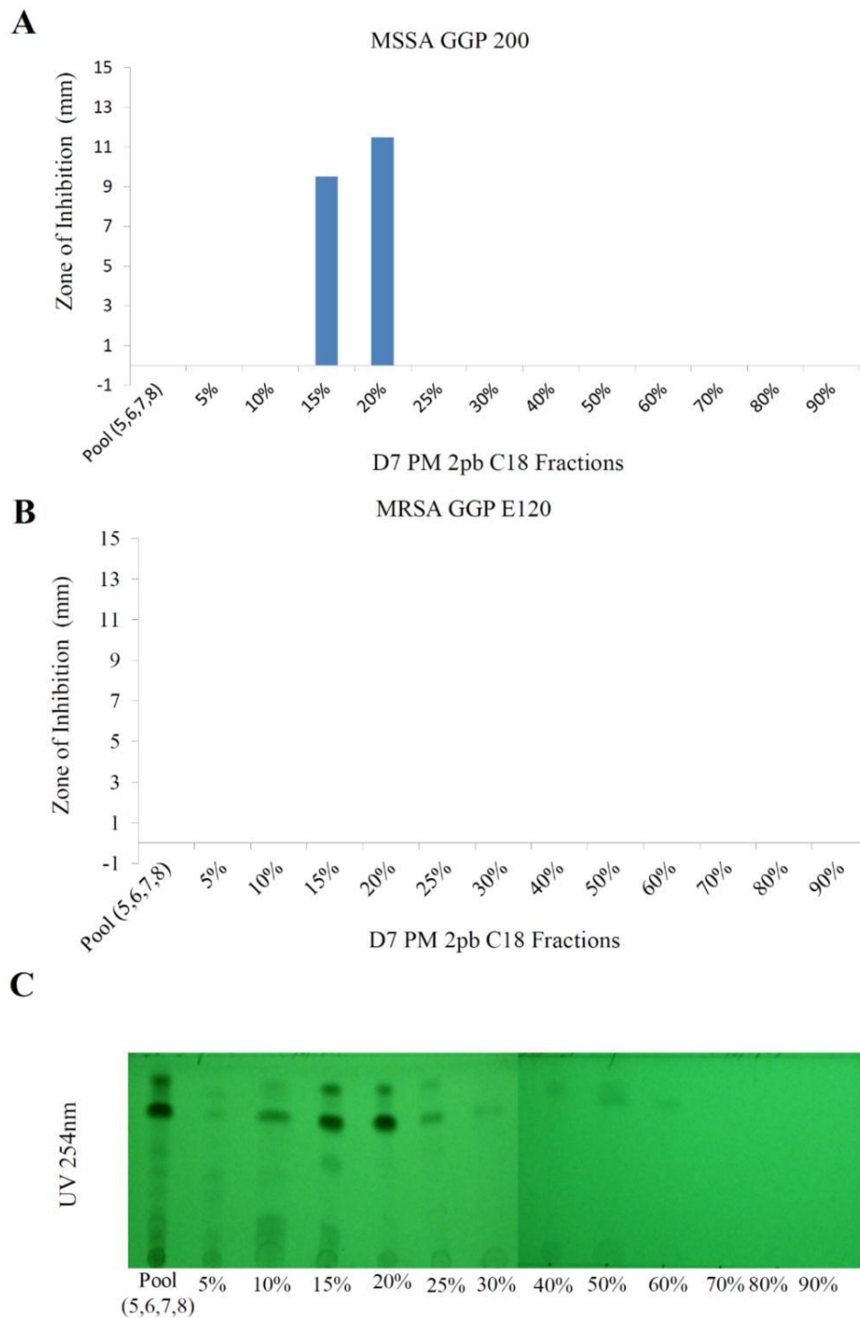

Figure 4-35: Antibacterial activity of LH-20 pooled fractions (5, 6, 7, and 8) of D7 PM 2pb extract and corresponding C-18 fractions against MSSA GGP 200 (A) and MRSA GGP E120 (B). TLC profile of LH-20 pooled fractions (5, 6, 7, and 8) and corresponding C-18 fractions. Pooled fractions of D7 PM 2pb were concentrated and taken through reverse phase chromatography using C-18 as stationary phase and water- acetonitrile gradient as the solvent phase. 5%-90% are increasing percentages of acetonitrile used in the elution process. The disc diffusion method was used to assay the antibacterial activity of 20ul of each fraction and zones of inhibition obtained after 12hours of incubation at 37°C was recorded.5μl of fractions were spot on silica gel TLC plates and developed with (Ethyl acetate: petroleum ether: acetonitrile 7:2:1). TLC plate was dried and viewed under UV 254nm.

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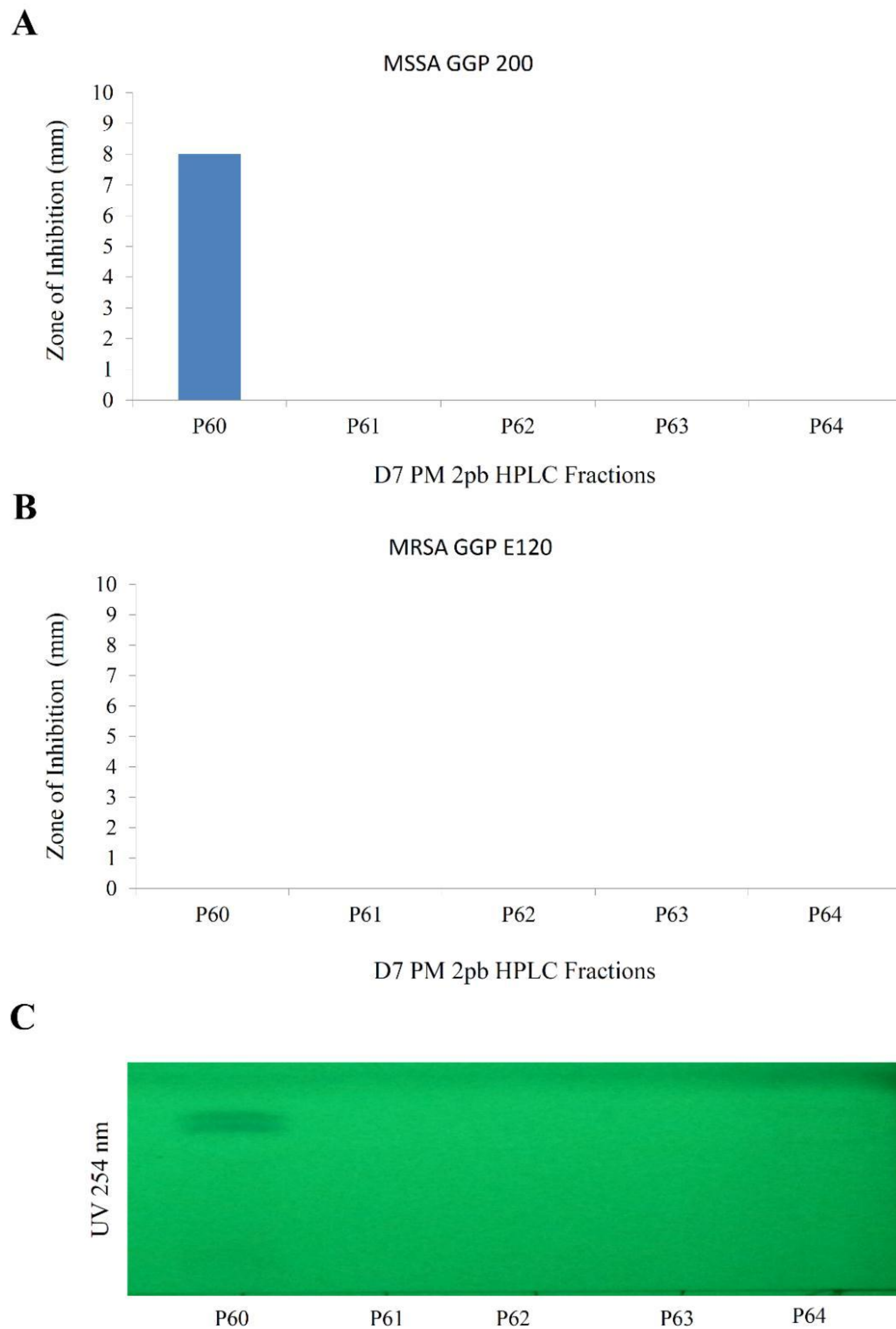

Figure 4-36: Antibacterial activity of HPLC fractions of D7 PM 2pb active fractions from C-18 fractionation of Pool (3 and 4) against MSSA GGP 200 (A) and MRSA GGP E120 (B). TLC profile of HPLC fractions of D7 PM 2pb. Fractions from the C-18 fractionation of pool (3 and 4) with antibacterial activity were taken through High performance liquid chromatography (HPLC). The resultant fractions were assayed for antibacterial activity using the disc diffusion method.5μl of fractions were spot on silica gel TLC plates and developed with (Ethyl acetate: petroleum ether: acetonitrile 7:2:1). TLC plate was dried and viewed under UV 254nm

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4.12 Large Scale fractionation of B7 NEW BM and D7 PM 2pb

Using all the improved media formulation and fermentation techniques developed in this project, the two top WDF candidates which were fractionated were set up on a large scale. The two WDF produce five antibacterial compounds as determined by the fractionation experiment, therefore much more of their extracts needs to be generated in order to obtain sufficient quantities of extract to isolate those five compounds. Six 10L cultures of each fungus were set up initially. Based on the results from earlier experiments on the optimum media for culturing fungi, Yeast Peptone Malt extract and Dextrose (YPMD) Broth was used.

The fungi were first grown on Yeast Peptone Dextrose Agar (YPDA). Mycelia discs from ten plates were used to inoculate each 10L culture. The fermentation vessels were capped with a perforated insert overlaid with filter paper and fungi were cultured at room temperature. Pieces of mycelia were observed in the first week of culture for Tank 1 of B7 NEW BM. By the second week of culturing a mycelia carpet had formed which increased in size progressively through the subsequent weeks. By week 4, a full mycelia carpet was observed that covered the entire surface of the broth. The growth seemed to increase further even after the whole surface was covered. By week 8, a second layer of mycelia carpet was observed. By this time the carpet had curved down the side of the fermentation vessel (Figure 4.38 A). The growth only seemed to slow down and stabilize after 12 weeks of culturing. Tanks 2 and 5 followed the same pattern of growth until the end of the second week when they had developed a full carpet. By the third week, the broth was foamy, there were light brown sediments at the bottom of the vessel and the carpet of mycelia appeared as fragments of mycelia. Tank 3, 4 and 6 experienced these same characteristics in the first week of culturing and only had fragments of mycelia. (Figure 4.38B).

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Three more 10L cultures of B7 NEW BM was set up and they mirrored the growth pattern observed in the first tank (Figure 4.38 A). The growth pattern of D7 PM 2pb was different from that observed in B7 NEW BM. For the first three weeks of culturing, only pieces of mycelia were observed with no carpet forming. By the second week, the broth was observed to have a thin layer of foam. After four weeks of culturing, two tanks (3 and 5) developed a carpet of mycelia, which is yet to cover the entire surface of the broth as at the 8th week of culturing. The other four tanks still have pieces of mycelia (Fig 4.38 C). Work on these large cultures is beyond the scope of this thesis and will be continued in the laboratory.

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A

B

C

Figure 4-38: Large scale cultures of B7 NEW BM pure cultures (A), B7 NEW BM contaminated cultures (B) and D7 PM 2pb pure culture (C). Mycelia disc cultured on 10 Yeast peptone dextrose agar (YPDA) plates were used to inoculate 10L of yeast, peptone, malt extract and dextrose (YPMD) broth and incubated at room temperature. B7 NEW BM cultures are at least 10 weeks of incubation. D7 PM 2pb are 8 weeks of incubation

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CHAPTER FIVE

5.0 DISCUSSION

This project was carried out in response to the urgent need for the discovery of new antibiotics. The "golden age" of antibiotic research made scientists believe the menace of infectious pathogens was over. This golden age, however, seems to have come to a halt as bacteria have evolved over the years to become resistant to almost all antibiotics (Sing h *et al.,* 2006). According to the 2014 WHO report on antimicrobial surveillance, we may soon be re-living the pre-antibiotic era where common wounds led to the death of many (World Health Organization, 2014).

Natural products, even though sidelined for a while, are known globally to be an important source of anti-infective compounds against infectious pathogens (Mishra *et al.,* 2011). They have a diversity of compounds, which are not easily produced by chemical synthesis due to their complexity and the presence of multiple chiral centers (Lahlou, 2013). Since the discovery of penicillin, microorganisms, especially fungi, have been explored for the production of bioactive compounds (Cragg *et al.,* 2013). The discovery of new antibiotics from fungi is not possible without the development of fermentation techniques (Subramaniyam *et al.,* 2012).

The findings of this study come at a very essential period in the overall work of the laboratory. The laboratory has a library of 3000 fungi, majority of which are yet to be screened. This study has revealed key processes in the development of fermentation techniques for optimum production of antibacterial metabolites, and the optimum media for production of fungal bioactive metabolites. Specifically, mycelia discs cultured on Yeast Peptone Malt extract agar (YPDA) was established to be a better source of inoculum for fermentation of fungi compared to submerged mycelia from a mature fungal broth culture. Liquid-state fermentation was also shown to be better

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than solid-state fermentation in the culturing of our fungal library for antibacterial metabolites. Finally YPMD proved to be the optimum media for culturing WDF for high and consistent antibacterial activity.

A Phenotypic array assay has been developed to prioritize WDF with promising antibacterial activity for product isolation. This assay led to the selection of two WDF, B7 NEW BM and D7 PM 2pb. Fractionation of these two fungi revealed five antibacterial compounds. All the findings in this study were employed in setting up 6 large scale (10L) cultures for the top two selected WDF. This was done to obtain more crude extract in order to characterize the five compounds.

5.1 Screening of 180 WDF confirm WDF produce antibacterial secondary metabolites

Fungi is a rich source of antibacterial compounds. (Gloer, 2007). Efforts by our laboratory have sought to explore some of these antibacterial compounds. Previously, a 68% hit -rate was obtained against MSSA from an initial screen of 189 Wood Decay Fungi (WDF). This supports the claim that fungi are capable of producing antibacterial compounds. To obtain larger quantities of the crude extract for more advanced studies, the top twenty WDF were grown on a large scale.

In this project, another library of 180 Wood Decay Fungi (WDF) was screened against MSSA and two strains of MRSA; MRSA GGP E120 and MRSA Noguchi. A hit rate of 48% was obtained. From this pool, 59.2% recorded antibacterial activity against all the strains of *S. aureus* tested whilst 1.2% of the hits demonstrated selective antibacterial activity for only the susceptible strain of *S. aureus*. This is similar to results obtained in Thialand where 377 fungal extracts were screened and an 18.6% anti antimicrobial activity was observed with a 6-10% hit rate against

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S.aureus (Phongpaichit *et al.,* 2006). It also confirms that fungi have the potential to produce antimicrobial compounds. This study revealed a set of fungal extracts, (23.45% of the total hits) that had selective antibacterial activity against the resistant strains of *S. aureus*, but not the sensitive strain (Fig 4.2). Both resistant strains of *S. aureus* show decreased sensitivity to ampicillin and amoxicillin, which are beta lactam antibiotics and target the cross linking of the peptidoglycan cell wall. Thus, this pool of extracts which target the resistant strains may contain new compounds with a different mechanism of action, unlike ampicillin and amoxicillin .This observation is particularly significant because most of the strains of *S. aureus* posing a threat are resistant strains (World Health Organization, 2014). Having confirmed that fungi produce antibacterial compounds, the next step was to optimize the fermentation technique.

5.2 Mycelia disc inoculum, filter paper covered fermentation vessels and Yeast, Peptone, Malt extract, Dextrose (YPMD) broth prove to be optimum conditions for fermentation

There are a number of challenges that confronts fermentation of fungi in the laboratory, first, not all fungi can be grown under laboratory conditions. Those that grow under laboratory conditions are influenced by factors such as temperature, aeration, nutrient diversity, and pH (Jain *et al.,* 2011). Studies done on culture media, pH and temperature of fermentation show that these factors have an impact on the production of bioactive compounds. These were however found to be tailored for individual fungi (VanderMolen *et al.,* 2013; Fiedurek *et al.,* 1996; Bode *et al.,* 2002; Miao *et al.,* 2006; Bills *et al.,* 2008; Xu *et al.,* 2008; Mohanty & Prakash, 2009; Kossuga *et al.,* 2012; Shang *et al.,* 2012).

The most important elements of fermentation development are strain selection and optimization; media and process development; and scaling-up the fermentation process to maximize productivity.(www.sigmaaldrich.com, 2012) The scarcity of the fungal fruiting body

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was the first challenge our laboratory encountered. Adadey in 2014, developed a protocol employing plate mycelia (PM) and broth mycelia (BM) to address the issue of having a continuous source of inoculum for culturing fungi (Adadey 2014). However, challenges due to long-term storage of isolates on slants at 4°C were encountered. To optimize the inoculum, submerged mycelia from fungal cultures were used as inoculums for subsequent fungal cultures and assayed for the production of bioactive metabolites. The results obtained did not favor production of bioactive metabolites. The antibacterial activity observed in Step 1 cultures decreased as the culture steps increased. This indicates that fresh mycelia discs cultured on plates are a better source of fungal inoculum compared to mycelia from a mature broth culture.

A possible explanation for this observation may be the fungal culture set-up. Fungi were cultured until the stationary secondary metabolism stage. This implies that, the earlier primary metabolism stage where nutrients like glucose and proteins are metabolized, was not actively in process when fungal cultures matured. When mycelia from mature cultures are put into fresh broth, the cycle begins all over again. Usually, it takes a while to revive cultures at stationary phase to actively divide and metabolize nutrients. Since all steps were cultured for the same period, subsequent steps may not have fully reached secondary metabolism to produce metabolites before they were halted. This can be compared to what Gerbi *et al.,* observed for the anti-cellulose activity of fungi. Similar to the observation made in this work, as the developmental cycle came to an end, there was a decrease in total activity (Gerbi *et al.,*1996).

Establishing that mycelia discs grown on agar plates were a better form of inoculum than submerged mycelia, another important component of the fermentation process; aeration, was investigated.

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In the fermentation process, aeration is a critical factor (Wise, 1951). Previous work in the laboratory has studied the effect of varying the aeration to culture ratio on metabolite production (Adadey, 2014). In his study, higher aeration to culture ratio gave higher antibacterial activity, but a lower yield, whereas lower aeration to culture ratio gave a higher yield, but lower antibacterial activity. Since both yield and activity are crucial in this study, it was necessary to optimize the aeration conditions. To have optimum aeration in fungal cultures, fermentation vessel closing mechanisms were varied employing tight capping, loose capping, foam capping and filter paper capping. Antibacterial activity of extracts generated from each vessel type was used as a read out to determine the best closing mechanism.

The fermentation vessel closure mechanism study showed that filter paper capped vessels produce consistent bioactive metabolites. Even though extracts from other capped vessels showed higher antibacterial activity, they were not replicated in their duplicates broths. Filter paper (pore size 2.5 micron) favors gaseous exchange between fermentation vessel and external environment, and acts as an effective barrier to foreign agents that contaminate the fungal culture. Foam capped vessels similarly afford this gaseous exchange and barrier; however, the structure of foam is more dense and compact than filter paper, and thus gaseous exchange is reduced compared to filter paper. Although loosely capped vessels arguably favor gaseous exchange best, they are not an effective barrier since foreign agents can easily contaminate the broth. This was observed in the antibacterial activity pattern of the control vessels, which had no inoculum. For these vessels without inoculum, it was observed that extracts from the loosely capped vessels exhibited some antibacterial activity. This unexpected result suggests that the observed antibacterial activity was possibly due to the introduction of a foreign agent in the course of incubation.

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Media is another essential component of the fermentation process (VanderMolen *et al.,* 2013; Fiedurek et a1., 1996). The media used determines largely which secondary metabolites the fungi produce (Bode *et al.,* 2002). Two available and widespread methods of fermentation are solid and liquid state fermentation. Varying reports have been given as to which of the two methods are better. Fang and Zhong in 2001 reported the extraction of ganoderic acid from Ganoderma lucidum using submerged fermentation whilst studies done by Maghsoodi and Yaghmaei favour solid state fermentation over submerged fermentation in the production of chitosan from Aspergillus niger (Maghsoodi and Yaghmaei, 2010). Submerged cultures (liquid-state fermentation) is the method employed in our laboratory; however due to challenges encountered in achieving consistency in duplicate cultures and in progressing to large-scale fermentation, the need arose to compare solid-state and liquid-state fermentation, and to analyze the production of bioactive metabolites.

In general, submerged broth cultures consistently produced bioactive metabolites in this study. In the test organisms, the zone of inhibition generated by extracts of submerged broth cultures was higher than solid-state cultures. Work done by Subramaniyam and colleagues suggest that the advantage of solid- or liquid-state cultures over each other is dependent on the compounds produced and the strain of fungi employed (Subramaniyam *et al.,* 2012). Thus, for the set of fungi employed in this study, submerged broth cultures encouraged the production of antibacterial compounds.

Mineral supplementation of PDB has previously been investigated for its effect on the production of bioactive metabolites from fungi. Soil extract increased bioactivity slightly although batch to batch inconsistencies were still present (Adadey, 2014). With the knowledge that manipulating the nutrition of fungal broth impacts metabolite production (Fiedurek *et al.,* 1996), the best media for production of fungal bioactive metabolites was investigated.

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Six different media (PDB; PDB+2X Soil Extract; PDB+2X SE; Yeast, Peptone, Malt extract, Dextrose; YPMD, YPMD+PB, YPMD+2XSE, YPMD+PB+2X SE) were set up and tested. The objective of this study was to identify the best media for a primary drug screen programme. This is because, consistent activity across different fungi is a more important factor to assess than excellent activity in only one fungi.

YPMD aided the production of antibacterial compounds in both B7 NEW BM and D7 PM 2pb. PDB matched the activity of YPMD in B7 NEW BM. However, in the second fungi, D7 PM 2pb, YPMD extracts had antibacterial activity almost two fold higher than PDB against MSSA GGP 200. Against the resistant strain of *S. aureus*, PDB extracts lost their activity except for one replicate which had a low zone of inhibition. Soil extract-supplemented PDB however showed strong activity against MSSA and MRSA in one of the four replicates. YPMD was thus observed to aid production of bioactive metabolites from the two fungi. This is similar to what Fang and Zhong found in their studies. A combination of yeast and peptone in fungal growth media generated better yield and growth relative to yeast or peptone as the sole nitrogen source (Fang and Zhong, 2001). YPMD consists of yeast extract, peptone, malt extract and dextrose. Peptone is a source of carbon, nitrogen, and amino acids, which supports growth of moulds and yeasts in general (www.thermoscientific.com, 2011). Malt extract also supplies nutrients necessary for the growth of fungi (Fullmer and Grimes, 1923). Dextrose is a source of carbon energy and yeast extract is known to supply B-complex vitamins to fuel the growth of yeasts and moulds (www.thermoscientific.com, 2011).This implies that YPMD has a varied nutrient supply, and hence its ability to support the growth and bioactive metabolite production of different fungi. This is relative to PDB, which in addition to dextrose only has potato as a carbohydrate source. Soil extract, known to contain potassium phosphates among other essential elements, inhibited the activity of B7 NEW BM and D7 PM 2pb observed in YPMD. It however boosted the activity of

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D7 PM 2pb when added to PDB. The additional nutrients and elements introduced by soil extract aids some species of fungi when the media is not an optimal one. However, for an optimum media with rich and varied source of nutrients like YPMD, soil extract tends to inhibit the production of bioactive compounds. Gao *et al.,* showed that the carbon-nitrogen ratio of growth media is essential for optimum growth and production of fungal secondary metabolites (Gao *et al.,* 2000). In this ratio, the carbon component is usually higher than the nitrogen component. PDB has only a carbon component, supplementation with soil extract adds a nitrogen component which enhances growth and production of bioactive metabolites. YPMD however already has both carbon and nitrogen components and addition of soil extract destabilizes this ratio and might be the reason for decreased production of bioactive compounds. This phenomenon is however strain-specific, and might not apply for all strains of fungi.

Even though studying the effect of different media on antibacterial metabolite production singled out YPMD as the best media type, it is known that fungi have gene clusters of secondary metabolism which are silent under laboratory conditions (Zutz *et al.,* 2013). The media type alone is not sufficient to activate these silent gene clusters. Epigenetic modifications have been studied to activate these silent genes (Reyes-Dominguez *et al.,* 2012). Small chemicals which inhibit histone deacetylases have also been studied to activate these genes (Cole, 2008)

Media (YPMD+SE+PB) was thus modified with six small chemicals (valproic acid, SAHA, sodium butyrate, sodium-4 phenyl butyrate, Trichostatin A and 2-hexyl-4 pentynoic acid) which are inhibitors of histone deacetylases. This was an attempt to explore silent gene clusters of secondary metabolism in fungi. Trichostatin A, valproic acid and 2-hexyl-4-pentynoic acid modification of fungal broth caused an increase in the antibacterial activity. Trichostatin A

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increased the activity of B7 NEW BM against MRSA. Valproic acid and 2-hexyl-4-pentynoic acid increased the antibacterial effect of B7 OLD PM against MSSA.

The increase in antibacterial activity caused by all three chemicals were at least 5mm more than the zone observed in the untreated control. This is significant and could mean a number of things. First, data obtained from Minimum inhibitory concentration studies of standard antibiotics in our lab against MSSA (Table 5.1 Appendix) suggests that, a two fold increase in concentration does not necessarily mean a two fold increase in the zone of inhibition. When a concentration that gives a zone ranging from 10 to 15mm is doubled, it usually gives a corresponding doubling of the zone of inhibition (Tetracycline and chloramphenicol data, Appendix). However, after a point, doubling the concentration does not reflect in the zone of inhibition. For both valproic acid and 2-hexyl-4 pentynoic acid, the zone of inhibition generated by the control untreated extract increased from an average of 13.5mm to 20mm when broth was treated. This increase is similar to what was observed with the standard antibiotics (Table 5.1 Appendix)) when the concentration that gave a zone of 12.5 was doubled. This might suggest that, the concentration of the initial compound causing the zone of inhibition was very low. In this case, treatment with the iHDACs, could have increased the concentration of that specific compound significantly. There is also the possibility of production of another compound which is synergizing with the compound in the control untreated extract, to generate the high zone of inhibition.

Against MRSA, doubling the concentration was not observed to double the zone of inhibition. This implies that very high concentration of the antibiotic is required to double the zone of inhibition. Trichostatin A treated extracts of B7 NEW BM however cause a 6mm increase in

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the zone of inhibition against MRSA. This same increase is not observed for MSSA. This suggests that a compound specific to MRSA may have been induced by treatment with Trichostatin A

An outlier was observed in the SAHA-treated extracts. Broth A of untreated plate inoculum and treated broth exhibited a two-fold increase in the zone of inhibition against MSSA GGP 200 compared to the control, and a threefold increase in zone of inhibition against MRSA GGP E120. This, however, was absent in its duplicate broth and the other SAHA-treated broths. The lack of reproducibility of this activity makes it difficult to determine if the observed activity is due to SAHA treatment. However, the improved antibacterial activity it generates especially against MRSA GGP E120 is very impressive and worth investigating.

Even though these observed results are not conclusive in themselves as to the effect of these small chemicals (iHDAC) in exploring silent gene clusters, it is a big stride and serves as a platform for further investigation. Zutz et al. did extensive work on studying small chemicals and their ability to activate silent gene clusters. After screening 1,512 treated fungal samples, only 30 showed ability to improve the antibacterial activity (Zutz *et al.,* 2013). Petit et al. emphasize that these small chemicals are target-specific and induce specific secondary metabolite production (Petit *et al.,* 2011). Therefore, to maximize the effect of these small chemicals, extensive study on several fungal strains must be done. It is also worth mentioning that the media used for this study was found later not to be the most optimum for metabolite production, more work should be done using the optimum media (YPMD).

At the end of the studies done to optimize the fermentation technique, mycelia disc inoculum were observed to be the favorable form of inoculum for the fungal cultures. Liquid-state cultures using Yeast, peptone, malt extract and dextrose (YPMD) also proved to be better

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than solid-state cultures. Inhibitors of histone deacetylases were confirmed to have the potential of improving the production of antibacterial compounds from fungi.

The next step was to develop an assay that helps to prioritize which of the WDF hit extracts has promising activity. This was to make way for antibacterial compound isolation.

Table 5-1: Determination of minimum inhibitory concentration (MIC) of ampicillin, tetracycline and chloramphenicol against MSSA and MRSA using the disc diffusion assay

Concentration (µg/disc)	MSSA	MRSA
Ampicillin	Zone of inhibition (mm)	
0.1	9	0
0.3	19	10
0.5	21.5	10.5
Chloramphenicol	Zone of inhibition (mm)	
1	0	0
2	7.5	0
5	12	0
10	20	6.5
20	23	7
30	25	9
Tetracycline	Zone of inhibition (mm)	
0.1	0	0
0.3	8	0
0.5	10	0
1	11	0
2	13.5	6
5	18	7
20	20.5	15.5
30	23	17

MSSA- Methicillin susceptible *Staphylococcus aureus,*

MRSA-Methicillin resistant *Staphylococcus aureus*

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5.3 Phenotypic array assay identifies two WDF extracts with promising antibacterial activity against MSSA GGP 200 and MRSA GGP E120

One challenge that pharmaceutical companies sometimes encounter is the rediscovery of existing antibiotics from a natural products screen. (Mahajan and Balachandrancite, 2014). This has caused a shift to synthetic chemistry and structure activity relationship (SAR) experiments to finding new antibiotics. A chemical phenotypic screening assay was developed to minimize the risk of rediscovery of antibacterial compounds. Standard antibiotics and other phenotype modulating compounds were incorporated into the media for the disc diffusion assay. Each WDF extract generated a profile of antibacterial activity when assayed on each of the chemically modified media. Extract profiles were compared to the profile generated by standard antibiotics. Only extracts with unique activity profiles were prioritized for product isolation.

Standard antibiotics (14) and twenty WDF fungi selected from the primary screen were subjected to a synergy array with different chemicals (Ampicillin, Clavulanic Acid, Chloramphenicol, Tetracycline, Streptomycin, N-acetyl glucosamine and Arginine) incorporated into the growth media (Mueller Hinton Agar, MHA). Chloramphenicol influenced the activity of most of the antibiotics. It increased the activity of ampicillin, ethambutol and cycloserine whilst it had a suppressive activity on vancomycin and streptomycin. Against MRSA, it also increased the activity of tetracycline and erythromycin. Chloramphenicol is an inhibitor of protein synthesis in bacteria (Gale and Folkes, 1953; Maxwell and Nickel, 1954;Wisseman *et al.,*1954)

Ampicillin, ethambuthol and cycloserine are all inhibitors of cell wall synthesis. The enhanced antibacterial effect may be a combined effect; as the cell wall inhibitors disrupt the formation of the cell wall, chloramphenicol gets easy access into the cell and inhibits protein synthesis. The combined effect makes the bacterial cell more sensitive to the antibiotic

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Arginine also had a suppressive effect on Linezolid, causing it to totally loose antibacterial activity in its presence. Linezolid is a known oxazolidinone which binds to the 50S subunit of the prokaryotic ribosome, preventing formation of the initiation complex for protein synthesis. Arginine influences biofilm formation and pathogenesis (Zhu et al, 2007). It might have enhanced *S. aureus* biofilm which blocked linezolid from entering the cell and thus reach its target. Hence the loss of activity of linezolid in arginine modified Mueller Hinton agar.

The extracts were also taken through this synergy assay, one extract that stood out in its activity across all the differently modified media was B7 NEW BM. Unlike the standard antibiotics, the chemicals barely affected its activity. Chloramphenicol and arginine seemed to boost it up by increasing its zone of inhibition to 30 and 29.5mm respectively when its sensitivity reduced against MRSA from the 24 mm on the control plate. The activity profile of B7 NEW BM makes it a good candidate for product isolation since it does not mirror the activity pattern of the standard antibiotics used. Unlike vancomycin and streptomycin which had their zones of activity decreasing in the presence of chloramphenicol, the activity of B7 NEW BM is enhanced. It also maintains strong activity for both susceptible and resistant strains of *S. aureus.*

Other extracts, B8 2pb e3, D7 2pb e2 and D7 2BB_A e1 also exhibited high activity and were further boosted to varying extents by the chemical modifiers in instances where their activity reduced against the resistant *S. aureus*. This is good sign, implying that even against the resistant strains, interventions such as combination therapy with these extracts can help reduce the severity of infection. Based on the activity profile of the extracts, eight were selected for further study. Standard antibiotics were also assayed in the presence of key ions known to play a role on the pathogenicity of *S. aureus*. The effect on their antibacterial activity profile was observed. Zinc (Zn), Iron (Fe) and Calcium were investigated. High zinc concentration is known to have

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antibacterial activity (Södeberg *et al.,* 1991). Increasing concentration of zinc was observed to boost the activity of Rifampicin and Chloramphenicol against MSSA. This could be a complementary activity since zinc acts as an antibacterial agent by disruption of the cell membrane and inducing oxidative stress (Xie et al. 2011). Rifampicin and chloramphenicol are DNA and protein synthesis inhibitors respectively. Both targets are found within the cell. The effect of zinc in disrupting the cell wall could possibly have enabled them get to their target in the cell more easily and hence the increase in antibacterial activity. Zinc was observed to reduce the zone of inhibition of moxifloxacillin and streptomycin. However this was minimal by a 2mm difference and may be insignificant.

Iron and Calcium are known to influence biofilm production in *S. aureus*. (Lin et al, 2012; Shukla *et al.,* 2014).The effect they had on majority of the antibiotics was a suppressive one. Biofilm formation increases resistance of antibiotics by preventing entry into the cell. Thus their reduced susceptibility. Ampicillin was however observed to increase slightly in its zone of inhibition. This might be due to the target of ampicillin. Its target is in the cell wall and not the internal part of the cell. Thus it maintains its antibacterial activity.

In general, DNP and DCCD did not have a significant effect on *S. aureus*, even though a slight increase and decrease were observed for Rifampicin and Linezolid by DNP and DCCD respectively. DNP and DCCD target the mitochondria and cause decoupling of the electron transport chain. This process is essential for the survival of the cell, to assay the effect of these compounds on *S. aureus*, sub inhibitory concentrations were applied.

The pattern of activity observed by the standard antibiotics in the presence of DNP, DCCD and ions adds on to the phenotypic array assay in a complementary way. In future, extracts which

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are able to maintain or improve their antibacterial activity in the presence of iron and calcium for instance; which induce biofilm formation and make cells resistant, may be good lead compounds with antibacterial activity against resistant strains of bacteria.

The phenotypic array assay, aided in the selection of WDF that stood out with promising antibacterial activity. However these extracts were crude and there was a need to investigate the specific compound(s) in the crude extract that produced the antibacterial activity. Thus chromatographic separation techniques were employed.

5.4 Fractionation of WDF extracts B7 NEW BM and D7 PM 2pb reveal 5 potential antibacterial compounds

To isolate the specific compounds responsible for the antibacterial activity of B7 NEW BM, the crude extract was separated employing size exclusion (LH-20) chromatography, reverse phase C-18 chromatography and High performance liquid chromatography (HPLC). Eleven fractions were obtained in the size exclusion fractionation which were tested for antibacterial activity against MSSA GGP 200 and MRSA GGP E120. Fractions 6,7,8,9 were observed to have activity with the activity peaking at fraction 7. Fraction 7 had similar activity as the crude, and this activity was maintained in both sensitive and resistant strains of *S. aureus*. The peak observed in fraction 7 suggests that the compound started to elute in the sixth fraction, peaked in the seventh and tailed off in the eighth and ninth fractions. In size exclusion chromatography, large size molecules elute first followed subsequently by small size molecules.

The fractions with antibacterial activity suggest that the compound is relatively small. The ability of the fraction to inhibit growth of the resistant *S. aureus* strain to the same extent as the sensitive strain makes it a good drug candidate capable of containing a novel compound with

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different mechanism of action compared to available antibiotics which are failing against resistant bacteria.

To do further separation, fractions 6,7,8 and 9 from the LH-20 fractionation were pooled together and separated using reverse phase C-18 chromatography. The bioactivity against MSSA revealed the bioactive compound eluted in 5% Acetonitrile fraction alone. Against MRSA however, basal activity in the 10%, 15% and 50% fractions were observed. This shows that, in addition to the compound(s) eluted in the 5% fraction which levels off in the 10% and 15% fractions, another compound(s) elutes at the 50% fraction. Also, 5%, 10% and 15% ACN fractions have more water and thus are relatively polar than the 50% ACN fraction. A compound eluting in these polar fraction suggests that the compound is relatively polar since polar compounds dissolve better in polar solvents than non-polar compounds. The 5% fraction was further separated using HPLC but due to low concentration of the compound, no antibacterial activity was observed.

The second fungal extract to be separated was D7 PM 2pb, from the size exclusion LH-20 chromatography, fraction 3, 5, 6, 7 and 8 out of the eleven fractions had antibacterial activity. Comparing the activity profile of the fractions with the TLC band patterns, two peaks were identified, one at fraction 3 and another at fraction 6 implying the elution of two compounds. Since size exclusion chromatography elutes larger molecules first, followed by smaller molecules, it suggests that the compound(s) eluted at fraction 3 has a larger size relative to the compound(s) eluted at fraction 5 to 8. Upon further separation of pooled LH-20 fractions 3 and 4, using reverse phase C-18 chromatography, fractions 5% and 50% Acetonitrile fractions exhibited antibacterial activity. This suggests that the fraction actually contained two distinct compounds, one which is relatively polar (5% fraction) than the other (50%) fraction. C-18 chromatography of the pool (5,

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7, and 8) revealed activity against MSSA in the 15% and 20% fractions. This suggests the elution of one compound which is relatively polar. Thus from the fractionation of D7 PM 2pb, 3 compounds were eluted with antibacterial activity.

Table 5-2: Summary of fractionation of WDF extracts B7 NEW BM and D7 PM 2pb

WDF extract	LH-20 (Active fractions)	C-18 (Active fractions)	Number of peaks	Number of potential compounds
B7 NEW BM	5,6,7,8	5%, 10%, 15%, 50%	2	2
D7 PM 2pb	3	5%, 50%	2	2
	5,6,7,8	15%, 20%	1	1
Total				5

WDF- Wood decay fungi

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5.5 Large scale Culturing of B7 NEW BM and D7 PM 2pb

Employing the improved media formulation and fermentation techniques developed in this project, the two top WDF candidates which were fractionated were set up on a large scale. The growth pattern of B7 NEW was different from that of D7 PM 2pb. B7 NEW BM, grew steadily, forming fragments of mycelia in the first week which joined to become a carpet by the second week and progressed steadily in growth till it stabilized at week twelve. D7 PM 2pb however exhibited a different pattern of growth. Fragments of mycelia were observed by the first week but a carpet of mycelia was not observed until the fourth week. At the eighth week, the carpet had not fully covered the surface of the broth. These discrepancies in the growth of fungi are species specific. All fungi do not grow at the same rate (Meletiadis *et al.,* 2000). D7 may be a typical slow-growing fungi. It also differed in growth from B7 NEW BM by foam formation, which did not happen for B7 NEW BM. Nobel et al, studied that, in two fungi fermentation, extracellular proteins, lipophilic compounds and α- keto acids partitioned into a foam layer above the surface of the broth. This could be the situation for D7 PM.

Fungi goes through primary phase of metabolism in the initial culturing phase when nutrients are in abundance. During this stage, primary metabolites needed for the growth of the fungi is produced. This was evident in the first two weeks of culturing for B7 NEW BM as fragments of mycelia gradually formed a carpet of mycelia by the fourth week of culturing. After primary metabolism, secondary metabolism sets in when nutrients are depleted and growth conditions become limited. This was observed after twelve weeks of culturing B7 NEW BM. Growth slowed down after 12 weeks of culturing, when most of the nutrients in the media was used up. At secondary metabolism stage, primary metabolites for growth are converted to secondary metabolites, thus no significant growth in terms of mycelia mass is observed rather, secondary metabolites, some of which are antibacterial in nature, are produced (Brakhage, 2013).

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CONCLUSION

The major conclusions of this study are that;

- WDF are capable of producing anti-Gram positive compounds
- Optimum fermentation conditions has a direct impact on production of antibacterial compounds. The optimum conditions revealed in this study include
  - mycelia discs cultured on agar as a source of inoculum.
  - liquid-state cultures are more consistent in producing antibacterial secondary metabolites than solid-state cultures.
  - Yeast, peptone, malt extract and Dextrose (YPMD) broth is the optimum media for production of antibacterial secondary metabolites from WDF
  - chemicals which inhibit histone deacetylases have the potential of enhancing the production of antibacterial secondary metabolites from fungi
- Phenotypic array assay has the potential to select WDF extracts with promising activity for antibacterial compound isolation
- Two WDF, B7 NEW BM and D7 PM 2pb, produce 5 potential antibacterial compounds

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RECOMMENDATIONS

- The two WDF which produced potential anti-Gram positive activity should be further characterized by both morphological and molecular techniques
- Further separation and NMR studies should be performed to obtain pure compounds and to determine the structure of these compounds responsible for anti Gram-positive activity
- Minimum inhibitory concentration of pure compounds as well as toxicity studies should be done to characterize the antibacterial compounds
- Further studies should be performed using the inhibitors of histone deacetylases (iHDAC).
    - the optimum media (YPMD) as seen in this work should be used to culture the fungi in the presence of iHDACs
    - the iHDACs which showed potential to improve the antibacterial activity of the fungi should be repeated with different concentrations to verify and validate the primary data obtained

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