SCREENING AND IDENTIFICATION OF ANTIBIOTIC PRODUCING FUNGI FROM THE SOIL OF BAYERO UNIVERSITY KANO, NIGERIA.

BY

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BEING A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, BAYERO UNIVERSITY KANO, IN PATIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF MASTER OF SCIENCE IN MEDICAL MICROBIOLOGY

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DECLARATION

I herebydeclarethat this research work entitled Screening and identification of antibiotic producing fungi from the soil of Bayero University Kano reportedinthisthesis, was the research effort undertaken under the supervision of Dr. Sani Yahaya and has not been presented elsewhere for the award of degree or certificate. All sources have been duly acknowledged.

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CERTIFICATION

This is to certify that the research work for this Desertation write-up by Muhammad Kabiru Rabiu with registration number SPS/13/MMB/00038 were carried out under my supervision.

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DEDICATION

This work is dedicated to my family.

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ABSTRACT

This study was conducted to isolate antibiotic producing fungi from the soil samples collected from Bayero University, Kano. The samples were collected from Botabic Garden, Ecological Garden, Sport Complex, and B.U.K Staff School. The soil samples were suspended in distilled water, serially diluted and then inoculated on potato dextrose agar by pour plate method. Antibiotic-producing fungifrom these four different locations were isolated and identified through primary screening of the fungi by agar well diffusion method. Out of the 60 fungi isolated, six were selected (coded A6: *A. flavus* A10: *A.fumigatus* B2: *A. flavus* B6: *A.fumigatus*and C2: *A. niger*) for the extraction of metabolites using ethyl acetate and liquid fermentation methods. The extract were dried and subjected to disc diffusion assay against *Streptococcus pneumoniae*, *Staphylococcus aureus, E.coli* and *Pseudomonas aeruginosa*. Result showed that, the extracts revealed inhibitory activity against all the test organisms at concentration of 1000µg,(27.66±0.57), 800µg and (12.33±0.57). The highest activity was shown by five extracts on the test organisms with MIC values of 250µg/ml. Thin layer chromatography of all the five extracts revealed four sports per chromatogram. Showingthat the extractscontain many bioactive compounds. The Gas Chromatography Mass spectra analysis reveals compound such as Hexadecanoic acid, Oleic acid with higher percentage, while propane, eicosanoic acid, methyl tetradecanoate with low percentage area. Also the extracts shows significant toxicity to brine shrimp larvae, with high toxicity in isolate coded B2, therefore the extracts should be subjected to further bioassay such as anticancer,and antioxidant. All the isolates were in the genera of *Aspergillus*.

1.0 INTRODUCTION

1.1 Background

The term 'antibiotic' literally means 'against life'(Schlegel, 2003). An antibiotic was originally defined as a substance, produced by one microorganism (Denyer *et al.,* 2004; Brooks *et al.,* 2015), or of biological origin (Schlegel, 2003) which at low concentrations can inhibit the growth of other microorganisms or infectious organisms (Carrol *et al.,* 2016; Hugo and Willey, 2008). Antibiotics include a chemically heterogeneous group of small organic molecules of microbial origin that, at low concentrations, are deleterious to the growth or metabolic activities of other microorganisms (Carrol *et al.,* 2016; Thomashow and Weller, 1995). According to Talaro and Chess (2015), antibiotics are substances produced by natural metabolic processes of some microorganisms that can inhibit or destroy other microorganism. Dutta (2005) defined antibiotics as the miracle drugs of modern times that act as magic bullets shooting down the infective organisms that have invaded the human body and caused infections. Originally, the term antibiotics referred only to organic compounds, produced by bacteria and fungi, which are toxic to other microorganisms (Willey *et al.,* 2008; Hugo and Russell, 1998). Antibiotics represent the single contribution of drug therapy for the health care of increasing population of the world, and provide effective control of many microbial pathogens that have been the cause of death of human and animals (Robbert*et al.,* 1996). The advent of synthetic methods has, however, resulted in a modification of this definition and an antibiotic now refers to a substance produced by a microorganism, or to a similar substance (produced wholly or partly by chemical synthesis), which in low concentrations inhibits the growth of other microorganisms (Willey *et al.,* 2008; Denyer *et al.,* 2004).

Antibiotic, generally, refers to antibacterial. However, because the term is loosely defined, it is preferable to specify compounds as being antibacterials, antifungals and antivirals (Brooks *et al.,* 2015). Originally, the term antibiotics referred only to organic compounds, produced by bacteria and fungi, which are toxic to other microorganisms (Hugo and Russell, 1998). It represent the single contribution of drug therapy for the health care of increasing population of the world, and provide effective control of many microbial pathogens that have been the cause of death of human and animals (Robbert, 1996).

Fungi were proven to be a rich source of antibiotics. Beyond antibiotics, fungi provide a number of drugs that were part of our current therapeutically tools, such as antiparasitic, antitumor and hypercholesterolemic agents. Of late, although there was strong competition with synthetic products, natural products, especially from microbes, remain promising source of novel bioactive compounds (Muhsin *et al.,* 2011; Rajaseker and Balaji, 2012). Microbial metabolites derived from the secondary metabolism of microorganisms evolve in response to the needs and challenges of the natural environment. An obvious explanation was that microbial products, primarily meant for the defense against competitors, may mimic metabolites of a variety of living systems, including mammals. Most of them have drug-like properties and as a result, require minimal modifications to be developed as effective, orally active and marketable products. Indeed, ascomycetes such as *Aspergillus, Penicillium,* and *Fusarium* were the most frequent producers of bioactive compounds among the fungal species (Berdy, 2005). In addition, a large number of the compounds isolated from soil fungi show potent biological activities that can be exploited as antibacterial, antifungal and antitumor in drug discovery programs. Nowadays, the emergence of pathogenic bacteria resistant to most of the currently available antimicrobial agents has become a critical problem in modern medicine. The discovery of new

bioactive compounds that would address these issues was much sought after (Kishore *et al.,* 2007). For the reasons mentioned above, soil microorganisms from forests could become a source of choice for the identification of new lead compounds in terms of drug discovery. Furthermore, fungi isolated from ordinary surroundings such as soil and plants have been the source of a wide range of bioactive natural products. Drugs derived from fungal origin mainly come from fungi from temperate climate countries. However, tropical regions offer a much larger wealth of biotopes and biodiversity. Yet, very little work has been carried out on tropical m icrobes in general.

1.2 Statement of the Problem

Fungi are known producers of antibiotics and enzymes used in pharmaceuticals and industries respectively. However, the emergence of multidrug resistance pathogens has point the need to discover new antibiotic from the environment. Low discovery rate of these antibiotics is attributed to standard cultivation methods that use nutrient rich media (Strohl, 2000). General purpose media has therefore been used in this study to cultivate the soil fungi for antibiotic production. The finding of this study will therefore shed more light on the availability of fungi and the metabolites the produce in the sample site.

1.3 Justification of the Study

Emerging antibiotic resistance has created a major public health dilemma, compounded by a dearth of new antibiotic options. Increasing rates of bacterial resistance among common pathogens are threatening the effectiveness of even the most potent antibiotics. The introduction of new antibiotics has not kept pace with the increasing rate of resistance, leaving clinicians with

fewer treatment options. Therefore there is need to search for new and potent antibiotic so as to replace the position of current ones.

1.4 Aim

This research is aim at screening for the potential antibiotic-producing soil fungi at different location in Bayero University Kano Old Campus, Nigeria.

1.5Objectives

The objectives of this study were to:

i. Isolate and identify fungi from soil samples using soil dilution techniques.
ii. Extract metabolites from pure cultures of isolated fungi, using liquid fermentation.
iii. Assay extracted metabolites for antimicrobial activity.
iv. Conduct toxicity assay of the extract, using brine shrimp.

CHAPTER TWO

2.0 LITERETURE REVIEW

2.1 Fungi

Fungi are microscopic plant-like cells that grow in long threadlike structures or hyphae that make a mass called mycelium. The mycelium absorbs nutrients from the roots it has colonised, surface organic matter or the soil (Tabasso and Simon, 2006). Fungi are a group of simple plants that have no chlorophyll. There are some species of fungi that are single celled organisms, and there are other kinds of fungi that are multi-cellular organisms. It produces special hyphae that create the reproductive spores. Fungi have many different structures but they can act in similar ways. Since the discovery of penicillin, a number of antibiotics have been discovered mostly from soil inhabiting fungi.

2.2 Structure and composition of fungal cells

The structure of the fungal cell itself has a significant outcome on the diverse medicinal application of the organism, and so it is important from a pharmacological perspective to understand this biologically significant structure-function relationship. Altogether, the fungal cell wall has a multifunctional role. It is involved in the regulation of morphogenesis, fungal reproduction, determination of the antigenic and adhesive characteristics, control of dimorphism, and transfer to the membrane and intracellular messengers (Feofilova, 2010).

The cell wall plays a fundamental role in the development and the integrity of the structural requirements for survival and proliferation. It is extremely protective, in that, it shields the fungus from abrasion, screens out poisons from the environment and by restricting the inflation of the cytoplasm, allows the cell to become highly pressurised (Osherov *et al.*, 2010). It also

serves as a scaffold for proteins that protect the inner polysaccharide layers and provides a dynamic interface with its surroundings (Osherov *et al.*, 2010). However, despite much information being known about the protective role of the cell wall, there is little known about its complete biosynthesis, particularly among filamentous fungi (Esser *et al.*, 2006). The physical and biological properties of the fungal cell wall are determined by the composition and arrangement of their structural components, varying amongst species (Fontaine *et al.*, 1997). It is these variations which led to the various taxonomic classifications between fungal species in the past (Roncero, 2002). As well as taxonomic differences, the dynamic cell wall structure is subject to continuous change in response to culture conditions and environmental stresses, throughout the growth of the fungus (Latge, 2007).

Essentially, two types of components form fungal cell walls; fibrous polymers, which constitute the structural scaffold of the wall and gel-like polymers, which act as the interconnecting molecule. It is the relationship between these components which is responsible for the dynamic properties of the cell wall, required for its survival under diverse environmental conditions (Roncero, 2002).

2.3 Nutritional requirement, metabolism and development

All fungi are heterotrophic organisms and as such, obtain their energy and carbon supply from other organisms for metabolism and survival. Most fungi and yeast have basic nutritional needs and could survive alone once supplied with an aerobic environment, glucose, ammonium salts, inorganic ions and a few growth factors (Kavanagh, 2011). Carbon sources such as simple hexoses like glucose or complex polysaccharides such as starch and cellulose can be utilised by filamentous fungi for growth. The range of a species in the environment is likely to reflect the availability of carbon sources (Carlile *et al.*, 2000). In higher fungi, development takes place

following uptake of nutrients from the environment by cellular components which stimulate septation and biomass.

The first stage of primary vegetation is the formation of branched filamentous structures of fungi known as hyphae, a mass of which is known as the mycelium. Prior to the development of the fruiting body (which is required for reproduction), fungi perform an important role in the decomposition of organic matter and have a fundamental role in nutrient intake and exchange. The mycelium penetrate the substrate and continually interacts with the surrounding environment, breaking down waste products such as; nitrogen, carbon, plant and animal debris, converting them into assimilable nutrients. Mycelia have also shown the ability to break down pesticides, dioxin, chlorine and hydrocarbons which form the majority of heavy metal pollutants from industrial production (Stamets, 2011).

Initial growth and biomass production of fungi is dependent on the availability of primary metabolites (protein, carbohydrates, nucleic acids and lipids). These metabolites are associated with the rapid initial growth phase of the organism and maximum production occurs near the end of this phase (Kavanagh, 2011). This phase of growth is known as the exponential phase and is often termed primary metabolism.

Nutritional requirements are important in an industrial sense, with regard to cultivation and optimization of growth conditions for increased commercial biomass or metabolite production. Some primary metabolites are produced commercially, such as citric acids (food and soft drink manufacturing), ethanol (alcoholic drink production), enzymes (food processing) and amino acids, and vitamins (food supplements). When the supply of essential nutrients is exhausted, the specific growth rate of the culture enters the stationary phase, entering a stage of secondary

metabolism. As fungi enter into the stationary phase, compounds (secondary metabolites) which are not required for active growth and are not essential for vegetative proliferation are produced. A range of secondary metabolites have been found to contain substantial biological activity and so a lot of research has focused on the production, isolation and extraction of these compounds (Mizuno, 1999).

One of the major benefits and advances of microbial technology in commercial scale operations is the ability to produce high-nutritional and pharmaceutical value biomass using abundant agro-industrial residues (Akyuz *et al.*, 2009; Philippoussis *et al.*, 2011). Submerged liquid fermentation (SLF) has in recent years been utilized for this purpose. Mycelial biomass produced using SLF in high quantities for therapeutic downstream processes, such as isolation of bioactive metabolites, may be generated in a short period of time. Simultaneous valorization of important agro-industrial liquid residues such as waste glycerol, sugar cane and soya molasses, as well as olive-mill wastes or thin sillage may be used as a substrate medium for fungal mycelia or fruit body formation (Tang *et al.*, 2007; Diamantopoulou *et al.*, 2014). There is increased interest in the use of mycelial biomass as a possible foodstuff, not only because of its high nutritional value, but also due to its therapeutic potential, due to the presence of bioactive proteins, lipids, polyunsaturated fatty acids, sterols and polysaccharides (Pedneault *et al.*, 2008; Diamantopoulou *et al.*, 2012). Thereby, both intracellular and extracellular polysaccharides produced by SLF are of considerable industrial and economical significance. Intracellular carbohydrates are recognised as containing immunomodulating, antitumor and hypoglycaemic factors (Zhong *et al.*, 2004; Tang *et al.*, 2007), and extracellular polysaccharides are recognised as also having a range of biological activities (Wasser, 2002). Microscopic fungi have been shown to produce bioactive metabolites and with improved possibilities for genetic, pharmacological and chemical

analysis, the therapeutic potential of fungi and fungal metabolites is promising (Lindequist *et al.*, 2005).

A number of metabolites were isolated from fungi which found their way into medical applications as natural products, starting material for pharmaceuticals or as lead structures for the development of pharmaceutical products (Kavanagh, 2011). For example, nowadays, research has been improved in order to isolates novel active metabolites which are produced by fungi. This fact, together with the knowledge about the great potential of microscopic fungi for production of bioactive metabolites e.g. penicillin from *Penicilliumnotatum,* ergotamin from *Claviceps purpurea,* cidosporin from *Tolypocladium inflatum* (Tabasso and Simon, 2006).

2.4 Soil as reservoir of antibiotic-producing fungi

Soil is commonly used as medium of finding a new antibiotic as it is rich in diversity of microorganism including fungi and bacteria. Soil has capacity to supply adequate nutrients to meet the needs of growing microorganism. Fungi are an important component of the soil microbiota typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions. The role of fungi in the soil is an extremely complex and is fundamental to the soil ecosystem. They perform ecological services that strongly impact the quality of human life and have enormous potential for providing economic benefits, e.g., the isolation and identification of the soil fungus Penicillium leading to a large pharmaceutical industry of antibiotics (Takashi *et al.,* 2008).

As fungi play a major role in soil ecosystems along with bacteria, protists, small invertebrates and plants, through complex trophic interactions. Most soil fungi are regarded as saprobes, decomposing organic matter and contributing to nutrient cycling, while several species form

mycorrhizal associations with plants or are plant pathogens (Pfenning and Abreu, 2006). Also recognized as prolific secondary metabolite producers, fungi have provided several bioactive compounds and chemical models currently used as pharmaceuticals, and soils are traditionally the main source of fungal genetic resources for bio prospection programs (Adrio and Demain, 2003). Despite that, the biodiversity and biotechnological potential of the soil mycobiota in many tropical regions is still poorly studied. This environment is thought to select species with adapted metabolism and good potential for delivering new bioactive metabolites.

2.5 Secondary Metabolites of Filamentous Fungi

Natural product5 is mainly attributed to a group of compounds known as secondary metabolites. Some of the most powerful secondary metabolites that have been developed into therapeutic agents are derived from filamentous fungi. Metabolites formed during primary metabolism are converted into secondary metabolites; which are not produced or essential during active growth (Zjawiony, 2004; Zhong *et al.*, 2009). Many of these fungal metabolites have shown activity against a number of diseases. Generally, immunomodulating secondary metabolites provide their bioactivity by stimulation of the host defenses *via* several signal transduction pathways (Zhang *et al.*, 2007). These substances of therapeutic interest stem from five main metabolic sources (Zaidman *et al.*, 2005): amino-acid derived pathways; the shikimic acid pathway for the biosynthesis of aromatic amino acids; acetate-malonate pathway from acetyl coenzyme A; the mevalonic acid pathway from acetyl coenzyme A, which plays a role in the primary synthesis of sterols; and polysaccharides or peptidopolysaccharides (Wasser, 2011). The products of these pathways include pharmaceutically important products, such as the plant secondary metabolites morphine, quinine and digitalis, and the fungal secondary metabolites penicillin, ergotrate, statins and cephalosporin. Some substances are also derived from these pathways such as

aflatoxins, trichothecenes and ergot alkaloids which may be construed as toxic or pharmacologically useful (Keller *et al.*, 2005). The production of economically important metabolites such as antibiotics by microbial fermentation is one of the most important activities of the bioprocess industry. Keller *et al.* (2005) described secondary metabolites as bioactive substances (usually of low molecular weight), which are produced as families of related compounds at restricted parts of the life cycle, with production often correlated with a specific stage of morphological differentiation.

Since their discovery, secondary metabolites have demonstrated a range of bioactivities including antimicrobial activity towards bacteria, fungi, protozoa, parasites, insects and viruses, as well as antitumor activity. The following section describes the most significant secondary metabolites isolated from fungi.

2.6 Discovery of antibiotic

The discovery of penicillin, the first, best-known and most widely used antibiotic was incidental (Schlegel, 2003) in 1928 by an English Bacteriologist, late Sir Alexander Fleming that the first clinical trials of penicillin were tried on humans. This antibiotic was obtained from a blue green mould of the soil called *Penicillium notatum* (Dutta, 2005). Penicillin was discovered accidentally in 1928 by Fleming, who showed its efficacy in laboratory cultures against many disease producing bacteria. This discovery marked the beginning of the development of antibacterial compounds produced by living organisms (Taylor *et al*., 2003).

Another antibiotic, streptomycin was isolated in 1944 by Waksman, a Microbiologist, from a species of soil bacteria, called *Streptomyces griseus*, particularly tubercle bacilli, and has proved to be very valuable against tuberculosis. A vigorous search for more antibiotics was on at this time and in 1947, another antibiotic, chloramphenicol was discovered by Burkholder. It was

isolated from *S. venezuelae*. It has a powerful action on a wide range of infectious bacteria both Gram positive and Gram negative (Dutta, 2006).

The ability to produce antibiotics has been found mainly in fungi of the group *Aspergillales*, and in a few other bacteria (Schlegel, 2003). Streptomycetes are remarkable for the chemical diversity of antibiotics that they produce (Talaro, and Talaro 2002). Altogether about 2,000 antibiotics have been characterized so far; but only 50 are used therapeutically (Schlegel, 2003). As more antibiotics were discovered, designed and studied, scientists found that they had different properties. Some of these properties include their source, range of activity and their kinds. These were used to classify them (Sommer, 2006).

Perhaps one of the few most important discoveries regarding the beneficial use of fungi for humans was the identification in 1928 by Sir Alexander Fleming, that an isolate of *Penicillium notatum* produced a substance capable of killing Gram positive bacteria (Walsh, 2003). This compound was subsequently identified as penicillin and was the first member of the β-lactam class of antibiotics to be discovered. These compounds function by inhibiting peptidoglycan synthesis in bacteria and their use has reduced the importance of the Gram positive as a cause of disease (Walsh, 2003). Subsequent to the identification of penicillin production by *P. notatum*, screening experiments revealed that *P. chrysogenum* was a superior producer of penicillin. A typical fermentation yields three types of Penicillin, namely, Penicillin F, Penicillin G and Penicillin V (Schlegel, 2003).

Antibiotics produced by fungi, are widely used in current chemotherapy especially the penicillin, cephalosporin and fusidic acid, which have antibacterial and antifungal activity (Dobashi *et al*., 1998). A number of antibiotic drugs have been discovered from soil-inhabiting microorganisms

which include fungi (20% of isolated antibiotics), actinomycetes (70%) and eubacteria (10%) (Bredy, 1974; Lechevalier, 1975).

2.7 Microbial metabolites

Microbial metabolites are derived from the secondary metabolism of microorganisms. Secondary metabolites generally have low molecular weight and are not essential for growth, reproduction and development of cells (Verpoorte, 1998). Microbial metabolites have been adopted by human for use in treatment of some diseases because the metabolites have toxic or inhibitory effect on other organisms, fungal metabolites are used as antibacterial, antifungal, as shown in Table 2.1. They also have a potential to give negative impact to the human, animal and plant by producing mycotoxin (Shwab and Keller, 2008). The largest groups of microorganisms producing bioactive microbial metabolites are the filamentous actinomycetes, a source of 45% of the total number of known compounds. *Bacillus* and *Pseudomonas* species are among the major producers of bioactive microbial metabolites in a prokaryotic group. Fungi contributed approximately to 38% of all isolated microbial products and 17% by bacteria (Berdy, 2005).

2.1: Some Valuable Secondary Metabolite Produces from Fungi

Metabolite	Fungal source	Application
Penicillins	*P. chrysogenum*	Antibacterial
Cephalosporins	*Acremonium chrysogenum*	Antibacterial
Griseofulvin	*P. griseofulvun*	Antifungal
Fusidin	*Fusidium coccineum*	Antibacterial
Ciclosporins	*Tolypocladium* spp	Immunosuppresants
Zearalanone	*Gibberella zeae*	Cattle growth promoter
Gibberellins	*Gibberella fujikuroi*	Plant Hormone
Ergot alkaloid	*Claviceps purpurea*	Antimigrains

Decon 2010

2.8 Drugs derived from natural products

There are around 207,000 secondary metabolites isolated from natural sources (Taylor and Francis Group, 2009), but only few percent of them are the source of useful drugs to treat infections or other diseases. Secondary metabolites that are biologically active in some *in-vitro* assay are generally small molecules (molecular weight <500). Drugs can be categorized based on their sources such as natural product origin, drugs derived from natural products, biological, synthetic drugs, vaccines and natural product mimics (Newman *et al.,* 2003). Between 2000 and 2002, drugs derived from natural products were among the top 35 worldwide selling drugs sales (Butler, 2004). Examples are paclitaxel, augmentin, cyclosporine and simvastatin. Natural products play an important role in the discovery of leads for the development of a number of drugs, including antimicrobial, antitumor, immunosuppressive, antidiabetic, enzyme inhibitor and hypercholesterolemic drugs. They became the basis for semisynthetic and synthetic derivatives with significant improvement in pharmacological properties and activity against pathogenic fungi. Here, the main attention is focused on the identification of antibacterial and antifungal drugs derived from natural soil.

2.9 Concepts of industrial research in antibiotic production

The industrial fermentation industry received its greatest impetus for expansion and profits with the advent and exploitation of antibiotics as chemotherapeutic agents. The demand for penicillin during World War II, and later for Streptomycin and other antibiotics, brought on the undertaking of intensive research programs designed to find organisms capable of producing good antibiotics, and oriented toward the development of means for producing antibiotics on a large scale(Kavanagh 2011). New cultural procedures were devised, and the technique of submerged agitated aerated fermentations in deep-tank fomenters came into action. As a result

much of the knowledge gained during the development of antibiotic fermentation processes then became available for the commercial development of other new antibiotic fermentation processes on a large scale production (Singh and Kapoor 2010; Kavanagh 2011)

Screening of antibiotics has been widely performed for about 30 years, and new antibiotics are still being found. However, the possibility of discovering new antibiotics merely by random screening is reduced now a days and new approaches are required for finding new antibiotics efficiently (Kavanagh 2011).

In screening of new antibiotics, major factors must be considered such as, detection method, selection methods. These are closely related to each other, and their efficient combination is indis pensable for successful screening (Singh and Kapoor, 2010).

These days, new strain development for antibiotic production has been essential prerequisite for scale up of antibiotic production and also for search of new antibiotics. Current industrial practices involve natural selection, mutation and protoplast fusion for Streptomyces and other related genera. A major feature of industrial antibiotic production is directed to the screening programmes to generate new potent antibiotics producer microorganisms either from natural sources or from established culture (Kavanagh, 2011)

Screening for antibiotic producing microorganisms can be considered as the use of highly selective procedures to allow the detection and isolation of only those microorganisms of interest from a large population. Screening can be direct or indirect. Direct screening involves assay of product either by bioassay or by chemical means, in contrast indirect screening do not rely on

assay of product but rather on some other characteristics of strains which is correlated with antibiotic production (Kavanagh, 2011).

2.10Microbial fermentation

It is an enzymatic decomposition and utilizations of nutrients, particularly carbohydrate by microbes. Fermentation is used more broadly to refer to the bulk growth of microorganisms on a growth medium, often with the goal of producing a specific chemical product. There are five major groups of commercially important fermentations (Stanbury*et al.,* 2003; Singh and Kapoor 2010; Kavanagh 2011). They are as follows:

I) Fermentation that produce microbial cells (Biomass) as product.

II) Fermentation that produce microbial enzymes

III) Fermentation that produce microbial metabolites

IV) Fermentation that produce recombinant products.

V) Those that modify a compound which is added to the fermentation – transformation process.

2.10.1. Fermentation that produce microbial biomass

Commercial production of microbial biomass may be divided in to two processes. The production of yeast to be used in the baking industry, and the production of microbial cells to be used as a human or animal food. Bakers yeast have been produce on large scale since early 1900′s and yeast was produce as human food in Germany during first world war. However it eases not until 1960′s that the production of microbial biomass as a source of food protein was explored to any great depth (Stanbury, 2003).

2.10.2 Fermentation that produce microbial enzymes

Enzymes are considered as a potential biocatalyst for a large number of reactions. Particularly, the microbial enzymes have widespread uses in industries and medicine. The microbial enzymes are also more active and stable than plant and animal enzymes. In addition, the microorganisms represent an alternative source of enzymes because they can be cultured in large quantities in a short time by fermentation and owing to their biochemical diversity and susceptibility to gene manipulation. Enzymes have been produced commercially from plant, animal and microbial source. However microbial enzyme has the enormous advantage of being able to produced large quantities by established fermentation techniques (Stanbury, 2003; Kavanagh, 2011)..

2.10.3. Fermentation that produce metabolites

Metabolites are intermediates and products of metabolism, and typically characterized by small molecules with various functions. Metabolites can be categorized into primary and secondary metabolites. These metabolites can be used in industrial microbiology to obtain amino acids, developed vaccines and antibiotics (Stanbury *et al.,*2003), and isolated chemicals necessary for organic synthesis.

Primary metabolites: These are involves in growth, development and is typically a key component in maintaining normal physiological processes; thus it is often referred to as a central metabolite. Primary metabolites are typically formed during growth phase, deemed essential for proper growth. Example includes Alcohol, lactic acid and certain amino acids.

Using fermentation techniques product like l-lysine, l-glutamate are produce and isolated via the mass production of specific microorganisms. Example *A. niger* is one of the most widely used in food production it is also commonly used in pharmaceutical and cosmetic industries as well.

Secondary metabolites: Are typically organic compounds produce through the modification of primary metabolites synthesis. It does not play any role in growth, development and reproduction like primary metabolites do; and it formed during the stationary phase of the growth, some of the identified secondary metabolite have a role in ecological function, including defense mechanism, by serving as antibiotics and by producing pigments. Mostly antibiotics such as penicillin are secondary metabolite (Stanbury *et al.,* 2003; Kavanagh, 2011)..

2.10.4. Fermentation that producerecombinant product

The advent of recombinant DNA technology has extended the range of potential fermentation products. Genes from higher organism may be introduced into microbial cells such that the recipients are capable of synthesizing foreign proteins. A wide range of microbial cells have been used as hosts for such synthesis include *Escherichia coli*, *Sacchromyces cerevisiae* and filamentous fungi. Products produce by such genetically engineered organisms include interferon, insulin, human serum albumin etc (Singh and Kapoor, 2010).

2.10.5. Transformation processes

Microbial cells may be used to convert a compound into structurally related, financially more valuable compounds. Because microorganisms can behave as chiral catalysts with high potential specificity and stereo specificity, microbial process are more specific than purely chemical ones and enable the addition, removal or modification of functional groups at specific site on a complex molecules without the use of chemical protection. The reactions which may be

catalyzed include oxidation, dehydrogenation, hydroxylation, dehydroxylation and condensation. Microbial processes have the additional advantage over chemical reagents of operating at relatively low temperature and pressure without requirement for potentially polluting heavy metal catalysts.(Kavanagh, 2011)

The anomaly of the transformation fermentation process is that a large biomass has to be produced to catalyze a single reaction. Thus many processes have been streamlined by immobilizing either the whole cells, or the isolated enzymes which catalyze the reaction on the inert support. The immobilized cells or enzymes may then be considered as catalyst which may be reused many times. (Stanbury *et al.,*2003)

2.11 Antimicrobial agents and their modes of action.

The mechanism of action of antimicrobial agents can be discussed under four heading.

i. Inhibition of cell wall synthesis
ii. Inhibition of cell membrane synthesis
iii. Inhibition of protein synthesis
iv. Inhibition of nucleic acid synthesis
v. Inhibition of folic acid synthesis

2.11.1 Cell wall synthesis inhibitors

Cell wall synthesis inhibitors generally inhibit some step in the cell wall synthesis of bacterial peptidoglycan. Generally they exert their selective toxicity against eubacteria because human cell lack cell wall. Drugs that act in this way include:

A)Beta lactam antibiotic Chemically these antibiotic contain a 4-membered beta lactam ring. They are the products of two group of fungi, *Penicillium* and *Cephalosporium* molds, and are correspondingly represented by the penicillins and cephalosporins. The *B*eta lactan antibiotic inhibit the last step in peptidoglycan synthesis, the final cross-linking between peptide side chains mediated by bacterial carboxypeptidase and transptidase enzymes. Beta lactam antibiotic are normally bactericidal and require that cell be actively growing in order to exert their toxicity (Willey *et al.,* 2008; Talaro and Chess, 2015).

Penicillins can be natural when its soley synthesized by the *penicilliumchrysogenum* through the fermentation processesexample penicillins G and penicillin V. They are effective against *Streptococcus*, *Gonococcus* and *Staphylococcus*(Carrol *et al.,* 2016)

Semi synthetic penicillins in this the mold produce the main part of the molecule (6-aminopenicillanic acid) which can be modefied chemically by the addition of side chains. Many compounds have been developed to have distinct benefit or advantage over penicillin G. Such as increase in spectrum of activity, resistance to penicillase, effectiveness when administered orally etc. Amoxycillin and ampicillin are among (Brook *et al.,* 2015; Nester *et al.,* 2007; Willey *et al.,*2008).

B) Cephalosporins Are Beta lactam antibiotics with similar mode of action to penicillins, they are produced by species of *Cephalosporium*.they have low toxicity and a somewhat broader spectrum than natural penicillins. They are often used as penicillin substitute, against Gram – negative bacteria, and in surgical prophylaxis. They are subjected to degradation by some bacterial beta lactamases, but they tend to be resistant to beta lactamases from *S.aureus*. (Carrol *et al.,* 2016; Willey *et al.,* 2008)

2.11.2 Cell membrane inhibitors: They disorganized the structure or inhibit the function of bacterial membranes. The integrity of the cytoplasm and outer membranes is vital to bacteria, and compounds that disorganized the membrane rapidly kill the cell. However, due to the similarities in phospholipid in eubacteria and eukaryotic membrane, this action is rarely specific enough to permit these compound to be used systematically. The only antibacterial antibiotic of clinicalimportance that act by this mechanism is polymyxin, produce by *B. polymyxa*.It is effective mainly against Gram-negative bacteria and usually limited to topical usage. Polymyxin bind to membrane phospholids and there by interfere with membrane function (Brook *et al.,* 2015; Carrol *et al.,* 2016; Talaro and Chess 2015; Willey *et al.,* 2008).

2.11.3 Protein synthesis inhibitors: Many therapeutically useful antbiotics owe the action to inhibition of some step in the complex process of translation.their attack is always at one of the events occuring on the ribosome and rather than the stage of amino acid activation or attachment to a particular tRNA. Most have an affinity or specificity for 30s and 50s (as opposed to 80s) ribosomes, and they achieve their selective toxicity in this manner. The most important antibiotic with this mode of action are the Tetracyclilline, Chloramphenicol, the macrolides and the aminoglycosides (Brook *et al.,* 2015; Carrol *et al.,* 2016; Talaro and Chess 2015; Willet *et al.,* 2008).

2.11.4 Nucleic acid inhibitors: Some chemotherapeutics agents affect the synthesis of DNA OR RNA or can bind to DNA or RNA so that their message cannot be read. Either case, of course can block the growth of cells. The majority of these drugs are unselective, however, and affect animal cells and bacterial cells alike and therefore have no therapeutic application. Two nucleic acid synthesis inhibitors which have selective activity against prokaryotes and some medically utility are nalidixic acid and rifamycins (Willey *et al.,* 2008).

Nalidixic acid is a synthetic chemotherapeutic agents which has activity mainly against Gram-negative bacteria. Nalidixic acid belong to a group of compounds called quinolones. Nalidixic acid is bactercidal agent thatbind to the DNA gyrase enzymes (topoisomerase) which is essential for DNA replication and allows supercoils to be relaxed and reformed (Brook *et al.,* 2015; Carrol *et al.,* 2016).

Some quinolones penitrate macrophages and neutrophils better than most antibiotics and are thus useful in the treatment of infection cause by intracellular parasites. However the main use of nalidixic acid is in the treatment of lower urinary tract infection. However a floroquinolones, are mostly in used in the clinical treatments such as Sperfloxacin, Ciprofloxacin as recently derive from the modification of nalidixic acid.

Rifamycins also produce by *Streptomyces*. Rifamficin is a semi synthetic derivatives of rifamycin that is active against Gram-positive bacteria (including *Mycobacterium tuberculosis*) and some Gram- negative bacteria. Rifamficinact quite specifically on eubacterial RNA polymerase and is in active toward RNA polymerase from animal cells or toward DNA plymerase. The antibiotic bind to the beta sub unit of the polymerase and apparently blocks the entry of the first nucleotide which is nessary to activate the polymerase, there by blocking mRNA synthesis (Brook *et al.,* 2015;Talaro and Chess 2015; Coyler 2005).

2.11.5 Competitive inhibitors

the competitive inhibitors are mostly all synthetic chemotherapeutic agents. Most are "growth factors analogs" which are structurally similar to a bascterial growth factor but which do not fulfill its metabolic function in the cell. Some are bacteriostatic and some are bactericidal.

Sulfonamides were introduce as chemotherapeutic agent by Domagk in 1935, who showed that one of the compound (prontosil) had the effect of curing mice infected with haemolytic *Streptococcal* infections (Carrol *et al.,* 2016; Coyler, 2005).

2.12 Antibiotic-Resistance

Antibiotic resistance occurs when pathogenic microorganism are capable to inactivate antibiotics or survive under the selective pressure of antibiotics (Gillespie, 200I). Resistance is a condition in which the antibiotic fails to harm the pathogen enough to cure diseases. Emergence of resistance often begins with a large pathogen population in which a tiny fraction is naturally resistant the antibiotic, either through spontaneous changes or through the acquisition of resistance genes from other microbes (Drlica and Perlin, 2004).The emergence of antibiotic resistance in bacteria has been mainly attributed to the overuse of antibiotics and indiscriminate antibiotic treatment are often the main cause that contribute to the emergence of bacterial resistance to antibiotics (Eileen *et aI.,* 2010).

This emergence of resistance in bacteria towards antibiotics has posed a big challenge in the treatment of infectious diseases worldwide (Eileen *et al.,* 20 I0). The resistance has arisen not only against both natural and semi-synthetic antibiotics, but also towards pure synthetic chemical compounds. Some of the bacteria even become multi-drug resistant, where resistance has been developed against several drugs.

The antibiotic resistance property can be transmitted to the next generation through vertical transmission of inherited mutations or by horizontally exchange of genetic materials (Gillespie, 2001). Occasionally, spontaneous mutations, which occur in receptor or binding site of the bacteria, often leads to the change that renders antibiotics to become ineffective (FDA, 2009).

Besides, transferring of mutant resistance-causing genes from one bacterium to another can also cause a bacterium to become resistant rapidly. For example, horizontal transfer of *mecA* gene from *Staphylococcus sciuri* results in the emergence of MRSA (Carlos and Cuevas, 1996).

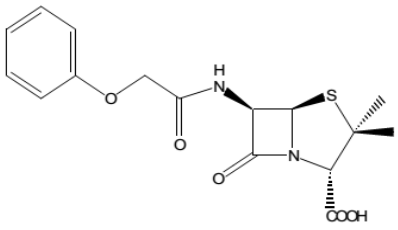

Figure 2.1: Chemical structure of penicillin V

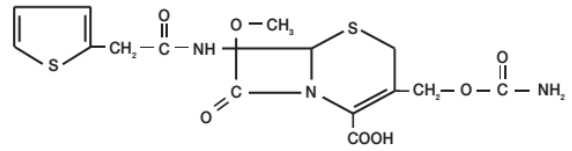

Figure 2.2: Cefoxitin

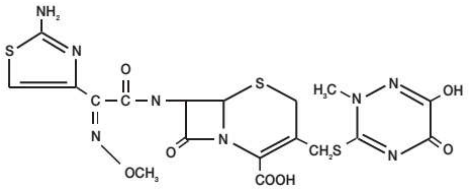

Figure 2.3: Ceptriaxon

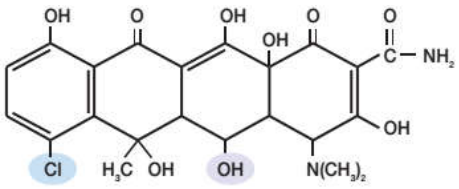

Figure 2.4:Tetracyclin

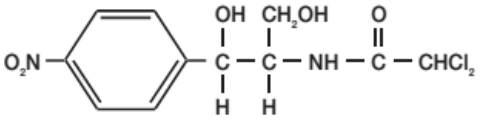

Figure 2.5:Chloramphenicol

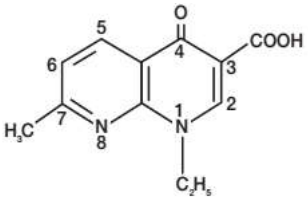

Figure 2.6:Nalidixin acid

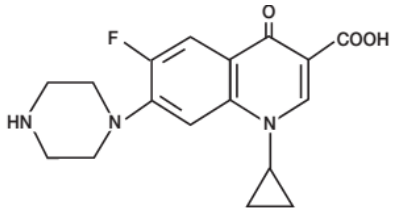

Figure 2.7:Ciprofloxacin

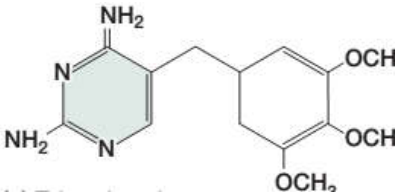

Figure 2.8:Trimethoprim

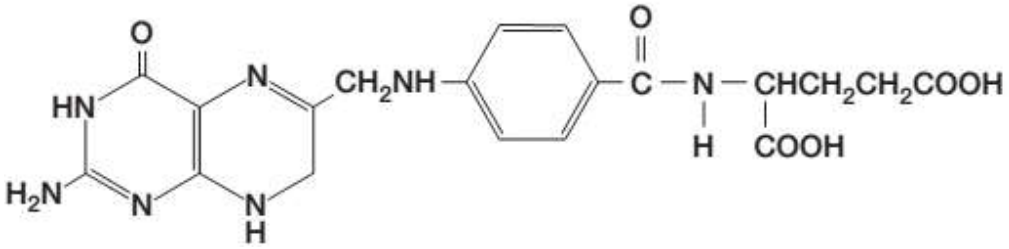

Figure 2.9:Dihyrofolic acid

Structures of some antibiotics

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The study was conducted in Kano State of the Federal Republic of Nigeria, which is located at north –west zone of Nigeria. Most of its area lies within Sudan Savanna with edges boarded to the north and east with Jigawa State, on the west by Katsina State and on the south by Kaduna State. It is located on northern high plain of Hausa land at about latitude 12º-12º 15¹ north and longitude 8º 30¹-8º 45¹, and has an elevation of 525 meters above sea level (Olofin, 2000).

3.2 Study site

The samples were collected from four different location of Bayero University Kano Old Site, Kano, Nigeria.The locations were Botanic Garden, Ecological Garden, Sport Complex and Staff School compound.

3.3 Sample collection

Soil sample were collected as described by TiwarI *et al.*(2009). All the vegetation was removed from the surface of the soil. Spatula was used to collect the soil samples into glass containers of 200ml capacity which were previously sterilized. Soil was dug to 5 centimeter deep, and 50g of the soil each was collected aseptically from four different locations in Bayero University Kano old site (Biologic Garden, Ecological Garden, Staff School Compound and Sports Complex Compound). The samples were taken immediately to the Microbiology Research Laboratory, Bayero University Kano, for analyses.

3.4 Determination of soil temperature

The temperature of the soil at the four different sites was determined during sampling (at the site) by the use of mercury in glass thermometer. A thermometer was inserted into the soil up to depth of 5cm and allowed to stay for 10minutes, after which the temperature reading was obtained. The average of three consecutive readings were calculated and recorded for each site (Dix, 1995).

3.5 Determination of soil pH

The soil pH values were determined using digital pH meter in accordance with the standard methods of Watson and Brown (1998). Using this method, 3g of soil sample was weighed into a beaker containing 3ml of distilled water, and was stirred for five seconds and allowed to stand for 10minutes. The electrode of the pH meter was then inserted into the slurry and swirled carefully. The reading was taken and the average of three consecutive readings was recorded for each site (Makut and Owolewa, 2011).

3.6 Isolation of fungi from the soil sample

The soil fungi were isolated using the Soil Dilution Techniques and Pour Plate methods. One gram of each sample was suspended in 9ml of sterile water. From the stock solution, 1 ml was used to prepare the final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} by serial dilution method. Potato Dextrose Agar (PDA) was prepared in accordance with the manufacturers instruction, The media was sterilize and poured in to three petri dish to 5mm depth, using pour plate method. From solution of 10^{-3} 10^{-4}, and 10^{-5}, an aliquot (100 µL) of the diluted filtrate was spread evenly on PDA medium supplemented with 50µg/mL Streptomycin in a petridish and incubated at 37°C for 5 days. Individual fungal colonies were sub-cultured to obtain the pure fungal cultures (Ogbonna *et al.*, 2013).

3.7 Identification of the fungal isolate

Pure cultures of fungal isolates were identified using both macroscopic (cultural) and microscopic (morphological) features with reference to the method described by Barnett and Hunter (2004);David *et al.* (2007).

3.7.1 Direct microscopic mounts

Using sterile technique, a small portion of the isolated fungal colony was removed with an inoculation needle and mount in a drop of Lactophenol Cotton Blue on a clean microscope slide. Covered with a cover slip, the preparation was squashed with the butt of the inoculation needle and then blots off the excess fluid, then mount under low power of X 40 (David *et al.*, 2007).

3.7.2 Test organisms and their reconfirmation

The test organisms that were used in this study included*Candida albicans*, *Aspergillus niger, Escherichia coli*, *Pseudomonas aeruginosa, Streptococcus spp.* and *Staphylococcus aureus*. The organisms were obtained from Microbiology Laboratory at Bayero University Kano and were reconfirmed with reference to Cheesbrough (2006). These included Germ tube, Gram stain, Catalases, Coagulase, Oxidase and Indole tests.

A: Germ tube test

This is the technique employed for rapid identification of *C. albicans*. Half (0.5) ml of human serum was pipetted and transferred into sterile glass test-tube. With a sterile wire loop, a yeast colony from the culture was inoculated into the serum and incubates at 37ºC for 2 hours at room temperature. Using a Pasteur pipette, a drop of the serum yeast culture was place on a glass slide and covered with coverslip. The set up was examined under X10 and X40 objectives respectively

with the condenser closed sufficiently to give a good contrast. Sprouting yeast cells indicates the presence of *C. albicans* (Cheesbrough, 2006)

B: Gram's stain

Using an inoculating needle a colony was taken aseptically and put on a clean slide and heat fixed. Crystal violet was added on the slide and allowed to stands for 60 sec and washed with distilled water. Gram's iodine was added and left for 30 seconds then the decolourizer was added from one side of the slide till it become light violet. Safranin was added on the slide and allowed to stand for 1 min, then, the slide was washed with distilled water. Slide was then air dried and observed under the microscope (X 100)(Cheesbrough, 2006).

C: Coagulase

A drop of distilled water was place on the two end of glass slide; the colony of the test organisms (previously gram stain) was emulsified to make thick suspension. A loopful of plasma was added to one suspension and mix gently. Clumping of the organisms within 10 seconds indicate positive result(Cheesbrough, 2006).

D: Catalase test

Two (2) mls of hydrogen peroxide was poured into a test tube, using a glass rode a colonies of the test organisms were removed and immersed in the hydrogen peroxide solution, immediate bubbling indicate catalase positive results.

E: Indole test

Peptone water containing tryptophan was inoculated with a smooth colony of the suspected colony and incubate at 35-37^{o}c overnight. A positive result showed erect pink color at the top by the addition of Kovacs reagent.

F: Oxidase test

3 drops of oxidase reagent (tetra- methyl paraphenylene-diamine hydrochloride) was placed on a clean filter paper, a colony of the test organism was smeared and observed. A positive reaction turn the filter paper dark purple in 5-15 second (Cheesbrough, 2006).

3.8 Preparation of McFarland standard

One percent (1%) v/v solution of sulphuric acid was first prepared by adding 1ml of concentrated sulphuric acid to 99ml of distilled water, and mixed well. Also 1% w/v solution of barium chloride was prepered by dissolving 0.5g of dehydrate barium chloride ($BaCl_2 .2H_2O$) in 50ml of distilled water. To 99.4ml of the diluted sulphuric acid, 0.6ml of the barium chloride solution was added. The solution was stored in well closed dark container at room temperature before used. (Cheesbrough, 2006).

3.8.1 Preparation of inocula

A loop of the confirmed test isolates from an overnight culture of the test bacteria were picked using a sterile wire loop and emulsified in 2ml of physiological saline until its density matched with density of McFarland (0.5) standard of turbidity, as described by Kirby Bauer (1993)

3.9 Plate preparation

Nutrient Agar and PDA were prepared according to manufacturer's instruction. Sufficient molten agar was be poured in to sterile Petri dishes at a depth of 4mm and each plate was labeled with the test organism to be inoculated.

3.10 Primary Screening of isolates for inhibitory action

Methods used by Williams and Cross (1971) was employed to determine the *in vitro* antibiotic production potential by fungal isolates against the test organisms. The test organisms used for the study include; *Escherichia coli,Staphylococcus aureus* and *Candida albicans*. Each fungal isolate was streaked on Nutrient Agar as a straight line and incubated at 30°C. After two days of incubation, test organisms were streaked perpendicular to the streaked line of the growing fungus. These were then incubated at 37°C for 24 hours, after which the zone of Inhibition of each test organism from the streaked line of the growing fungus was observed.

3.11 Secondary screening of the isolates

The isolated fungi were tested for their antimicrobial activity against the test strains. The colonies of the isolated fungi were subcultured in to the potato dextrose broth for five days; the filtrate was absorbed on the disc. The discs were place on the culture of the test organisms and incubated over the night. The isolates that produce zones of inhibition were taken for the fermentation process (Sandhya *et al.,* 2015).

3.12.1 Preparation of culture filtrates of fungi

The procedure described by Shamim *et al.* (2011) was adopted. Test fungi were grown in conical flasks (500 ml) containing 300 ml Potato Dextrose Broth, plugged with cotton wool and autoclaved at 121°C for 20 minutes. After cooling the medium, each flask was inoculated with 5 mm disc, cut from the margin of growing culture of test fungi. Each test fungus had 5 flasks. These flasks were incubated for 15 days at room temperature (25°C) as stationary mat culture. After 15 days, test fungi were filtered through Whatman No.1 filter paper.

3.12.2 Extraction of bioactive metabolite

After filtration of the fermented broth, it was extracted by solvent extraction method (Chacko *et al.,* 2012). Ethyl acetate was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 1 hour for complete extraction. The organic phase having antibacterial property and it was evaporated in water bath at 40ºC. The residue obtained was weighed. To assure that extracts wer e derived solely from the cultured fungi of interest, an aliquot of the fermentation culture was pipette and its contents cultured on PDA. Fungal strains were authenticated after microscopic visualization. All cultures were found to contain only the fungi of interest and harbored no additional microbe.

3.13 Preparation of Sensitivity Disc

Sensitivity discs of 6.0mm in diameter were punched from Whatman No.1 filter paper with the aid of paper puncher. The discs were placed in screw capped Bijou bottles and sterilized by autoclaving at 121^{o}C for 15 min. Sensitivity discs were prepared by gently dispensing 100 improvised paper discs into various test concentration. Amoxicillin (30µg) was used as positive control (Vallekobia *et al.*, 2001).

3.14.1 Preparation of test concentration for sensitivity

Various test concentration of 1000µg, 800µg, and 600µg, were prepared from dry fungal metabolites extract in accordance with the dilution method described by Baker *et al.* (1990). Stock solutions of each extracts were prepared by dissolving four milligram (4mg) of the extract in a bijou bottle containing 2ml of distilled water to give 4000µg/2ml solution and labeled as the stock solution. The working solutions were prepared from the stock solution. From the stock solution 0.5ml was transferred to a bijou bottle containing 0.5ml of distilled water, 0.4ml to 0.6ml of distilled water, 0.3ml to 0.7ml of distilled water, and 0.2ml to 0.8ml of distilled water this gives a concentration of 1000µg/ml, 800µg/ml, and 600µg/ml respectively to which 10 discs were added such that after even distribution, each of the discs is equivalent to discs potencies of 100µg/disc, 80µg/disc, and 60µg/disc respectively for each of the six extracts.

3.14.2 Sensitivity testing

Nutrient agar plates were prepared and dried in an oven at 45^{o}C to remove moisture on the surface of the agar plates. Using a sterile swab stick, standardized inocula of each isolates were swabbed on the surface of Nutrient agar in separate plates. This was followed by application of the prepared discs of the four different concentrations of the extracts (1000µg/ml, 800µg/ml, and 600µg/ml) and standard antibiotics (Amoxicillin 30µg) onto the center of the surface of the inoculated media to serve as positive control, using a sterile pointed forceps. The discs prepared were kept at distance apart to prevent any overlapping of zones formed. The plates were inverted and allowed to stand for 10mins to allow pre-diffusion of the extract into the agar and subsequently incubate at 37^{o}C for 18hours. This was followed by measurement of the diameter of zone of inhibition formed by each of the test organisms and standard antibiotic using ruler in

millimeter. These test was performed in duplicate, result were expressed as the mean of two independent experiments, and standard antibiotic discs (Mukhar and Okafor, 2002).

3.14.3 Determination of minimum inhibitory concentration

The fungal metabolite extracts that showed significant antibacterial activity by the discs diffusion assay were subjected to minimum inhibitory concentration (MIC) assay by using tube doubling dilution technique, using distilled water to arrive at concentrations of 1000µg/ml, 500µg/ml, 250µg/ml and 125µg/ml. Two milliliter (2mls) of extract and Nutrient broth was mixed and 0.1ml of standardized inocula was added to each of the test tubes above. The tubes were incubates aerobically at 35^{o}C for 24hours. Tubes containing broth and extracts without inoculla which served as positive control while a tube containing broth and inocula served as negative control for comparison. The tubes were observed after 24 hours of incubation to determine and record the presences of growth (Turbidity), or absence of growth (clear solution) at the end of the incubation period, the lowest concentration that showed no evidence of growth (turbidity) was regarded as minimum inhibitory concentration (MIC) (Beker *et al.*, 1993; Vallekobia *et al.*, 2001).

3.14.4 Minimum bactericidal concentration

The minimum bactericidal concentration (MBC) of the fungi extracts were determined by sub culturing from the test tubes from each of the MIC test tube that showed no evidence of growth (turbidity). The plates were further incubated at 37^{o}C for 24 hours to determine the MBC, as the highest dilutions that yielded no single bacterial colony on the soil medium was regarded as the minimum bactericidal concentration (Beker *et al.*, 1993; Vallekobia *et al.*, 2001).

3.15 Thin Layer chromatography (TLC) method

Extract of each of the six metabolites were dissolved in separate test tubes and were sported on the TLC plate by using capillary tubes and making the distance of the spotting point of the extract on the plate to be 2cm from the base of the plate. This was followed by running the chromatogram with a suitable solvent system in developing chamber which contained the mobile system of chloroform, methanol (9:1). After running the chromatography, plates were then removed and the solvent evaporated in air. Different bands of the bioactive compounds were observed by illuminating the plates with ultraviolent light (UV) at 254 and 365nm. Retention factor values of each spot were calculated.

3.16 Gas chromatography-mass spectra analysis

The metabolites were subjected to quantitative analysis using the GC-MS analyzer (**GC-MS QP2010 Plus Shimadzu, Japan**) to quantify the compounds contained in each of the metabolite produced, and determine the proportion as well as to identify the chemical constituents in the metabolite. The metabolites were analyzed using GC-MS while the mass spectra of the compounds found in the extract were compared with the National Institute of Standard and Technology (NIST) library.

3.17.1 Preparation of artificial sea water

Artificial sea water was made by dissolving 38g of sodium chloride per liter of distill water (Daruliza *et al.,* 2012)

3.17.2 Test sample preparation

The recovered metabolites were dissolved in sterile distilled water to obtain the stock solution from which various concentrations of 10, 50, and 100µg were made, concentration by serial dilution after dissolving 1mg of each metabolite in 10ml of water, and artificial sea water serves as a negative control.

3.17.3 Hatching of brine shrimp eggs

Brine shrimp eggs were obtained from Department of Chemistry, Bayero University Kano, and hatched in an improvised tank containing artificial sea water. The nauplii were hatched within three days (Gufta *et al.,* 1996).

3.17.4 Brine shrimp lethality test

The toxicity of the metabolites was adopted from Zafar *et al.* (2015) at various concentrations of 10, 50, and 100µg in artificial seawater contained in test tubes, ten (10) active shrimp larvae were added in to each test tube. Tube contained 5ml of artificial sea water was used as control, after 24hrs surviving shrimp were counted using hand lens, and the Percentage of the Mortality of each dose was calculated (Gufta *et al.,* 1996).

3.18 Statistical analysis

All of the data were expressed as a mean ± s.d. from at least three separate experiments, each performed in triplicate. The ANOVA test for unpaired observation between controls and experimental samples and Tukey´s test for multiple comparisons were conducted to evaluate statistical differences; *p* values of 0.05 or less were considered statistically significant.

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CHAPTER FOUR

4.1 RESULTS

A total of twelve samples were collected from four different sites of Bayero University Kano, and analyzed for physicochemical parameters. From the analysis, temperatures of the sampling sites ranged between 25°C and 37°C (Table 4.1). The temperatures were measured to help determine the initial incubation conditions. Soil textural properties from Botanic and Ecological Garden were identified to be loamy, while that from Sports complex and Staffs School Compound are sandy in their texture. The pH of the sampling sites was found to range between 8.3-6.8. (Table 4.1)

The occurrence of fungi in this study sites was presented in Table 4.2. From the results, the number of colonies (mean ± S.D) counted from Botanic Garden, Ecological Garden, Sport Complex and Staff School were observed to be 6.43±6.51cfu, 5.66 ±3.05 cfu, 5.10 ±7.53 cfu and 3.00 ± 2.69 cfu respectively. There was no statistical difference between the mean fungal colony counts of sites A, B and C ($p \geq 0.05$).

Table 4.1: Physical parameters of the soil samples at various locations

Location	Soil type	pH	Temperature (^{o}C)
Biologic Garden	Loamy	8.3	25
Ecological Garden	Loamy	7.4	26
Sports Complex	Sandy	6.9	26
Staff School Compound	Sandy	6.8	26

Table 4.2: Mean soil fungal counts of the sample sites at Bayero University, Kano

Sample Site	Fungal Count (cfu)
A	$6.43\times 10^4 \pm 6.51^a$
B	$5.66\times 10^4 \pm 3.05^a$
C	$5.10\times 10^4 \pm 7.53^a$
D	$3.00\times 10^4 \pm 2.69^b$

Values are mean ±S.D, values with similar letter within the same column were observed to be not significantly different ($p > 0.05$).

Keys: A= Biological garden

B = Ecological garden

C =Sport complex

D =Staff school

During this study, Screening and Identification of Antibiotic Producing Fungi was conducted to assess soil fungi associated with the antibiotic production. A total of 60 fungal were isolated. Twenty one (21) isolates from Biological Garden (35%), 20 from Ecological Garden (33.33%), 11 from Sport Complex Field (18.33%), and 7 from Staff School Play Ground (13.33%). (Table 4.3). The 60 isolates were subjected to primary screening in which eight 8 shown positive response coded (A6, A8, A10, B2, B6, C1, C2, and C4. The secondary Screening confirmed 5 fungi coded (A6, A10, B2, B6, and C2) in Table 4.4.

The residue obtained from the fermentation of fungi in the potato dextrose broth, in which strain C2 metabolite had the highest yield of five gram (1.43g/l), the least was A10 with (0.6g/l), as shown in (Table 4.5)

Table 4.3: Occurrence of fungi in the sample sites at Bayero University, Kano

Location	Total fungal isolate	Percentage (%)
Biologic Garden	21	35.00
Ecological Garden	20	33.33
Sport Complex	11	18.33
Staff School Compound	8	13.33
Total	60	99.9

Table 4.4: Primary screening of the isolates for antibiotic production.

Test organisms	Sensitive to metabolites	Percentage sensitive	Resistance to metabolites	Percentage Resistance
E. coli	20	33.33	40	66.66
S. aureus	18	30	42	70
Candida albicans	0	0	60	100

Table 4.5: Recovered active metabolites from the isolated fungal strains at different locations

S/N	Isolate code	Isolated Species	Yield of metabolite (g/l)
1	A6	*A. flavus*	1.25
2	A10	*A. fumigates*	0.68
3	B2	*A. flavus*	0.8
4	B6	*A. niger*	3.7
5	C2	*A. fumigates*	1.43

Antimicrobial activity of metabolites produced by fungi from Botanic Garden against four bacterial strains by disc diffusion method is presented in Table 4.5. The activity of conventional antibiotic shows a significance difference between the concentrations of the metabolites ($p<0.05$), also the extracts shows significance difference between difference concentrations in each test bacterium ($p<0.05$). For the metabolite from*A. flavus*, has the highest activity with zone of inhibition of 17mm in *Staphylococcus* and *E. coli*, followed by *Streptococcus* with 15mm, and the least was 8.66 ±2.318 in *P. aeruginosa* shown in (Table 4.6). *A. fumigatus*produced metabolites that shows activity in the concentration of 1000µg *S. aureus* has the highest zone of 16.33±0. The least zone was in *P. aeruginosa* 8.66 ±0.57mm (Table 4.5)

The result of antimicrobial activity of metabolites extracted in fungi isolated from Ecological Garden shows that, the conventional antibiotic activity shows a significance difference in activity in against all the test bacteria ($p>0.05$), also the result indicates significance difference between difference concentrations among the tested metabolite ($p>0.05$). In the metabolite extracted from *A. flavus* the highest zone of inhibition was 18.66±1.15 while the least zone was6.33±0.57. Also in the sites *A. niger* produced metabolites that shows high activity in *E.coli* with one of inhibition of 27.33±0.57 and the lowest zone of inhibition shown in *Pseudomonas aeruginosa* was 6.00±0.00. (Table 4.7)

The metabolites from *A. fumigatus* isolated from Sport Complex shows that, the highest activity with the zone of inhibition of 21.66±3.78mm in *Streptococcus* then while *Streptococcus* shows no activity 6.00±0.00 as shown in (Table 4.8).

Table 4.6 Antibacterial Activity of Fungal Metabolites from Site (A) against Test Microorganisms (zones in mm)

Concentration	*E. coli*		*P. aeruginosa*		*S. aureus*		*S.pneumoniae*	
	A6	A10	A6	A10	A6	10	A6	A10
AMX 30 µg	27.66 ±0.57[a]	30.00±1.00[a]	22.66±1.15[a]	22.00±2.00 [a]	25.66±0.57 [a]	23.33±0.57 [a]	25.33±0.57 [a]	24.33±1.15 [a]
Disc	6.00 ±0.00[b]	6.00 ±0.00[b]	6.00 ±0.00[b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]
1000µg	15.66±1.15[c]	17.66±0.57[c]	12.66±0.57[c]	12.66±1.15 [c]	16.33±0.57 [c]	17.66±0.57 [c]	15.66±0.57 [c]	15.6 ±1.53 [c]
800 µg	12.33±0.57[d]	12.33±0.57[d]	8.66 ±0.57[d]	8.66 ±2.31 [d]	12.00±0.57 [d]	12.00±1.00 [d]	12.66±1.15 [d]	9.66 ±0.57 [d]
6000 µg	6.33±0.57[e]	6.66 ±0.57[e]	6.00 ±0.00[e]	6.00 ±0.00 [e]	6.00 ±0.00 [e]	7.00 ±1.72 [e]	6.00 ±0.00 [e]	6.33 ±0.57 [e]

Values are mean ±SD, values with different superscript within the same column are considered significantly different (P<0.05)

KEY- A6-*A.fumigatus*

A10- *A.flavus*

Table 4.7:Antimicrobial Activity of Fungal Metabolites from Site (B) against Test Microorganisms (zones in mm)

Concentration	*E. coli*		*P. aeruginosa*		*S. aureus*		*S. pneumoniae*	
	B2	B6	B2	B6	B2	B6	B2	B6
AMX 30 µg	29.66±0.57 [a]	31.33±0.57 [a]	22.00±2.00 [a]	22.00±1.73 [a]	22.66±0.57 [a]	22.00±0.00 [a]	23.00±1.00 [a]	23.00±1.00 [a]
Disc	6.00 ±0.00 [b]	6.00±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]
1000µg	16.33±0.57 [c]	27.33±0.57 [c]	11.66±0.57 [c]	19.33±0.57 [c]	18.66±1.15 [c]	21.00±1.00 [c]	18.66±1.15 [c]	21.66±0.57 [c]
800 µg	11.33±0.57 [d]	15.66±0.57 [d]	9.66±0.57 [d]	13.66±0.57 [d]	17.00±0.00 [d]	13.66±0.57 [d]	15.33±0.57 [d]	13.33±0.57 [d]
600 µg	8.66 ±0.57 [e]	7.33 ±0.57 [e]	6.33 ±0.57 [e]	6.00 ±0.00 [e]	7.66 ±0.57 [e]	23.00±1.00 [e]	8.33 ±0.47 [e]	7.00 ±0.00 [e]

Values are mean ±, values with different super scripts within the same column are considered significantly different ($P<0.05$)

KEY-B2- *A.flavus*

B6- *A.niger*

Table 4.8: Antimicrobial Activity of Fungal Metabolites From site (C) against Test Microorganisms (zones are in mm)

Concentration	*E. coli*		*P. aeruginosa*		*S. aureus*		*S. pneumoniae*	
	C2	C4	C2	C4	C2	C4	C2	C4
AMX 30 µg	30.33±0.57 [a]	29.66±0.57 [a]	19.66±0.57 [a]	22.66±1.15 [a]	24.00±1.00 [a]	30.00±1.00 [a]	25.66±1.53 [a]	23.00±1.73 [a]
Disc	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00 ±0.00 [b]	6.00±0.00 [b]	6.00±0,00 [b]	6.00±0,57 [b]	6.00±0.00 [b]	6.00±0.00 [b]
1000µg	16.66±0.57 [c]	12.00±1.00 [c]	13.66±1.15 [c]	9.00±1.00 [c]	16.66±1,15 [c]	6.66±0.57 [c]	21.66±3.78 [c]	6.33±0.57 [c]
800 µg	12.33±0.57 [d]	7.66±0.57 [d]	12.33±0.57 [d]	6.00±0.00 [d]	10.33±0.57 [d]	6.00±0.00 [d]	12.00±1.00 [d]	6.00±0.00 [d]
600 µg	7.33 ±0.57 [e]	6.00±0.00 [e]	6.00±0.00 [e]	6.00±0.00 [e]	7.33±1.15 [e]	6.00±0.00 [e]	7.00±1.00 [e]	6.00±0.00 [e]

Values are mean ±, values with different super scripts within the same column are considered significantly different (P<0.05).

KEY-C- *A. fumigatus*

The result of the Thin Layer Chromatography of the extracted metabolites shows four fractions in each metabolite except in C2 (*A. fumigatus*) which had three fractions (Table 4.9).The screening of bioactive metabolites fractions for antibacterial activity shows that, fraction 2 of A6, and A10 shows wider zones of inhibitions among their respective fractions, also fraction 4 and 1 had more zones in B6 and C2, and are used for GC-MS analysis, (Table 4.9).

Table 4.9: Thin Layer Chromatography of Purified Antimicrobial Compound

Isolates	Fractions/ Rf values			
	1	2	3	4
A6	0.23	0.31	0.67	0.78
A8	0.42	0.67	0.75	0.83
B2	0.24	0.33	0.71	0.98
B6	0.28	0.59	0.82	N/A
C2	0.5	0.58	0.6	0.81

Key: Rf = Retention Factor

Table 4.10: Screening of Bioactive Metabolite Fractions for Antibacterial Activity

Test organism	A6				A8				A10				B6				C2			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
E. coli	-	++	+	+	-	+	-	+	-	++	+	++	-	+	+	++	++	-	+	NA
P. aeruginosa	-	++	-	-	+	+	+	+	+	++	+	+	-	+	-	++	+	+	+	NA
S. aureus	+	++	-	-	-	++	+	-	-	++	-	+	+	-	-	++	++	-	-	NA
S. pneumoniae	+	+	-	+	-	++	+	+	-	++	-	++	+	+	-	+	++	-	-	NA

Key ++ zones (12-above mm)

\+ zone(7 – 9 mm)

\- no zones

N/A – not available

The metabolites studied in this work showed significant lethality against brine shrimps (table 11). The maximum percentage mortality (%) was observed after 24 hrs at 1000 ppm in each extract (A6-80, A10-100, B2-90, B6-70, and C2-100), while minimum percentage mortality was observed at 10 ppm (A6-0, A10-20, B2-60, B6-30, and C2-40). The LC_{50} of the extract against brine shrimps was calculated, from the result the following are the values of the LC_{50} (A6-114.82, A10-31.112, B2-4.172, B6-67.589, and C2-20.947).

The organic extract studied in this work showed significant lethality against brine shrimps as the results are shown in table 2. The maximum mortality was observed after 24 hrs at 1000 ppm (70%) while minimum at 10 ppm (36.7%). The LC_{50} of the extracts against brine shrimps was calculated.

Table 4.11: Brine shrimp lethality assay of the different fungal metabolites

Sample	Concentration(µg)	No of organisms	Mortality	% Mortality	LC_{50}
A6	1000	10	8	80	114.82
	100	10	4	40	
	10	10	0	0	
A8	1000	10	6	60	152.02
	100	10	6	60	
	10	10	2	20	
B2	1000	10	9	90	4.172
	100	10	7	70	
	10	10	6	60	
B6	1000	10	7	70	67.589
	100	10	6	60	
	10	10	3	30	
C2	1000	10	10	100	20.947
	100	10	7	70	
	10	10	4	40	

Aspergillus genera were found to be the active genus among the isolated fungi from the study area. Morphologic and microscopic appearance of the fungal isolate were used for the identification, *A.fumigates* and *A. flavus* were found in the soil samples from Biological garden, while *A. flavus* and *A. niger* were identify in the soil sample from Ecological garden and *A. flavus* were isolated from the soil sample of Sport complex (Appendice 7).

In this study, the use of organic solvent in the extraction of metabolite from *Aspergillus* led to the identification of more compounds by GC-MS. During GC-MS analysis of solvent (ethyl acetate) extract of *Aspergillus* showed 26 compounds, *Aspergillus niger* from the Botanic Garden, 19 compounds in A*spergillusflavus* from the Ecological Garden, 21 compounds in A*spergillusfumigatus* also from the Ecological Garden, the *Aspergillus fumigatus* and A*spergillus flavus* from Sports Complex showed 20 compounds. The mass spectra was used in compound identification (App 1). A total of 26 different compounds were identified in the fungal extract of the isolate (Table 4.13). The concentrations of compounds for these isolates ranged between 0.1% - 30%. Oleic acid (30%). The least three in abundance were undecanal (0.1%). The (App 2) of mass spectra was used in compound identification. A total of 26 different compounds were identified from the fungal extracts of the isolated Table 4.15. The concentrations of compounds for this isolate ranged between 0.2% - 35%. Oleic acid,(35%). The least one in abundance was undecanoic acid (0.2%).

A total of nineteen 19 different compounds were identified in the fungal extract of the isolate (Table 4.16). The concentrations of compounds for this isolate ranged between 0.1% - 33%. Oleic acid (33%). The least in abundance was Methyl tetradecanoate (0.1%). Table 18 was used to identify a total of 20 different compounds produced in the fungal extracts. The concentrations of compounds for these isolates ranged between 0.1% - 32%. Oleic acid,(33%). The least in

abundance was Methyl tetradecanoate (0.1). A total of 20 different compounds were identified in the fungal extract of the isolate (Table 4.16) using mass spectra. The concentrations of compounds for this isolate ranged between 0.1% - 32%. Oleic acid,(30%). The least in abundance were Propane, 1,1-dimethoxy-2-methyl (0.18).

Table4. 13: Chemical Composition of the Extracts of *A. fumigatus* (GC-MS analysis) A10

S/N	Retention time	Compound name	Molecular formula	Molecular weight	Area %
1	3.609	Hexenal	$C_8H_{14}O$	126	0.67
2	4.417	Nonanal	C_9H18O	142	0.65
3	4.658	Cyclopentasiloxane	$C_{10}H_{30}O_5Si_5$	370	0.28
4	5.202	Decanal	$C_{10}H_{20}O$	156	0.36
5	5.674	Propane	$C6H_{14}O_2$	118	0.41
6	6.731	Undecanal	$C_{11}H22O$	170	0.19
7	7.686	Hexadecanal	$C_{16}H_{32}O$	240	0.47
8	7.781	Dodecanoic acid	$C_{13}H_{26}O_2$	214	0.51
9	8.864	Decanal	$C_{10}H_{20}O$	156	0.25
10	10.145	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	1.10
11	10.741	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.69
12	11.492	1-Octadecyne	$C_{18}H_{34}$	250	0.85
13	11.983	Phytol	$C_{20}H_{40}O$	296	0.40
14	12.208	13-Docosenoic acid	$C_{23}H_{44}O_2$	352	0.40
15	12.433	Pentadecanoic acid	$C_{17}H_{34}O_2$	270	5.55
16	13.033	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	15.29
17	14.208	:9,12-Octadecadienoic acid	$C_{19}H_{34}O_2$	294	3.66
18	14.275	6-Octadecenoic acid	$C_{19}H_{36}O_2$	296	11.50
19	14.517	Octadecanoic acid	$C_{19}H_{38}O_2$	298	2.71
20	14.950	Oleic Acid	$C_{18}H_{34}O_2$	282	30.15
21	15.142	Octadecanoic acid	$C_{18}H_{36}O_2$	284	11.60
22	16.042	1,1,1-Trifluoroheptadecen-2-one	$C_{17}H_{31}F_3O$	308	3.66
23	16.692	Hexadecanoic acid	$C_{37}H_{74}NO_8P$	691	1.20
24	17.150	Eicosanoic acid	$C_{21}H_{42}O_2$	326	0.28
25	18.908	(E)-13-Docosenoic acid	$C_{22}H_{42}O_2$	338	4.78
26	19.608	9-Octadecenal	$C_{18}H_{34}O$	266	2.37

Table 4.14: Chemical Composition of the Extract of *A. flavus* (GC-MS analysis) A6

S/N	Retention time	Compound name	Molecular formula	Molecular weight	Area %
1	3.575	Hexenal	$C_8H_{14}O$	126	1.01
2	4.417	Propane	$C_6H_{14}O_2$	118	0.33
3	5.667	Propane	$C_6H_{14}O_2$	118	0.74
4	7.300	Ethanol	$C_{10}H_{22}O_4$	206	0.43
5	9.750	7-(1,3-Dimethylbuta-1	$C_{15}H_{22}O_2$	234	0.26
6	10.142	Undecanoic acid	$C_{12}H_{23}BrO_2$	287	0.21
7	10.492	2-Octanol	$C_{10}H_{20}O$	156	0.24
8	10.750	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.39
9	11.983	alpha.-D-Glucofuranosidurono-6	$C_{16}H_{16}O_7S$	352	0.67
10	12.425	Pentadecanoic acid	$C_{17}H_{34}O_2$	270	6.14
11	13.017	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	18.50
12	14.200	9,12-Octadecadienoic acid	$C_{19}H_{34}O_2$	294	1.75
13	14.258	11-Octadecenoic acid	$C_{19}H_{36}O_2$	296	9.70
14	14.517	Octadecanoic acid	$C_{19}H_{38}O_2$	298	2.92
15	14.942	Oleic Acid	$C_{18}H_{34}O_2$	282	35.74
16	15.133	Octadecanoic acid	$C_{18}H_{36}O_2$	284	10.17
17	16.042	1,1,1-Trifluoroheptadecen-2-one	$C_{17}H_{31}F_3O$	308	2.77
18	16.692	Decane, 1-fluoro	$C_{10}H_{21}F$	160	1.25
19	18.900	(E)-13-Docosenoic acid	$C_{22}H_{42}O_2$	338	4.34
20	19.617	9-Octadecenal	$C_{18}H_{34}O$	266	2.13
21	:22.675	Tetracosanoic acid	$C_{25}H_{50}O_2$	382	0.32

Table 4.15: Chemical Composition of the Extract of *A. flavus* (GC-MS analysis) B2

S/N	Retention time	Compound name	Molecular formula	Molecular weight	Area %
1	3.592	2-Hexenal, 2-ethyl-	$C_8H_{14}O$	126	0.84
2	4.425	Propane, 1,1-dimethoxy-2-methyl-	$C_6H_{14}O_2$	118	0.27
3	5.675	Propane, 1,1-dimethoxy-2-methyl-	$C_6H_{14}O_2$	118	0.60
4	7.300	Ethanol, 2-[2-(2-butoxyethoxy)ethoxy]-	$C_{10}H_{22}O_4$	206	0.27
5	10.150	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	0.16
6	10.742	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.58
7	11.492	1-Octadecyne	$C_{18}H_{34}$	250	0.31
8	12.208	2-Heptadecanone	$C_{17}H_{34}O$	254	0.44
9	12.433	Pentadecanoic acid	$C_{17}H_{34}O_2$	270	5.21
10	13.033	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	16.93
11	14.275	11-Octadecenoic acid	$C_{19}H_{36}O_2$	296	8.30
12	14.517	Octadecanoic acid	$C_{19}H_{38}O_2$	298	2.52
13	14.967	Oleic Acid	$C_{18}H_{34}O_2$	282	33.79
14	15.150	Octadecanoic acid	$C_{18}H_{36}O_2$	284	20.19
15	16.042	1,1,1-Trifluoroheptadecen-2-one	$C_{17}H_{31}F_3O$	308	2.57
16	16.700	Hexadecanoic acid	$C_{37}H_{74}NO_8P$	691	0.95
17	18.908	9-Octadecenal	$C_{18}H_{34}O$	266	3.40
18	19.617	(E)-13-Docosenoic acid	$C_{22}H_{42}O_2$	338	1.98
19	22.067	E-11-Hexadecenal	$C_{16}H_{30}O$	238	0.69

Table 4.16: Chemical Composition of the Extract of *A. niger* (GC-MS analysis) B6

S/N	Retention time	Compound name	Molecular formula	Molecular weight	Area %
1	3.585	2-Hexenal, 2-ethyl-	$C_8H_{14}O$	126	1.41
2	4.423	Propane, 1,1-dimethoxy-2-methyl-	$C_6H_{14}O_2$	118	0.39
3	5.668	Propane, 1,1-dimethoxy-2-methyl-	$C_6H_{14}O_2$	118	0.76
4	7.311	Ethanol, 2-[2-(2-butoxyethoxy)ethoxy]-	$C_{10}H_{22}O_4$	206	0.36
5	10.148	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	0.18
6	10.743	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.53
7	12.208	Cyclopropaneoctanoic acid, 2-hexyl-, methyl ester	$C_{18}H_{34}O_2$	282	0.55
8	12.434	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270	7.11
9	13.034	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	16.44
10	14.206	9,12-Octadecadienoic acid, methyl ester, (E,E)	$C_{19}H_{34}O_2$	294	2.24
11	14.279	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	10.56
12	14.518	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	4.03
13	14.951	Oleic Acid	$C_{18}H_{34}O_2$	282	32.17
14	15.142	Octadecanoic acid	$C_{18}H_{36}O_2$	284	13.40
15	16.040	16-Hexadecanoyl hydrazide	$C_{16}H_{34}N_2O$	270	3.51
16	16.698	Palmitin, 1,2-di-, 2-aminoethyl hydrogen phospha	$C_{37}H_{74}NO_8P$	691	0.91
17	17.156	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326	0.44
18	18.901	5-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	2.95
19	19.616	2-Methyl-Z,Z-3,13-octadecadienol	$C_{19}H_{36}O$	280	1.58
20	22.680	Tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$	382	0.46

Table 4.17: Chemical Composition of the Extract of *A. fumigatus* (GC-MS analysis) C2

S/N	Retention time	Compound name	Molecular formula	Molecular weight	Area %
1	3.582	2-Hexenal, 2-ethyl-	$C_8H_{14}O$	126	0.7
2	4.421	Propane, 1,1-dimethoxy-2-methyl	$C_6H_{14}O_2$	118	0.18
3	5.668	Propane, 1,1-dimethoxy-2-methyl-	$C_6H_{14}O_2$	118	0.38
4	10.148	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	0.25
5	10.742	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.44
6	11.294	Isopropyl Myristate	$C_{17}H_{34}O_2$	270	0.25
7	12.209	2-Tridecanone	$C_{13}H_{26}O$	198	0.50
8	12.434	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270	5.97
9	13.035	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	15.51
10	14.209	9,12-Octadecadienoic acid, methyl ester, (E,E)	$C_{19}H_{34}O_2$	294	3.88
11	14.279	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	8.95
12	14.519	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	5.22
13	14.956	Oleic Acid	$C_{18}H_{34}O_2$	282	30.88
14	15.145	Octadecanoic acid	$C_{18}H_{36}O_2$	284	12.89
15	16.042	1,1,1-Trifluoroheptadecen-2-one	$C_{17}H_{31}F_3O$	308	5.70
16	16.702	Palmitin, 1,2-di-, 2-aminoethyl hydrogen phospha	$C_{37}H_{74}NO_8P$	691	1.99
17	17.157	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326	0.62
18	18.904	(E)-13-Docosenoic acid	$C_{22}H_{42}O_2$	338	3.56
19	19.620	9-Octadecenal	$C_{18}H_{34}O$	266	1.72
20	22.680	Tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$	382	0.39

4.2 DISCUSSION

The result of physical properties of soil samples revealed that soil environments of Botanic Garden, Ecological Garden are loamy, while the soil samples from Sport Complex and Staff School are sandy; this indicates why Botanic Garden and Ecological Garden has a high fungal count as loamy soil contain more organic matter which serves as a nutrient than sandy soil. The pH values of the soil sample showed that Sport Complex and Staff School are slightly acidic with pH of 6.9 and 6.9 respectively and Ecological Garden was slightly alkaline (pH 7.4) while Botanical garden was slightly alkaline with pH of 8.3. The temperature of the soils ranged between 25^{o}C and 26^{o}C, this agreed with Nester *et al* (2007). Various species of fungi can grow at temperature ranging from -6^{o}c to 50^{o}c, however the optimum temperature for the majority of fungi is in this range of 20^{o}c to 35^{o}c. Also pH which different fungi can grow varies widely, ranging from as low as 2.2 to as high as 9.6. The pH can limit the activity of enzymes with which an organism is able to synthesize new protoplasm. As in the case of temperature, there exists for each organism an optimum concentration of hydrogen ions in which it grows bests. The pH values above and below which an organism fails to grow are, respectively, referred to as the minimum and maximum hydrogen ion concentrations. (Harold 2002)

Table 4.2: showed the result of total fungal counts of Botanic Garden, Ecological Garden, and Sport Complex with counts of $6.3x10^{-3}$, $5.8x10^{-3}$, and $4.3x10^{-3}$ cfu/ml respectively while Staff School Compound had the counts of $2.3x10^{-3}$ CFU/ml which was the least count.

The results from this study revealed the presence of fungi capable of producing antimicrobial metabolites, in the study site. A number of isolates (8) exhibited inhibitory action in the primary screening and the high proportion of antibiotic producers isolated from the primary screening

may be associated with its ecological role, serving as a defensive mechanism to maintain their niches, or enabling the invasion of an established microbial community (Brandelli, 2004). Although six (6) eight (8) isolates amounting to 75% showed antimicrobial activity in this study. However some inhibitory screening investigations have recorded values closed to what was obtained in this study while other recorded different values either higher or lower than this study. In a study, carried out by Adelaide (2011) reported that out of 119 isolates from soil source 23% of the isolates were active against test organisms. Ivanova *et al*. (1998) reported that out of the 491 bacteria isolated from different marine sources, 26% of the isolates shows activity on the test bacteria. Zheng *et al.* (2005) also reported that eight out of twenty-nine (29) strains, representing 28 % of the microbes considered in their study were able to inhibit the growth of at least one of the target microorganisms. Another study carried out by Brandelli *et al*. (2004) recorded 70% of the isolates that are able to produce bioactive metabolites, from the Amazon Basin whilst O'Brien *et al*. (2004) recorded as low as 0.29% (13 out of 4496) of active microbes from soil samples collected at different locations in the Antarctica. The differences among the detection rates reported in literature strongly depend on the isolation and assay procedures, test organisms, type of media used, as well as the sources of bacterial isolates (Giudice *et al*., 2007).

The implication of these findings is that, the genus of *Aspergillus* present in the soil environment of Bayero University Kano was found to be of significant to this study interms of antimicrobial metabolites. Aneja (2003) reported that soil is the best economical source of antibiotic producing microorganisms, and the soils around the world are continuously being screened for novel antibiotics. The soil is thus a veritable source of antibiotic producing microorganisms from which novel antibiotics can be sought. The result of this investigation has further confirmed that soil dwelling antibiotic producing fungi are mostly in the genus *Aspergillus* as reported by

several workers (Schlegle 2003). Therefore the antibiotic producing isolate from the soil environment of Bayero University Kano can be harnessed for the production of novel antibiotics.

It was observed that, the isolates inhibited Gram-positive bacteria better than the Gram-negative bacteria. This could be attributed to the differences in the sensitivities of Gram-positive and Gram-negative bacteria, largely due to the differences in the structure and composition of their cell walls. Gram negative bacteria possess a thin peptidoglycan layer and a unique outer membrane which consists of lipo-polysaccharide (LPS) components. This outer membrane makes the cell wall impermeable to lipophilic solutes thus blocking certain antibiotics such as penicillin, dyes, and detergents from penetrating the cell, like wised these metabolites being it is lipid in nature. Gram-positive bacteria only possess the peptidoglycan layer which is not a very effective permeability barrier (Scherrer and Gerhardt, 1971).

Some of the isolates exhibited inhibitory activities in the primary screening but when they were isolated into pure cultures, they lost this effect. Several reasons could account for the presence of inhibition zones exhibited by isolates in the master plates which later disappeared when the isolates were grown as pure cultures. According to Bushell and Grafe (1989), in the master plates the isolates exhibited a higher degree of competition for space which provoked the cells to secrete diverse compounds to serve as competition mechanisms. However in the absence of competition they were unable to secrete compounds in the liquid media. Also as cells in the master plates were closer to each other, chemical signals could have been emitted which could induce the isolates to secrete inhibitory substances (Dobashi*et al*,. 1998). Also the genes that synthesize antimicrobial agents may have to be activated by diverse signals, for example the presence of a protein or enzyme, so that if the target that was used does not synthesize the same inducers that were synthesized in the master plates then the antagonistic substance will not be

produced. The antimicrobial activity exhibited by the supernatant solutions of isolates A 10, A6, B2, B6, C2 and C4 showed that antimicrobial agents are not produced only in the presence of competition. In some organisms, these metabolites are produced perhaps to serve as regulators for cellular differentiation processes and in other instances, as a means of avoiding predation on themselves (Dobashi *et al.,*1998) and also increase predation on other bacteria. The stability study of the cell-free filtrates indicated that the antimicrobial agents of these isolates can gradually deteriorate with time.

The quantity of the metabolites recovers from the fermented broth varies among the fungi, in which B6 has high yield here 1.43g/liter of the broth was recover, follows by A6 with 1.25g/liter, and the least was A10 and B2 with 0.68g/liter and 0.8g/liter respectively. Ghada *et al.* (2011) have recovered 500mg and 400mg of crude extract from *A. niger* and *A. flavus*, this result is a little lower than this finding, probably the difference comes due to the difference in the media composition, they made use of malt extract broth and this research potato dextrose broth was employed. Also Zafar *et al.* (2015) in their research using *A. niger* recovered 300mg/liter, this also is slightly lower than this result, and it may be due to the difference of incubation period, where they incubated the broth for five days and the incubation period of this research was fourteen days.

The crude antibiotic extract of isolate A6 exhibited activity against all the test organisms except *Pseudomonas aeruginosa*. These results indicated that the antagonistic activity was due to the production of an antimicrobial compound which can be extracted from the growth medium with organic solvents. MIC of the crude extract ranged from 0.25mg/ml to 4mg/ml. The extract appears to be bacteriostatic in its mode of action. Bacteriostatic agents (e.g. β-lactams, chloramphenicol, clindamycin, macrolides and linezolid) have been effectively used for

treatment of a range of bacterial infections, including endocarditis, meningitis, and osteomyelitis (Pankey and Sabath, 2004). Furthermore, a bacteriostatic agent like clindamycin has been shown to completely inhibit the toxic shock syndrome toxin-1 production by *Staphylococcus aureus* (Van Lagevelde *et al.*, 1997) and toxin production in both *Streptococci* and *Staphylococci* (Russell and Pachorek, 2000). These reports suggest that the active constituents present in this crude extract have the potential of being efficacious in the treatment of various infectious. The extract is therefore a rich source of bioactive compounds which can serve as leads for the development of efficacious antibiotics.

Ndwigah *et al*. (2016) in his research found seventeen compounds in the extract with high percentage abundance in Isopentyl alcohol (3.9%), and least abundance of N-(1-Cyclopenten-1-yl)-morpholine (0.1%). Khatijah *et al.* (2014) stated that there were four major compounds when he used chloroform as a solvent for extraction that include dodecanoic acid, methyl ester (17.76%), cyclododecacane (15.038%), octadecanoic acid (11.059%) methyl tetradecanoate (6.16%) and hexadecanoic acid (5.077%), the result varies from the finding of this studies in with methyl teradecanoate(0.16%) in B2 and 0.18% in C2. Octadecanoic acid showed higher percentage with 20.19% in B2, and 11.6%, 10.17% and 13.40% in the extract of A6, which indicates more percentage abundance; this is probably due to difference in the media composition or their source, or even period of incubation. Agoramoorthy *et al*. (2007) find out that the GC-MS analysis of the extract of the screen fungi has fourteen compounds which include; Palmitic acid (56.02%), steric acid (2.8%), oleic acids (1.7%). While in my study A10 has oleic acid of (30%), A.6 (35%), C2 32.17%, and C4 30%, which all shows greater percentage abundance. Udgire and Pathade (2013) in his research the chemical analysis made shows ten

compounds and among the most abundance include pentadecanoic acid, 9, 12-octadecanoic acid which they are not most abundance in my findings.

The Agoramoorthy *et al*. (2007) found that compounds palmitic acids, oleic acid from *A. fumigatus and A.flavus* have antibacterial and antifungal activities, also Subbiahanadar *et al.*(2014) states that the hexadecanoic acid and octadecanoic acid were found to be a powerful antibacterial and antioxidant and ant cancer agents.

From the result of the brine shrimp lethality assay, the organic extract studied in this work showed significant lethality against brine shrimp as the result shown (Table 9), as the values of Lc_{50} is lessthan 1000ppm (Mc Laughlin *et al*,.1991) the result agreed with that of Zafar *et al.* (2015), as *Aspergillus* genus produce toxic metabolites.

CHAPTER FIVE

5.1 Conclusion

It was concluded from this study that, the antibiotic producing fungi are present in the soil of Bayero University, Kano. Out of the 60 fungi isolated from the study sites, 6 of them were active against all the test organisms of which all exhibited zones of inhibition greater than 15 mm in the concentration of 1000µg, except against *Pseudomonas aeruginosa*. The fungal isolates from Biologic Garden were higher than what was recorded in Ecological Garden, with Staff School Compound having the least fungal isolates.

From the preliminary screening, Out of the 60 isolated fungi from the study sites, only six (6) were found as potential antibiotic producing fungi, and isolate from Sport Complex yielded higher metabolite of 5.7g from four liter (4L) of the fermented broth, followed by that from Biologic Garden with 5g, and the least was from Ecological Garden with 3.2g.

The chemical analysis revealed that the extracts consist of many bioactive compounds, as it shows many bands from the TLC results, and more than 20 compounds from the GC-MS analysis, some of the metabolites found includedOleic acid, n-Hexadecanoic acid, and Octadecanoic acid. And the metabolites are found to be toxic from the result of brine shrimp assay.

5.2 Recommendation

The present studies show that, the metabolites exhibited antibacterial activity however, the finding of this study, the following recommendations can be:

i. Further studies are recommended on the use of metabolites in cancer bioassay due to its toxicity.

ii. Physical agents are to be used to induce mutationin the isolated fungi for possible yield improvement, and possible identification of any new bioactive compounds.

iii. Also the metabolites should be subjected to other biological assays such as antifungal

iv. . There is also the need to evaluate the metabolites for stability in solution, pH, and temperature as potential antibiotics.

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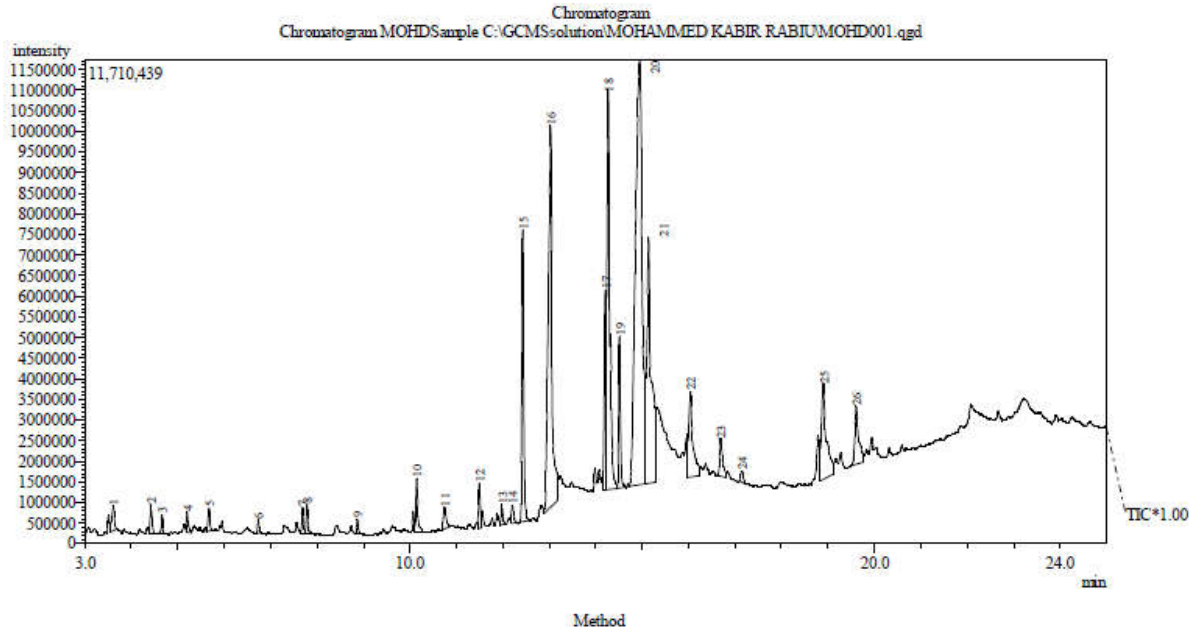
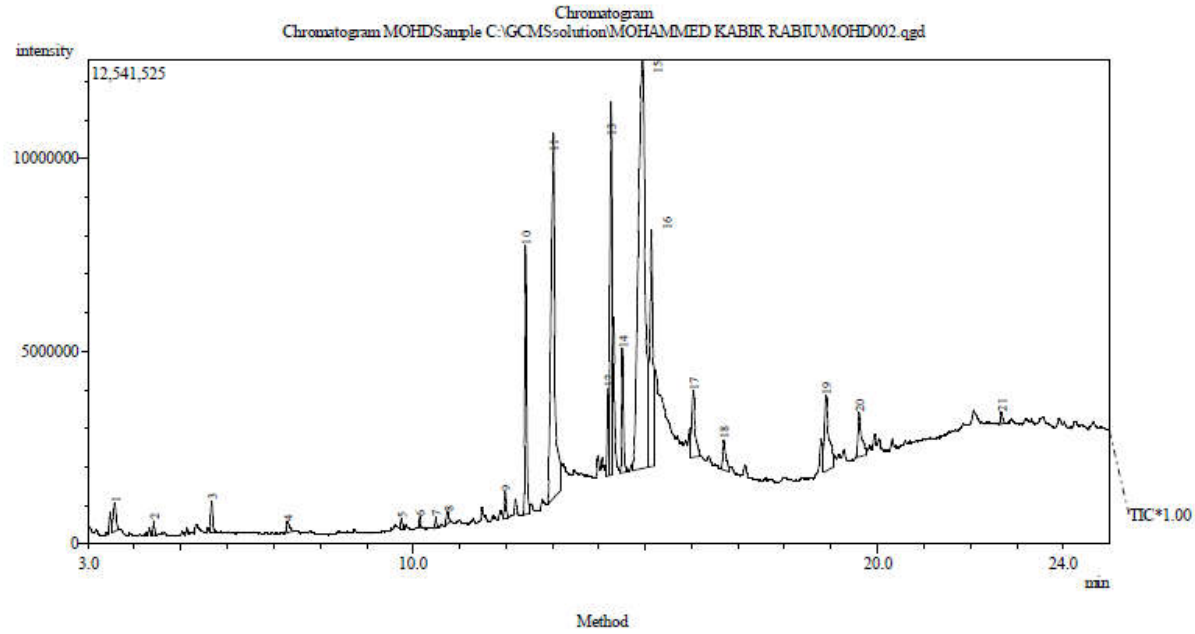
APPENDICES

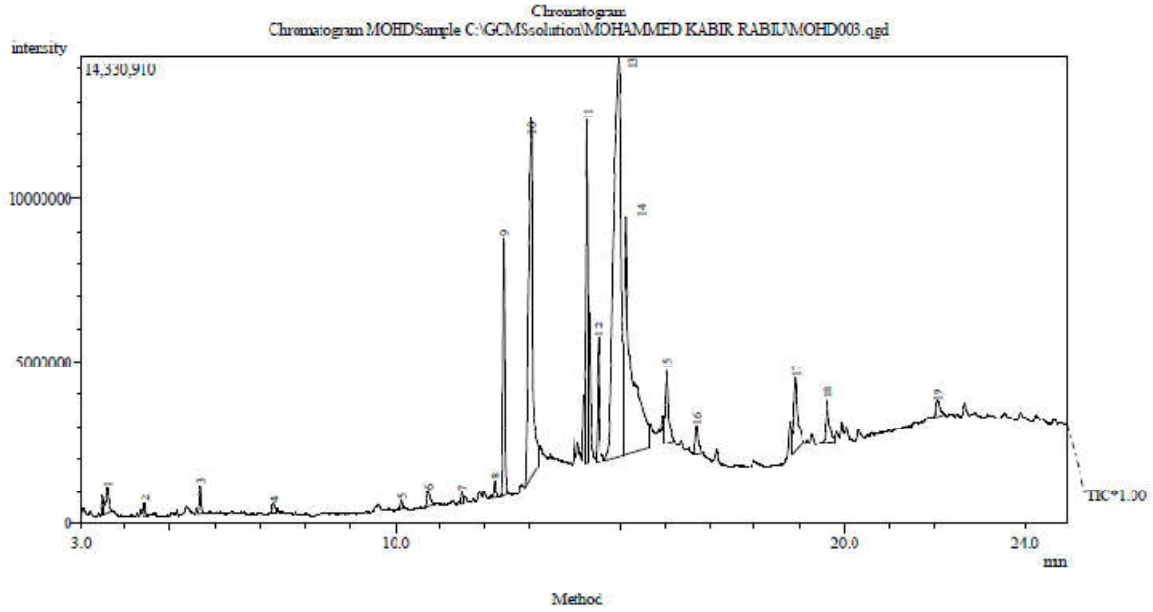
Appendix 1: GC-MS chromatogram of sample A6



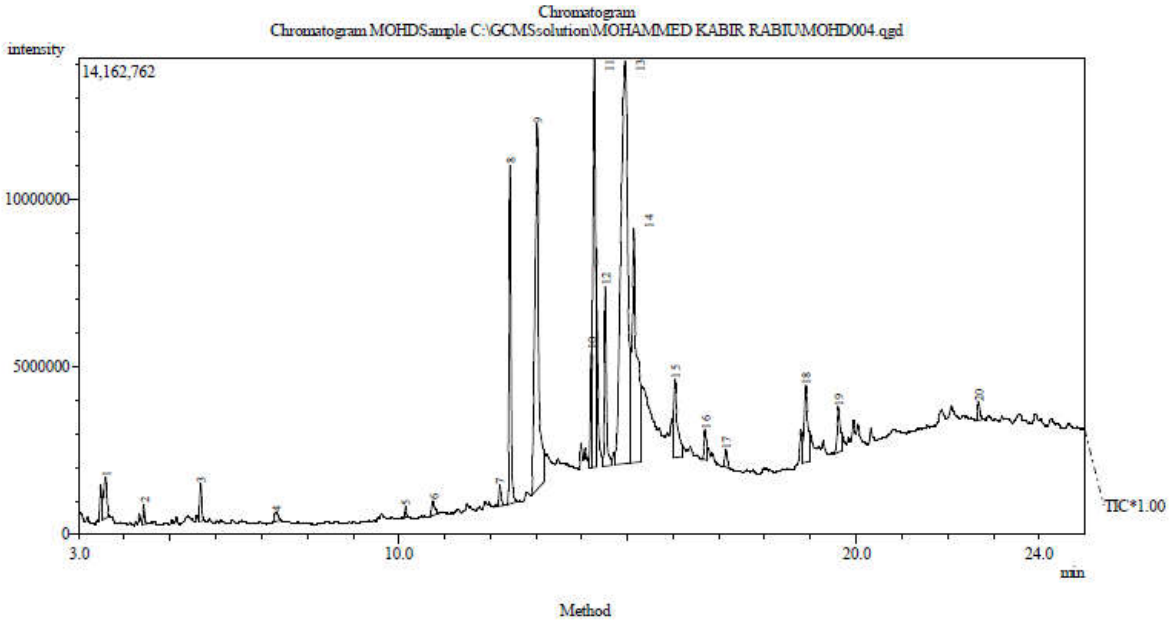
Appendix 2: GC-MS chromatogram of sample A10



Appendix 3: GC-MS chromatogram of sample B2



Appendix 4: GC-MS chromatogram of sample B6



Appendix 5: GC-MS chromatogram of sample C2

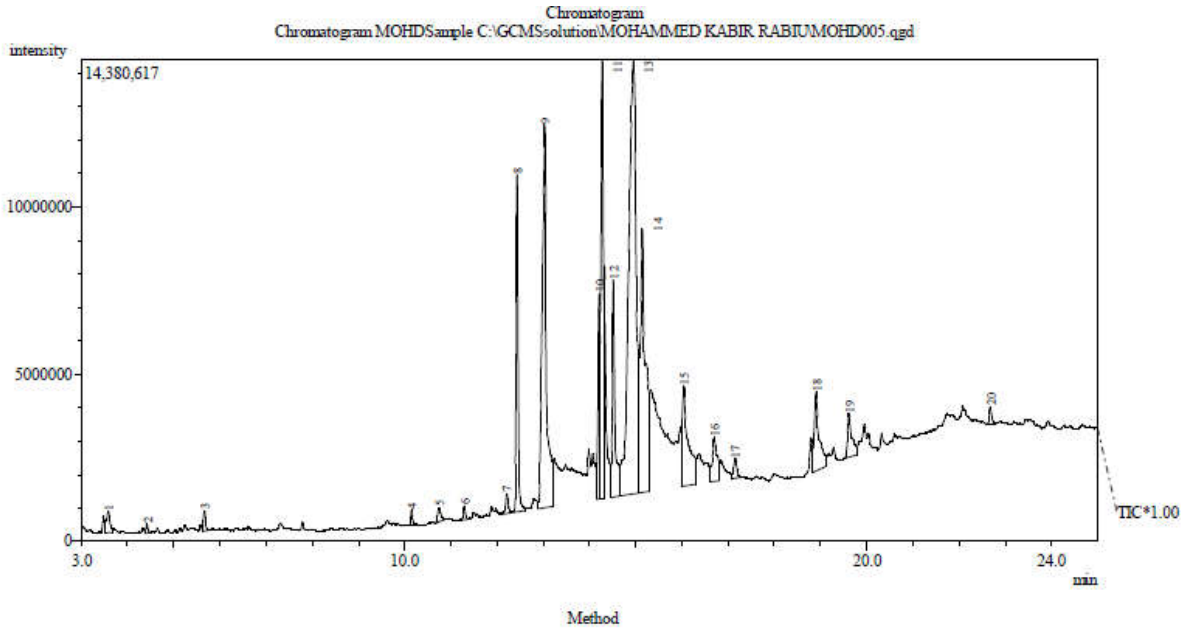


Figure 6: Identification of the fungal isolate

Isolated code	Source	Color of hyphae	Color of substrate hyphae	Nature of hyphae	Shape of hyphae	Appearance of conidiospore	Inference
A6	Biological garden	gray	Tan	Septate and branch	Oval conidiospore	Long and erect non conidiospore	*A.fumigatus*
A10	Biological garden	green	Red-brown	Septate and branch	Spherical conidiospore	Long and erect non conidiospore	*A.flavus*
B2	Ecological garden	green	Red-brown	Septate and branch	Spherical conidiospore	Long and erect non conidiospore	*A.flavus*
B6	Ecological garden	black	Yellow	Septate and branch	Spherical conidiospore	Long and erect non conidiospore	*A.niger*
C2	Sport complex	green	Red-brown	Septate and branch	Spherical conidiospore	Long and erect non conidiospore	*A.flavus*

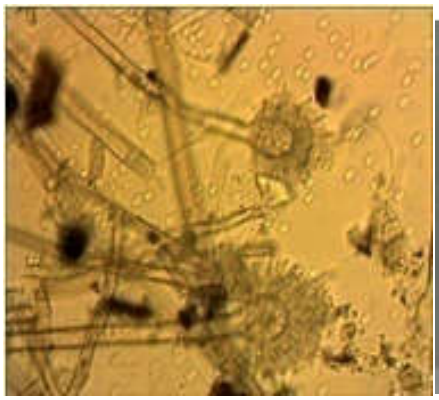



Plate 1A: Microscopic appearance of *A. flavus*Plate 1B: Colonial appearance of *A. flavus*

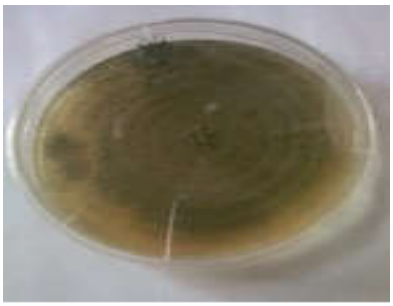



Plate 2A: Microscopic appearance of *A. fumigatesPlate 2B:* Colonial appearance of *A. fumigates*

Plate 3A: Microscopic appearance of *A. niger*Plate 3B: Colonial appearance of *A. niger*