MOLECULAR CHARACTERIZATION OF FUNGI ISOLATED FROM PHARMACEUTICAL PRODUCTS

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15010101013

A PROJECT REPORT SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE, (MICROBIOLOGY) MOUNTAIN TOP UNIVERSITY, OGUN STATE, NIGERIA.

JULY, 2019

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CERTIFICATION

I hereby certify that OTAIKU, EYIOWUAWI TOLUWASE dully carried out this research under my supervision.

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DEDICATION

I dedicate this project to the Almighty God, who has been my strength, provider and my sustainer. Also to my mum (Mrs. Olawole) and my sister and her husband (Oluwaseyi and Oluwaseyi) for their unending support.

ACKNOWLEDGEMENT

I give all glory to God Almighty, who has been the solid foundation that has kept me standing through all my days in as a student in Mountain Top University, all adoration be unto his name. I would also want to thank my supervisor Dr. Oyebanji for pushing me past my limits. Also to all academic staff in the department of Biological sciences.

My profound gratitude goes to my loving and ever supporting mother Mrs Olawole, my sister Oluwaseyi and her husband Oluwafemi for their unending support. Also, my dearest appreciation goes to Ayomide for always helping me with prayers, a big thank you to everyone who as by any means helped me, may the Lord never forget your labour of love.

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ABSTRACT

Studies on Fungi isolated from pharmaceutical products from pharmacy and stores were carried out between the month of April 2019 and July 2019. Collection, isolation, characterization and identification of the isolated Fungi were achieved using both conventional laboratory methods and molecular methods (DNA extraction, PCR and DNA sequencing).Thirteen fungi was isolated out of which seven fungal species were identified and they include Aspergillus tamari strain A, Aspergillus tubingenesis strain, Aspergillus tamari strain B, Aspergillus ellipticus, Aspergillus niger strain A, Aspergillus niger strain B, Aspergillus niger strain C, from the pharmaceutical drugs sampled using 3 protocols. The result of the BLAST DNA sequence database of the isolated fungi revealed that no sequence data was 100% homologous with those in the Gene bank.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF STUDY

Pharmaceutical medicines are used to eradicate so many illnesses in humans (Mugoyela, 2010) and they are one of Nigeria's rapidly growing and expanding economies. The quality of drugs available at home varies considerably as they are predominantly retail oriented. Oral medicines are highly prone to large microbial flora owing to production circumstances (Aulton, 2002). Contaminations of raw materials, sweeteners, techniques of restoration, incorrect storage and impacts of handling can contribute to the growth of microbes (Nirmala, et al., 2012) therefore, continuous examination of the pharmaceutical products and the microbial quality (Mwambete et al., 2009. Kallings et al., 1996), particularly of the oral drugs that are made for mostly children, is therefore of significant to consumer safety demand. Some of the dosage forms of oral dugs, if stored in favourable environment, can serve as substrates for microorganism (Hossain, 2009. Shaikh, et al., 1998. Lund, 1994. Lowe, 2001). Unfortunately, some studies in this area have not been conducted in Nigeria (Moniruzzaman et al., 2012). Microbial contaminations may ultimately contribute to secondary bacterial infections in pediatric patients. (Adeshina et al., 2009)

Fungal contamination in pharmaceutical products is of major concern. Firstly, it can cause spoilage of the product, (Sandle, 2014) spoilage not only affects the product's parts and efficacy, but also discourages the patient from taking the drugs. Secondly, product contamination represents a health hazard for the patient and the extent of the hazard may vary from product to product and patient to patient depending on the micro flora present, the route of administration and the patients resistance. All pharmaceutical products manufactured in the sector and any other means of manufacturing have the potential for microorganism contamination. In the case of sterile products, any microbial contamination poses an unacceptable danger, in the case of non-sterile products, the contamination implications are based on whether the microorganisms can be deemed objectionable and to the extent that it can harm the patient (Sandle et al., 2014).

Non-sterile fungal contamination is one of the key variables in product recalls, abandonment of production and manufacturing losses. All these lead to decreased in shelf life by compromising the integrity of the item or presenting patients with potential health hazards (Sandle, 2015).

Most of the reports related to pharmaceutical product contamination were mainly about bacterial contamination. There has been an overview of fungi that also poses the same threat as bacteria to the pharmaceutical product. This study considers fungi an issue and asserts that the danger posed to pharmaceutical goods by fungi are greater than those suggested by industrial level. This research is therefore intended to determine the microbial quality of two different types of oral drugs, specifically paracetamol syrup and cough syrup, primarily used for children in Nigeria by various reputable pharmaceutical company.

1.2 STATEMENT OF PROBLEM

During production, microorganisms may contaminate pharmaceutical products, which may result in the spoilage and rejection of the contaminated product or may even cause impairment or death to the consumer (Denyer et al., 2004). The major problem of contamination is microorganisms such as bacteria and fungi. There are three major laws that analyze all the safe process components which are:

- Health and Safety at Work Act 1984
- Control of Substances Hazardous to Health (COSHH) 1944
- Genetically Modified Organisms (Contained Use) Regulations 1992 (amended in 1996) (Baird et al., 2000)

Microbial contamination from raw materials, as well as from process operators, packaging materials and environments can act as contaminants, thus ensuring proper hygiene of the manufacturing equipment at all stages of the processes should be of high priority and importance.

During production, storage and use is not only cause for microbial spoilage in pharmaceutical products, but also the resulting behavior of microorganisms within products. The entry of microorganisms into the products is aided by many physiochemical factors like packaging, addictive's etc. (Friedel, 1998). This leads to loss of potency of active ingredients (Coker, 2005).

1.3 AIM AND OBJECTIVES OF STUDY

The aim of this study is to identify and characterize at molecular at molecular level fungi contaminants associated with some products in Nigeria.

The specific objectives are to:

1. Isolate and identify fungi contaminants from the pharmaceutical products.
2. Characterization of fungal contaminants from the pharmaceutical products using three (3) protocols.

1.4 SIGNIFICANCE OF STUDY

Based on the paucity of report and research study on fungal contaminant in pharmaceutical products. This study will provide accurate information on fungal identification and fungi causing contamination.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 FUNGI

A fungus is any member of a large group of eukaryotic organisms that include microorganisms such as yeasts as well as the mushrooms (Moore, 1980). These organisms are classified under the Kingdom Fungi. What makes a fungus unique and different from animals, plants, bacteria and some protists is that their cell walls contain chitin (Bowman, 2006). Fungi are the principal decomposers in ecological systems. These and other differences place fungi in a single group of related organisms, named the Eumycota (true fungi), which share a common ancestor (form a monophyletic group), an interpretation that is also strongly supported by molecular phylogenetic. This fungal group is distinct from the structurally similar myxomycetes (slime molds) and oomycetes (water molds). The study of fungi is known as Mycology.

They are abundant worldwide. Most fungi are inconspicuous because of the small size of their structures, and their cryptic lifestyles in soil, on dead matter, and as symbionts of animals, some other fungi and plants. They may become noticeable when fruiting, either as mushrooms or as moulds. Fungi perform an essential role in the decomposition of organic matter and have fundamental roles in nutrient cycling and exchange. They have long been used as a direct source of food, such as mushrooms and truffles, as a leavening agent for bread, and in fermentation of various food products, such as beer, wine, and soy sauce. Since the 1940s, fungi have been used industrially and in detergents. Fungi are also used as biological pesticides to control weeds, plant disease and insects pests. Many species produce bioactive compounds called mycotoxins, such as alkaloids and polypeptides, that are toxic to animals including humans. The fruiting structures of a few species contain psychotropic compounds and are consumed recreationally or in traditional spiritual ceremonies. Fungi can break down manufactured materials and buildings, and become significant pathogens of humans and other animals. Losses of crops due to fungal diseases (e.g., rice blast disease) or food spoilage can have a large impact on human food supplies and local economies.

The fungus kingdom encompasses an enormous diversity of taxa with varied ecologies, life cycle strategies, and morphologies ranging from unicellular aquatic chytrids to large mushrooms. However, little is known of the true biodiversity of Kingdom Fungi, which has been estimated at 2.2 million to 3.8 million species. Of these, only about 120,000 have been described, with over 8,000 species known to be detrimental to plants and at least 300 that can be pathogenic to humans. Ever since the pioneering 18th and 19th century taxonomical works of Carl Linnaeus, Christian Hendrik Person and Elias Magnus Fries, fungi have been classified according to their morphology (e.g characteristics such as spore color or microscopic features) or physiology. Advances in molecular genetics have opened the way for DNA analysis to be incorporated into taxonomy, which has sometimes challenged the historical groupings based on morphology and other traits. Phylogenetic studies published in the last decade have helped reshape the classification within Kingdom Fungi, which is divided into one sub-kingdom, seven divisions, and ten sub-division. A group of fungi present in a particular area is known as mycobiota (Bruns, 2006).

2.1.1 ETYMOLOGY OF FUNGI

The English word fungus is directly adopted from the Latin fungus, used in the writings of Horace and Pliny. This in turn is derived from the Greek word sphongos (sponge), which refers to the macroscopic structures and morphology of mushrooms and molds, the root is also used in other languages, such as the German Schwamm (sponge) and Schimmel (mould). The word mycology was derived from the Greek mykes (mushroom) and logos (disclosure), to denote the scientific study of fungi (Hawksworth, 2006).

2.1.2 CHARACTERISTICS OF FUNGI

Before the introduction of molecular methods for phylogenetic analysis, taxonomists considered fungi to be members of the plant kingdom because of similarities in lifestyle: both fungi and plants are mainly immobile, and have similarities in general morphology and growth habitat. Like plants, fungi often grow in soil and, in the case of mushrooms, form conspicuous fruit bodies, which sometimes resemble plants such as mosses. The fungi are now considered a separate kingdom, distinct from both plants and animals, from which they appear to have diverged around one billion years ago (Baldauf, 1993) Some morphological, biochemical,

and genetic features are shared with other organisms, while others are unique to the fungi, clearly separating them from the other kingdoms:

Shared features of fungi with other organisms:

With other eukaryotes: Fungal cells contain membrane-bound nuclei with chromosomes that contain DNA with noncoding regions called introns and coding regions called exons. Mitochondria sterol-containing membranes and ribosomes of the 80S type are types of membrane-bound cytoplasmic organelles present in fungi. They have a characteristics range of soluble carbohydrates and storage compounds including sugar alcohols (e.g., mannitol), disaccharides (e.g., trehalose) and polysaccharides (e.g., glycogen, which is also found in animal). (Shoji., et al 2006).

With animals: Energy sources are performed with organic compounds required by fungi because they are heterotrophic organisms that lack chloroplasts.

With plants: A cell wall and vacuoles are present in fungi (Zabriskie, 2000). They reproduce by both sexual and asexual means, and like basal plant groups (such as ferns and mosses) produce spores. Similar to mosses and algae, fungi typically have haploid nuclei.

With euglenoids and bacteria: Higher fungi, euglenoids, and some bacteria produce the amino acid L-lysine in specific biosynthesis steps, called the α-aminoadipate pathway (Zabriskie, 2000).

The cells of most fungi grow as tubular, elongated, and thread-like (filamentous) structures called hyphae, which may contain multiple nuclei and extend by growing at their tips. Each tip contains a set of aggregated vesicles cellular structures consisting of proteins, lipids, and other organic molecules called the Spitzenkörper. Both fungi and oomycetes grow as filamentous hyphal cells. In contrast, similar-looking organisms, such as filamentous green algae, grow by repeated cell division within a chain of cells. There are also single celled fungi (yeasts) that do not form hyphae, and some fungi have both hyphal and yeast forms.

In common with some plant and animal species, more than 70 fungal species display bioluminescence (Desjardin, et al., 2008).

2.1.3 UNIQUE FEATURES OF FUNGI

Dimorphic fungi can switch between a yeast phase and a hyphal phase in response to environmental conditions. Some species grow as unicellular yeasts that reproduce by budding or fission (Alexopolous, et al., 2007).

The fungal cell wall is made up of glucans and chitin; while glucans are also found in plants and chitin in the exoskeleton of arthropods, these two structural molecules are only combined in the cell wall of fungi . Fungal cell walls do not contain cellulose unlike those of plants and oomycetes (Keller, et al., 2005).

Most fungi lack an efficient system for the long-distance transport of water and nutrients, such as the xylem and phloem in many plants. To overcome this limitation, some fungi, such as *Armillaria*, form rhizomorphs (Wu et al., 2007) which resemble and perform functions similar to the roots of plants. Mevalonic acid and pyrophosphate are used as chemical building blocks for producing terpenes in the biosynthetic pathway possessed by a fungi as an eukaryotes. Fungi produce several secondary metabolites that are similar or identical in structure to those made by plants. Many of the plant and fungal enzymes that make these compounds differ from each other in sequence and other characteristics, which indicates separate origins and convergent evolution of these enzymes in the fungi and plants.

2.1.4 DIVERSITY OF FUNGI

Fungi have a worldwide distribution and grow in a wide range of habitats, including extreme environments such as deserts or areas with high salt concentrations or ionizing radiation, as well as in deep sea sediments. Some can survive the intense UV and cosmic radiation encountered during space travel. Most grow in terrestrial environments, though several species live partly or solely in aquatic habitats, such as the chytrid fungus *Batrachochytrium dendrobatidis*, a parasite that has been responsible for a worldwide decline in amphibian populations. This organism spends part of its life cycle as a motile zoospore, enabling it to propel itself through

water and enter its amphibian host. Other examples of aquatic fungi include those living in hydrothermal areas of the ocean.

Around 120,000 species of fungi have been described by taxonomists, but the global biodiversity of the fungus kingdom is not fully understood. A 2011 estimate suggests there may be between 2.2 and 6.8 million species (Hibbert, et al., 2007). In mycology, species have historically been distinguished by a variety of methods and concepts. Classification based on morphological characteristics, such as the size and shape of spores or fruiting structures, has traditionally dominated fungal taxonomy.

Species may also be distinguished by their biochemical and physiological characteristics, such as their ability to metabolize certain biochemicals, or their reaction to chemical tests. The biological species concept discriminates species based on their ability to mate. The application of molecular tools, such as DNA sequencing and phylogenetic analysis, to study diversity has greatly enhanced the resolution and added robustness to estimates of genetic diversity within various taxonomic groups.

2.1.5 MORPHOLOGY OF FUNGI

2.1.5.1 MICROSCOPIC STRUCTURE OF FUNGI

Most fungi grow as hyphae, which are cylindrical, thread-like structures 2–10 µm in diameter and up to several centimeters in length. Hyphae grow at their tips (apices), new hyphae are typically formed by emergence of new tips along existing hyphae by a process called *branching*, or occasionally growing hyphal tips fork, giving rise to two parallel-growing hyphae. Hyphae also sometimes fuse when they come into contact, a process called hyphal fusion. These growth processes lead to the development of a mycelium, an interconnected network of hyphae. Hyphae can be either septate or coenocytic. Septate hyphae are divided into compartments separated by cross walls, with each compartment containing one or more nuclei, coenocytic hyphae are not compartmentalized. Septa have pores that allow cytoplasm, organelles, and sometimes nuclei to pass through; an example is the dolipore septum in fungi of the phylum Basidiomycota. Coenocytic hyphae are in essence multinucleate supercells.

Many species have developed specialized hyphal structures for nutrient uptake from living hosts; examples include haustoria in plant-parasitic species of most fungal phyla, and arbuscules of

several mycorrhizal fungi, which penetrate into the host cells to consume nutrients (Stevens, et al., 2006).

Although fungi are opisthokonts a grouping of evolutionarily related organisms broadly characterized by a single posterior flagellum all phyla except for the chytrids have lost their posterior flagella (Ferguson, et al., 2003). Fungi are unusual among the eukaryotes in having a cell wall that, in addition to glucans (e.g., β-1,3-glucan) and other typical components, also contains the biopolymer chitin.

2.1.5.2 MACROSCOPIC STRUCTURE OF FUNGI

Fungal mycelia can become visible to the naked eye, for example, on various surfaces and substrates, such as damp walls and spoiled food, where they are commonly called molds. Mycelia grown on solid agar media in laboratory petri dishes are usually referred to as colonies. These colonies can exhibit growth shapes and colors (due to spores or pigmentation) that can be used as diagnostic features in the identification of species or groups. Some individual fungal colonies can reach extraordinary dimensions and ages as in the case of a clonal colony of *Armillaria solidipes*, which extends over an area of more than 900 ha (3.5 square miles), with an estimated age of nearly 9,000 years.

The apothecium(a specialized structure important in sexual reproduction in the ascomycetes) is a cup-shaped fruit body that is often macroscopic and holds the hymenium, a layer of tissue containing the spore-bearing cells. The fruit bodies of the basidiomycetes (basidiocarps) and some ascomycetes can sometimes grow very large, and many are well known as mushrooms.

2.1.6 GROWTH OF PHYSIOLOGY OF FUNGI

The growth of fungi as hyphae on or in solid substrates or as single cells in aquatic environments is adapted for the efficient extraction of nutrients, because these growth forms have high surface area to volume ratios. Hyphae are specifically adapted for growth on solid surfaces, and to invade substrates and tissues. They can exert large penetrative mechanical forces, for eample, many plant pathogens including Magnaporthe grisea form a structure called an appressorium that evolved to puncture plant tissues. The pressure generated by the appressorium, directed against the plant epidermis, can exceed 8 megapascals (1,200 psi). The filamentous fungus *Paecilomyces lilacinus* uses a similar structure to penetrate the eggs of nematodes.

The mechanical pressure exerted by the appressorium is generated from physiological processes that increase intracellular turgor by producing osmolytes such as glycerol. Adaptations such as these are complemented by hydrolytic enzymes secreted into the environment to digest large organic molecules such as polysaccharides, proteins, and lipids into smaller molecules that may then be absorbed as nutrients. The vast majority of filamentous fungi grow in a polar fashion by elongation at the tip of the hypha. Other forms of fungal growth include intercalary extension (longitudinal expansion of hyphal compartments that are below the apex) as in the case of some endophytic fungi, or growth by volume expansion during the development of mushroom stipes and other large organs. Growth of fungi as multicellular structures consisting of somatic and reproductive cells a feature independently evolved in animals and plants has several functions, including the development of fruit bodies for dissemination of sexual spores and biofilms for substrate colonization and intercellular communication.

The fungi are traditionally considered heterotrophs, organisms that rely solely on carbon fixed by other organisms for metabolism. Fungi have evolved a high degree of metabolic versatility that allows them to use a diverse range of organic substrates for growth, including simple compounds such as nitrate, ammonia, acetate, or ethanol. In some species the pigment melanin may play a role in extracting energy from ionizing radiation, such as gamma radiation. This form of "radiographic" growth has been described for only a few species, the effects on growth rates are small, and the underlying biophysical and biochemical processes are not well known. This process might bear similarity to CO_2 fixation via visible light, but instead uses ionizing radiation as a source of energy.

2.1.7 REPRODUCTION

Fungal reproduction is complex, reflecting the differences in lifestyles and genetic makeup within this diverse kingdom of organisms. It is estimated that a third of all fungi reproduce using more than one method of propagation; for example, reproduction may occur in two stages within the life cycle of a species, the teleomorph and the anamorph. Environmental conditions trigger genetically determined developmental states that lead to the creation of specialized structures for sexual or asexual reproduction. These structures aid reproduction by efficiently dispersing spores or spore-containing propagules.

2.1.7.1 ASEXUAL REPRODUCTION

Asexual reproduction occurs via vegetative spores conidia or through mycelial fragmentation. Mycelial fragmentation occurs when a fungal mycelium separates into pieces, and each component grows into a separate mycelium. Mycelial fragmentation and vegetative spores maintain clonal populations adapted to a specific niche, and allow more rapid dispersal than sexual reproduction. The "Fungi imperfecti" (fungi lacking the perfect or sexual stage) or Deuteromycota comprises of all the species that lack an observable sexual cycle. Deuteromycota is not an accepted taxonomic clade, and is now taken to mean simply fungi that lack a known sexual stage.

2.1.7.2 SEXUAL REPRODUCTION

Sexual reproduction with meiosis has been directly observed in all fungal phyla except Glomeromycota. It differs in many aspects from sexual reproduction in animals or plants. Differences also exist between fungal groups and can be used to discriminate species by morphological differences in sexual structures and reproductive strategies. Mating experiments between fungal isolates may identify species on the basis of biological species concepts. The major fungal groupings have initially been delineated based on the morphology of their sexual structures and spores, for example, the spore containing structures, asci and basidia, can be used in the identification of ascomycetes and basidiomycetes, respectively. Fungi employ two mating systems: heterothallic species allow mating only between individuals of opposite mating type, whereas homothallic species can mate, and sexually reproduce, with any other individual or itself.

Most fungi have both a haploid and a diploid stage in their life cycles. In sexually reproducing fungi, compatible individuals may combine by fusing their hyphae together into an interconnected network; this process, anastomosis, is required for the initiation of the sexual cycle. Many ascomycetes and basidiomycetes go through a dikaryotic stage, in which the nuclei inherited from the two parents do not combine immediately after cell fusion, but remain separate in the hyphal cells.

In ascomycetes, dikaryotic hyphae of the hymenium (the spore-bearing tissue layer) form a characteristic *hook* at the hyphal septum. During cell division, formation of the hook ensures proper distribution of the newly divided nuclei into the apical and basal hyphal compartments.

An ascus is then formed, in which karyogamy occurs. Asci are embedded in an ascocarp, or fruiting body. Karyogamy in the asci is followed immediately by meiosis and the production of ascospores. After dispersal, the ascospores may germinate and form a new haploid mycelium.

Sexual reproduction in basidiomycetes is similar to that of the ascomycetes. Compatible haploid hyphae fuse to produce a dikaryotic mycelium. However, the dikaryotic phase is more extensive in the basidiomycetes, often also present in the vegetatively growing mycelium. A specialized anatomical structure, called a clamp connection, is formed at each hyphal septum. As with the structurally similar hook in the ascomycetes, the clamp connection in the basidiomycetes is required for controlled transfer of nuclei during cell division, to maintain the dikaryotic stage with two genetically different nuclei in each hyphal compartment. A basidiocarp is formed in which club-like structures known as basidia generate haploid basidiospores after karyogamy and meiosis. The most commonly known basidiocarps are mushrooms, but they may also take other forms.

In fungi, formerly classified as Zygomycota, haploid hyphae of two individuals fuse, forming a gametangium, a specialized cell structure that becomes a fertile gamete-producing cell. The gametangium develops into a zygospore, a thick-walled spore formed by the union of gametes. When the zygospore germinates, it undergoes meiosis, generating new haploid hyphae, which may then form asexual sporangiospores. These sporangiospores allow the fungus to rapidly disperse and germinate into new genetically identical haploid fungal mycelia.

2.1.8 SPORE DISPERSAL OF FUNGI

Asexual as well as sexual spores or sporangiospores are often actively spread from their reproductive structures by forceful ejection. This ejection guarantees that the spores are exiting from the reproductive structures and traveling lengthy distances through the atmosphere.

2.1.9 ECOLOGY

Although often unnoticeable, mushrooms happen in every Earth setting and in most ecosystems play very significant roles. In most terrestrial habitats, fungi, along with bacteria, are the main decomposers and thus play a critical part in biogeochemical cycles and many food webs. They play an essential role as decomposers in nutrient cycling, particularly as saprotrophs

and symbionts, degrading organic matter into inorganic molecules, which can then reenter anabolic metabolic pathways in plants or other organisms.

2.1.10 MYCOTOXINS

Many fungi produce biologically active compounds, several of which are toxic to animals or plants and are therefore called mycotoxins. Of particular relevance to humans are mycotoxins produced by molds causing food spoilage, and poisonous mushrooms. Particularly infamous are the lethal amatoxins in some *Amanita* mushrooms, and ergot alkaloids, which have a long history of causing serious epidemics of ergotism in people consuming rye or related cereals contaminated with sclerotia of the ergot fungus, Claviceps purpurea. Other notable mycotoxins include the aflatoxins, which are insidious liver toxins and highly carcinogenic metabolites produced by certain *Aspergillus* species often growing in or on grains and nuts consumed by humans, ochratoxins, patulin, and trichothecenes (e.g., T-2 mycotoxin) and fumonisins, which have significant impact on human food supplies or animal livestock.

Mycotoxins are secondary metabolites, and research has established the existence of biochemical pathways solely for the purpose of producing mycotoxins and other natural products in fungi. Mycotoxins may provide fitness benefits in terms of physiological adaptation, competition with other microbes and fungi, and protection from consumption. Many fungal secondary metabolites are used medically.

2.1.11 HUMAN USES

Human use of fungi for food preparing or conservation is vast and has a lengthy history. Mushroom farming and the collection of mushroom in many nations are big sectors. Ethnomycology is known as the research of historical uses and sociological effect of fungi. Because of this group's ability to produce a wide range of natural products with antimicrobial or other biological activity, many species have been or are being developed for the industrial production of antibiotics, vitamins and anticancer and cholesterol lowering drugs.

Methods for genetic engineering of fungi have lately been created to enable metabolic engineering of fungal species. Fungi can be of use in so many ways whether beneficial or pathogenic which include ;Therapeutic uses, Traditional medicine, Cultured foods, Foods, Poisonous fungi, Pest control, Bioremediation.

Fungi are widely used in the production of industrial chemicals such as citric, gluconic, lactic and malic acids and Industrial enzymes such as lipases used in biological detergents, cellulases used in the manufacture of cellulosic ethanol and jeans, amylases, invertases, proteases and xylanases.

2.2 PHARMACEUTICAL PRODUCT

Any chemical substance formulated or compounded as a single active ingredient or inc onjunction with another pharmacologically active substance is a pharmaceutical drug. It may be packed separately but as a combination product intended for internal or external use in the field of medical diagnosis, cure, treatment or disease prevention. Medicines are categorized in different respects. One of the key divisons is between traditional small molecule medicinal products usually derived from chemical synthesis and biopharmaceuticals, which incombinant proteins, vaccines, blood products such as gene therapy and cell therapy. Another important difference is the amount of command that seperates prescription drugs from over the counter drugs. Other methods of classifying medicinal products include mode of action, route of admission, impacted biological system or therapeutic impacts. The anatomical chemical classification system is an elaborate and commonly used in classification. The World Health Organization keeps a list of essential medicines (Steven, et al., 2010).

Drug discovery and drug development are complex and costly efforts of pharmaceutical companies, scholarly researchers, and governments. In general, governments regulate what drugs can be marketed, how drugs are sold, and drug pricing in some jurisdictions. There has been controversy over the pricing of medicines and the disposal of used medicines.

2.2.1 CLASSIFICATION OF PHARMACEUTICAL PRODUCTS

Pharmaceutical drugs are classified on the basis of their origin.

1) Drug from natural origin: Plant or mineral origin, some drug substances are of marine origin.
2) Drug from chemical as well as natural origin: derived from partial herbal and partial chemical synthesis chemical.
3) Drug derived from chemical synthesis.
4) Drug derived from animal origin: hormones and enzymes

5) Drug derived from microbial origin: antibiotics
6) Drug derived by biotechnology genetic engineering, hybridoma technique sample
7) Drug derived from radioactive substances.

A sampling of classes of medicine includes:

1) Antimalarial drugs: treating malaria
2) Antiseptics: prevention of germ growth near wounds, burns and cuts
3) Antibiotics: inhibiting germ growth
4) Hormone replacements: premarin
5) Stimulants: methylphenidate
6) Tranquilizers: chlorpromazine, diazepam and alprazolam
7) Analgesics: reducing pain
8) Antipyretics: reducing fever
9) Oral contraceptives: biphasic pill, triphasic pill and enovoid
10) Mood stabilizers: valpromide and lithium
11) Statins: lovastatin, simvastatin and pravastatin

2.2.2 TYPES OF MEDICINES

For the central nervous system

Drugs affecting the central nervous system include: Psychedelics, hypnotics, anesthetics, antipsychotics, antidepressants (including tricyclic antidepressants, monoamine oxidase inhibitors, lithium salts, and selective serotonin reuptake inhibitors (SSRIs), antiemetics, anticonvulsants\antiepileptics. Anxiolytics, barbiturates, movement disorder (e.g., Parkinson's disease) drugs, stimulants (including amphetamines), benzodiazepines, cycloprrolones, dopamine antagonists, antihistamines, cholinergic, anticholinergics, emetics, cannabinoids, and 5-HT (serotonin) anatagonists.

The main categories of drugs for mtisculoskeletal disorders are: NSAIDs (including COX-2 selective inhibitors), miscle relaxants, neuromuscular drugs, and anticholinesterases.

For the respiratory system

Mucolytics, anticholinergics, mast cell stabilizers, bronchodilators, antitussives.

For contraception

Hormonal contraception, spermicide, ormeloxifene.

For ear, nose and throat (ENT)

Antibiotics, corticosteroids, antihistamines, local anesthetics, antifungal, cerumenolytic, antiseptics.

For the eye

General; adrenergic neurone blocker, ocular lubricant, astringent.

Diagnostics; topical anesthetics, paprasympatholytics, mydriatics, cycloplegics.

Antibacterial; antibiotics, sulfa drugs, aminoglycoside, topical antibiotics.

Antiviral drug

Anti-fungal: polyenes, imidazoles.

Anti-inflammatory; NSAIDs, corticosteroids.

Anti-allergy: mast cell inhibitors

Anti-glaucoma: adrenergic agonists, beta-blokers, carbonic anhydrase inhibitors, cholingerics, miotics, prostaglandin inhibitors.

For the cardiovascular system

General: β-receptor blockersm(beta blockers), calcium channel blockers,diuretics, cardiac glycosides, antiarrhythmics, nitrate, antianginals, vasoconstrictors, vasodilators.

Affecting blood pressure/(antihypertensive drugs): ACE inhibitors, angiotensin receptor blockers, beta-blockers, α blockers, calcium channel blockers, thiazide diuretics, loop diuretics, aldosterone inhibitors

Coagulation: anticoagulants, heparin,antiplatelets drugs,fibrinolytics, anti-hemphilic factors, haemostatic drugs

HMG-CoA reductase inhibitors (stains) for lowering LDL cholesterol inhibitors: hypolipidaemic agents.

For digestive system

Upper digestive tract: antiflatulents, cytoprotectants, prostaglandin analogues, antidopaminergics and reflux suppressants.

Lower digestive tract: laxatives, opioid, antidiatthoeals, bile acid sequestrants.

For reproductive system or urinary system

Antifungal, quinolones, antibiotics, cholinergics, anticholinergics, antispasmodics, slidenafils, fertility medications.

For endocrine problems

Androgens, antiandrogens, gonadotropin, corticosteroids, human growth hormones, insulin, antidiabetics (sulfonylureas, biguanides/metformin, thiazolidinediones, insulin), thyroid horomes, antithyroid drugs, calcitonin, diphosponate, vasopressin analogues.

For the reproductive system or urinary system

Antifungal, alkalinizing agents, quinolones, antibotics, cholinergics, anticholingrgics, antispasmodics, 5-alpha reductase inhibitor, selective alpha-1 blockers, sidenafils, fertility medications.

For obstetrics and gynaecology

NSAIDs anticholingergics, haemostatic drugs, antifibrinolytics, Hormones Replacement Therapy (HRT), bone regulators, beta-receptor agonists, follicle, tamoxifen, gonadorelin, gamolenic acid, follicle stimulating hormones, Diethylstilbestrol, luteinizing hormone, LHRH gamolenic acid, gonadotropin release inhibitor, Clomhene,

For nutrition

Tonics,electrolytes and mineral preparations (including iron preparation and magnesium preparations), parental nutritions, vitamins, anti-obesity drugs, anabolic drugs, haematopoietic drugs, food product drugs

For the immune system

Monoclonal antibodies, vaccines, immunosuppressants ,interferons, immunoglobulins

For neoplastic disorders

Recombinant interleukins, G-CSF, erythropoietin, cytoxic drugs, sex hormones, aromatase inhibitors, somatostain, cytotoxic drugs

For infection and infestations

Antibiotics, antifungal, antileprootics, antitiberculous drugs, antimalarial, anthelmintics, ameobicides, antivirals, antiprotozoals, probiotics and prebiotics.

For the skin

Tar products, systemic antibiotics, topical antibiotics, hormones, fibrinolytics, proteolytics, anti pruritics, antifungal, disinfectants, scabicides, abrasives, systemic antibiotics, keratolytics and immune modulators.

For euthanasia

A euthanaticum is used for euthanasia and physician assisted suicide. Euthanasia is not permitted by law in many countries, and consequently medicines will not be licensed for this use in those countries.

For allergic disorders

Anti-allergic, NSAIDs, corticosteroids, antihistamines.

2.2.3 ADMINISTRATION

Administration is supplying a patient with a pharmaceutical drug. There are three main categories of drug administration: parenteral (bringing the drug straight into the circulatory system), enteral (oral medicine) and other (including intranasal, tropical, inhalation and rectal medicine). (Stevens, et al., 2010). it can be performed in different dosage forms such as tablets capsules or pills. There are many differences in administration paths, including intravenous blood through the vein and oral mouth administration.

2.2.4 DRUG DISCOVERY

In the fields of medicine, pharmacology and biotechnology, drug discovery is the process by which new candidate medications are discovered. Historically, drugs were discovered through identifying the active ingredient from traditional remedies or by serendipitous discovery. Later

chemical libraries of synthetic small molecules, natural products or extracts were screened in whole organisms or intact cells to identify substances that have a desirable therapeutic effect in a process known as classical pharmacology.

2.3 CLASSES OF ANTIFUNGAL DRUGS FOR HUMANS

2.3.1 IMIDAZOLE, TRIAZOLE AND THIAZOLE ANTIFUNGALS

Azole antifungal drugs (except for abafungin) inhibit the enzyme lanosterol 14 α-demethylase, the enzyme necessary to convert lanosterol to ergosterol. Depletion of ergosterol in fungal membrane disrupts the structure and many functions of the fungal membrane leading to the inhibition of fungal growth examples of Imidazole include butoconazole, econazole, isoconazole, luliconazole, omoconazole, sulconazole, oxiconazole, miconazole, econazole, clotrimazole, tioconazole and ketoconazole. Examples of Triazoles are

Posaconazole
Fluconazole
Itraconazole
Epoxiconazole
Albaconazole
Isavuconazole
Terconazole
Propiconazole
Voriconazole
Ravuconazole
Efinaconazole
Allylamines

Allylamines . Inhibit squalene expoxidase, another enzyme required for egosterol synthesis.
Examples are Amorolfin, Butenafine, Terbanafine and Naftifine.

2.3.2 ECHINOCANDINS

Echinocandins may be used for synthetic fungal infections in immunocompromised atient, they inhibit the synthesis of glucan in the cell wall through the enzyme 1,3-β-glucan synthase examples include: Micafungin, Anidulafungin and Caspofungin

Echinocandins are poorlyabsorbed when administered orally. When admistered by the injection they will reach most tissues and organs with concentrations sufficient to treat localized and systemic fungal infections.

2.3.3 POLYENE ANTIFUNGALS

A polyene is a molecule with multiplied conjugated double bonds. A polyene antifungal is a macrocyclic polyene with a heavily hydroxylated region on the ring opposite the conjugated system. This makes polyene antifungals amphiphilic. The polyene antimycotics bind with sterols in the fungal cell membrane, principally ergosterol. This changes the transition temperature (Tg) of the cell membrane, thereby placing the membrane in a less fluid, more crystalline state. In ordinary circumstances membrane sterols increase the packing of the phospholipid bilayer making the plasma membrane more dense. As a result, the cell's contents including monovalent ions (K^+, Na^+, $H^+$and Cl^-), small organic molecules leak and this is regarded one of the primary ways cell dies. Animal cells contain cholesterol instead of ergosterol and so they are much less susceptible. However, at therapeutic doses, some amphotericin B may bind to animal membrane cholesterol, increasing the risk of human toxicity. Amphotericin B is nephrotoxic when given intravenously. As a polyene's hydrophobic chain is shortened, its sterol binding activity is increased. Therefore, further reduction of the hydrophobic chain may result in it binding to cholesterol, making it toxic to animals. Examples include Amphotericin B,Candicidin Filipin-35 carbons, binds to cholesterol (toxic), Hamycin Natamycin-33 carbons, binds well to ergosterol Nystatin and Rimocidin.

2.3.4 OTHER ANTIFUNGAL DRUGS INCLUDE:

Haloprogin: discontinued due to the emergence of more modern antifungals with fewer side effects.

Crystal Violet: a triarymethane dye, it has antibacterial, antifungal and anthelmintic properties and was formerly important as a topical antiseptic.

Flucytosine or 5-fluorocytosine: an antimetabolite pyrimidine analog.

Undecylenic Acid: an unsaturated fatty acid derived from natural castor oil: fungistatic, antibacterial, antiviral and inhibits Candida mophogenesis.

Benzoic acid: has antifungal properties, but must be combined with a keratolytic agent such as in Whitfield's ointment.

Tolnafate: a thiocarbonate antifungal, which inhibits fungal squalene epoxidase (similar mechanism to allylamines like terbinafine)

Griseofulvin: binds to polymerized microtubules and inhibits fungal mitosis

Ciclopirox: is a hydroxypyridone antifungal that interferes with active membrane transport, cell membrane integrity and fungal respiratory processes. It is most useful against tinea versicolour.

2.4 MECHANISM OF ACTION

Antifungal work by exploiting differences between mammalian and fungal cells to kill the fungal organism with fewer adverse effects to the host. Unlike bacteria, both fungi and humans are eukaryotes. Thus, fungal and human cells are similar at the biological level. This makes it more difficult to discover drugs that target fungi without affecting human cells. As a consequence, many antifungal drugs cause side effects. Some of those side effects can be life threatening if the drugs are not used properly.

2.5 ADVERSE EFFECTS OF ANTIFUNGAL AGENT

Apart from side effects like liver damage or affecting oestrogen levels, many antifungal medicines can cause allergic reactions in people. For example, the azole group of drugs is known to have caused anaphylaxis.

There are so many drug interactions. Patients must read in detail the enclosed data sheets of the medicine. For example, the azole antifungals such as Ketoconazole or Itraconazole can be both substrates and inhibitors of the P-glycoprotein, which (among other functions) excretes toxins and drugs into the intestines. Azole antifungals also are both substrates and inhibitors of the cytochrome P450 family CY3A4. Causing increased concentration when administering, for example, macrolides, tricyclic antidepressants, benzodiazepines, calcium channel blockers, immunosuppressants, chemotherapeutic and SSRIs.

Before oral antifungal therapies are used to treat nail disease, a confirmation of the fungal infection should be made. Approximately half of suspected cases of fungal infection in nails have a non-fungal cause. The side effects of oral treatment are significant and people without an infection should not take these drugs.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 DRUG SAMPLE COLLECTION

Twenty-five samples of two different categories of oral liquid drugs (paracetamol and cough syrup) used by children with appropriate dates of manufacturing and expiration were procured from five different pharmaceutical shops in Lagos, Nigeria every week in the month of April. The pharmaceutical products were collected in sterile Ziploc bags from one location to the other. The Ziploc bags were labeled with necessary information concerning the product (information such as manufacturing date, expiry date and manufacturers name were recorded). All samples were transported to the laboratory under septic conditions in order to assess the growth of fungi.

3.2 ISOLATION AND IDENTIFICATION OF FUNGI

The agar used was potato dextrose agar (PDA) because it contains the specific nutrients needed to grow fungi. Chloramphenicol Antibiotics was added to it to prevent bacterial contamination. Thirty-nine grammes (39g) of commercially produced potato dextrose agar was measured and dissolved in a litre (1litre) of sterile distilled water and boiled to dissolve the medium completely. The agar was sterilized by autoclaving at 121°C for 15 minutes and allowed to cool to 50°C before pouring the melted medium into a sterile petri dish (15cm diameter petri plates at a depth of 4.0mm). While the medium was in the autoclave, 1ml of the pharmaceutical products were serially diluted in 9ml of distilled water inside the testubes and 1ml was taken from each of the bottles and added into another (9ml) for 10^{-1} and another 1ml was taken from each 10^{-1} and added to another 9ml of distilled water for 10^{-2} and then another 1ml was taken from the 10^{-2} and added to another bottle of distilled water of 9ml to get 10^{-3} After this procedure petri dishes were labeled 10^{0} to 10^{-3} with the acronyms of each products. After this procedure 0.1ml was taken from each bottles of the dilution and added to the center of the empty petri dishes and then the agar was poured after it had been cooled. The cultures were incubated at room temperature and left for 3 days before been examined.

Once there is presence of growth, the topography, texture and pigmentation of each type specific type of colony is noted in order to identify the fungi accurately. A little portion of the growth of the colony was teased out using the borer and an inoculating needle and mount on the slide with

a drop of lactophenol on it and covered with a cover slip. The preparation was examined under a light microscope with an attached camera (Motic Mc Camera 2.0 megapixel digital colored camera) connected to a computer for the microscopic photography of the fungi. The essence of this was to observe the exact arrangement of the conidiospores and the way the spores are produced. The identities of these fungi were certified using cultural, morphological as well as comparing them with confirmed representatives of different species in relevant texts. Alexopolous et al., 2007 and Ellis et al., 2007.

3.4 DNA EXTRACTION

Extraction of fungal DNA was done using three different protocols which are as follows:

Zymo kit, Cetyltrimethyl ammonium bromide (CTAB) protocol and Modified CTAB protocol.

3.4.1 DNA EXTRACTION USING ZYMO KIT

Ten grammes of pure fungal isolate were prepared and suspended in 50µl of water to the tubes of ZR Bashing Bead lysis rack. After which 400µl of lysis solution was added to each tube. The tube cap was tightened to prevent leakage. The ZR Bashing Bead lysis rack was centrifuged at5,500rpm for 5minutes. 250µl of the supernatant was transferred into each well of a deep well block followed by 750µl of fungal DNA binding buffer after which was covered with cover foil and mixed thoroughly by vortexing block for 2minutes. The deep well block was centrifuged at 5,000rpm for 5minutes. 500µl of each supernatant was transferred to the wells of silicon-A on a collection plate. The assembly was then centrifuged at 5,00rpm for 5minuted. 200µl of DNA prewash buffer was added to the wells of the silicon plate on the emptied collection plate and centrifuged the assembly at 5,000rpm for 5minutes. 500µl fungal DNA wash was added to the well on the collection plate and centrifuged the assembly at 5,000rpm for 5minutes. The silicon plate was transferred to an elution plate and 100µlof DNA elution buffer was added directly to the matrices in the plate. The whole assembly was then centrifuged at 5,000rpm for 5minutes. The DNA is now ready for PCR and sequencing.

3.4.2 DNA EXTRACTION USING CTAB PROTOCOL

Ten milliliters of sterilized distilled water were added to a freshly pure culture of each isolates to be extracted, a suspension was made by gently probing the colon with the tip of a sterilized

Pasteur pipette. The suspension was transferred into an eppendorf tube and was spun for 30seconds. The supernatant was decanted and 400µl of CTAB buffer and 75µl of 10% SDS were added to the pellet. This was then incubated in water bath at a temperature of 65°C for 15minutes. 500µl of chloroform was added and this was mixed by rocking on an orbital shaker at 100rpm for 5minutes, which was then spun 10,000rpm for 10minutes. The supernatant was collected into the fresh eppendorf tube and 500µl of isolated propanol was added to the supernatant. This was kept at -20°C for one hour after which it was spun at 14,000rpm for 10minutes. The supernatant was decanted gently and the pellet (DNA) was washed with 70% ethanol then the pellet was air dried for 30minutes. The dried pellet was then re suspended in 200µl of sterile distilled water.

3.4.3 DNA EXTRACTION USING MODIFIED CTAB PROTOCOL

Ten millilitres of isolation buffer (10x CTAB) containing 80µl of mercaptorthanol in 50ml blue cap tube was pre heated in 65°C water bath. One gramme of freshly scrapped pure culture plates of each fungal isolate was added to the preheated isolation buffer in each tube. The mixture was then incubated at 65°C for 15minutes. Ten milliliters of SEVAG (24:1chlorform: Isoamyl alcohol), then mixed gently but thoroughly. The cap of the tubes containing the mixture was then opened to release gas, which was then re-tightened then the tubes were rocked using an orbital shaker at 100rpm for 60minutes. After rocking, the tubes were spun at 4000rpm at 25°C for 20minutes. The mixture gave a clear and colorless aqueous solution at the top containing the DNA, the clear colorless aqueous solution was removed into another set of tubes with the aid of a plastic transfer pipette. Two third volume of isopropanol was added to the aqueous solution. The mixture was then mixed gently and stored in a freezer (-20°C) for 24hours. This was allowed the DNA in it to precipitate, and the liquid in the tube was discarded. Then 3ml of 70% ethanol was added to dislodge the pellet in order to facilitate washing. It was spun again at 3,000rpm for 5minutes, the liquid was discarded and the alcohol was allowed to evaporate by leaving the tubes open and on its side. The DNA was suspended in 1.5ml of water and stored at (-20°C) prior to shipment for PCR and Sequencing.

3.4.4 DNA VERIFICATION ON 1% AGAROSE GEL ELECTROPHORESIS

1% of Agarose gel was prepared by mixing 1.5g Agarose with 150ml 1X TBE buffer. The mixture was boiled in a microwave until all Agarose was dissolved usually for 2minutes. This was cooled down under running cold tap water; after which 6µl of Ethidium Bromide (done in the fume cupboard) was added and swirled to mix. The gel was then poured into the tray and allowed to stand for at least 30minutes before removing the crumbs. The gel was then placed into an electrophoresis tank. 5µl of loading dye was for 10µl of each DNA samples on paraffin paper. Using a pipette, 15µl of each sample was loaded into each well of the prepared 1% Agarose gel; one half microgram of 1kn ladder (Lambda DNA Hind 111) was loaded into the marker well. It was then ran for 45minutes at 110 Milliamps.

3.4.5 POLYMERASE CHAIN REACTION (PCR)

The PCR was carried out in other to remove unutilized Dntps, primers, polymerase and other PCR mixture compounds to obtain a highly purified DNA template for sequencing. The extracted DNA samples were contracted out to Inqaba biotech Pretoria, South Africa for the PCR and DNA sequence analysis. The Polymerase Chain Reaction – Restriction fragment length Polymorphism (PCR-RFLP) is used to amplify the internal transcribed spacer (ITS) region of the rDNA. The PCR was performed using the universal primer pair ITS1-ITS4 (Jackson et al., 1999). The thermal cycler was programmed for 4minutes at 94°C followed y 35cycles of 1minute at 72°C. the nucleotides sequences were determined automatically using the cycle sequencing protocol by the Bid Dye Terminator v3.1 Cycle sequencing kit in the ABI PRISM 377-DNA Sequencer. The generated sequence data from the DNA extraction were sent to Inqaba Biotechnology for further identification and BLASTED to show relationship between sequences as well as to help to confirm identity. This was done by comparing nucleotide sequence data that was produced from the extracted fungal DNA at the genebank and to determine the statistical significance matches.

CHAPTER FOUR

4.0 RESULTS

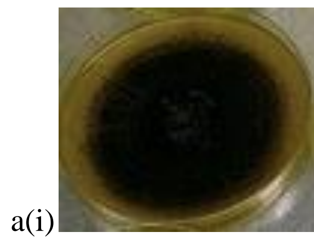
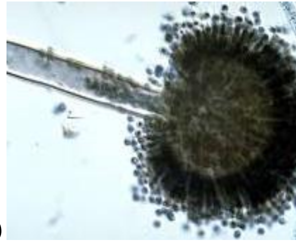
The collection of pharmaceutical products in the month of April produced a total of 25 samples. These products PC, EC, FC, MP, EP, SP, EI, MM, EI, EC, VM, EM, VI, were collected from three different pharmacy shop.

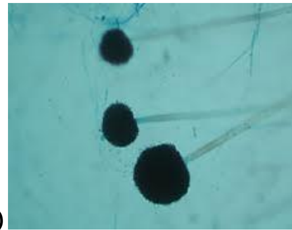
4.1 ISOLATION AND IDENTIFICATION OF THE FUNGI

Out of twenty-five samples, twelve did not yield any fungal growth irrespective of the sampling. The remaining thirteen produced seven morphologically different species of fungi. Morphological comparison was done with that of standard mycology text books such as (Alexopolous et al., 2007) and (Ellis et al., 2007). These fungi were later confirmed by molecular characteristics as Aspergillus tamari strain A, Aspergillus tubingenesis strain, Aspergillus tamari strain B, Aspergillus ellipticus, Aspergillus niger strain A, Aspergillus niger strain B, Aspergillus niger strain C (table 1). Plates 2a – g shows the photographs and the photomicrographs of each of the isolated fungi species and strain cultures. Table 1 shows the fungal morphology and the microscopic description of isolates from pharmaceutical products. The isolated fungi are all filamentous.

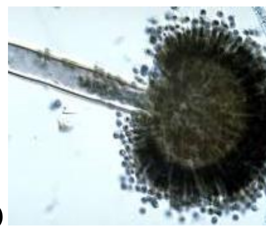
Table 1: fungal morphology and microscopic description of isolates from pharmaceutical product

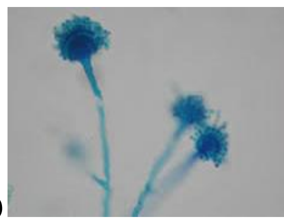
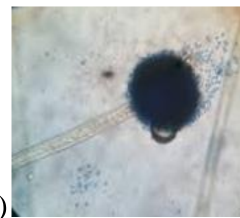
FUNGI	CHARACTERISTICS OF THE ISOLATED FUNGI
Aspergillus niger strain A	Colony is a fast growing one. Black in color. Conidial head is short. Conidiospores is erect, simple with thick wall. (plate 2b)
Aspergillus niger strain B	Colony is a fast growing one formed in group. Black in color. Conidial head is short. Conidiospores is erect, simple with thick wall. (plate 2c)
Aspergillus niger strain C	Colony is typical black in color. Conidial heads are compact with phalides borne directly on vesicle. Conidial heads are long columnar and biserate with long and thin blue smooth wall stripes. (plate 2f)
Aspergillus tamari strain A	Colony is typically suede like and buffy to sand brown in color. Conidial heads are short columnar and biserate, with short brownish and smooth walled stipes. (plate 2d)
Aspergillus tamari strain B	Colony is typically black in color. Conidial heads are short columnar and uniserate with short and black smooth wall stipes. (plate 2e)
Aspergillus tubingenesis strain	Colony is fast growing. Conidiospores upright, simple terminating in a globose shape with spores at the apex and the entire surface. (plate 2a)
Aspergillus ellipticus	Colony is fast growing. Conidiospores upright, simple terminating in a globose shape with spores at the entire surface. (plate 2g)

a(i)  a(ii) 

b(i) b(ii)  c(i)

c(ii) d(i) d(ii)

e(i) e(ii)  f(i)

f(ii)  g(i) g(ii) 

a(i): culture plate of *Aspergillus tubingensis*
b(i): culture plate of *Aspergillus niger strain A*
c(i): culture plate of *Aspergillus niger* strain B
d(i): culture plate of *Aspergillus tamari strain A*
e(i): culture plate of *Aspergillus tamari* strain B
f(i): culture plate of *Aspergillus niger strain C*
g(i): culture plate of *Aspergillus ellipticus*

a(ii): photomicrograph of *Aspergillus tubingensis*
b(ii): photomicrograph of *Aspergillus niger* strain A
c(ii): photomicrograph of *Aspergillus niger* strain B
d(ii): photomicrograph of *Aspergillus tamari*
e(ii):photomicrograph of *Aspergillus tamari* strain B
f(ii): photomicrograph of Aspergillus niger strain C
g(ii): photomicrograph of *Aspergillus ellipticus*

4.2 MOLECULAR CHARACTERIZATION

The extracted genomic DNA from each of the isolated fungus which was size separated on 1% agarose gel was viewed under ultraviolet trans-illuminator for DNA quality and yield assessment and the photomicrograph of the gel was taking as shown in the electrophorogram. Plate 2a shows the electrophorogram of DNA extracted with Zymo kit, plate 2b shows the electrophorogram of DNA extracted with CTAB protocol, plate 2c shows the electrophorogram of DNA extracted with modified CTAB protocol.

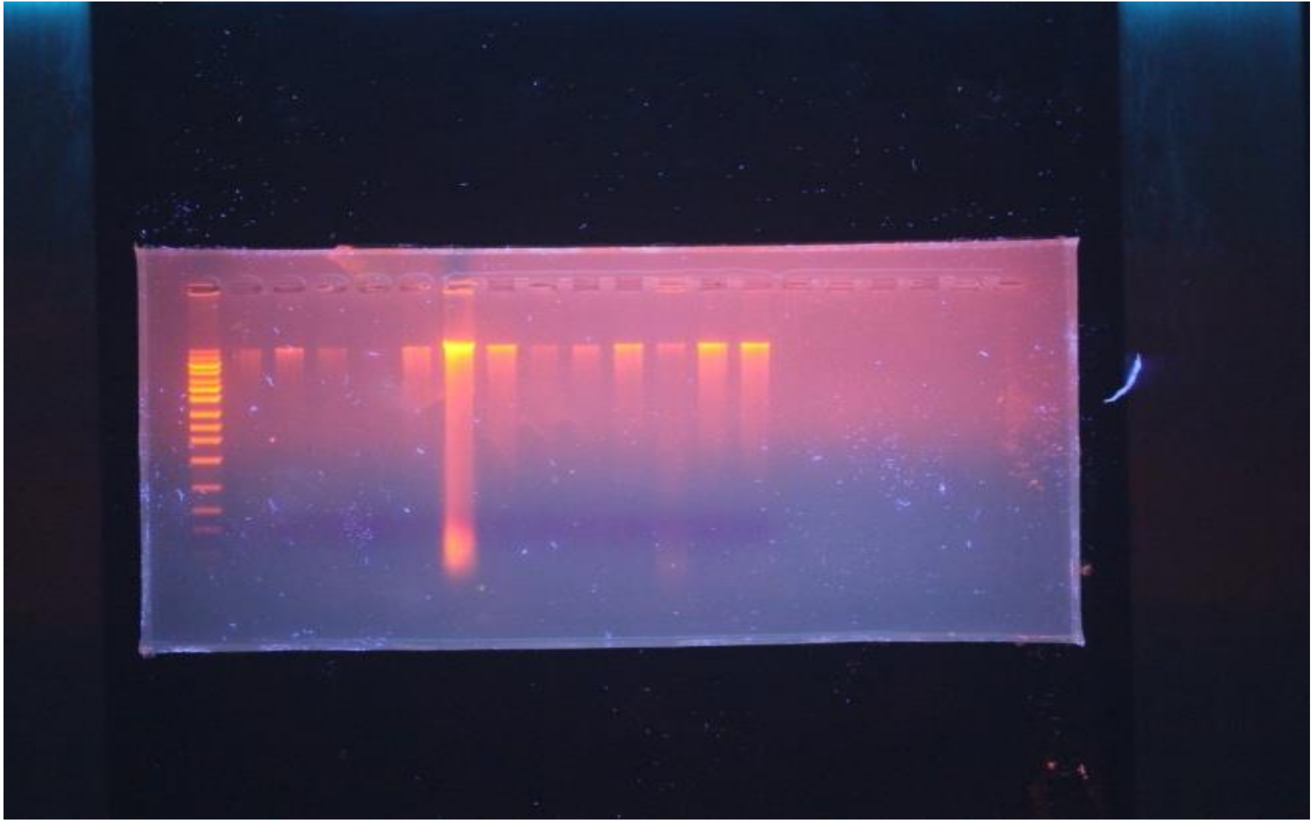


Plate 2a: Electrophorogram of extracted DNA samples of PC, EC, FC, MP, EP, SP, EI, MM, EI, EC, VM, EM, VI, using Zymo kit.

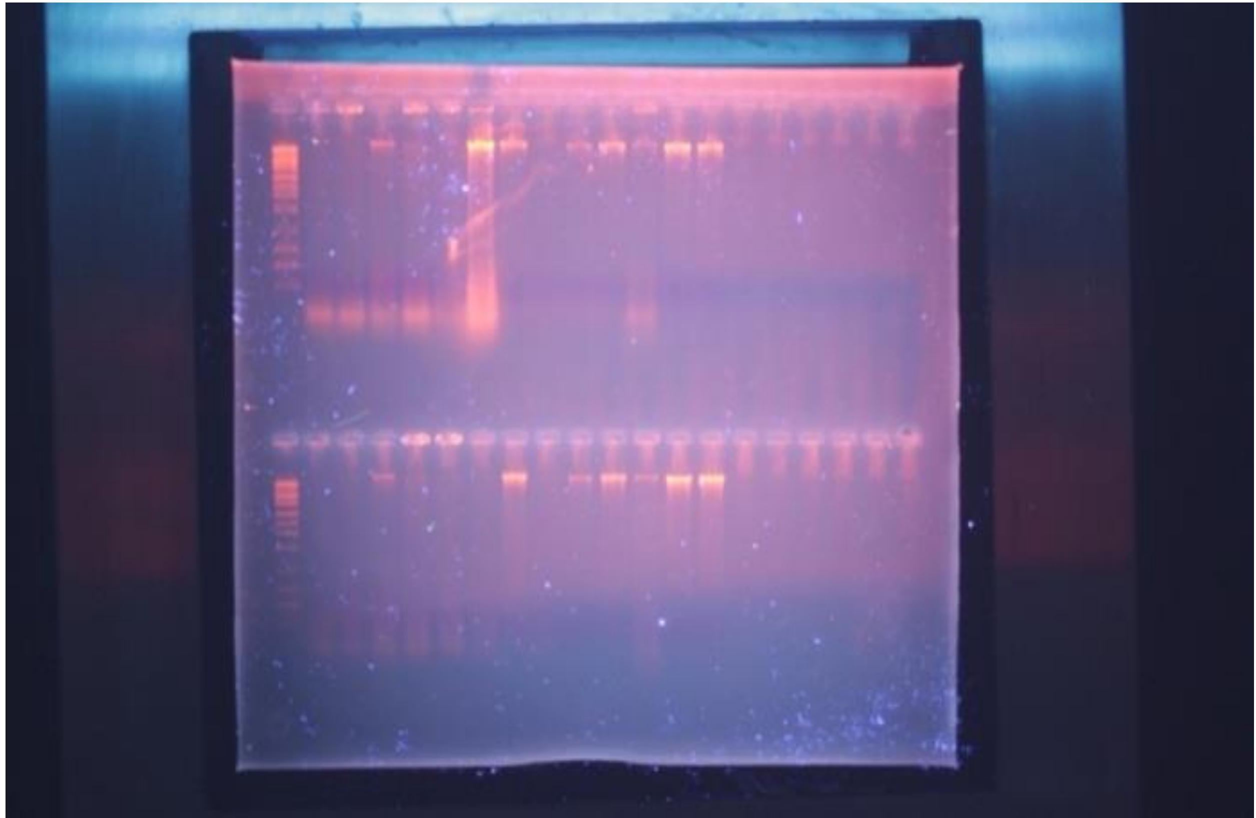


Plate 2b: Electrophorogram of extracted DNA samples of PC, EC, FC, MP, EP, SP, EI, MM, EI, EC, VM, EM, VI, using CTAB protocol.

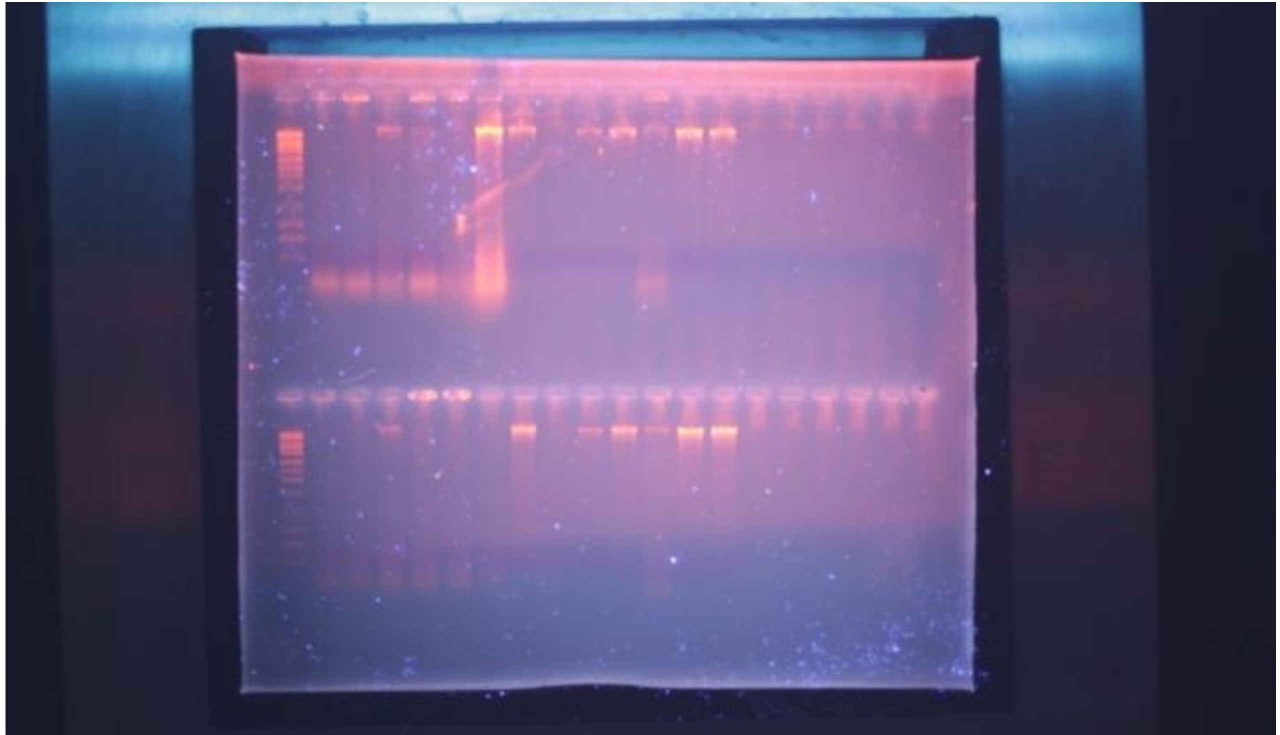


Plate 2b: Electrophorogram of extracted DNA samples of PC, EC, FC, MP, EP, SP, EI, MM, EI, EC, VM, EM, VI, using Modified CTAB protocol.

CHAPTER FIVE

In developing countries, the possibility of disease incidence is very high due to unstable environmental conditions, poor hygienic practices and consumption of contaminated food and water (Pruss-ustun and Corvalan 2006). Smaller numbers of opportunistic pathogens become infectious when resistance mechanisms are impaired either by severe underlying disease or by use of immunosuppressive drugs (Manu-Taiwah et al., 2001). Contamination of pharmaceutical products specifically pediatric formulation can be contaminated with microorganism irrespective of where it is produced (Rahman et al., 2014). Microbial contamination in non-sterile oral drugs rather claimed more significance as the patients, who are taking the drug are already diseased. Therefore, it is very necessary to examine the efficacy or potency of some drugs used for disease medication most especially in children.

A total of seven different strain of fungi were isolated in this study and they include Aspergillus Tamari strain A, Aspergillus tubingenesis strain, Aspergillus tamari strain B, Aspergillus ellipticus, Aspergillus niger strain A, Aspergillus niger strain B, Aspergillus niger strain C. the application of molecular techniques was employed in the accurate identification of this organisms. The result from the DNA sequence data revealed that no single organism sequence data was 100% homologus with those in the genebank when compared with the significance matches with the sequence database at the genebank which means that the isolated organism are of different strain. This is probably the first work to report the studies of fungi contaminants in pharmaceutical products using molecular techniques. According to Adekunle (2011) the major challenge in curing disease caused by fungi is correct identification in order to determine the appropriate drug to administer. (Allen, 1991, Dix and Webster, 1995). Reported quick and simple methods for determining the species composition of fungal communities based n sequencing of particular regions of the fungal genome have proven a reliable alternative to traditional methods.

In this current study, all the tested samples were not found to be highly contaminated with fungi. Aspergillus niger strain were found to be present in 3 out of the total syrup samples while others samples were found to habor Aspergillus Tamari strain A, Aspergillus tubingenesis strain, Aspergillus tamari strain B, Aspergillus ellipticus. Raw materials, ingredients unhygienic

environmental condition and lack of septic handling would be the main factors for the observed microorganism growths in the samples studies (Parker, 2000). To minimize the load of microorganism and the possibility of spoilage during the preparation of liquid drugs, different antimicrobial agent or chemical preservatives (parabens, quarternary ammonium compounds, sorbic acids, formic acids etc.) may be used (Denyer et al., 2004). Unhygienic environmental condition and improper handling of raw materials, ingredients and products might be the cause.

In the classification of fungi, conventional methods are not sufficient in identifying fungi because of the emergence of different strains of fungi including yeasts. Molecular identification clarifies a more realistic separation of genera and species than the phenotypic characterization. DNA sequence was achieved through DNA extraction using Zymo kit, CTAB prtotocol and modified CTAB protocol. Amplification of the rDNA and internal transcribed spacer sequence (ITS) and purification of the PCR product. The universal primer for the identification of fungi which are the ITS1 and ITS4 was used for the amplified region (Trios et al., 2004). The comparison of the rDNA is important in relationship of many organisms. Phylogenetic study helps in the evolutionary relationship between different species and population of organism based on gene analysis by grouping them in clusters. The phylogenetic shows how different or the similar the species are in the course of evolution.

(Fredricks et al., 2005) reported that no single DNA extraction protocol can analyse fungi completely. (Muller et al., 1998) reported that the major problem for isolation of good quality DNA and quantity from fung lies in the braking down of the rigid cell walls due to the fungal nucleases and high polysaccharides content of the cell wall. The work of (Melo et al., 2006) agreed with (Zang et al., 1996) and (Muller et al., 1998) by finalizing that isolation of DNA from some fungal species is hard because they have cell wall that are relatively unsusceptible to breaking down. The result from this study support all the above statement because no single DNA extraction protocol can isolate fungal DNA from the fungi isolated. Out of the three DNA extraction protocol used in this study, Zymo kit was the only one to have extracted the DNA from the fungi isolated with clear band when viewed under the electrophogram.

(Horton and Bruns, 2001) reported that the use of molecular methods for determining the species of a fungus based on amplification and sequencing of the internal transcribed spacer (ITS) region

of the fungal rRNA operon using PCR with universal primers has proven a reliable alternative to traditional methods. Ribosomal genes and spacers regions with the fungal genome have proven good candidates for amplification via PCR because they are comprised of highly conserved tracts with heterogenous regions in between. The conserved tracts are ideal for universal primer design that can allow for the amplification and sequencing of the internal transcribed spacer (ITS) region of the fungal genome, which is highly variable among species or even populations of the same species (Horton and Bruns, 2001). This region lies between the 18S small subunits and 28S large subunit ribosomal RNA genes and contains two noncoding spacer regions (ITS-A and ITS-B) separated by the 5.8S rRNA gene. In fungi, the ITS 1 and ITS 4 designed by (white et al., 1990) the findings in this present study agrees with all the above statement.

A total number of seven different strains were isolated from the pharmaceutical products sampled which support the work of (Baird et al., 2000) that in the process of manufacturing pharmaceutical products, microorganisms can occur as contaminants in the microbiology.

CONCLUSION

From this study, fungi contaminants in pharmaceutical products in Nigeria were isolated, identified, characterized and documented. This was made possible by the use of conventional laboratory and molecular methods. The combination of the two methods employed in this study brought to light the issue of emergence of the new strains of fungi contaminants in pharmaceutical products in Nigeria. In addition, the findings from DNA extraction which is the first approach in all molecular studies showed that one DNA extraction method might not be able to extract all fungal DNA.

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