MOLECULAR CHARACTERIZATION OF MYCOTOXIN PRODUCING MOULDS FROM GRAINS AND LEGUMES AND THEIR SUSCEPTIBILITY TO PLANT EXTRACTS

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CERTIFICATION.

This is to certify that this work **"Molecular characterization of mycotoxin producing moulds from grains and legumes and their susceptibility to plant extracts"** was carried out by **Ohabughiro, Ndidi Blessing (20134877908)** in partial fulfilment for the award of the degree of Ph.D in Medical Microbiology in the Department of Microbiology of the Federal University of Technology Owerri.

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DEDICATION.

I dedicate this work to my loving and kind family members, my parents for their unflinching love and support.

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ABSTRACT

Molecular characterization of mycotoxin producing moulds from grains and legumes and their susceptibility to plant extracts was studied. Grains and legumes were stored for a period of two and four months. They were analysed for the presence of moulds and mycotoxin producing moulds. They were also analysed for the production of different mycotoxins. Sabouraud dextrose agar, Sabouraud dextrose broth, potato dextrose agar and malt extract agar were used in this study. The methods of isolation used were direct physical examination and direct plate count method. Moulds were identified based on their colony morphology, cultural characteristics and molecular characterization. Mycotoxin analysis was done using Liquid chromatography tandem mass spectrometry. Antifungal susceptibility test was done using the Clinical Laboratory and Standards Institute approved methods for testing of moulds using disk diffusion method. The following four oxoid antifungal agents were used: Amphotericin B, fluconazole, ketoconazole and voriconazole. The following medicinal plants: *Ocimum gratissimum* (scent leaves), *Vemonia amygdalina* (bitter leaves), *Gongronema latifolium* (utazi leaves) and *Piper guineense* (uziza leaves) were assessed for their phytochemical properties and antifungal susceptibility profile against various moulds. The organic solvents used for antifungal susceptibility profile were methanol and hot water. One way analysis of variance, Duncan tests and descriptive statistics were used for statistical analysis. The moulds isolated and identified culturally were *Aspergillus* sp. *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Penicillium chrysogenum*, *Fusarium* sp, *Rhizopus stolonifer*, *Rhizopus nigricans* and *Mucor* sp. while those identified molecularly were *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum.* The genus *Aspergillus* was the most prevalent mould. Groundnut had the highest number of isolates followed by maize, beans, rice while wheat had the least number of isolates. The numbers of colony forming units were more in Potato dextrose agar, Sabouraud dextrose agar and least on malt extract agar. Potato dextrose agar recovered the highest number of isolates from both unstored and stored samples followed by sabouraud dextrose agar then malt extract agar. The following mycotoxins were detected Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1, Aflatoxin G_2, Ochratoxin A, Citrinin, Dihydrocitrinone, Fumonisin B_1, Fumonisin B_2, Fumonisin B_3, Fumonisin B_4, Zearalenone, Deoxynivalenol and Nivalenol. Aflatoxin was the most frequent mycotoxin detected. Rice (1286.3 ± 29.689 µg/kg) had the highest rate of recovery of mycotoxins followed by wheat (1166.8 ± 0.901 µg/kg) and groundnuts (1142.9 ± 10.488 µg/kg) while maize (1111.6 ± 9.810 µg/kg) had the least concentration of mycotoxins. Ketoconazole (39 ±1.000 mm) had the highest minimum inhibitory concentration among different anti-fungal agents, followed by voriconazole (36 ± 2.000 mm), Amphotericin B (30 ±2.000 mm) and then Fluconazole (21 ± 12.124 mm). Susceptibility profile using only tetrazolium gave no zone of inhibition. Antifungal agents alone gave a better zone of inhibition than the combination of antifungal agents with tetrazolium. The medicinal plants such as scent leaves, bitter leaves, uziza leaves and utazi leaves analysed contained the following phytochemicals tannin, alkaloid, steroid, saponin and flavonoid. Percentage growth inhibition of moulds by methanol and hot water

extract of scent leaf gave the highest inhibition followed by bitter leaf, utazi and uziza gave the least growth inhibition. Methanol extract gave a better inhibition than hot water extract at 100 mg/ml and 50 mg/ml. One-way analysis of variance and Duncan tests showed that there was a significant difference ($P < 0.05$) between the isolates found in stored and unstored grains and legume. There was also a significant difference ($P < 0.05$) between the antifungal agents and media used. Potato dextrose agar should be used frequently since it gave the highest rate of mould recovery. Ketoconazole remains the best drug of choice among other antifungal agents against moulds. Medicinal plants like scent leaves, bitter leaves, utazi leaves and uziza leaves gave varying levels of growth inhibition against various isolates and should be used both at home and clinical settings. The grains and legumes studied were contaminated with various species of moulds and contained many mycotoxins of public health importance.

Keywords: Grains and legumes, moulds, 18S rRNA sequencing, mycotoxins, antifungal agents and medicinal plants.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND INFORMATION.

Fungi have been characterized as non-photosynthetic and as such need to get supplements from life forms' natural matter. Most fungi are fundamentally saprophytes and accordingly get sustenance from dead natural matter created by different creatures. Few fungi are parasites and hence obtain nourishment from other living organisms (Royse, 2003). Fungi are members of the subdivision of the subkingdom Thallophyta. Fungi lack chlorophyll which is used to characterize most plants, and they have well defined nuclei (Mavor & Harold, 1966). Fungi are made up of multicellular branching filaments known as hyphae. The hyphae form a mesh of network known as mycelium. The hyphae reproduce asexually by means of spores. Fungi do not have vascular tissue, though their body may be amoeboid or unicellular in few species (Talbot, 1971). Fungi are known as heterotrophs feeding on plants and animal substrates. Moulds and yeasts forms are the morphological and predominant forms of fungi which have no significance taxonomically. Some fungi exhibit dimorphism in that they can exist as moulds or yeasts depending on certain environmental or nutritive conditions (Moore & Jaciow, 1979).

Fungi are used by man in various ways; in the industry they can be used as yeast for baking, they can be used as food yeast (which is a source of amino acids, proteins and vitamins) and sources of food like mushrooms. On the other hand fungi can cause great economic loss when pathogenic fungi grow on food, thus reducing the economic value, causing great harm to health of people by producing secondary metabolic products like mycotoxins. They also attack and destroy raw and manufactured products. Fungi can also cause disease in man and animals (Talbot, 1971).

Fungi can grow in a variety of habitats and can be found worldwide. Fungi can be found in deserts, hyper saline climate (Sancho *et al*., 2007), remote ocean (Hawksworth, 2006), on rocks (Mueller & Schmit, 2006), boundaries of temperatures like low and high temperatures. Fungi can be found in extraordinary ultraviolet and enormous radiation experienced during space travel (Alexopoulos *et al*., 1996).

Mould is a sort of organism that forms long multicellular fibers called hyphae (Moore & Robson, 2011). Fungi species of moulds are taxonomically diverse and large; the growth of hyphae causes a discolouration and fuzzy appearance mainly on grains and legumes. Hyphae network known as mycelium, which is usually transparent appears like fine fluffy white thread over surfaces. Septa that are cross-walled may delimit connected compartments of hyphae, each compartment having one or multiple genetically identical nuclei. The production of many asexual spores (conidia) formed by differentiation at the end of hyphae is responsible for the dusty texture of many moulds. The mode of formation and shapes of fungi spores were traditionally used to classify them (Ryan & Ray, 2004).

Examples of common genera of moulds are: *Acremonium, Alternaria, Aspergillus, Cladosporium, Fusarium, Mucor, Penicillium, Rhizopus, Stachybotrys, Trichoderma, Trichophyton* (Moore & Robson, 2011). Moulds do not just only cause direct losses but can damage the health of both man and animals. Damages caused by moulds are not quickly noticed until it has reached an advance stage. Moulds produce secondary metabolites known as mycotoxins, which are poisonous and can damage the health of man and animals. Mycotoxins are generally produced by the genus of *Aspergillus, Penicillium* and *Fusarium.* They are extremely heat stable and can be resistant to ultraviolet light inactivation (Kiark, 2002).

Mycotoxicoses is the disease that results because of the presence of mycotoxins. Mycotoxins can be acutely or chronically toxic or both depending on the type of toxin and its dosage. Acute diseases in animals include the kidney and liver damage, central nervous system attack, skin diseases and hormonal effects. Among the mycotoxins, the most potent natural carcinogenic compound causing mutation (transversion) of 249^{th} codon of P53 gene is aflatoxins produced by *A. flavus, Aspergillus parasiticus* and *Aspergillus nomius* (Deng & Ma, 1998).

According to researchers, there are 300 to 400 estimated mycotoxins presently identified with more being isolated as new techniques and processes evolve (Kiark, 2002). Some mycotoxins impacting significantly on agricultural commodities include include aflatoxins produced by *A. flavus,* and *A. parasiticus,* zearalenone and trichothecenes (particularly deoxynivalenol) produced by *Fusarium* spp., ochratoxin produced by *A. ochraceus* and fumonisins produced by *Fusarium moniliforme* (Deng & Ma, 1998).

It is estimated that about 25% estimate of the world's food crops are affected yearly by mycotoxins (Cole & Cox, 1981). Mycotoxins may develop in almost any foodstuff and feedstuff during the growing season, during harvest or during storage. Moist or wet weather favours *Fusarium* toxins, while hot and humid weather encourages aflatoxin formation.

Among by-products affected by mycotoxins, like grains, oilseeds, wet brewer's grains, food wastes, forages, by-product feeds, protein concentrates and finished feeds, attention is given mostly to grains. Most fungal toxins occur in families of chemically related metabolites, with about 300 to 400 compounds known as mycotoxins, and a dozen groups regularly seen as normal threats to human and animal health (Cole & Cox, 1981). While all mycotoxins are of fungal origin, not all toxic compounds produced by fungi are known as mycotoxins.

1.2 PROBLEM STATEMENT.

Molecular techniques that would characterize mycotoxin producing moulds from grains and legumes are relevant in the identification of different species of mould that produce mycotoxins. The presences of mycotoxins cause reduction in quantity and quality of grains and legumes during storage. This lowers their market value. There is need to know the different mycotoxins in grains and legumes that has adverse effect on the health of consumers.

1.3 AIM:

Molecular characterization of mycotoxin producing moulds from grains and legumes and their susceptibility to plant extracts.

1.4 OBJECTIVES:

1. To extract and amplify genomic deoxyribonucleic acid (DNA) of isolated moulds from grains and legumes (rice, maize, wheat, groundnut and beans).
2 To characterize isolates using 18S rRNA gene sequencing method.
3 To determine the Phylogenetic tree analysis of moulds.
4 To identify and quantify mycotoxins in grains and legumes.
5 To determine the occurrence of mycotoxins producing moulds in grains and legumes.
6 To test the *in vitro* antifungal activities of some selected conventional antifungal drugs against the isolated organisms.
7 To ascertain the antifungal of some medicinal plant against the mycotoxin producing fungi.

1.5 JUSTIFICATION OF STUDY.

There will be knowledge of the molecular characterization of mycotoxin producing moulds from different grains and legumes.There is need to create awareness of mycotoxin producing moulds infestation of grains and legumes. The different mycotoxins found in grains and legumes will be known.

1.6 SCOPE OF STUDY.

1. Sampling of unstored and stored grains and legumes.
2. Isolation and characterization of moulds.
3. Molecular analysis:

   Deoxyribonucleic acid (DNA) extraction, Polymerase Chain Reaction, Sequencing and Phylogenic tree analysis.
4. Mycotoxin analysis (Liquid chromatography tandem mass spectrometry).
5. Antifungal susceptibility test (Disk diffusion method).
6. Antifungal susceptibility test using Medicinal plants.
7. Statistical analysis.

CHAPTER TWO

2.0 LITERATURE REVIEW.

2.1 GRAINS AND LEGUMES.

The botanical names for some grains and legumes are Rice (*Oryza sativa*), Maize *(Zea mays*), Wheat (*Triticum aestivum*), Groundnut (*Arachis hypogaea*) and Cowpea (*Vigna sinensis*) (Kassam, 2016). In Africa, cereals (Rice, maize, wheat) and legumes (groundnut, and beans) are the major food sources for people because they are cheap and readily available. They also have the potential to become a food of the future (Kassam, 2016)

2.2 STORAGE FUNGI.

Most Fungi found in grains and legumes can be divided into two groups, the "field fungi" and the "storage fungi". It may not be easy to ascertain a sharp distinction as whether fungal growth started in the field or during storage, though, fungal growth usually starts in the field. Fungi found in stored grains and legumes include all species of *Aspergillus, Fusarium* and *Penicillium* (Pitt & Hocking, 1985). There are factors that necessitate the growth of fungi in stored grains and legumes and they are:

(1) Nutrient contents of grains.

(2) Temperature and moisture conditions

(3) Biotic factors like competition or the presence of insects in stored product.

Storage fungi are seen more frequently in grains and legumes infested with insects. This is because insects produce moisture and spread fungi spores in the grains and legumes.

For fungi to grow in storage, they require a relative humidity of 65% (or a water activity of aw = 0.65) which is equal to an equilibrium moisture content of 13% in cereal grain, and temperatures of between 10°C and 40°C though every fungal specie, has its own optimal

temperature and climatic growth requirement. Table 2.1 shows the minimum moisture requirement in grains for the growth of some important storage fungi (Multon, 1988).

TABLE 2.1: TEMPERATURE AND WATER ACTIVITY OF VARIOUS FUNGI.

Microorganism (mycotoxin)	Temperature ^{o}C	Water Activity (A_w)
Aspergillus flavus, A.parasiticus (aflatoxin)	33	0.99
Aspergillus ochraceus (ochratoxin)	30	0.98
Penicillium verrucosum (ochratoxin)	25	0.90 to 0.98
Aspergillus carbonarius (ochratoxin)	15 to 20	0.85 to 0.90
Fusarium verticilloides, F. proliferatum (fumonisim)	10 to 30	0.93
Fusarium verticilloides, F. proliferatum (DON)	11	0.90
Fusarium graminearum (zearalenone)	25 to 30	0.98
Penicillium expansum (Patulin)	0 to 25	0.95 to 0.99

Source: (Multon.1988)

2.3 FACTORS INFLUENCING THE GROWTH OF FUNGI IN STORED GRAINS AND LEGUMES.

2.3.1 Water activity.

This is a chemical concept introduced by Scotts in 1957 to microbiologists, which efficiently quantifies the relationship between moisture content in food and the ability of microorganisms to grow on them. Water activity (Aw) can be defined as a ratio: aw=p/po (Atanda *et al*., 2011).

Here, P is the partial pressure of water vapour in the test material and Po is the saturation vapour pressure of pure water under the same condition. Water activity is numerically equal to equilibrium relative humidity (ERH) expressed as a decimal. When a food product is held at a constant temperature in a sealed area until the water in the product has reached an equilibrium with the water in the surrounding air, then a_w (food) = ERH (air)/100. In a practical sense, this is the main environmental factor controlling fungal growth and this is used to determine the stability of stored grains and legumes. There can be prediction of storage life or shelf life of commodities with the knowledge of fungal water relations. Also an understanding of the water relations of mycotoxin production will assist in knowing the ability for mycotoxins to be formed in stored grains and legumes. Fungi like every other microorganism require water for optimal growth though some fungal species can tolerate low water activity. Certain fungi have the ability to grow at low water activity level which is why storage fungi can grow in dried foods. The tolerance degree level to low water activity is usually expressed in terms of the minimum fungal spore germination and hyphal growth that occurred.

Xerophiles (dry loving) are fungi that are able to grow at low water activity level of below 0.85 with one set of environmental conditions (Pitt, 1973). Few xerophiles are able to grow at or below O.75a_w within 6 months (Pitt and Hocking, 1985). Food products stored between 75 and 85% humidity are usually affected by presence of water on the a_w scale, life exist over

the range 0.9999+ to 0.60. Growth of animals is usually 1.0 – 0.99 a_w while most microorganisms cannot grow below 0.95 a_w (Pitt & Hocking, 1985).

2.3.2 Concentration of hydrogen ion.

Fungi growths are usually affected by the concentration of hydrogen ion in stored grains and legumes. pH of stored grains and legumes affects the growth of most fungi species, with most fungi growing optimally at hydrogen ion concentration of 4 and 6.5 (Pitt & Hocking, 1985).

2.3.3 Temperature.

Temperature is usually an essential factor to be considered in the growth of fungi; moreover, food products are usually stored under conditions suitable for fungal growth. Temperatures below 20°C always favour cold–tolerant fungi like *Penicillium* and *Cladosporium* while *Aspergillus* species are favoured by higher storage temperatures. In tropical areas, stored grains and legumes are more susceptible to *Aspergillus* species than other fungi. Many aspergilli are favoured by the combination of low and relatively high storage temperatures of between 8-45°C. Temperature range of -7 to 0°C has been reported to be the lowest temperature for the growth of *Fusarium* species *and Cladosporium* species (Pitt & Hocking 1985; Alan, 1999).

2.3.4 Gas tension.

Reduction in oxygen combined with increase in carbon dioxide concentration can have a great effect on the growth of fungi. These factors are necessary in the storage of food products, where such conditions are primarily for the control of insects (Hocking, 1991). Filamentous fungi like food spoilage moulds have absolute requirement for oxygen. Even though many fungi species appear to be efficient oxygen scavengers so that growth will be determined by the total amount of oxygen available and not by the level of oxygen tension. Most of the food spoilage moulds appear to be sensitive to high levels of carbon dioxide content. The growth of fungi species like *Aspergillus* and *Penicillium* are encouraged by

increase in the uptake of carbon dioxide up to 15% in air, but greatly reduced by high levels (Hocking, 1991).

2.3.5 Consistency.

Liquid products are more readily spoiled by yeast because single celled microorganisms are able to disperse more readily in liquids than solids. However, a liquid substrate is more likely to give rise to anaerobic condition and fermentation is usually seen in liquids than in solids. Though filamentous fungi that are assisted by a firm substrate have ready access to oxygen.

2.3.6 Specific solute effect.

Growth of fungi on a particular food can be influenced by the type of solutes present in that food. Scott (2012), reported that *Aspergillus* grew 50% faster at its optimal a_w of 0.96 when a_w was controlled by glucose rather than by magnesium chloride, sodium chloride or glycerol.

2.3.7 Preservatives.

Normally, stored grains and legumes are free of preservatives when preserved for human consumption. At times, Insecticides and fumigants may be present though little knowledge is known about it (Hocking, 1991). Food products used as animal feeds may sometimes contain weak acid preservatives like propionates usually sold as proprietary chemical.

2.3.8 Combinations of factors.

The various factors just discussed above do not act independently, and indeed often are synergistic. If two or more factors act simultaneously, it may be possible to store commodities for longer than would otherwise be expected. The combination of low water activity, reduced oxygen and/or increased carbon dioxide levels may greatly influence the growth of fungi in stored foods. In a natural storage conditions, water activity is the predominant factor determining the storage life of food products. The most effective way of ensuring that fungi do not invade stored grains and legumes is to dry the products as quickly and evenly as possible and keeping them dry (Akerstrand, 1995).

2.4 Damages caused by storage fungus.

(1) Nutrients loss.

(2) Grain discoloration.

(3) Reduction in germination ability

(4) Caking of stored grains

(5) Increase in the temperature of the stored goods up to spontaneous combustion

(6) Moldy smell and taste

(7) Production of mycotoxins

(8) Creation of environment for the development of special insect species (= indicator for low grain and legume quality) (Christensen & Meronuck, 1986).

2.4.1 Ways of minimizing/controlling storage fungus.

(1) Drying of the produce as quickly and evenly as possible after harvesting up to the critical moisture/safe moisture level. The critical water content for safe storage corresponds to a water activity of about 0.7 (Akerstrand, 1995).

(2) Careful prevention of grains and legumes damage during harvest, handling, threshing or drying by keeping them in a cool and dry place.

(3) Prevention of condensation (Sealed storage under a modified atmosphere). On a large scale, areas should be equipped with instruments for measuring ambient conditions: keep temperatures in the store as constant as possible and carry out regular controls.

(4) Prevention of moisture reabsorption resulting from incorrect ventilation or water entering the store.

(5) Ensuring that the development of high insect population ("hot spots") in grains and legumes are avoided.

(6) Proper arrangement of food products, re-drying of parts of the stack with unacceptable high moisture content (Christensen & Meronuck, 1986).

2.5 MYCOTOXINS.

Various fungi produce mycotoxins which are toxic secondary metabolites. Mycoxins remain in stored grains and legumes as residues. After about a period of 24 hours infestation of stored grains and legumes by fungi, mycotoxins can be produced. The optimum climatic conditions for the growth of fungi and the production of mycotoxins are not always the same but depends on various other unidentified factors. Mycotoxin contamination in stored grains and legumes can only be stated with certainty by means of laboratory examination (Anon, 1989).

Filamentous fungi therefore produce low molecular weight natural products as secondary metabolites. These metabolites constitute a toxigenically and chemically heterogeneous assemblage that is grouped together only because the members can cause disease and death in human beings and other vertebrates. Mycotoxins display different levels of toxicites in invertebrates, plants and microorganisms (Bennett, 1987).

Losses that result as a production of mycotoxins are as follows:

i It lowers the animal production and any human toxicity is attributable to the presence of the toxin,

ii The presence of the toxin in the affected commodity definitely lowers its market value.

iii There is a secondary effect on agriculture production and agricultural communities.

Mycotoxin production affects many areas of economic aspect of agriculture; the production sectors and consumption of grains and legumes production via grains and legumes producers, handlers, processors, consumers and society as a whole. The producers of grains and legumes are affected by limited yields, restricted end markets due to contamination and price discounts. Grains and legume producers' are mainly affected by few storage facilities, high cost of testing grains and legumes and generally loss of market. Those that produce grains

and legumes as a result incur high cost in grains and legume production because they lose a lot of grains and legumes in the process; there is the issue of monitoring cost and market availability. The consumers therefore pay a lot for the grains and legumes because of increased cost of monitoring at all levels of production and in rare cases, death can occur from the consumption of contaminated grains and legumes. When the products cause an increase in regulation, it will make the consumers in the society to pay higher for the grains and legumes. Other factors that contribute to increase in cost are lower cost of exports, higher cost of import and research that need to be done. Research done over the years especially by Nigerian Stored Products Research Institute (NSPRI) has revealed the presence of aflatoxin in Nigerian groundnut and livestock feed, others include maize, millet and sorghum (Oyeniran, 1978; Opadokun & Ikeorah, 1979; Stoloff *et al*., 1991; Chu, 1996; Stroka & Anklam, 2002).

2.5.1. Occurrence.

Normally mycotoxins occur on the field prior to harvesting. Post-harvest contamination can occur when proper and even drying is delayed and also during storage when the water contents exceeds the critical level for mould growth. Stored grains and legumes can easily be invaded by moulds when they are infested by insects or rodents during storage. Products like cheese, milk, eggs have been contaminated as a result of consumption of aflatoxin contaminated feeds by livestock because aflatoxin M_1 has been found in human milk. It is usually unstable in processes used in making tortillas that uses alkaline conditions or oxidizing steps (Goldblatt, 1969).

2.5.2 Production.

Majority of fungi are aerobes, they use oxygen and are ubiquitous in very small quantities because of the minute size of their spores. In conducive environment where there is available temperature and humidity, fungi consume organic matter. In favourable conditions, fungi

proliferate and the level of mycotoxins production becomes high (Małgorzata, *et al*., 2013). The reason for the high production of mycotoxins is not yet known. Some fungi do not need mycotoxins to grow or develop. Knowing that mycotoxins weaken the receiving host, the fungi will use the host to make the emvironment conducive for further fungal proliferation. The production of mycotoxins depends on the available intrinsic and extrinsic environment and the mycotoxicosis varies greatly in their severity, depending on the type of organism infected and its susceptibility, metabolism and defense mechanisms (Małgorzata, *et al*., 2013).

2.5.3 MAJOR GROUPS OF MYCOTOXINS.

i) Aflatoxins.

One of the difuranocumarin derivatives is aflatoxins. The major naturally produced aflatoxins based on their natural fluorescence (blue or green) are called B_1, B_2, G_1, and G_2. Aflatoxin M_1 is a monohydroxylated derivative of AFB_1 which is usually formed and excreted in the milk of lactating animals (Zain, 2011). Aflatoxins (AF_s) are freely soluble in moderately polar organic solvents (e.g. chloroform and methanol); are very slightly soluble in water (10–30 μg/mL); and extremely soluble in dimethyl sulfoxide and insoluble in non-polar solvents (Richard, 2007). They are unstable under the influence of ultraviolet light in the presence of oxygen, to extremes of pH (< 3, > 10) and to oxidizing agents according to International Agency for Research on Cancer (IARC, 2002).

Aflatoxins are usually produced only by a closely related group of aspergilli *like*: *Aspergillus flavus*, *A. parasiticus* and *A. nomius* strains (Moss, 2002). The term aflatoxin normally refers to four different types of mycotoxins produced, which are B_1, B_2, G_1, and G_2. Aflatoxin B_1, the most toxic, which is a potent carcinogen, has directly seen to have a correlation to adverse health effects, like carcinogenic diseases in many animal species (Małgorzata, *et al*., 2013). In the tropics and subtropical areas, aflatoxins are usually produced by products like rice,

maize, cotton, peanuts, spices, pistachios etc. Rice is normally an important dietary source of aflatoxins. Many complications are found related to aflatoxin contamination in grains and legumes. Maize is usually colonized by *A. flavus* and related species in the field (Rajankar *et al*., 2007; Richard, 2007; Zain, 2011; Małgorzata, *et al*., 2013).

ii) Ochratoxin.

This mycotoxin is ususally in three secondary metabolic forms, A, B and C. *Penicillium* and *Aspergillus* species produce all these secondary metabolic forms. The difference between the three forms lies in the fact that ochratoxin B (OTB) is a nonchlorinated form of ochratoxin A (OTA) and that ochratoxin C (OTC) is an ethyl ester form ochratoxin A (Leslie, 1990). *Aspergillus ochraceus* can be seen in a lot of products like beverages like beer and wine. *Aspergillus carbonarius* is the main fungi specie found on vine fruit which during its process of juice making release its toxins (Ivić, *et al.,* 2009). OTA has been known and labeled a carcinogen and a nephrotoxin and has been linked to tumors in the human urinary tract (Ivić, *et al.,* 2009).

iii) Ochratoxin A (OTA).

Ochratoxin A is a chlorinated isocumarin derivative, and it contains a chlorinated isocoumarin moiety linked through a carboxyl group to L-phenylalanine via an amide bond (Moss 2002). This mycotoxin is relatively more stable in the environment than aflatoxins. Ochratoxin is also soluble in polar organic solvents, colourless and crystalline (Larsen, 2001). According to the study of Moss (2002), ochratoxin A is destroyed after exceeding a heat temperature of 250^{o}C. Ochratoxin A is normally produced by *Penicillium* species such as *P. verrucosum, P. auriantiogriseum, P. nordicum, P. palitans, P. commune, P.variabile* and by *Aspergillus* species e.g. *A. ochraceus, A. melleus, A. ostanius*, as well as the *Aspergilli* species (Larsen, 2001).

When the climate is moderate, *Penicillium* species are the main producers of Ochratoxin A, while *Aspergillus* species are the main producers of Ochratoxin A in tropical and subtropical climates (Moss 2002). Citrinin is often found with Ochratoxin A and produced by *Penicillium aurantiogriseum*, *P. citrinum*, and *P. expansum* (Larsen, 2001). Ochratoxin A is found in reasonable quantity in wine, grape juice, coffee, spices, dried fruits and cereal-based products, e.g. whole-grain breads and also from farm products of animal origin, like pork and pig blood-based products (Larsen, 2001). The Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA) has estimated a tolerable weekly intake of Ochratoxin A to be on the level of 120ng/kg. The IARC has also labeled it a possible human carcinogen (Group 2B). Ochratoxins are usually the cause of urinary tract cancer and also cause kidney damage. In ruminants, it is divided into non-toxic ochratoxin alfa and phenylalanine (IARC, 2002).

iv) Citrinin.

Citrinin is a polyketide nephrotoxin usually produced by several species of the genera *Aspergillus*, *Penicillium* and *Monascus*. Some fungi that produce citrinin can also produce ochratoxin A or patulin. Citrinin is insoluble in cold water though soluble in aqueous sodium hydroxide, sodium carbonate or sodium acetate, ethanol, methanol, acetonitrile and some other polar organic solvent (Pereyra *et al*., 2011).

Above or under 175 °C in dry conditions and at or above 100 °C in the presence of water thermal decomposition of citrinin occurs. Citrinin H_2 which does not show significant cytotoxicity is one of the decomposition products of citrinin (Barros, 2011). Citrinin H_1 shows a reasonable increase in cytotoxicity when compared to the parent compound (Pereyra *et al*., 2011). Citrinin was first isolated from *Penicillium citrinu* has been identified in many species of *Penicillium* and several species of *Aspergillus* (Barros, 2011). Some of these species have been used to produce human foodstuffs like cheese (*Penicillium*

camemberti), miso, sake, and soy sauce (*Aspergillus oryzae*) (EFSA.2012). Citrinin produces a toxin known as nephrotoxin in all animal species tested and is responsible for the yellowed rice disease in Japan (Pereyra *et al*., 2011). It is also associated with many human foods (wheat, rye, barley, oats, rice, corn and food colored with Monascus pigment). Citrinin also acts synergistically with ochratoxin A to depress RNA synthesis in murine kidneys (Barros, 2011). The full effect on human health is yet to be known (Barros, 2011). The most commonly contaminated products of grains include corn, oats and barley while plant products include fruits juices and vegetables, beans, herbs and spices (EFSA.2012).

v) Ergot alkaloids.

Ergot Alkaloids are compounds which are usually produced by a toxic mixture of alkaloids in the sclerotia of species of *Claviceps*, which are common pathogens of various grass species. The ingestion of ergot sclerotia from infected cereals, usually used to make bread that are produced using contaminated flour causes ergotism the human disease. This disease is historically known as St. Anthony's fire (Barros, 2011).

There are two forms of ergotism; gangrenous, that affects the blood supply to extremities and convulsive that affects the central nervous system (Barros, 2011). The use of modern methods to clean grains and legumes has reduced the incidence of ergotism as a human disease; however, it is still an important veterinary problem. Pharmaceutically, ergot alkaloids have been used in producing industial products (Barros, 2011).

vi) Patulin.

The following fungi species; *P. expansum*, *Aspergillus*, *Penicillium* and *Paecilomyces* are known to produce patulin as a toxin. Fungal species, like *P. expansum* is usually associated with a range of mouldy fruits and vegetables especially rotting apples and figs (Weidenborner, 2000; Biernasiak, 2012). Fermentation usually destroys the toxin patulin, so it is not found in beverages or in apples like cider. Patulin has been reported to damage the

immune system in animals though it has not been shown to be carcinogenic (Weidenborner*et al.*, 2000). The European community in 2004 set limits to the concentration of patulin in food produce at 50 µg/kg in all fruit juice concentrations, 25 µg/kg in solid apple products used for direct consumption, and 10 µg/kg for children's apple products, including apple juice (Biernasiak, 2012).

vii) Fumonisins (F_S).

This is a group of diester compounds with different tricarboxylic acids and polyhydric alcohols and primary amine moiety. Fumonisins have different types though only fumonisins B_1 (FB_1) and B_2 (FB_2) have been found in significant amounts. When the tricarboxylic acid chain in fumonisin is hydrolyzed, the toxin becomes more toxic than fumonisin (Scott, 2012). FB_1 is produced by fungi from *Fusarium* genera, especially by *F. moniliforme* and *F. proliferatum.* The risk of contamination with *Fusarium* toxins is higher for maize and wheat than for soybean and pea according to the study of Ivić, *et al*., (2009). Hot and dry weather followed by periods of high humidity are usually associated with high concentrations of fumonisins. Complete studies on fumonisin residues in milk, meat and eggs are yet to be established (IARC, 2002; Carvet & Lecoeur 2006). The assessment of fumonisin B_1in human exposure has rarely been reported. The mean daily intake in different countries has been established as follows; In South Africa, the estimates ranged from 14 to 440 µg/kg bw/day, in Switzerland. It is estimated to be 0.03 µg/kg bw/day while in the Netherlands the exposure estimates ranged from 0.006 to 7.1 µg/kg bw/day (Carvet & Lecoeur 2006). This shows that the exposure to FB_1 is higher than in the other countries in which exposure assessments were performed (Yazar & Omrtag, 2008).). The study concluded that for fumonisins there was not enough evidence in humans for carcinogenicity. Therefore, the IARC classified *Fusarium monilliforme* toxins, including fumonisins, as potential carcinogens to humans (group 2B) (IARC, 2002).

Fusarium toxins are produced by more than 50 species of *Fusarium*. It has been known to infect grains of developing cereals like maize and wheat (Pacin *et al*., 2001; Torres, *et al*., 2010). Some of the major types of *Fusarium* toxins include: beauvercin and enniatins, butenolide, equisetin, and fusarins (Pacin, 2001; Torres, *et al.,* 2010; Błajet-Kosicka *et al*., 2010; Opinion of the Scientific Committee on food on Fuarium toxin, 2000)

viii) Zearalenone.

Zearalenon is a macrocyclic lactone with high binding affinity to oestrogen receptors. Zearalenon is mainly produced by *Fusarium graminearum* and *F. sporotrichoides.* They are usually produced in the field and during storage of grains and legumes like maize, sorghum, barley and soybean. The carcinogenicity of zearalenone has been analyzed by the IARC and has been found to be carcinogenous and a possible human carcinogen (group 2B). Residues of zearalenone in meat, milk and eggs do not appear to be a practical problem (Yazar & Omrtag, 2008).

ix) Trichothecenes.

A group of 50 mycotoxins produced by *Fusarium*, *Cephalosporium* and *Stachybotrys* genera in various grains and legumes constitutes Trichothecenes. They include deoxynivalenol, T-2 toxins, nivalenol, and diacetoxyscirpenol. Aside from trochothecenes, deoxynivalenol (DON, womitoxin) is probably the most widely distributed in cereal and soybean foods and feeds. In cereals that have been contaminated, DON derivatives such as 3-acetyl DON and 15-acetyl DON may occur in significant amounts (10 – 20%) with deoxynivalenol. Deoxynivalenol is produced by closely related *Fusarium graminearum, F. culmorum* and *F. crokwellense* species (Yazar & Omrtag, 2008). Fungi species of *F. sporotrichoides* and *F. poae* normally produce T-2 d toxin. It can be found in mouldy wheat, rye, oats, millet and buckwheat. The toxin can be transmitted from dairy cattle feed to milk (EFSA, 2012).

x) Alternaria toxins.

Grains like soybean and cereals has been known to be infested by fungi of *Alternaria* species, besides *Fusarium.* There are other known several species that can produce Alternaria mycotoxins. The most important *Alternaria* mycotoxins include alternariol (AOH), alternariol monomethyl ether (AME), altertoxins I, II, and III (ATX-I, -II, III), tenuazonic acid (TeA), and altenuene (ALT) (Ostry, 2008). The three structural classes which they belong to are: dibenzopyrone derivatives, perylene derivatives, and tetramic acid derivatives. Fungi species of *Alternaria alternate, A. brassicae, A. citri, A. cucumerina, A. dauci, A. kikuchiana, A. solani, A. tenuissima,* and *A. tomato* can produce alternariol and related metabolites (AME and ALT) (Ostry, 2008). These *Alternria* strains are known as plant, especially fruits and vegetable pathogens. AOH, AME and ALTare produced in cereals, oilseeds and soybean. The fungi species responsible for their production include *Alternaria alternata, A. tennuisima,* and *A. infectoria.* AOH has been studied and found to possess cytotoxic, mutagenic, genotoxic, carcinogenic, and oestrogenic properties (Scott, 2012). Tenuazonic acid (TeA) is a mycotoxin and phytotoxin produced primarily by *Alternaria alternata* and other phytopathogenic *Alternaria* species (Ostry, 2008).

xi) Sterigmatocystin.

Sterigmatocystin (STC) is an aflatoxin precursor which is mainly produced by *Aspergillus* species such as *A. versicolor, A. ruber, A. aureolatus A. quadrilineatus, A. chevalieri A. sydowi, Eurotium of amstelodami* and less often by *Penicillium, Chaetomium, Bipolaris* and *Emericella* genera (Versilovskis, 2010). Sterigmatocystin has been studied and reported as a fungal metabolite in mouldy wheat, rice, corn, peanut, cheese or salami, rapessed and barley. The contamination level of STC in cereals and beans based foods and feeds as the STC producers, occurrence and toxic properties has been reviewed (Versilovskis, 2010 ; EU, 2002).

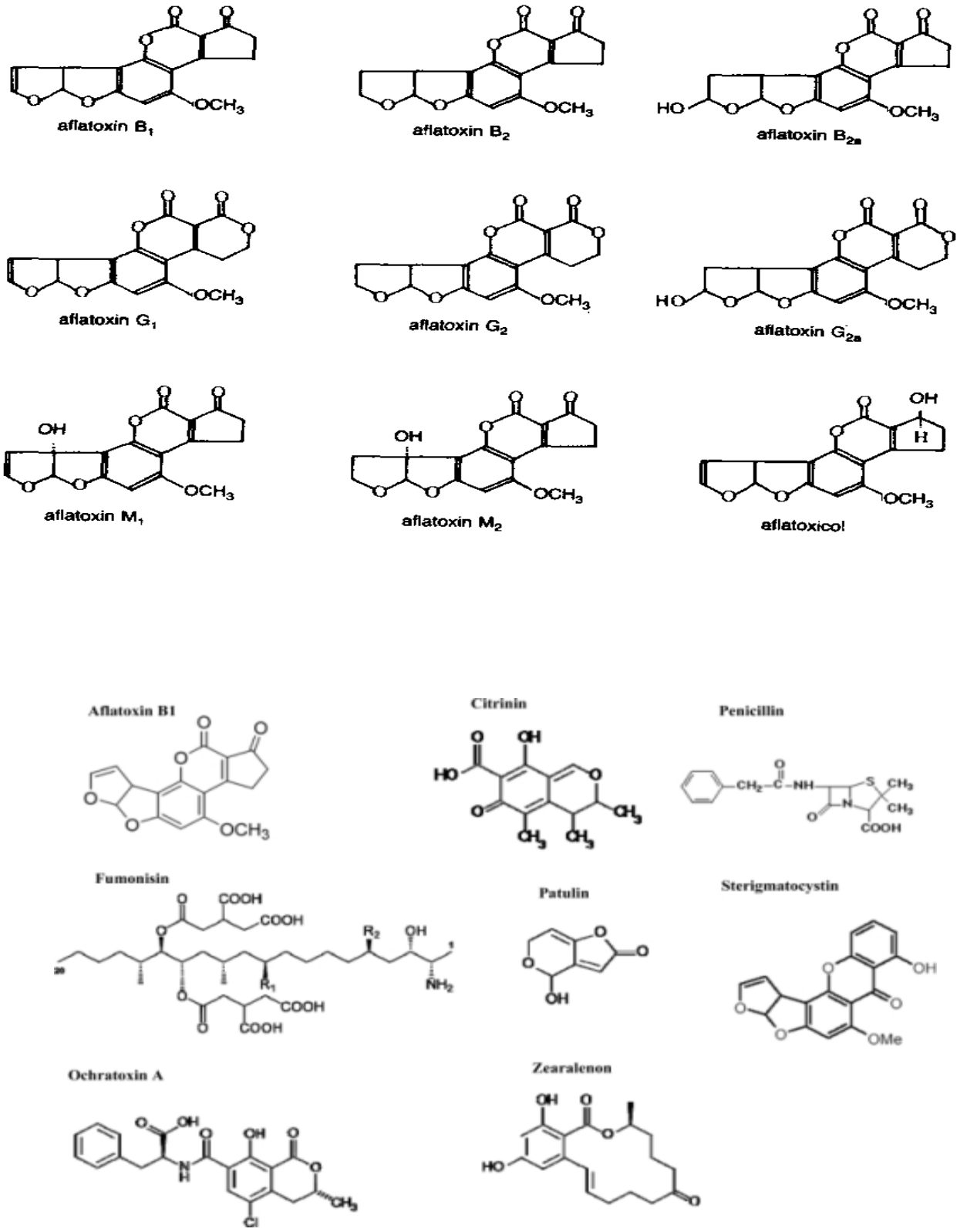


Figure 2.1: Structures of different Mycotoxins.

Source : Małgorzata, *et al*. (2013).

TABLE 2.2 MYCOTOXINS PRODUCERS AND COMMODITIES AFFECTED.

Mycotoxin	**Producers**	**Commodity**
Aflatoxins	*Aspergillus flavus, A.parasiticus, A.nomius, A.bombycis, A.ochraceoroseus, A.pseudotamari*	Cereals, Nuts, spices, maize, soybean, rice
Ochratoxin A	*Penicillium verrucosum, P.auriantiogriseum, P.nordicum, P.palitans, P.commune, P.variabile, Aspergillus ochraceus, A.melleus, A.niger, A.carbonarius, A.sclerotiorum, A.sulphureus*	Cereals, spices, coffee, fruits Food of animal origin
Citrinin	*Penicillium citrinum, P.verrucosum, P.viridicatum, Monascus purpureus*	Oats, rice, corn, beans, fruits, fruit and vegetable juices, herbs and spices
Sterigmatocystin	*Aspergillus versicolor, A.nidulans, A.chevalieri, A.ruber, A.aureolatus, A.quadrilineatus, Eurotium amstelodami*	Cereals, cheese
Zearalenone	*Fusarium graminearum, F.sporotrichoides,*	Maize, soybean, cereals

Mycotoxin	Producers	Commodity
	F.culmorum, F.cerealis, F.equiseti, F.incarnatum	
Deoxynivalenol	*Fusarium graminearum, F.culmorum, F.crokwellense*	Maize, soybean, cereals
Fumonisins	*Fusarium proliferatum, F.verticillioides,*	Maize, soybean, cereals
Alternariol, alternariol monomethyl ether	*Alternaria alternata, A.brassicae, A.capsici-anui, A.citri, A.cucumerina, A.dauci, A.kikuchiana, A.solani, A.tenuissima, A.tomato, A.longipes, A.infectoria, A.oregonensis*	Vegetables, fruit, cereals, soybean
Tenuazonic acid	*Alternaria alternata, A.capsici-anui, A.citri, A.japonica, A.kikuchiana, A.mali, A.solani, A.oryzae, A.porri, A.radicina, A.tenuissima, A.tomato, A.longipes*	Vegetables, fruit, cereals, soybean

Source: IARC, (2002).

TABLE 2.3: GROUPS OF MYCOTOXINS MOST HARMFUL TO MAN OR ANIMALS AND POSSIBLE DISEASE SYMPTOMS.

Mycotoxin	Toxicity class according to International Agency for Research on Cancer (IARC)	Symptoms and diseases
Aflatoxins	I *	Aflatoxicosis, liver cancer, lung neoplasm, lung cancer, failure of the immune system, vomiting, depression, hepatitis, anorexia, jaundice, vascular coagulation.
Ochratoxins	II B **	Renal diseases, nephropathy, anorexia, vomiting, intestinal haemorrhage, tonsillitis, dehydration
Fumonisins	II B **	Diseases of the nervous system, cerebral softening, pulmonary oedema, liver cancers, kidney diseases, oesophagus cancers, anorexia, depression, ataxia, blindness, hysteria, vomiting, hypotension

Mycotoxin	Toxicity class according to International Agency for Research on Cancer (IARC)	Symptoms and diseases
Zearalenone	-	Reproduction disruptions, abortions, pathological changes in the reproductive system
Trichothecenes		Nausea, vomiting, haemorrhages, anorexia, alimentary toxic aleukia, failure of the immune system, infants' lung bleeding, increased thirst, skin rash

Key:

[i] - *The agent (mixture) is carcinogenic to humans. The exposure circumstance shows exposures that are carcinogenic to humans

[ii] - **The agent (mixture) is possibly carcinogenic to humans. The exposure circumstance shows exposures that are possibly carcinogenic to humans.

Source: IARC, (2002).

2.6 HEALTH EFFECTS OF MYCOTOXINS.

Health problems, weak immune system, allergens or irritants identifiable diseases or even death are some of the health effects of mycotoxins found in animals and humans. Some mycotoxins are harmful to other microorganisms like fungi or bacteria (Škrinjar, 1995). It has been stated that mycotoxins found in stored animal feed are the cause of rare phenotypical sex changes in hens that causes them to look and act like male chicken (Montville & Shih 1991; Lee & Magan, 1999).

2.6.1 In humans.

Mycotoxicosis is a term generally used to denote poisoning associated with exposures to mycotoxins. The type of mycotoxin determines the symptoms shown, the length of exposure, the concentration, as well as the health status , age and sex of the exposed individual (Barros *et al.*, 2011). The synergistic effects associated with several other factors such as genetics, diet and interactions with other toxins have not been sufficiently studied.Therefore, vitamin deficiency, alcohol abuse, caloric deprivation and infectious disease status can all have compounded effects with mycotoxins (Barros *et al.*, 2011).

Mycotoxins have the potential to cause both acute and chronic health effects via ingestion, skin contact, (Green *et al.*, 1999) and inhalation. Mycotoxins have the ability to inhibit protein synthesis, inhibit particle clearance of the lung, damage macrophage systems, and increase sensitivity to bacterial endotoxin when they enter the blood stream and lymphatic system (Chelkowski, 1992).

2.6.2 Mitigation.

Mycotoxins have great resistance to decomposition or being broken down in digestive system, which enables them to remain in the food chain of meat and dairy products. Even

temperature treatments like cooking and freezing do not destroy some mycotoxins (Vaamonde & Bonera 1987; Wisniewska*et al*., 2011).

2.6.3 Removal.

It has become a tradition to add mycotoxin binding agents such as montmorillonite or bentonite clay in order to affectively adsorb the mycotoxins in food and feed industries. Mycotoxin deactivation becomes the latest approach to mycotoxin control by means of enzymes (esterase, de-epoxidase), yeast (*Trichosporon mycotoxinvorans*) or bacterial strains (Eubacterium BBSH 797), where mycotoxins can be reduced during pre-harvesting contamination. Other removal methods include washing, physical separation, milling, heat-treatment, radiation, extraction with solvents, and the use of chemical or biological agents. Irradiation methods have proven to be an effective treatment against the growth of moulds and the production of toxins (Song & Karr 1993).

2.7 MYCOTOXIGENIC FUNGI.

Mycotoxigenic fungi are used to refer to fungi that produce mycotoxins. The main mycotoxigenic fungi involved in the human food chain belong to three genera: *Aspergillus*, *Fusarium* and *Penicillium* (Scott, 1993), though mycotoxins have been detected from other fungi under certain growth conditions. The fungi strain determines the amount and kind of toxin produced other factors include the growing conditions as well as the presence or absence of other organisms. The United Nations Food and Agriculture Organization (UNFAO) has assisted most countries in sub-Saharan Africa to enter Alimentarius Standards (CODEX) into law, though monitoring of food quality for foods destined for local consumption is rare. It has promoted and supported the benefits from the economy and health of people of all the nations (Scott, 1993).

There are government laboratories for testing for the presence of mycotoxins especially for grains and legumes that are to be exported. Thus like in the case of peanut export to Europe, the best quality peanuts are exported then the left over may not be monitored for quality. It becomes necessary to have a system that will make monitoring possible and sustainable by using a good cost sharing system and sampling protocols. The need for regular surveillance of high risk agricultural products by checking for selected mycotoxins and also monitoring the human population for diseases attributable to mycotoxins have to be carried out throughout the world to ensure a supply of safe food which is free from naturally occurring contaminants. This will ensure better human and animal health, improved financial and human investments and reduce economic losses. There is accepted knowledge that exposure to high level of food borne mycotoxins poses a serious threat to public health. It is a developmental problem that emcompases childhood survival, demographics, immune system function, drainage of the economic and human resources as a result of diseases like cancers, as well as food security where livestock feeds are contaminated.

Research should be carried out using inexpensive and appropriate sampling and testing protocols. Research should also be carried out on identification and application of appropriate technologies for obtaining low grain moisture during harvest and maintaining low grain and legume moisture during storage. More studies are needed for traditional food preparation technologies like fermentation and nixtimalization, or chelating additives like clays or yeast that may lower mycotoxins in prepared foods (Atanda, *et al*., 2011).

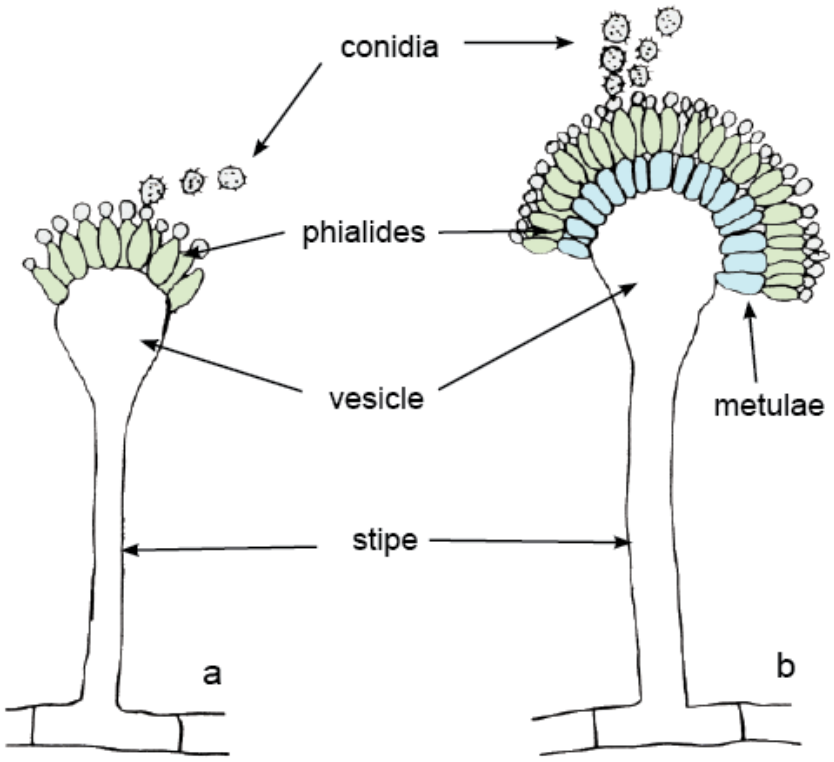
2.7.1 Aspergillus.

The fungi *Aspergillus* is a very large genus containing about 250 species. These species are currently classified into seven subgenera which are further subdivided into several sections comprised of related species. They produce different mycotoxins like aflatoxins, ochratoxins,

citrinin, zearalenone and patulin (Raper and Fennell 1965, Gams *et al.*, 1985, Geiser *et al.*, 2007; Gautam & Bhadauria 2009).

Morphological description: The colonies are usually fast growing, yellow, yellow-brown, white, green, brown to black shades of green usually consisting of dense felt of erect conidiophores. The conidiophores terminate in a vesicle covered with either a single layer of Phialides (uniseriate) or a layer of subtending cells (metulae) bears small whorls of phialides (biseriate structure). The conidia head is formed by the combination of the conidia and vesicle, phialide, metulae (if present). Conidia are one-celled, smooth or rough-walled, hyaline or pigmented, which are produced in long dry chains, which may be divergent (radiate) or aggregated in compact columns (columnar). Some species may produce Hülle cells or sclerotia.

To identify fungi morphologically, the isolates are usually inoculated on three points on Czapek Dox agar and 2% malt extract agar and incubated at 25^{O}C. Most of the species sporulate within 7 days. Fungi descriptions are primarily based on colony pigmentation and morphology of conidial head. Microscopic mounts are usually done using slide culture preparations mount in lactophenol cotton blue or using cellotape flag. A drop of alcohol is usually used to remove bubbles and excess conidia (Samson *et al*., 2014).



Conidial head morphology in *Aspergillus* (a) uniseriate, (b) biseriate.

Figure 2.2: Morphology of the Conidial head of *Aspergillus* (a) uniseriate, (b) biseriate

Source: Scott (1993).

2.7.2 Penicillium.

Penicillium is a very large and ubiquitous genus which currently contains 354 accepted species (Visagie *et al*., 2014). Many species of *Penicillium* are potential producers of mycotoxins like ochratoxins, citrinin, patulin, zearalenone and are usual contaminants on various substrates. When studying *Penicillium* contamination of food, proper identification is important. Though human pathogenic species are rare, there are opportunistic infections leading to mycotic keratitis, otomycosis and endocarditis (following insertion of valve prosthesis) which has been reported (Beuchat, 1992; Lyratzopoulos *et al*., 2000; Samson *et al*., 2014).

Morphological description:

The colonies are usually fast growing in shades of green, at times white and mainly consisting of dense felt of conidiophores. Phialide is seen microscopically as a chain of single-celled conidia which are produced in basipetal succession from a specialized conidiogenous cell. Basocatenate is often used to describe such chains of conidia where the youngest conidium is at the basal or proximal end of the chain. Phialides may be produced singly, in groups or from branched metulae, giving a brush-like appearance (a penicillus) in *Penicillium*. All the cells between the metulae and the stipes of the conidiophores are referred to as branches. The penicillus may contain both branches and metulae (penultimate branches which bear a whorl of phialides). The branching pattern may either be simple (non-branched or monoverticillate), one-stage branched (biverticillate-symmetrical), two-stage branched (biverticillate-asymmetrical) or three- to more-staged branched. Phialides are usually flask-shaped, consisting of a cylindrical basal part and a distinct neck, or lanceolate (with a narrow basal part tapering to a somewhat pointed apex).

Conidiophores are hyaline, smooth or rough-walled. Conidia are in long dry chains, divergent or in columns, globose, ellipsoidal, cylindrical or fusiform, hyaline or greenish, smooth or rough-walled. Some species produce sclerotia (Visagie *et al*., 2014).

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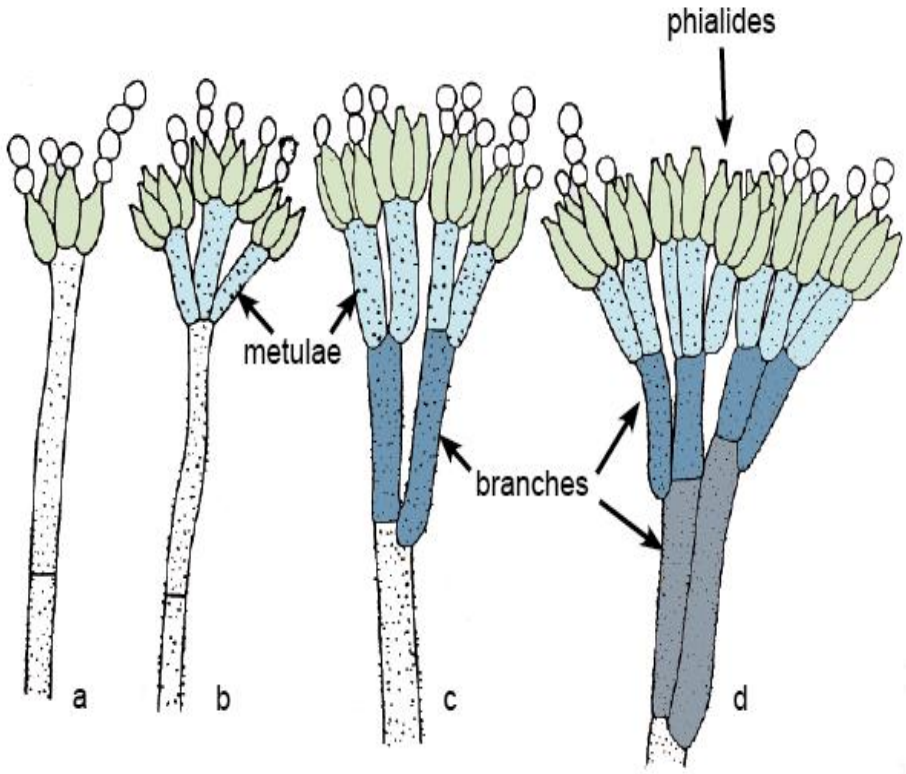


Figure 2.3: *Penicillium;* Morphological structures and types of conidiophore branching; (a) Monoverticillate; (b) Biverticillate; (c) Terverticillate; (d) Quaterverticillate.

Source: Visagie *et al.* (2014).

2.7.3 Fusarium.

Most *Fusarium* species are soil fungi and are distributed worldwide. Some *Fusarium* species are plant pathogens, causing root and stem rot, vascular wilt or fruit rot, while other species have emerged as important opportunistic pathogens in humans causing hyalohyphomycosis (especially in burn victims and bone marrow transplant patients), mycotic keratitis and onychomycosis (Guarro 2013). Some other species produce mycotoxins and cause storage rot. Analysis of the Multi-locus sequence of EF-1α, β-tubulin, calmodulin, and RPB2 have also shown the presence of multiple cryptic species within each "morphospecies" of medically important *fusaria* (Balajee *et al.,* 2009). An example, with *Fusarium solani* represents a complex (i.e. *F. solani* complex) of over 45 phylogenetically distinct species of which at least 20 are associated with human infections. Likewise, members of the *Fusarium oxysporum* complex are phylogenetically diverse, as are members of the *Fusarium incarnatum-equiseti* complex and *Fusarium chlamydosporum* complex (Balajee *et al*., 2009, Tortorano *et al*., 2014, Salah *et al*., 2015). Currently, *Fusarium* genus comprises of at least 300 phylogenetically distinct species, with 20 species complexes and nine monotypic lineages (Balajee *et al*., 2009, O'Donnell *et al*., 2015).

Majority of the opportunistic *Fusarium* pathogens identified belong to the *F. solani* complex, *F. oxysporum* complex and *F. fujikuroi* complex. While the less frequently identified *Fusarium* are members of the *F. incarnatum-equiseti, F. dimerum and F. chlamydosporum complexes*, or species such as *F. sporotrichioides* (*O'Donnell et al.,* 2015, Van Diepeningen *et al.,* 2015).

To identify isolates, the isolates are normally inoculated on Czapek Dox agar and 2% Malt extract agar and incubated at 25^{O}C, most of the species sporulate within 7 days. Microscopic mounts are usually done using a slide culture preparation mounted in lactophenol cotton blue

or mounts are made using a cellotape flag. A drop of alcohol is usually needed to remove bubbles and excess conidia (Samson *et al*., 2014).

Morphological description: The Colonies are usually fast growing, pale or bright-coloured (depending on the species) with or without a cottony aerial mycelium. The colour of the thallus varies from whitish to yellow, pink, red or purple shades. Different species of *Fusarium* typically produce both macro- and microconidia from slender phialides. Macroconidia are hyaline, two to several-celled, fusiform to sickle-shaped, mainly with an elongated apical cell and pedicellate basal cell. Microconidia are one or two-celled, hyaline, smaller than macroconidia, pyriform, fusiform to ovoid, straight or curved. There may be presence of Chlamydospores. It is often difficult to identify *Fusarium* species because of the variability between isolates (e.g. in shape and size of conidia and colony colour) and because not all features required are always well developed (e.g. the absence of macroconidia in some isolates after subculture) (Van Diepeningen *et al*., 2015).

Fusarium species can be identified based on important characteristics like;

1. The colony growth diameters on potato dextrose agar and/or potato sucrose agar after incubation in the dark for four days at 25^0C.
2. The culture pigmentation on potato dextrose agar and/or potato sucrose agar after incubation for 10-14 days with daily exposure to light.
3. The microscopic morphology which includes the shape of the macroconidia; presence or absence of microconidia; and presence or absence of chlamydospores, shape and mode of formation of microconidia; nature of the conidiogenous cell bearing microconidia;, (Guarro, 2013; Geiser *et al*., 2013; Tortorano *et al*., 2014; Salah *et al*., 2015; Van Diepeningen *et al*., 2015).

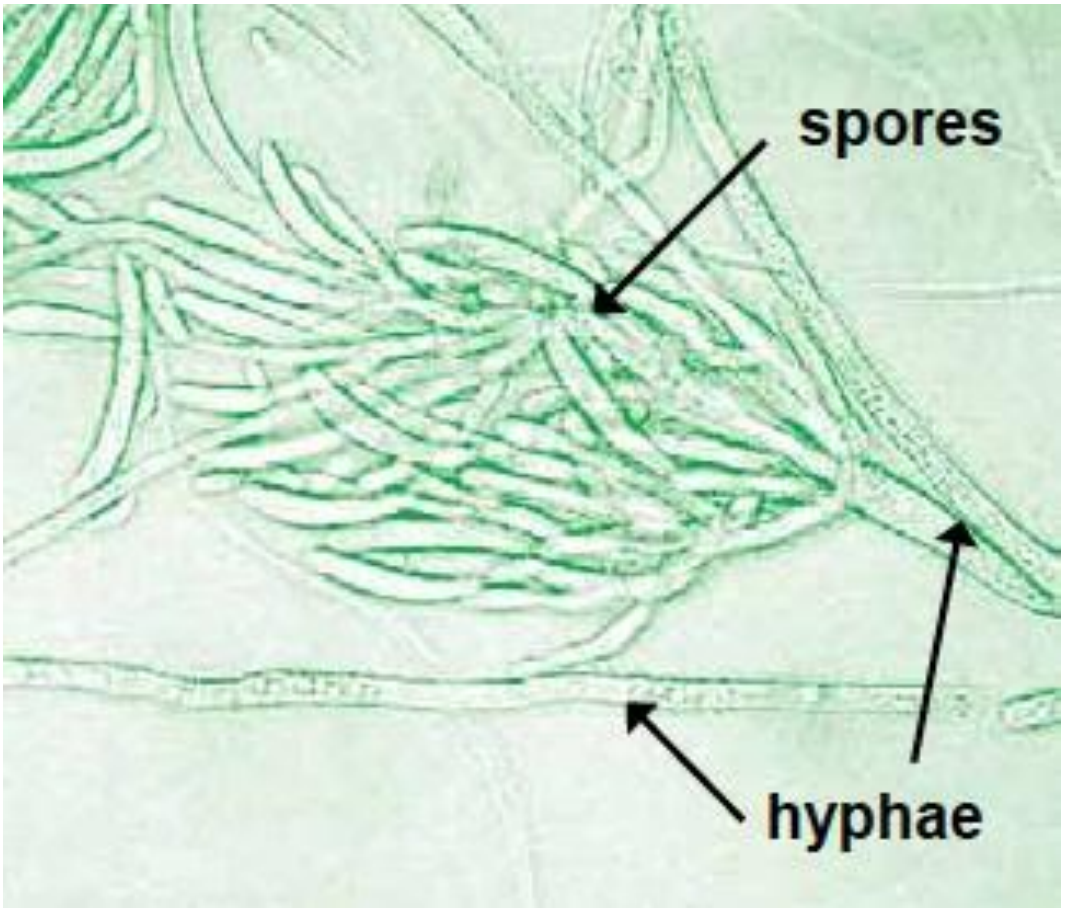


Figure 2.4: Spores and Hyphae of *Fusarium.*

Source: Balajee *et al*. (2009) O'Donnell *et al*. (2015).

2.7.4 Molecular techniques used in the characterization of mycotoxigenic fungal isolates.

Classical culture-based methods for detection and identification of the main mycotoxigenic fungi occurring on grains and legumes (belonging to *Aspergillus*, *Fusarium* and *Penicillium* genera, as well as to *Alternaria, Claviceps, Monascus, Phoma, Phomopsis, Pithomyces, Stachybotrysgenra*) present numerous limitations, including being time-consuming and labourious to use (Thomas & brain 2014). Modern molecular techniques are rapid, specific and sensitive and their application is being rapidly used for identification of microbial species. Molecular techniques have been used to ascertain the aflatoxigenicity of *A. flavus* and *A. niger* fungi in food. (Toma & Abdulla, 2013).

Molecular methods for identifying fungi can be categorized into two subsets: Non-culture-based molecular diagnostic methods and culture-based molecular diagnostic methods (Thomas & brain 2014).

a) Non culture based diagnostic methods;

These methods do not require the use of a culture. It goes on directly before ussing molecular tools.

b) Culture-Based Molecular Diagnostic Methods;

The gold standard for the identification of fungi has been the establishment of a pure culture from a given microbial sample. The advantages of pure cultures are that there are enough materials to perform an assay, assays can be repeated in case of failure, culture can be sent to another location where the analysis can be done, and they can allow unrelated assay to be carried out.

In this era of molecular techniques, the major molecular taxonomic tool, ribosomal sequencing, is now the major molecular tool for identification of fungi (Thomas & brain 2014).

Most of the molecular detection procedures are now DNA-based, with the use the polymerase chain reaction (PCR) based-methods. This has protocols that allows the identification of a single specie or multiple species that belongs to the same genus or to mixed population of different genera. The PCR has two main complementary approaches: The first targets conserved functional mycotoxigenic genes or regions of taxonomical interest or by focusing on the mycotoxigenic genes. The different PCR methods like real-time PCR technology and multiplex PCR which monitors the DNA (or RNA) amplification products and allows fast detection and identification of the fungal content in a food sample during a single assay. Other technologies like 'basic' PCR, such as PCR-restriction fragment length polymorphism and PCR and enzyme immunoassay, denaturing gradient gel electrophoresis PCR-based, PCR and single strand conformational polymorphism, microsatellite lengthpolymorphism, loop-mediated-isothermal amplification assay, can also be used to detect toxigenic fungi (Shehata *et al*., 2008; Munaut, *et al*., 2011).

Molecular technologies for sequencing the whole genome can provide adequate information for the detection or characterization of fungal species. (Munaut, *et al*., 2011).Sequencing of fungal ribosomal targets is a promising venture because of the following reasons; firstly the ribosomes genes of fungi and most eukaryotes are multicopy in nature, this increases their detection sensitivity during amplification of PCR because of more target sequences. Secondly, the multiple conserved ribosomes (18s, 5.8s, and 28s) subunit genes are in close proximity, (Thomas & brain 2014) increasing their chance of easy detection during PCR amplification. This offers conserved PCR primer sites that are positioned such that multiple

target sites are close enough to yield PCR products. The conserved nature of the subunits, and their primer annealing sites, makes identification of PCR and sequence possible, enabling virtually any unknown fungus to be amplified with universal primers targeted to those regions.

Thirdly, the organization of this region confers variability owing to the fact that variable regions eparate the key ribosomal units. These regions are known as the internal transcribed spacer (ITS) sequences, which consist of two regions (ITS1 and ITS2) that are not part of the fungal ribosome and are spliced out after transcription. The presence of variability to the sequence makes rDNA sequencing the most powerful nucleic acid-based diagnostic method available. The third variable region with a large 28s ribosomal subunit called the D1/D2 region exists in addition to the variable ITS1 and ITS2 regions. All the three regions provide information because of their variable nature which yields genus-specific and species-specific identifications.

Fourtly, any sequence data generated from an unknown fungus can be used to search the public database like the Genbank using the web-based Basic Local Alignment search tool Nucleotide (BLASTn) algorithm to identify the particular fungus (Thomas & brain 2014).

2.8 METHODS FOR THE DETECTION OF MYCOTOXINS.

2.8.1 Immunological methods.

These are binding assays based on monoclonal or polyclonal antibodies raised against toxins (antigens), which can be performed either as immunoaffinity column-based analysis (IAC) or enzyme-linked immunosorbent assay (ELISA) (Crowthe, 1995).

The principle behind ELISA test is the antigen-antibody reaction. The Commercially available kits normally used are the competitive assay format in which the toxin competes with the enzyme conjugate to the toxin for a specific immobilized antibody. Here, bound

enzyme conjugate converts the substrate into coloured, fluorescent or chemiluminescent active product. Another type of assay is the one based on the competition between free and immobilized toxin for the binding sites for the toxin on the specific antibodies. An enzyme (i.e., alkaline phosphatase) is used to label secondary antibodies. When this is added to the system it will interact with a chromogenic, fluorogenic, chemiluminescent or electrochemical substrate to give a measurable result. Common mycotoxins in fungi cultures can be measured using both direct and indirect competitive ELISA assays (Gheorghe *et al*., 2008).

Immunoassay critical points are stability of enzyme and tracer element which is the most sensitive reagent. Several methods can be used to achieve enzyme stability like immobilization to a solid phase, chemical or addition of stabilizing agents (Kolosova *et al*., 2006).

ELISA methods used in mycotoxins determination. Toxin Protocol for Aflatoxins Sequential injection immunoassay, Aflatoxin, uses Competitive ELISA. Deoxynivalenol, uses Competitive ELISA (Veratox, Neogen Corp., Lansing, MI), Fumonisin uses ELISA commercial kit (Ridascreen, R-Biopharm, Darmstadt, Germany), Ochratoxin uses Indirect ELISA, Trichothecenes and zearalenone use Commercial ELISA kits (R-Biopharm, Darmstadt, Germany), Trichothecenes use Commercial ELISA assay (QuantiToxTM Kit, Envirologix INC., Portland, Maine), Zearalenone uses Indirect ELISA . ELISA when combined to other techniques like electrochemical sensors (Piermarini *et al*., 2007) and surface plasmon resonance (SPR) produce result with higher sensibility. (Yuan *et al*., 2009) and have shown to produce a rapid and highly sensitive competitive immunoassay coupled to SPR for ochratoxin quantification in cereals and beverage.

To ensure high sensitivity, a toxin can be immobilized on a surface containing gold nanoparticles through its ovalabumin conjugate with polyethylene glycol linker. By applying

gold nanoparticles for signal enhancement, the limit of detection can be improved from1.5 ng/ml to 0.042ng/ml (Gheorghe *et al*., 2008).

Toxin detection can be done for trichothecences family by using an enzyme linked immunomagnetic assay based on magnetic beads as solid phase and screen printed electrodes as sensing platform for HT-2 and T-2 – toxins of trichothecenes family (Romanazzo *et al.*, 2009; Meneely *et al*., 2010).The use of an antibody clone with 100% cross-reactivity towards both toxins leds to their simultaneous and highly sensitive detection (Meneely *et al*., 2010). To provide a highly sensitive system, chemiluminescent (CL) and bioluminescent (BL) reactions are exploited in immunoassays. These involve either the use of components of CL (luminol) and BL (luciferase) reactions as labels, or the use of the reactions to monitor an enzyme label or its products. CL and BL methods have been developed for many enzymes, e.g., alkaline phosphatase, horseradish peroxidase (HRP), Renilla luciferase, xanthine oxidase. The most successful enzyme assay is the enhanced CL for HRP involving luminol, or isoluminol, hydrogen peroxide and p-iodophenol as enhancer. A direct CL method for alkaline phosphatase and adamantyl 1, 2-dioxetane phenyl phosphate substrate is also very sensitive (Romanazzo *et al.*, 2009).

According to Lee and Chu (1984) the CL ELISA has a similar sensitivity to that obtained by ELISA for ochratoxin analysis in wheat grain. Fluorescence polarization immunoassay (FP) is a method based upon the competition between free and fluorescein tracer-toxin for toxin specific monoclonal antibodies in solution. In this technique, the detection does not involve an enzyme. Also, there is no need to separate bound and free label. Such assay has also been developed for aflatoxin and deoxynivalenol quantification in grains (Hernandez *et al*., 2006).

The applications of immunoassay, like ELISA became popular because of the sensitivity, specificity, rapidity in screening of the different products of grains in addition to the relative

low cost and simplicity. Also, its detection limit is lower than those obtained with instrumental methods. A comparative study performed with ELISA and high performance liquid chromatography (HPLC) showed that immunoassay is more rapid in toxin detection than the HPLC. Immunoassay gives a less toxin content than the HPLC. Lately, ELISA is mostly used for rapid monitoring in industry, while HPLC methods are used for research purposes. Many methods used to detect mycotoxins are now available, including methods for measuring multiple toxins with high sensitivity from a single matrix (Hernandez et *al*., 2006).

The advantages of ELISA method are; Firstly, it is possible to perform the test on a 96-well assay platform; this means that many samples can be analyzed at the same time. Secondly, ELISA kits are cheap and easy to use and do not require extensive sample cleanup and thirdly, there is no known inherent hazard associated with the enzyme labels as there are for isotopes, though Elisa technique needs multiple washing steps, which is not only laborious but also time consuming (Hernandez et *al*., 2006).

2.8.2 Chromatographic techniques.

Thin layer chromatography (TLC) is a technique that is widely used for both quantitative and semi-quantitative measurements of mycotoxins with detection by fluorodensitometry or visual procedures (0.01 ppm detection limit) (Mohammed, 2015). Common mycotoxins (aflatoxins, citrinin, and fumonisin) have been detected using TLC based on silica gel, F254 fluorescent silica gel or silica gel impregnated with organic acid (Lin, *et al*., 1998). Though it is cost effective and suitable for rapid screening, its lack of automation has led to TLC being replaced by other techniques (De Saeger*et al.,* 2003). Compounds that are volatile and thermo stable use the gas Chromatography (GC) technique. Toxins are usually detected by linking the system to mass-spectrometry (MS), flame ionization or fourier transform infrared spectroscopy. Since most mycotoxins are not volatile, they need to be derivatised by

chemical reactions such as silylation or polyfluoroacylation in order to be quantified. Gas Chromatography method in tandem with MS has been used to measure trichothecenes in fungal cultures (Nielsen & Thrane 2001). Gas Chromatography technique is not suitable for commercial purposes because it needs volatile and thermostable compounds. High performance liquid chromatography (HPLC) is officially accepted method for detecting toxins and it can also be applied in conjunction with UV, fluorescence, amperometric or spectrofluorimetric detection. Both normal and reverse-phase HPLC are used for separation and purification (Jimenez *et al*., 2000; Trucksess *et al*., 2001; Cappiello *et al*., 2002; De Saeger *et al.,* 2003; Mohammed, 2015).

Some mycotoxins (ochratoxin, citrinin) naturally have fluorescence and as such can be detected directly using HPLC-fluorescence (HPLC-FD) (Toscani *et al.,* 2007). Others like fumonisin, require derivatisation that can be performed by employing o-phthalylaldehyde or 9-(fluorenylmethyl) chloroformate. Spectroscopy is commonly used. Recently, MS with electrospray or pressure chemical ionization interface is a configuration allowing an increase in sensitivity compared to HPLC-FD (Sorensen & Elbaek 2005). Immunochemical methods (like ELISA) can be used for rapid screening of mycotoxin presence, though for confirmation purpose, analytical methods based on high performance liquid chromatography (HPLC) are preferred, especially when coupled with tandem mass spectrometry (MS), which allows the determination of multiclass mycotoxins in a single analysis (Aldo, 2017).

The limit of detection varied between 0.1 and 1 µg/kg in all the tested matrices. HPLC as an analytical tool has the following advantages of having a high resolution, limit of detection, with the possibility to be coupled to multiple detection automated systems, accurate and specific. Its limitation is that chromatographic assays are expensive, time-consuming and they require a lot of clean-up procedures (Beltran *et al*., 2009).

2.8.3 Physico-chemical methods.

Capillary electrophoresis (CE) is an electrophoretic method leading to a fast separation of components based on charge and mass dependent migration in electrical fields. When this method is combined with fluorescence, CE allows the detection of mycotoxins at trace levels. A flow system coupled to CE for the screening of aflatoxins in feed samples has been reported (Pena *et al*., 2002).

2.8.4 Biological methods.

Mycotoxins can be analyzed using biosensors. Biosensors are rapid, sensitive, practical and convenient. They are made up of a recognition element, usually of biological origin, which produces a quantifiable response in a signal transduction element when in contact with the target analyte. Most of the signal transduction mechanisms are optical (colorimetric, fluorescence, enhanced chemiluminescence), electrochemical or surface plasmon resonance (Wang & wang 2008; Yuan, 2009).

Mycotoxins (ochratoxin, aflatoxins, fumonisin and deoxynivalenol) in food products have been detected using different biosensors like enzyme sensors, optical immune sensors, tissue biosensors, array and plasmon resonance biosensors, quartz crystal biosensors, and electrochemical sensors. For example, zearalenone and its derivatives have been detected in milk products with a yeast whole-cell bioluminescent sensor (genetically modified *Saccharomyces cerevisiae)* allowing detection at nanomolar concentrations (Valimaa *et al*., 2010).

Comparing biosensors to other traditional analytical techniques, biosensors can monitor a large number of samples hence being a convenient tool that can also be automated for screening of toxins in routine analysis. Its main drawback is the regeneration of the receptor surface. It has a promising future because of its benefits of specificity, sensitivity, reproducibility and stability (Valimaa *et al*., 2010).

2.8.5 Regulations.

Many international agencies are trying to achieve a universal standardization of regulatory limits for mycotoxins. Currently, more than 100 countries have regulations regarding mycotoxins in the feed industry, in which 13 mycotoxins or groups of mycotoxins are of concern (Glenn, 2007).

Different governments have set regulatory limits for mycotoxins in food and animal foodstuffs that are for export or import. For aflatoxin, guidelines range from 4 to 50 µg/kg (parts per billion). Regulatory limits for fumonisin are still under consideration. For all mycotoxins, it is likely that, as analytical techniques and knowledge of the toxins improve, allowable limits will fall (FAO, 2015).

Over the years, there have been need for some control on the quality of foodstuffs. Since the discovery of aflatoxins in the early sixties, health authorities in many countries have been earger to establish regulations to protect their citizens and livestock from potential harm caused by mycotoxins. Different mycotoxins like aflatoxins, ochratoxin A, fumonisin B1 and B2, deoxynivalenol (DON), and patulin can be seen in a number of food products. FDA has been saddled with an objective to remove those foods from inter-state commerce that contain aflatoxins "at levels judged to be of regulator significance. FDA workers were instructed to sample and analyze all products for different types of mycotoxins. All baby foods should always be analyzed for all type of mycotoxins. The following limits were established: The limits of aflatoxins B_1, B_2, G_2, and M_1 in foods and feed stuffs varies from 0-40 ppb for foods and 0-1000 ppb for food; for ochratoxin A is 0-50 ppb in food and 0-1000 ppb in feed; for Don 500-2000 ppb in food and 5-10,000 ppb in feed; for Zearalenone 0-1000 ppb in food; for patulin 0-50 ppb in foods, for diacetoxyscirpenol 0-100 ppb in feed; for chetomin 0 ppb in feed); for stachybotryotoxin (0 ppb in feeds and for fumonisins (0-1000 ppb in food, and 5000-50,000 ppb in feedstuffs) (Papiya & Sasmal 2001).

The process of mycotoxin regulation includes laboratory testing which includes extracting, clean-up and separation techniques (Desjardins & Proctor 2007). Most official regulations and control methods rely on high-performance liquid techniques (e.g., HPLC) through international bodies (Desjardins & Proctor 2007). It is implied that any regulations regarding these toxins will be in co-ordinance with any other countries with which a trade agreement exists. The European Committee for Standardization (CEN) has set the standard for the analysis of mycotoxins (Desjardins & Proctor 2007). However, one must take note that scientific risk assessment is usually influenced by culture and politics, which will definitely affect trade regulations of mycotoxins (Bullerman & Bianchini 2007).

Food-based mycotoxins have been studied worldwide throughout the 20th century. In Europe, statutory levels of a range of mycotoxins permitted in food and animal feed are set by a range of European directives and Commission regulations. The U.S. Food and Drug Administration has regulated and enforced limits on concentrations of mycotoxins in foods and feed industries since 1985. It is through various compliance programs that the FDA monitors these industries to guarantee that mycotoxins are kept at a tolerable and practical level. These compliance programs sample food products including rice, peanuts and peanut products, tree nuts, corn and corn products, cotton seed, and milk. Though more work has to be done because there is still a lack of sufficient surveillance data on some mycotoxins that occur in the U.S (IARC, 2002).

Figure 2.5 Discoloured grains and legumes.

Source: (Moore & Robson, 2011).

2.9 CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) REFERENCE SUSCEPTIBILITY TESTING ASSAYS.

The clinical and laboratory standard institute have approved the standard disk diffusion method to test antifungal drugs for non-dermatophyte filamentous fungi isolates.This document defined reference strains with ranges of Minimal Inhibitory Concentrations (MIC) and Break Points (BPs) for some antifungals and their action against yeasts and moulds which was published in 1997 by a subcommittee on antifungal susceptibility testing by the Clinical and laboratory standard institute (CLSI) formerly known as National Committee for Clinical Laboratory Standards (NCCLS) in 1985.

The standard disk diffusion method to test antifungal drugs for non-dermatophyte filamentous fungi isolates provides qualitative results in 8-24 h when caspofungin, triazoles, and amphotericin B are used, faster than the CLSI reference microdilution method. The studying of antifungal susceptibility tests led to the documentation of reference microdilution methodologies for yeasts (including *Candida* spp. and *Cryptococcus neoformans*) and moulds

(*Aspergillus* spp., *Fusarium* spp., *Rhizopus* spp., *Pseudallescheria boydii*, and the mycelial form of *Sporothrixschenckii*). The reference CLSI documents include antifungal susceptibility testing of amphotericin B, flucytosine, fluconazole, ketoconazole, itraconazole, and the new triazoles (posaconazole, ravuconazole, and voriconazole). The method is based on visual reading of minimum inhibitory concentration (MIC, µg/ml) values (CLSI, 2010).

A lower agreement result when compared to the reference method was seen by disk diffusion susceptibility tests for *A. flavus* and Amphotericin B or voriconazole. Amphotericin B to test *A. fumigates* susceptibility also showed a lower agreement when compared to the reference method. Amphotericin B disks usually show the lowest correlation between MICs

and inhibition zone diameters for filamentous fungi. Itraconazole disk percentage of major errors is usually similar to that obtained with the Amphotericin B or voriconazole, but the percentage of minor errors is higher. Even though breakpoints for filamentous fungi have not been defined, epidemiological cut-off values can be proposed to identify non-wild-type isolates. Disk diffusion method usually provides qualitative results which are useful in the clinical laboratory routine, though quantitative MIC data is sometimes critical for the management of invasive infections (CLSI, 2010; NCCLSI, 2002).

Breakpoints.

The main aim of antifungal susceptibility testing (AFST) is to select the best treatment for a given isolate. This method is also used to detect resistant strains, thus permitting the establishment of an epidemiology map of antifungal resistance which is an emerging problem in medical mycology. The two major factors are; firstly, the development of secondary resistance and secondly, the selection of species that are intrinsically resistant. AFST has become necessary for the selection of the best antifungal agent. Some fungal species and antifungals agents, in both CLSI and European committee on Antimicrobial susceptibility testing now have break points**.** Break points have been developed for the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods. These Break Pointshave categorize fungal isolates into (i) susceptible (the drug is an appropriate treatment); (ii) resistant (the drug is not recommended as a treatment), and (iii) intermediate (the drug may be an appropriate treatment, depending on certain conditions; e.g. fluconazole to treat a urinary infection caused by an intermediate strain).

The definition of Break Points can be a complex process based on the critical review of several aspects and data. CLSI thus evaluates Minimum Inhibitory Concentration distributions, the relationship between Minimum Inhibitory Concentrations and clinical

outcome, pharmacokinetics and pharmacodynamics. CLSI has proposed a single interpretative Break Points for fluconazole, itraconazole, voriconazole, and echinocandins for all *Candida* species. Latter, CLSI Break Points was revised to include a number of clinical studies and cases reporting strains classified as susceptible but associated with treatment failure, and as a consequence, species-specific Break Points were proposed, as had been previously established by EUCAST (CLSI, 2011).

Plate 2.1: Oxoid disc diffusion method.

Source: Espinel-Ingroff *et al.* (1999).

2.9.1 Tetrazolium test.

2, 3, 5-Triphenyltetrazolium chloride can also be known as Tetrazolium Red, Triphenyl tetrazolium chloride, (TTC), or Tetrazolium Chloride (off white to yellowish powder).Tetrazolium Chloride is normally colorless but changes to red when it comes into contact with hydrogen.Tetrazolium red is used in a biochemical viability test for seeds. The principle behind the test relies on dehydrogenase enzymes to release hydrogen ions which subsequently reduce the colorless tetrazolium salt solution to a red compound known as formazan. Red colour indicates living cells while dead cells remain colorless (Witty, 2012). In the TTC assay (also known as TTC test or tetrazolium test), TTC can also be used to differentiate between metabolically active and inactive tissues. It forms colorless to yellowish (practically clear) solution when dissolved in 5 % ethanol or water.The white compound is enzymatically reduced to red TPF 1, 3, 5-triphenylformazan (TPF) in living tissues due to the activity of various dehydrogenases (enzymes important in oxidation of organic compounds and thus cellular metabolism), while it remains colourless in its unreacted state in areas of necrosis since these enzymes have either been denatured or degraded.

For more than 60 years, tetrazolium salts have been known (Albert *et al*., 1956). Their biological benefits began only in 1939, when they were first used to determine the viability of seeds. They were known to reduce with ease colourless soluble compounds to pigmented insoluble formazans. Tetrazolium salts serves as a good indicator of dehydrogenase assay. This has made them found an increasing use in the study on the dehydrogenases of microbial, plant and animal tissues. They have also been adapted for the study of histochemical techniques, bioautography, isolated enzyme systems, tissue culture and insect studies (Albert *et al.,* 1956).

The numerous studies with tetrazolium derivatives have established their toxicity for microbial, higher plant and metazoan cells. Other studies have shown that they are readily

reduced by bacteria, on which they exert various effects by pleuropneumonia-like organisms, Mycobacteria, Streptomycetes and fungi. Other studies suggest that certain pathogens may be controlled by tetrazolium salts because of its dehydrogenase enzymes as the sites of tetrazolium inhibition and the wide distribution of these electron transport systems among microorganisms. Though these agents are very toxic for systemic administration, may be useful for topical treatment of dermatophytic infections where high concentrations can be applied locally (Albert *et al.*, 1956).

2.9.2 Current and emerging azole antifungal agents.

The azole antifungal agents have been useful in the treatment of systemic fungal infections. The available azoles can be grouped into two: the triazoles (fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole) and the imidazoles (ketoconazole). Research has been going on in the studies of azoles since the 1990s. Azoles have provided many options that can be used to treat many opportunistic and endemic fungal infections. Fluconazole and itraconazole have proved to be safer than both amphotericin B and ketoconazole. Nevertheless, most serious fungal infections remain difficult to treat, and resistance to azole drugs keep on emerging (Georgopapadakou and Walsh 1996).

Griseofulvin, which was the first antifungal agent, was isolated in 1939 (Fromtling, 1988) and the first azole and polyene antifungal agents were reported in 1944 and 1949, respectively (Fromtling, 1988). Oral griseofulvin and topical chlormidazole became available for clinical use in 1958 (Fromtling, 1988). The gold standard for the treatment of severe systemic mycoses (Georgopapadakou and Walsh 1996) remains amphotericin B which was introduced after griseofulvin in 1960 (Gupta *et al*., 1994). Miconazole and clotrimazole, both topical azole antifungal agents were introduced in 1969 and Econazole was introduced in 1974, (Gupta *et al*., 1994) and a parenteral formulation of miconazole in the late 1970s.

These days, these three agents remain the mainstay of topical therapy for many dermatophytoses.

The intensive efforts in research in the area of antibacterial therapy which began in the 1940s following the large-scale production of penicillin and also to the relatively low incidence of serious fungal infections when compared with that of bacterial infections slowed down the progress in the development of both topical and systemic antifungal agents. By 1980, the only new drug introduced for the treatment of systemic fungal infections was oral ketoconazole.The four major classes of antifungal agents—polyenes, azoles, morpholines, and allylamines have been known since 1980 (Kauffman & Carver 1997). It could take more than 10 years for either fluconazole or itraconazole to become available for the treatment of systemic mycoses. New advances were made in the 1990s that led to the introduction of a new allylamine, terbinafine, for the treatment of dermatophytoses and new lipid formulations of amphotericin B with improved safety profiles (Kauffman & Carver 1997). New classes of antifungal agents like candins (pneumocandins and echinocanidins), the nikkomycins, and the pradamicins-benanomicins are being studied (Kauffman & Carver (1997). Moreover, the azoles are currently the most widely used and studied class of antifungal agents among the 15 different marketed drugs worldwide (Gupta *et al*., 1994).

2.9.3 Mechanism of action.

Ergosterol which is a major component of fungal plasma membrane which needs to be prevented from synthesis by inhibiting the cytochrome P-450-dependent enzyme lanosterol demethylase (also referred to as 14α-sterol demethylase or P-450_{DM}) (Georgopapadakou & Walsh 1996). This enzyme also plays an important role in cholesterol synthesis in mammals. When azoles are present in therapeutic concentrations, their antifungal efficacy is attributed to their greater affinity for fungal P-450_{DM} than for the mammalian enzyme. When fungi are exposed to an azole, there is depletion of ergosterol in fungi and accumulation of 14α-

methylated sterols (Georgopapadakou & Walsh 1996). This interferes with the "bulk" functions of ergosterol in fungal membranes and disrupts both the structure of the membrane and several of its functions such as nutrient transport and chitin synthesis. The net effect is to inhibit fungal growth. Ergosterol also has a hormone-like ("sparking") function in fungal cells, which stimulates growth and proliferation. This function may be disrupted when ergosterol depletion is virtually complete (>99%) (Georgopapadakou & Walsh 1996).

2.9.4 Clinically important azole antifungal agents.

The azole antifungal agents contain two or three nitrogens in the azole ring that is known as imidazoles (e.g. clotrimazole, ketoconazole and miconazole) or triazoles (e.g., itraconazole and fluconazole), respectively. This antifungal agent is usually used in clinical settings. The use of imidazole is limited to the treatment of superficial mycoses except ketoconazole while the triazoles have a broad range of applications in the treatment of both superficial and systemic fungal infections. The triazole have another advantage which is their greater affinity for fungal rather than mammalian cytochrome P-450 enzymes, which contributes to an improved safety profile. Some members of the triazole family are the most widely used antifungal agents in clinical settings (Gallagher *et al.,* 2003). Triazoles offers susceptibility to many fungal pathogens without serious nephrotoxic effects which can be observed with Amphotericin B. Newer azole agents have emerged as first-line therapies for several severe fungal diseases like invasive Aspergillosis. Voriconazole has become the drug of choice for invasive Aspergillosis (Gallagher *et al.,* 2003).

2.9.5 Medicinal plants.

Medicinal plants can also be known as medicinal herbs. It has been discovered and used in traditional medicine practices since ancient times. Plants have a lot of functions against insects, microorganisms, diseases and herbivorous mammals because of their ability to synthesize hundreds of chemical compounds. A lot of phytochemicals with established biological activity have been identified. Moreover, a single plant can contain diverse

phytochemicals (Lichterman, 2004). Also the phytochemical content and pharmacological actions of some plants have remained unassessed and needs more scientific research to define their efficacy and safety (Ahn, 2017).

Herbal plants in Nigeria which are used in making home remedies and for traditional uses are getting more recognition and need to be screened for the antimicrobial properties of their extracts against known microorganisms. Thus, the medicinal values of plants lie in the content of their bioactive phytocomponents which are present in plants (Veermuthu *et al*., 2006; Okorondu *et al*., 2010b; Okorondu, 2011).

Nigeria has a wide variety of plants which are of both nutritional and medicinal value. Plants are used as a valuable source of food and medicine for the prevention of infections and maintenance of human health. There is now a global shift in the production of drugs from medicinal plants because of its potential herbal remedies for safety, efficacy and economy (Davis & Heywood 1963). Medicinal plants contain many phytochemicals like alkaloids, which have marked physiological effects on animals (Edeoga & Eriata, 2001) and has a lot of pharmaceutical activity (Davis & Heywood, 1963). Tannins are useful in medicine because of their astringent properties. Tannins and alkaloids are known to have anti-herbivore defense function in plants (Harbone, 1988). Furthermore, the presence of tannins and alkaloids in some medicinal plants could be a deterrent to grazers (Edeoga & Eriata, 2001). Saponins are glycosides which occur in a variety of plants. They prevent parasitic fungal from invading plants and causing diseases (Bidwell, 1979). In medicine, it can be used as an expectorant and emulsifying agent (Basu & Rastogi, 1967). Flavonoids are the commonest phenolic constituents having 15-compounds generally distributed throughout the plants kingdom (Harbone, 1988). Some flavonoids have antibacterial functions being more active against

gram-positive species than gram- negative species. Phenolic compounds are also known to have anti-fungal and anti-microbial effects (Harbone, 1988).

List and uses of some medicinal plants in Nigeria.

1). *Ocimum gratissimum.*

English Name: Clove Basil (Scent Leaves).

Local Names: Nchuawun (Igbo), Efirin (Yoruba), Daidoya (Hausa), Ntong (Efik), Aramogbo (Edo).

Scent leaves are used in the preparation of various foods like vegetable soups, yam porridge, pepper soup, (*ofe akwu*) etc. As the name implies it gives a delicious aroma to the meal prepared with it. It has a lot of uses; it serves as an herbal leaf, it is used to lower blood pressure and to reduce blood sugar level. It is also used to treat piles, can be given to people that have gonorrhoea infection, vaginal douches for vaginitis, diarrhoea, fungal infections and other infections. Its leaves can be squeezed to act as a strong repellent for mosquito and other insects. Its squeezed leaves can also be used to treat ringworm and other skin disease (Johnson, 2018).

2). *Vernonia Amygdalina*.

English Name: Bitter Leaf.

Local Name: Onugbu (Igbo), Efo Ewuro (Yoruba), Shuwaka (Hausa), Etidot (Calabar).

Like the name suggest; the leaves are bitter. It is usually used to prepare the popoular Nigeria bitter leaf soup. The leaves are large, leafy and deep green leaves. Boiling of the leaves removes the bitterness before the leaves are used to cook soup. The leaves are rich in vitamins and minerals. In traditional medicine, the leaves are used to treat fever, hepatitis, diarrhea, dysentery, cough, stomach ache, head ache, scabies and gastrointestinal disorders. It also reduces the sugar level of the blood and repairs the pancreas and kidney which makes it good for diabetic patients (Johnson, 2018).

3). *Gongronema latifolium.*

English Name: Bush buck

Local Name: Utazi (Igbo), 'arokeke'(Yoruba), Ayoyo (Hausa).

Utazi leaves have a very sharp-bitter and sweet taste. It is usually used in small quantity to prepare various types of soups like nsala, ugba sauce. It can also be used to prepare yam porriage and can be used to garnish dishes like abacha ncha, isi ewu, nkwobi etc. In traditional medicine, it can be used to treat malaria, intestinal worms, cough, increase appetite, dysentery, dyspepsia, diabetes and high blood pressure (Johnson, 2018).

4). *Piper guineense*.

English name False Cubeb leaves.

Local name Uziza leaves or *akwa ose* (Igbo), Kanunfari (Hausa), Iyere (Yoruba), Eche (Idoma). Ashanti pepper leaves/ Benin pepper leaves (Benin).

Uziza, can also be known as Guinean pepper and is a relative of black pepper. Uziza in addition to its leaves produce fruits or seeds that have various health benefits (Dian, 2018). Uziza leaves are pale green when fresh and will be darker when dried. Uziza leaves have both nutritive and medicinal benefits. Studies have shown that Uziza leaves contain a good number of vitamins, minerals, dietary fiber, protein, alkaloids, flavonoids, tannin and essential oil. Furthermore, the health benefits of uziza leaves are as follows; it improves fertility both in males and females. It relieves cough since the leaves might have peppery with slight bitter taste (Dian, 2018). It relieves pains like headache, toothache, menstrual pain and joint pains. This is possible since the leaves contain alkaloids which can act as pain killer. It also prevents mosquitoes, lose weight, constipation, cancer, treat diarrhea and relieve flatulence (Dian, 2018).

Scent leaves, (*Ocimum gratissimum*)

Bitter Leaves (*Vernonia Amygdalina)*

.

Utazi leaves (*Gongronema latifolium).*

Uziza leaves (*Piper guineense*).

Figure 2.6: Medicinal plants.

Source: (Johnson, 2018).

CHAPTER THREE

3.0 MATERIALS AND METHODS.

3.1 SAMPLE STORAGE IN DIFFERENT MATERIALS.

Whole grains and fine powder (blended grains and legumes) of Rice, Maize, Wheat, Groundnut and Beans were stored in 4 different storage materials such as sack, polyethene, plastic containers and metal containers ranging from 2 months to 4 months of storage. The samples were labelled as follows;

RGS - rice grain sac, RGC – rice grain cellophane, RGP –rice grain plastic, RGM –rice grain metal. RPS – rice powder sac, RPC – rice powder cellophane, RPP –rice powder plastic, RPM – rice powder metal.

MGS - maize grain sac, MGC- maize grain cellophane, MGP - maize grain plastic, MGM- maize grain metal, MPS - maize powder sac, MPC - maize powder cellophane, MPP - maize powder plastic, MPM - maize powder metal.

WGS - wheat grain sac, WGC- wheat grain cellophane, WGP - wheat grain plastic, WGM- wheat grain metal, WPS - wheat powder sac, WPC - wheat powder cellophane, WPP - wheat powder plastic, WPM - wheat powder metal.

GGS - groundnut grain sac, GGC- groundnut grain cellophane, GGP - groundnut grain plastic, GGM- groundnut grain metal, GPS - groundnut powder sac, GPC - groundnut powder cellophane, GPP - groundnut powder plastic, GPM - groundnut powder metal.

BGS - beans grain sac, BGC- beans grain cellophane, BGP - beans grain plastic
BGM beans grain metal, BPS - beans powder sac, BPC - beans powder cellophane, BPP - beans powder plastic, BPM - beans powder metal.
GS/PS –means storage in sac, GC/PC –means storage in cellophane, GP/PP –means storage in plastic, GM/PM –means storage in metal.

Grains and legumes had the date they were produced on their sacs. Grains and legumes were then stored for a period of time from the date they were produced. Unstored and Stored grains and legumes samples were labelled and transported immediately to laboratory and kept in a cool place for mycological analysis. A total of 400 samples were collected and used for the analysis.

3.2 Sterilization of materials.

The materials used for this study were sterilized using standard techniques. Different glass wares were sterilized in the hot air oven at 160°C for 1 hour. The various culture media were sterilized by autoclaving at 121°C and 15 psi pressure unit for 15 minutes. The inoculation wire loops were sterilized by flaming to red hot over a bunsen flame. Hockey sticks were sterilized by dipping in absolute alcohol and bringing it over a burning flame to burn off. Bench top, wire loop and working areas were disinfected with antiseptic, dettol and flooded with 75% ethanol. Laboratory coat, sterile disposable hand gloves and face masks were worn and changed after each procedure to ensure aseptic conditions (Cheesbrough, 1987; Ogbulie, *et al*., 1998).

3.3 Media preparation.

The media that were used for this study were prepared according to the manufacturers' instructions. Sabouraud dextrose agar (SDA), Sabouraud dextrose broth, Potato dextrose agar (PDA) and Malt extract agar (MEA) were used for the analysis. An antibacterial agent chloramphenicol 50 mg/l was added to the media to inhibit the growth of bacteria (Valerie *et al*., 2001), while 0.1ml of lactic acid was added to prevent the growth of yeast (Valerie *et al*., 2001).

3.4 ISOLATION OF FUNGI.

3.4.1 Physical method: Light and hand lens were used for direct physical identification for spores and mycelium.

3.4.2. Isolation of fungi from samples using serial dilution method.

Serial dilution method was used to determine total fungal counts in unstored and stored grains and legumes. Ten grams of each sample (grains and fine powder) were transferred into screw capped bijou bottles containing 90 mls of sterile distilled water and was mechanically homogenized at constant speed for 15min. The sample-water suspension was allowed to stand for 10 min with intermittent shaking before being plated. Appropriate tenfold serial dilutions (1:10) were prepared and 1 ml portion of suitable dilutions of the resulting sample suspension (10^{-3}) was used to inoculate Petri dishes each containing 15ml potato dextrose agar (PDA), malt extract agar (MEA) and Sabouraud dextrose agar (SDA) for each sample. Spread plate method was done from 10^{-3} ml dilutions prepared, by using sterile wire loop on the surface of the media with three replicates for each sample used, and then plates were incubated at 28°C ± 2°C for 7days. Three replicates plates per medium were used for each sample and the developing fungi were counted and identified according to several key processes. After incubation, the results were expressed in colony forming units (CFU) /g of samples; the colonies on each plate were counted and all plates were examined visually, directly and with a microscope (Sejiny*et al.*, 1989; Shahidi, 2004; Suleiman, 2010; Atanda, *et al.*, 2011; Avasthi & Bhadauria, 2011: Larone 2011).

3.5 IDENTIFICATION OF FUNGI.

The Fungi colonies were identified according to their morphological and microscopic characteristics (Pitt *et al*., 1992).

Morphological and cultural characteristics of the growing cultures were evaluated for preliminary identification. Then fungal colonies were subjected to Microscopic identification (Gilman, 2001).

3.5.1. Wet mount technique (microscopic identification).

The fungal isolates were subcultured and transferred to sterilized plates for further identification of spores and purification.

Wet mount technique.

The grown fungi were mounted on clean grease free slides, then stained with lacto phenol-cotton blue and covered with a cover slip inorder to detect fungal structures. They were then examined under the microscope. Identification was done on the basis of their colony morphology and spore characteristics (Pitt *et al*., 1992).

3.5.2 Slide culture technique.

1. A small square piece of agar block was cut (1cm square and 3mm deep) from a plate with a sterile scalpel and place on a sterile glass slide.
2. The sides of the agar block were inoculated lightly with the culture and the agar block was covered with a cover slip.
3. The slide mount was placed on a bent glass rod in a petridish containing some layers of blotting paper soaked in 20% glycerol in water to provide a moist atmosphere.
4. The petridish was covered with its lid and incubated at room temperature or 30^0C for 3-5 days.

5. The coverslip was gently lifted from the agar block and transferred to a few drops of lactophenol cotton blue on a clean glass slide. Growth was then observed microscopically for the characteristic shape and arrangement of spores (Pitt *et al*., 1992).

3.6 ANALYSIS OF MYCOTOXINS.

From the 400 samples collected for this analysis, 160 samples of Rice, maize, wheat and groundnut from the 3 senatorial zones were analyzed for the presence of mycotoxins. Samples were homogenized and kept in slant glass bottle and stored at 8°C until further analysis. For the quantitative analysis of mycotoxins (Aflatoxin, Ochratoxin, Fuminisin, Citrinin, Dihydrocitrinone, Zearalenone, Deoxynivalenol and Nivalenol. Multimycotoxin method based on liquid chromatograph /tanden mass spectrometry **(**LC-MS/MS) was applied to investigate the occurrence of mycototoxin fungal metabolites (Sulyok*et al*., 2006).

3.6.1 Liquid chromatography / tandem mass spectrometry (LC-MS/MS).

Samples were homogenized and carefully kept in slant glass bottle and stored at 8°C until further analysis. For the qualitative and quantitative analysis of mycotoxins.

3.6.2 Sample preparation and (LC-MS/MS) determination.

To 5g of each sample, 20 mL of extraction solvent (acetonitrile/water/acetic acid 79 : 20 : 1, v/v/v) was added into a 50 ml centrifuge tube. Samples were extracted in a turbine mixer for 1 minute which was followed by vigorous vortexing for 30 minutes in an automatic vibrator. The resulting extract was then centrifuged for 5 minutes at 10,000 r/minute and the supernatant was collected. A 1 ml aliquot of the supernatant was transferred into a 5 ml centrifuge tube and diluted to 3 ml with water. The sample solution (1ml) was then transferred directly into vials for LC-MS/MS analysis (Sulyok *et al*. 2006).

LC–MS/MS determination.

Chromatographic separation were performed at 25°C on a Gemini C18 column, 150 × 4.6-mmi.d., 5-µm particle size, equipped with a C18 4 × 3-mm-i.d. security guard cartridge (all from Phenomenex, Torrance, CA, US). Both eluents contained 5 mM ammonium acetate and composed of methanol/water/acetic acid 10 : 89 : 1 (v/v/v; eluent A) or 97 : 2 : 1 (eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 100% within 12 min, followed by a hold-time of 3 min at 100% B and 4-min column reequilibration at 100% A. The flow rate of 1 mL/min electrospray ionization (ESI)-MS/MS was performed in the multiple reaction monitoring (MRM) modes both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte (Sulyok *et al*., 2006).

3.7 ANTIFUNGAL SUSCEPTIBILITY TEST.

The Clinical Laboratory and Standards Institute (CLSI) have approved methods for testing of moulds. The reference method OXOID DISC DIFFUSION METHOD. It was performed according to National Committee for Clinical Laboratory Standards, (2002) proposed guidelines and as described by (Espinel-Ingroff *et al.*, 1999).

3.7.1 Antifungal susceptibility using antifungal agents.

The following four oxoid antifungal agents were used: Amphotericin B, fluconazole, ketoconazole and voriconazole.

1. The isolates were subcultured on sabouraund dextrose agar (SDA) at 35°C for 4 to 7 days.

2. After the growth, the conidia were harvested in sterile saline and using a spectrophotometer, the conidial suspension was adjusted to 4.0×10^6 sfu/ml at optical density of 530nm.
3. A swab dipped into the standardized inoculum suspension was evenly streaked on a sabouraud dextrose agar Plates.
4. Using a sterile pair of forceps, the drug-impregnated disks containing the test agents were placed on the surface of inoculated plates.

Minimum Inhibition Concentration (MIC) endpoint readings were as follows:

1. Susceptible, ≥17 mm (azoles) and ≥15 mm (amphotericin B);
2. Intermediate, 14 to 16 mm (azoles) and 13 to 14 mm (amphotericin B);
3. Resistant, ≤13 mm (azoles) and ≤12 mm (amphotericin B) (Espinel-Ingroff *et al.,* 1999).

3.7.2 Antifungal susceptibility using tetrazolium chloride and antifungal agents.

2, 3, 5, - triphenyltetrazolium chloride (TTC) is reduced to red formazan in the presence of moulds. The red formazan obtained indicates the activity and viability of the cells. TTC (0.5% W/V) was added, at concentration of 10% – agar was prepared by mixing TTC with molten SDA before the agar solidifies (Shaaban *et al.,* 2013).

Using a sterile pair of forceps, drug-impregnated disks (Oxoid) containing the test agents were placed on the surfaces of inoculated TTC plates. Plates were inverted and incubated at 28°C for 5 days to allow for fungal growth (Espinel-Ingroff *et al.,* 1999).

Minimum inhibition concentration reading

Minimum Inhibition Concentration (MIC) endpoint reading:

1 Susceptible, ≥17 mm (azoles) and ≥15 mm (amphotericin B);

2 Intermediate, 14 to 16 mm (azoles) and 13 to 14 mm (amphotericin B);

3 Resistant, ≤13 mm (azoles) and ≤12 mm (amphotericin B) (Espinel-Ingroff *et al.*, 1999).

3.7.3 Antifungal susceptibility using medicinal plants extracts.

3.7.3.1 Collection of samples and preparation.

Samples of the 4 fresh leaves were collected from Relief market in Owerri. The plants were identified at the Department of Plant Science and Biotechnology, Imo State University Owerri. The leaves were washed and air dried at room temperature for 14 days. The dried leaves were blended using a blender. The powdered samples were screened through 1mm sieve. The samples were used for extraction.

Extraction.

The organic solvents used were Methanol and Hot water.

Methanol Extraction.

Fifty grams (50g) of samples of each leaf were used. Using a soxhlex apparatus, the active ingredients of the ground particles were extracted. For extraction, 250 ml of methanol was used. The extract was filtered using sterile whatman filter paper no 42. The filtrate was evaporated to dryness using the rotary vacuum evaporator at the boiling temperature range of the solvent (79^{o}C). The solvent was recovered in the recovery flask while the extracts remained in the sample holder, which was collected and stored in the refrigerator at 4^{o}C. The extract was sterilized with UV light before use.

Hot water Extraction.

Fifty grams (50g) of samples of each leaf sample were used. Samples were soaked in hot water for 24 hours, after which the active ingredients of the ground particles were filtered and kept for use.

Fungal susceptibility testing.

The molten Sabouraud dextrose Agar - 10% was prepared and mixed with different concentrations of extract (12.5 mg/ml, 25 mg/ml, 50 mg/ml and 100 mg/ml) before the agar solidified. Fungus grown on SDA (3mm in diameter) were cut with a sterile cork borer and placed in the center of petri plates containing different concentrations of the extracts (12.5 mg/ml, 25 mg/ml, 50 mg/ml and 100 mg/ml) on different plates. Controls were SDA plates without the addition of the extracts. Replicates of each treatment were incubated at 28 °C ± 2 ^{o}C for 4 days. A metric rule was used to measure the radial growth inhibition in both the extract-treated and control plates. The radial growth inhibition was monitored in both the extract treated and the control and was expressed in percentage (Okorondu *et al*., 2012).

Minimum inhibitory concentration (MIC).

An extract of 200 mg was dissolved in 2 ml of diluent, to get 100 mg/ml, then 1ml of the diluent was put into another tube in 2 ml of diluent to get 50 mg/ml, 1 ml of the diluent was again transferred to another tube in 2 ml of diluent to get 25 mg/ml, 1 ml of the diluent was also transferred to another tube in 2 ml of diluent to get 12.5 mg/ml using the double dilution method.

The solid medium was inoculated with grown fungus and was incubated at 28 °C ± 2 ^{o}C for 4 days and observed for fungal growth. The lowest concentration of the extract that inhibited

the growth of fungi was recorded as the minimum inhibitory concentration. Plates without the test extracts were used as control (Okorondu *et al*., 2012).

3.7.3.2 Phytochemical analysis.

Determination of Phytochemical constituents of the plants was done according to (Trease & Evans, 1983).

Tannin.

Two grams of the samples were boiled in 20 ml of 45% ethanol for 5 minutes. The mixture was allowed to cool then the extract was used for analysis using the method of Trease and Evans (1983).

a. Ferric chloride test: One ml of filtrate was diluted with 2.0ml of distilled water and 2 drops of ferric chloride solution was added and observed for transient greenish to black colour.

Alkaloid.

One-tenth gram (0.1g) of the ground sample was boiled with 5ml of 2% hydrochloric acid on a steam bath. It was then filtered. 1ml portion of the filtrate reacted with 2 drops of the following reagents (Trease & Evans 1983).

a. Dragendroff's reagent (Bismuth potassium iodide solution) and was observed for orange precipitate.

Steroid.

The test for steroid was done by the Liberman acid test. A portion of the organic extract was treated with three drops of acetic anhydride. Then concentrated $H_2So_4$was carefully added by the side of the test tube. The presence of a brown colour at the boundary of the mixture was taken as positive result (Trease & Evans 1983).

Saponin.

One-tenth gram (0.1g) of the powdered samples were boiled with 5 ml of distilled water for 5 minutes and decanted while still hot. The filtrate was used for frothing and emulsion tests according to (Trease & Evans 1983).

Frothing test.

One milliliter (1 ml) of the filtrate was diluted with 4ml of distilled water and the mixture was shaken vigorously and observed on standing for suitable froth.

Flavonoid estimation.

Two grams (2g) of sample was heated with 10ml of 5% ethyl acetate in a boiling water bath for 3 minutes. The mixture was filtered and 4.0 ml filtrate was shaken with 1 ml of 1% aluminium chloride and 1 ml of 1% dilute ammonia solution. Yellow colouration of ammonia layer gave positive result ((Balagopalan *et al*., 1988; William *et al.*, 1996).

3.8 MOLECULAR ANALYSIS.

The isolates were identified using sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA of the isolate. All isolates were kept on Sabouraud's dextrose agar medium and stored at 4°C. The fungi were subcultured in Sabouraud dextrose broth, and

incubated at 28°C ±2 °C for 14 days. 200 mg to 300 mg of mycelia were harvested using 500 µl of DNA extraction buffer and centrifuged at 1600 for 10 mins, then washed twice with ice-cold sterile phosphate buffered saline (PBS) and finally stored at −70°C (Rezaie *et al.*, 2000).

3.8.1 DNA extraction.

DNA Extraction was done using a Zymo Research (ZR) fungal DNA mini prep extraction kit. Pure cultures of the fungal isolates were suspended in 200 microlitre of isotonic buffer into a ZR bashing bead lysis tubes. Then 750 microlitre of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for 5 min. The ZR bashing bead lysis tubes were centrifuged at 10,000 xg for 1 min. Four hundred (400) microlitres of supernatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 x g for 1 min. One thousand two hundred (1200) microlitres of fungal DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 microlitr. From this, 800 microlitre was transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 x g for 1 min, the flow through was discarded from the collection tube and the remaining volume transferred to the same Zymo-spin and spun.

Two hundred (200) microlitre of the DNA Pre-Wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000 x g for 1 min.This was followed by the addition of 500 microlitre of fungal DNA Wash Buffer and centrifuged at 10,000 x g for 1 min. The Zymo-spin IIC column was transferred to a clean 1.5 microlitre centrifuge tube. Thereafter, 100 microlitre of DNA elution buffer was added to the column matrix and centrifuged at

10,000 x g microlitre for 30 s to elude the DNA. The ultra-pure DNA obtained was then stored at -20^{0}C for other down stream reaction (Larry, 1994).

3.8.1.1 Agarose gel electrophoresis.

This derermines the qualitative estimation of Genomic DNA. To Analyse 3 µl of genomic DNA on a 0.8% - 1% agarose gel, Mix 3 µl genomic DNA with 2 µl loading dye. Run for about one hour at 100 v and view gel images using gel documentation system or transilluminator (Theodorakis & D' Sumey1994).

3.8.2 Polymerase Chain Reaction analysis.

The following 10 µl of One *Taq*Quick-Load 2X Master was mixed with Standard Buffer (New England Biolabs Inc.); 1µl each of forward and reverse primers; Internal transcribe space 1 and 4 (ITS 1 AND ITS 4). ITS 1 FORWARD PRIMER 5' – TCC GTA GGT GAA CCT GCG G-3' and the ITS 4 reverse primer 5' – TCC TCC GCT TAT TGA TAT GC -3'. The optimal concentration of primers used should be between 0.1 and 0.6 µM. This will ensure adequate yields of DNA amplicons (Larry, 1994).

The PCR cocktail was prepared by using 7 µl of nuclease free water and 1 µl of DNA template was used to prepare 20 µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to a pre heated thermo cycler. The amplification conditions for the PCR was as follows: 5 min at 94 ^{o}C to denature the DNA, followed by 35 cycles of denaturation at 94^{0}C for 30 secs, primer annealing at 50 ^{0}C for 30 seconds and strand extension at 68 ^{0}C for 10 minutes on an Eppendorf nexus gradient Mastercycler (Germany). PCR products were separated on a 2% agarose gel and DNA bands were visualized with syber gold (Larry, 1994).

3.8. 3 Sequencing.

PCR products were cleaned using ExoSAP Protocol as follows:

1. The Exo/SAP master mix was prepared by adding the following to a 0.6ml micro-centrifuge tube:

a. Exonuclease I (Catalogue No. NEB M0293L) 20 U/ul 50 µl.

b. Shrimp Alkaline Phosphatase (Catalogue No. NEB M0371) 1 U/ul 200 µl.

2. The following reaction mixture was prepared:

   Amplified PCR Product 10 µl; ExoSAP Mix (step 1) 2.5 µl.

3. The mixture was mixed well and incubated at 37°C for 15 min

4. The reaction was stopped by heating the mixture at 80°C for 15 min

Fragments were sequenced using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions:

The labelled products were then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053): The cleaned products were injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50cm array, using POP7: Sequence chromatogram analysis was performed using FinchTV analysis software (Platt *et al.*, 2007).

3.8.4 Basic local alignment search tool nucleotide (blastn).

The sequenced data obtained were uploaded to the database of National Center for Bioinformatics Information (NCBI) to identify corresponding organisms (Altschul, *et al,* 1990).

3.8.5 Phylogenic tree analysis.

The Phylogenic tree was constructed by using the geneious software version 4.0 (Matthew *et al*., 2012).

3.9 Statistical analysis.

Statistical analyses were carried out using statistically available software Statistical Package for the Social Sciences, (SPSS). The one way analysis of variance (ANOVA) was used to compare the mean occurrence of various organisms in the sample at 95% level of confidence, Duncan test was used to determine the mean occurrence among the various organisms as well as descriptive statistics.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION.

4.1 RESULTS.

4.1.1 Occurrence of morphological and microscopically identified isolates.

A total of nine moulds were identified morphologically and microscopically from the grains and legumes samples (rice, maize, wheat, groundnut and beans). The moulds are *Aspergillus* sp, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraecus*, *Penicillium chrysogenum*, *Fusarium* sp, *Rhizopus stolonifer*, *Rhizopus nigricans* and *Mucor* sp Table 4.1. The morphologically and culturally identified moulds were further confirmed with molecular identification by internal transcribed spacer (ITS) region. The moulds recovered were *Aspergillus flavus, Aspergillus tamarii, Aspergillus niger, Aspergillus brunneoviolaceus* and *Penicillium chrysogenum* Table 4.2. The gel band showing amplified deoxyribonucleic acid is on Figure. 4.1. The phylogenic tree, showing relationship among isolates is on Figure 4.2.

4.1.2 Occurrence of colony forming units of fungal isolates from unstored grains and legumes.

The colony forming units of fungal isolates from unstored grains and legumes are shown in Tables 4.3. Its shows the different colony forming units of fungal isolates in sac, cellophane, plastic container and metal container as well as from different media.

Table 4.1: Cultural and microscopic characteristics of identified isolates.

Cultural	Microscopic	Isolate
Colonies are greenish or yellow brown in colour	Hyphae are septate	*Aspergillus* sp
Colonies are green in colour	Hyphae are septate	*Aspergillus flavus*
Black colonies are seen.	Black spores , septate hyphae	*Aspergillus niger*
Colonies are fast growing from pinkisk to purple	Smooth or finely roughened phialides	*Aspergillus ochraceus*
Rusty brown or dark brown colonies	Conidia head with long chain of conidia	*Aspergillus tamarii*
Brown to dark brown colonies	Hyaline or pigmented longer stipes	*Aspergillus brunneoviolaceus*
Pale or bright colour conidia	Septate hyphae	*Fusarium* sp
White colonies becoming gray-brown	Rhizoids and stolons present	*Rhizopus stolonifer*
Colonies are black	Hyphae present are stolons, rhizoids	*Rhizopus nigricans*
White, gray to black colonies in old culture	Hyphae branched	*Mucor* sp

Table 4.2 : Molecular identification of isolates.

S/N	SAMPLE	SEQQUENCE ID	PERCENTAGE (%)	NCBI MATCH	ISOLATE
1	2	NR111041.1	99	*Aspergillus flavus* NR135325	*Aspergillus flavus*
2	11	NR138279.1	97	*Aspergillus brunneoviolaceus* NR138279	*Aspergillus brunneoviolaceus*
3	17	AY373852.1	91	*Aspergillus niger* AY373852	*Aspergillus niger*
4	22b	NR138306.1	99	*Penicillium chrysogenum* MH793845	*Penicillium chrysogenum*
5	23	AF004929.1	100	*Aspergillus tamarii* MN339986	*Aspergillus tamarii*

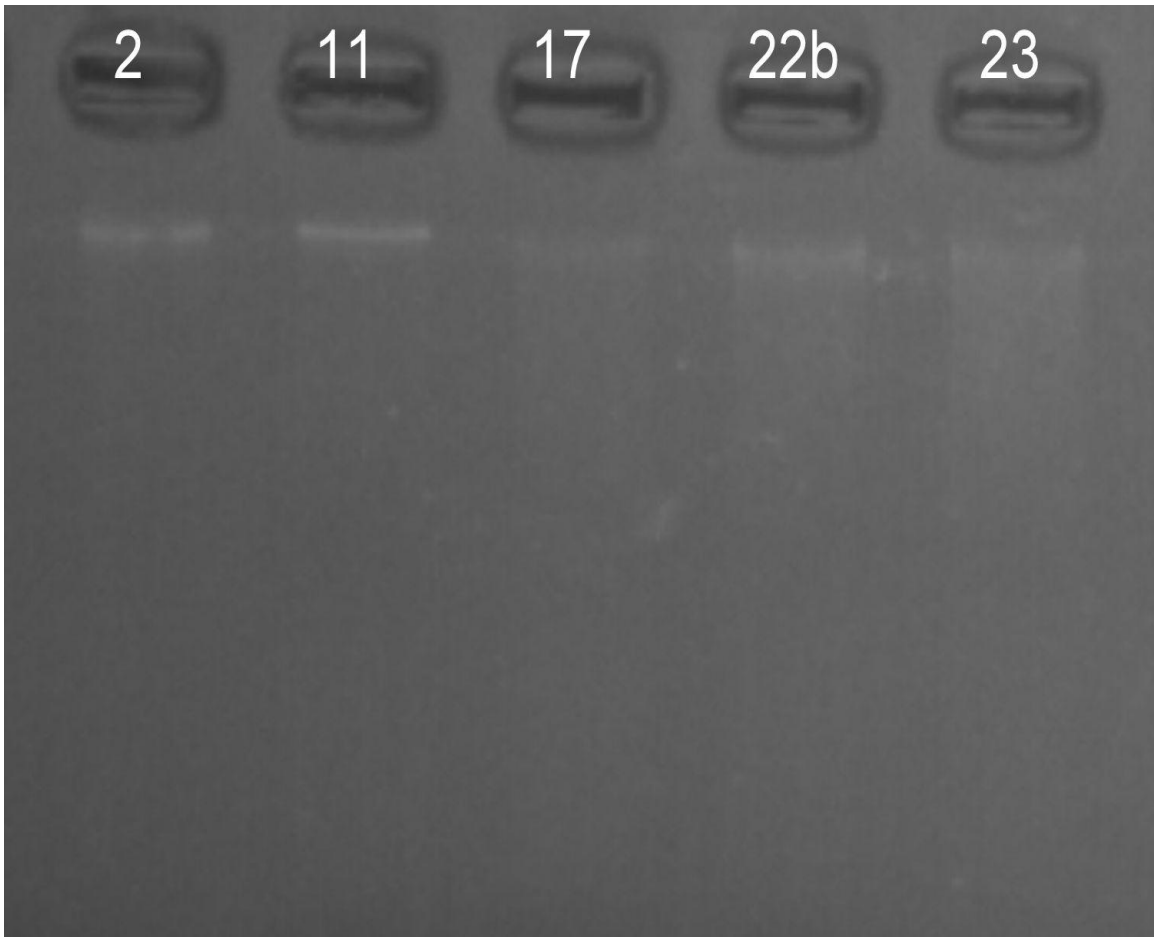


Figure 4.1: Gel bands.

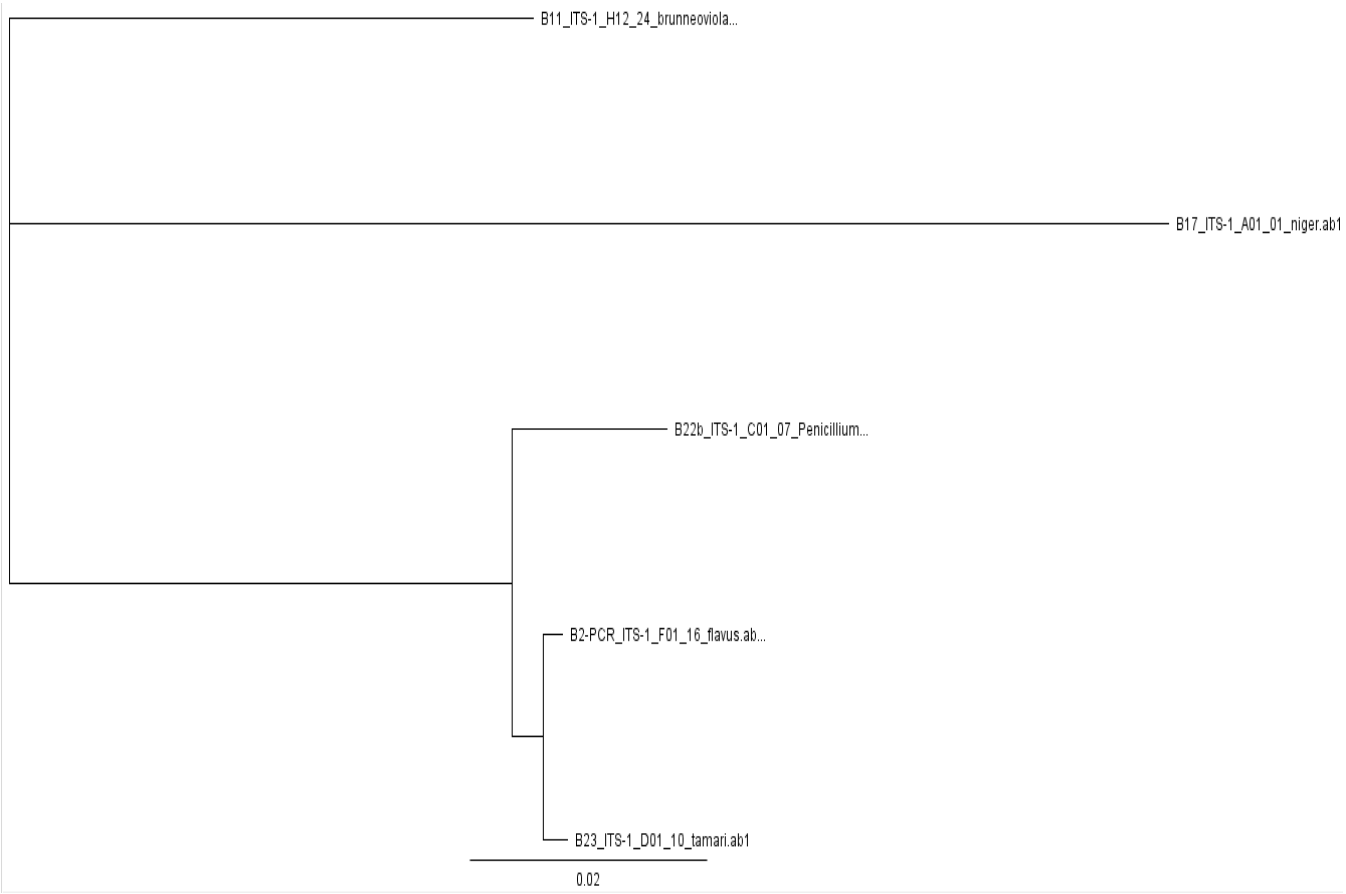


Figure 4.2: The Phylogenic tree was constructed by using the geneious software version 4.0.

Table 4.3: Colony forming units of fungal isolates from unstored grains and legumes.

Sample code / Storage material / Media			Count (cfu/ml) Rice Grain /Powder	Maize Grain / Powder	Wheat Grain / Powder	Groundnut Grain / Powder	Beans Grain / Powder
GS/PS	Sac	SDA	2.0×10^4 / NG	3.0×10^4 / 2.0×10^3	3.0×10^3 / NG	NG / 2.0×10^3	5.0×10^3/ NG
		PDA	NG / 2.0×10^3	7.0×10^3 / NG	3.0×10^3 / NG	6.0×10^3 / NG	1.0×10^3 / 1.0×10^3
		MEA	3.0×10^3 / NG	3.0×10^3 / 1.0×10^4	3.0×10^3 / NG	NG / NG	NG / NG
GC/PC	Cellophane	SDA	3.0×10^4 / NG	2.0×10^4 / 2.0×10^3	NG / NG	4.0×10^3 / 2.0×10^3	NG / 5.0×10^3
		PDA	NG / 2.0×10^3	3.0×10^3 / NG	2.0×10^3 / NG	2.0×10^3 / 7.0×10^3	4.0×10^3 / 3.0×10^3
		MEA	NG / 2.0×10^3	3.0×10^3 / NG	1.0×10^3 /NG	NG / NG	5.0×10^3 / 5.0×10^3
GP/PP	Plastic	SDA	2.0×10^4 / NG	NG / NG	NG / NG	4.0×10^3 / 1.0×10^3	NG / NG
		PDA	NG / 4.0×10^3	NG / NG	NG / NG	2.0×10^3 / NG	NG / 3.0×10^3
		MEA	NG / NG	NG / NG	1.0×10^3 / NG	1.0×10^3 / 4.0×10^3	NG / NG
GM/PM	Metal	SDA	NG / 2.0×10^3	3.0×10^3 / NG	3.0×10^3 / 2.0×10^3	NG / 1.0×10^3	NG / NG
		PDA	NG / 2.0×10^3	4.0×10^3 / 4.0×10^3	3.0×10^5 / 1.0×10^3	1.0×10^5 / 2.0×10^3	NG / 3.0×10^3
		MEA	NG / 4.0×10^3	4.0×10^4 / NG	1.0×10^3 / 2.0×10^3	4.0×10^3 / 3.0×10^3	NG / NG

Key: GS/PS –means storage in sac, GC/PC –means storage in cellophane, GP/PP –means storage in plastic, GM/PM –means storage in metal, NG – no growth, SDA sabouraud dextrose agar, PDA potato dextrose agar and MEA malt extract agar.

4.1.3 Occurrence (percentage) of isolates from unstored grains and legumes.

The percentage occurrence of fungal isolates on various grains and legumes ars shown on Table 4.4. This table contains the different fungal isolates as well as their frequency of occurrence and percentage occurrence from unstored grains and legumes. *Aspergillus* sp had the highest percentage occurrence while *Aspergillus ochraceus* had the least percentage occurrence.

4.1.4 Occurrence of colony forming units of fungal isolates on different storage materials and media from stored grains and legumes.

Its shows the different colony forming units of fungal isolates in different storage materials (sac, cellophane, plastic container and metal container). Metal containers had the highest number of fungal isolates followed by plastic containers and then cellophane while sac had the least number of fungal isolates as seen in Table 4.5. The colony forming units of fungal isolates on different media (potato dextrose agar, sabouraud dextrose agar and malt extract agar) of stored grains and legumes are also shown in Tables 4.5.

4.1.5 Occurrence (percentage) of isolates from stored grains and legumes.

The percentage occurrence of fungal isolates on various grains and legumes ars shown on Table 4.6. This table contains the different fungal isolates as well as their frequency of occurrence and percentage occurrence from stored grains and legumes. *Aspergillus* sp had the highest percentage occurrence while *Fusarium sp* and *Mucor sp* had the least percentage occurrence.

Table 4.4 Occurrence (percentage) of isolates from unstored grains and legumes.

Isolates	Rice	Maize	Wheat	Groundnut	Beans
	No. of Frequency/(%)	No. of Frequency/(%)	No. of Frequency/(%)	No. of Frequency/(%)	No. of Frequency/(%)
Aspergillus sp	6 (50)	5 (29)	2 (20)	2 (11)	4 (29)
Aspergillus flavus	-	1 (6)	4 (40)	3 (16)	3 (21)
Aspergillus niger	-	2 (12)	1 (10)		-
Aspergillus ochraceus	-	1 (6)	-		-
Penicillium chrysogenum	4 (34)	5 (29)	-	1 (5)	4 (29)
Rhizopus stolonifer.	-	1 (6)	3 (30)	7 (37)	2 (14)
Rhizopus nigricans	1 (8)	-	-	1 (5)	
Mucor sp	1 (8)	2 (12)	-	5 (26)	1 (7)

Key: Percentage - (%), No Isolate - (-)

Table 4.5: Colony forming units of fungal isolates on different storage materials from stored grains and legumes.

Sample code / Storage material / Media			Count (cfu/ml) Rice Grain / Powder	Maize Grain / Powder	Wheat Grain / Powder	Groundnut Grain / Powder	Beans Grain / Powder
GS/PS	Sac	SDA	4.0×10^5 / NG	1.0×10^5 / 1.0×10^5	3.0×10^5 / NG	2.0×10^5 / 2.0×10^5	NG / NG
		PDA	1.0×10^5 / 4.0×10^5	4.0×10^5 / 4.0×10^5	NG / 3.0×10^5	3.0×10^5 / 3.0×10^5	1.0×10^3/ 1.0×10^3
		MEA	4.0×10^3 /NG	3.0×10^5 /NG	NG / NG	1.0×10^5 / 2.0×10^5	NG / NG
GC/PC	Cellophane	SDA	NG /NG	1.0×10^5 / 2.0×10^5	1.0×10^5 / NG	3.0×10^5 / 4.0×10^5	NG / 4.0×10^2
		PDA	2.0×10^5 /1.0×10^5	2.0×10^6 / 4.0×10^5	1.0×10^5 / NG	2.0×10^5 / 7.0×10^5	4.0×10^3 / 3.0×10^3
		MEA	3.0×10^5 / 1.0×10^3	3.0×10^5 / 1.0×10^5	3.0×10^5 / NG	3.0×10^5 / 2.0×10^5	4.0×10^3 / 4.0×10^3
GP/PP	Plastic	SDA	3.0×10^5 / 2.0×10^6	1.0×10^5 / 5.0×10^5	1.0×10^5 / NG	3.0×10^5 / 3.0×10^5	4.0×10^5 / NG
		PDA	3.0×10^5 / 3.0×10^5	1.0×10^5 / 3.0×10^5	3.0×10^5 / 1.0×10^5	3.0×10^5 / 3.0×10^5	NG / 3.0×10^3
		MEA	NG / NG	3.0×10^5 / 2.0×10^5	2.0×10^5 / NG	3.0×10^5 / 3.0×10^5	NG / NG
GM/PM	Metal	SDA	NG / 3.0×10^5	4.0×10^5 / 4.0×10^5	1.0×10^3 / 4.0×10^5	3.0×10^5/ 4.0×10^5	3.0×10^5 / NG
		PDA	NG / 1.0×10^5	4.0×10^5/ 3.0×10^5	3.0×10^5 / 2.0×10^5	1.0×10^5 / 6.0×10^5	NG / 3.0×10^3
		MEA	2.0×10^5 / NG	2.0×10^5 / 2.0×10^5	3.0×10^5 / 2.0×10^5	3.0×10^5 / 3.0×10^5	NG / NG

Key: GS/PS means storage in sac, GC/PC –means storage in cellophane, GP/PP –means storage in plastic, GM/PM –means storage in metal, NG – no growth, SDA sabouraud dextrose agar, PDA potato dextrose agar and MEA malt extract agar.

Table 4.6 Occurrence (percentage) of isolates from stored grains and legumes.

Isolates	Rice	Maize	Wheat	Groundnut	Beans
	No. of Frequency/ (%)	No. of Frequency/ (%)	No. of Frequency/ (%)	No. of Frequency/ (%)	No. of Frequency/ (%)
Aspergillus sp	11 (37)	11 (22)	4 (15)	15 (27)	12 (37)
Aspergillus flavus	2 (7)	8 (16)	2 (7)	6 (11)	4 (13)
Aspergillus niger	-	-	4(15)		-
Aspergillus ochraceus	-	-	2 (7)	3 (5)	4 (13)
Penicillium chrysogenum	9 (30)	12 (25)	8 (30)	17 (30)	10 (31)
Fusarium sp	-	1 (2)	-	-	-
Rhizopus stolonifer.	7 (23)	16 (33)	6 (22)	14 (25)	-
Rhizopus nigricans	1 (3)	-	1 (4)	1 (2)	2 (6)
Mucor sp	-	1 (2)	-	-	-

Key: Percentage - (%), No Isolate - (-).

4.1.6 Frequency and percentage occurrence of mycotoxin producing moulds from stored grains and legumes.

Fungal isolates or mycotoxin producing moulds from stored grains and legumes (rice, maize, wheat, groundnut and beans) showed that *Aspergillus flavus* had the higest frequency of 140 (46%), *Aspergillus tamarii* 68 (23%), *Aspergillus niger* 53 (18%), *Penicillium chrysogenum* 27 (9%) while the least frequency was *Aspergillus brunneoviolaceus* 12 (4%) as shown in Table 4.7. For the stored grains and legumes, groundnut had the highest number of moulds frequency followed by maize, beans, rice and wheat had the least frequency of occurrence in Table 4.7. There was a significant difference ($P < 0.05$) among the isolates produced from the different samples. *Aspergillus* species had a higher significant rate compared to other isolates.

4.1.7 Analysis of the presence of mycotoxins.

The Samples (160) of Rice, maize, wheat and groundnut analyzed for the presence of mycotoxins had the following mycotoxins namely Aflatoxin B_1, Aflatoxin B_2 , Aflatoxin G_1, Aflatoxin G_2, Ochratoxin A, Citrinin, Dihydrocitrinone, Fumonisin B_1, Fumonisin B_2, Fumonisin B_3, Fumonisin B_4, Zearalenone, Deoxynivalenol and Nivalenol. There was a significant difference ($P< 0.05$) among the mycotoxins produced by the different samples Tables 4.8. Fumonisisns and Aflatoxin had a higher significant difference ($P < 0.05$) rate compared to other mycotoxins.

TABLE 4.7: Frequency / Percentage (%) occurrence of mycotoxin producing moulds.

Moulds	Rice No/Std/ (%)	Maize No/Std/(%)	Wheat No/Std/ (%)	Groundnut No/Std/ (%)	Beans No/Std/ (%)	Total No /Std	Total No (%)/Std
A .flavus	11 ± 1.000 (34%)	50 ± 5.773 (60%)	8 ± 0.577 (27%)	30 ± 3.000 (32%)	41 ± 2.309 (66%)	140 ± 5.773	46% ± 0.577
A. tamarii	7 ± 0.577 (22%)	20 ± 5.000 (24%)	1 ± 0.577 (3%)	25 ± 1.527 (28%)	15 ± 1.000 (24%)	68 ± 4.618	23% ± 2.516
A .niger	3 ± 1.000 (9%)	8 ± 0.577 (10%)	18 ± 2.000 (60%)	23 ± 1.527 (25%)	1 ± 0.577 (2%)	53 ± 4.041	18% ± 1.000
A.brunneoviolaceus	5 ± 0.577 (16%)	3 ± 0.000 (3%)	1 ± 0.577 (3%)	2 ± 0.577 (2%)	1 ± 0.577 (2%)	12 ± 0.00	4% ± 0.577
P.chrysogenum	6 ± 0.000 (19%)	3 ±1.000 (3%)	2 ± 0.577 (7%)	12 ± 1.000 (13%)	4 ± 0.577 (6%)	27 ± 1.000	9% ± 0.000
	32 ±1.154 (100%)	84 ±3.464 (100%)	30 ± 0.000 (100%)	92 ± 1.154 (100%)	62 ± 1.154 (100%)	300 ± 0.000	100% ± 5.773
	11%	28%	10%	31%	20%		

Key: No (%) – Percentage.

Table 4.8: Concentration of Mycotoxins in Wheat, Maize, Rice and Groundnut (µg/kg).

Mycotoxins	**Concentra tion (µg/kg)**			
	Wheat	**Maize**	**Rice**	**Groundnut**
Aflatoxin B_1	84.3 ± 2.100	58.2 ± 1.509	97.4 ± 2.500	81.5 ± 0.763
Aflatoxin B_2	86.1 ± 1.473	60.8 ± 1.750	96.3 ± 0.100	69 ± 2.000
Aflatoxin G_1	79.5 ± 2.500	84.7 ± 1.750	97.5 ± 1.500	65.3 ± 2.04
Aflatoxin G_2	80.5 ± 0.763	67.8 ± 1.400	94.2 ± 1.627	62.2 ± 1.101
Ochratoxin A	96.9 ± 1.300	87.8 ± 2.052	100.5 ± 3.500	86.6 ± 1.026
Citrinin	43.9 ± 0.550	27.1 ± 2.050	80 ± 1.700	98.8 ± 3.002
Dihydrocitrinone	115.5 ± 0.435	147.3 ± 4.000	100.1 ± 2.510	97.1 ± 2.510
Fumonisin B_1	85 ± 5.507	85 ± 2.081	85 ± 5.507	85 ± 3.214
Fumonisin B_2	85 ± 2.309	85 ± 1.527	85 ± 1.1554	85 ± 1.154
Fumonisin B_3	85 ± 3.214	85 ± 2.081	85 ± 2.081	85 ± 2.516
Fumonisin B_4	85 ± 1.154	85 ± 1.527	85 ± 3.214	85 ± 1.527
Zearalenone	100.1 ± 0.950	85 ± 2.081	99.2 ± 2.900	74.2 ± 0.642
Deoxynivalenol	80 ± 0.577	80 ± 2.309	80 ± 1.154	80 ± 0.577
Nivalenol	60 ± 2.00	72.9 ± 2.066	101.1 ± 2.066	88.2 ± 1.509

4.1.8 Moisture analysis of stored grains and legume.

The moisture contents of stored grains and legume (rice, maize, wheat and groundnut) from the three zone namely owerri, orlu and okigwe were analyzed. The moisture content of groundnut was highest after 6 hours of drying followed by maize, then rice while wheat had the lowest moisture content Tables 4.9.

4.1.9 Antifungal susceptibility profile.

Moulds were isolated from the different stored grains and legume which were collected from different markets. The isolated species were first identified morphologically and microscopically. They were further identified genetically by sequencing of 18S rRNA gene using ITS1 and ITS4 primers. The resulting mould species were *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum.* From Table 4.10, all the isolates were susceptible to ketoconazole and voriconazole though at different millimeters. According to Table 4.11, all the isolates were sensitive to ketoconazole and TTC.

Antifungal agents alone gave a better zone of inhibition than the combination of antifungal agents with tetrazolium test and tertazolium test alone. Comparing the susceptibility rates among the oxoid antifungal drugs, there was a significant difference ($P<.05$) between the susceptibility of oxoid antifungal agents. Ketoconazole had a higher significant rate when compared to fluconazole. Some of the plates containing various isolates with the different antifungal agents are equally shown.

Table 4.9: Moisture content of grains and legumes from different zones in Imo State.

Zone	Moisture content																			
	Wheat					Maize					Rice					Groundnut				
	Initial weight	2hr	4hr	6hr	100 µL	Initial weight	2hr	4hr	6hr	100 µL	Initial weight	2hr	4hr	6hr	100 µL	Initial weight	2hr	4hr	6hr	100 µL
Owerri	5.4	5.1	4.9	4.9	2.3	5.5	5.1	5.1	5.1	65.1	5.5	5.1	5.0	5.0	2.8	5.4	5.2	5.2	5.2	7.9
Orlu	5.4	5.1	5.0	5.0	1.0	5.5	5.1	5.0	5.0	22.1	5.4	5.0	4.9	4.9	3.3	5.5	5.2	5.2	5.2	5.0
Okigwe	5.7	5.2	5.2	5.2	0.1	5.5	5.2	5.1	5.1	111.5	5.5	4.9	4.8	4.8	12.1	5.4	5.3	5.2	5.2	70.7

Table 4.10: Susceptibiity profile using oxoid antifungal drugs in (mm).

ISOLATES	Amphotericin B (20 μg/ml)	Ketoconazole (10 μg/ml)	Flucanozole (25 μg/ml)	Vorioconazole (1 μg/ml)
Aspergillus flavus	20.00 ± 0.577 (S)	35.00 ±2.886 (S)	0.00 ± 0.000(R)	32.00 ±1.154 (S)
Aspergillus tamarii	23.00 ± 0.577 (S)	38.00 ± 2.081 (S)	0.00 ± 0.000(R)	36.00 ± 1.732 (S)
Aspergillus niger	11.00 ± 0.577(R)	20.00 ± 0.577(S)	0.00 ± 0.000(R)	25.00 ± 1.527(S)
Aspergillus brunneoviolaceus	30.00 ± 2.000(S)	39.00 ± 1.000 (S)	21.00 ± 12.124(S)	36.00 ± 2.000(S)
Penicillium chrysogenum	7.00 ± 0.577 (R)	20.00 ± 1.527 (S)	0.00 ± 000 (R)	17.00 ± 1.527 (S)

Minimum Inhibition Concentration (MIC) endpoint reading:

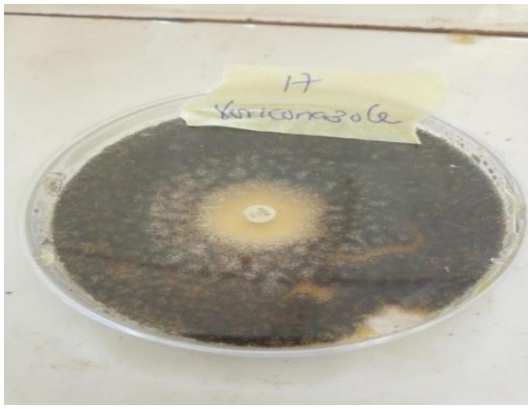
- susceptible, ≥17 mm (azoles) and ≥15 mm (amphotericin B);
- intermediate, 14 to 16 mm (azoles) and 13 to 14 mm (amphotericin B);
- resistant, ≤13 mm (azoles) and ≤12 mm (amphotericin B) [13].

Table 4.11: Susceptibiity profile using a combination of tetrazolium chloride and antifungal drugs in mm.

ISOLATES	Amphotericin B(20 μg/ml) + TTC	Ketoconazole (10 μg/ml) + TTC (mm)	Flucanozole (25 μg/ml) + TTC (mm)	Vorioconazole (1μg/ml)+ TTC (mm)
Aspergillus flavus	13.00 ± 0.577 (I)	25.00 ± 1.527 (S)	0.00 ± 0.000 (R)	15.00 ± 1.000 (I)
Aspergillus tamarii	13.00 ±1.154 (I)	28.00 ± 0.577 (S)	0.00 ±0.000 (R)	21.00 ± 1.000 (S)
Aspergillus niger	21.00±1.000 (S)	25.00 ± 2.000 (S)	8.00 ± 0.577(R)	30.00 ± 2.000 (S)
Aspergillus brunneoviolaceus	27.00 ±1.000 (S)	36.00 ± 2.000 (S)	16.00 ±0.577 (I)	32.00 ± 3.214(S)
Penicillium chrysogenum	9.00 ±0.000 (R)	19.00 ± 1.154 (S)	0.00 ±0.000 (R)	14.00 ± 0.577(I)

Minimum Inhibition Concentration (MIC) endpoint reading:

- susceptible, ≥17 mm (azoles) and ≥15 mm (amphotericin B);
- intermediate, 14 to 16 mm (azoles) and 13 to 14 mm (amphotericin B);
- resistant, ≤13 mm (azoles) and ≤12 mm (amphotericin B) [13].



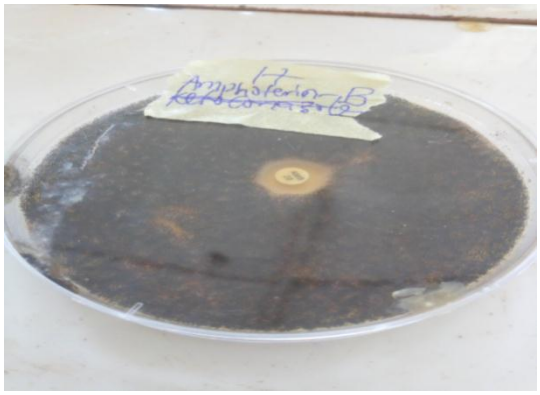


Plate 4.1: Susceptibility test of *Aspergilllus niger* on oxoid antifungal drugs.

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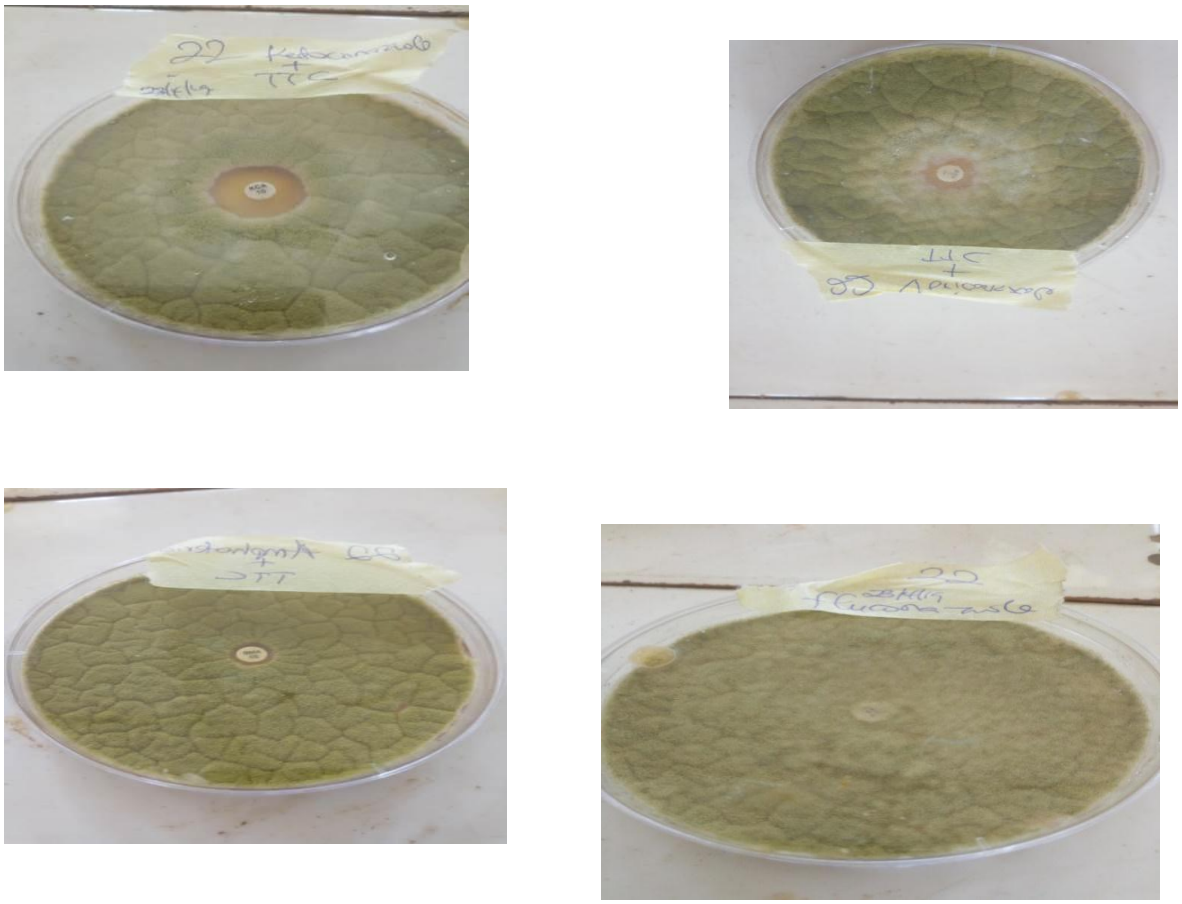

Plate 4.2: Susceptibility test of *Aspergilllus flavus* using a combination of Tetrazoliun chloride and antifungal drugs.

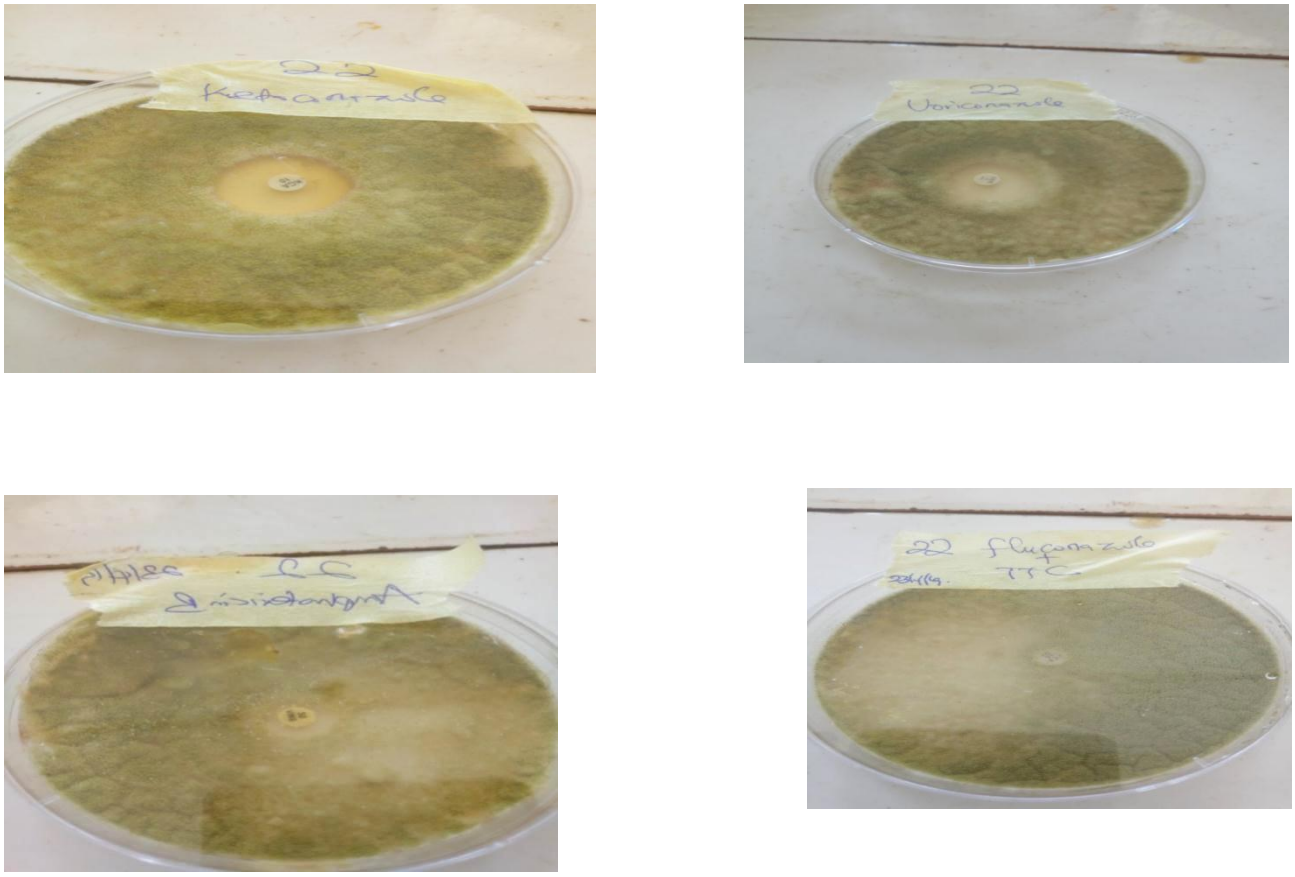

Plate 4.3: Susceptibility test of *Aspergillus flavus* on oxoid antifungal drugs.

4.1.10 Phytochemical screening of medicinal plants.

Qualitative determination of the phytochemical constitutes of the four medicinal plants used in this study showed that they contain secondary metabolites like tannin, alkaloids steroids, saponin and flavonoids as presented in Table 4.12.

4.1.11 Extraction yield of plant extracts.

A total of four different plant species were usedin this study. They include *Ocimum gratissimum, Vernonia amygdalina, Gongronema latifolium and Piper guineense.* The leaves of the various plants were used for the extraction. Methanol and hot water were used the solvent used in the extraction process. Percentage growth inhibition of moulds by methanol and hot water extract of scent leaf, bitter leaf, utazi leaf and uziza leaf is seen in Tables 4.13 - 4.16. Methanol had a higher extraction yield than hot water while *Ocimum gratissimum* gave the highest zone of inhibition and *Piper guineense* gave the least zone of inhibition at both 50mg/ml and 100mg/ml as seen in Table 4.13 - Table 4.16. Comparing the susceptibility rates among the various plant extracts, there was a significant difference ($P<.05$) between the susceptibility of scent leaves extract to uziza leave extracts.

Table 4.12: Qualitative analysis of phytochemical constituents.

Phytochemical screening of Plant extracts.

Plant extracts	Phytochemicals				
	Tannin	Alkaloid	Steroid	Saponin	Flavonoid
Scent leaf	+	+	+	+	+
Bitter leaf	+	+	+	+	+
Utazi leaf	+	+	+	+	+
Uziza leaf	+	+	+	+	+

Key : + Positive

Table 4.13: Percentage growth inhibition of moulds by methanol and hot water extract of scent leaf.

Isolates	Concentration of extracts (mg/ml)			
	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Control	0.00 ±0.00	0.00 ±0.00	0.00 ±0.000	0.00 ±0.00
Methanol				
Aspergillus flavus	0.00 ±0.00	0.00 ±0.00	8.00 ±0.577	14.00 ±0.577
Aspergillus tamarii	0.00 ±0.00	0.00 ±0.00	10.00 ±0.577	15.00 ±1.000
Aspergillus niger	0.00 ±0.00	0.00 ±0.00	4.00 ±0.577	7.00 ±0.577
Aspergillus brunneoviolaceus	0.00 ±0.00	0.00 ±0.00	14.00 ±0.577	19.00 ±2.081
Penicillium chrysogenum	0.00 ±0.00	0.00 ±0.00	2.00 ±0.577	3.00 ±1.000
Hot water				
Aspergillus flavus	0.00 ±0.00	0.00 ±0.00	4.00 ±0.577	7.00 ±1.000
Aspergillus tamarii	0.00 ±0.00	0.00 ±0.00	6.00 ±1.000	10.00 ±0.577
Aspergillus niger	0.00 ±0.00	0.00 ±0.00	2.00 ±0.577	3.00 ±0.000
Aspergillus brunneoviolaceus	0.00 ±0.00	0.00 ±0.00	10.00 ±0.577	12.00 ±1.000
Penicillium chrysogenum	0.00 ±0.00	0.00 ±0.00	1.00 ±0.577	3.00 ±0.577

Table 4.14: Percentage growth inhibition of moulds by methanol and hot water extract of bitter leaf.

Isolates	Concentration of extracts (mg/ml) 12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	0.00 ± 0.000
Methanol				
Aspergillus flavus	0.00 ±0.00	0.00 ± 0.00	6.00 ± 2.000	11.00 ± 0.577
Aspergillus tamarii	0.00 ± 0.00	0.00 ± 0.00	8.00 ± 2.708	15.00 ± 1.000
Aspergillus niger	0.00 ± 0.00	0.00 ± 0.00	2.00 ± 0.816	5.00 ± 0.577
Aspergillus brunneoviolaceus	0.00 ± 0.00	0.00 ± 0.00	10.00 ± 3.336	12.00 ± 0.000
Penicillium chrysogenum	0.00 ± 0.00	0.00 ± 0.00	2.00 ± 0.816	3.00 ± 0.000
Hot water				
Aspergillus flavus	0.00 ± 0.00	0.00 ± 0.00	3.00 ± 1.000	4.00 ± 0.577
Aspergillus tamarii	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 1.414	7.00 ± 0.577
Aspergillus niger	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.577	2.00 ± 0.577
Aspergillus brunneoviolaceus	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 1.414	6.00 ± 1.000
Penicillium chrysogenum	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	2.00 ± 0.577

Table 4.15: Percentage growth inhibition of moulds by methanol and hot water extract of utazi leaf.

Isolates	Concentration of extracts (mg/ml)			
	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	0.00 ± 0.000
Methanol				
Aspergillus flavus	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 0.577	7.00 ± 0.577
Aspergillus tamarii	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 0.577	9.00 ± 0.000
Aspergillus niger	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	3.00 ± 1.000
Aspergillus brunneoviolaceus	0.00 ± 0.00	0.00 ± 0.00	5.00 ±1.154	10.00 ± 0.577
Penicillium chrysogenum	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	1.00 ± 0.5777
Hot water				
Aspergillus flavus	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.000	2.00 ± 0.577
Aspergillus tamarii	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	3.00 ± 1.000
Aspergillus niger	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.000	0.00 ± 0.000
Aspergillus brunneoviolaceus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	3.00 ± 0.000
Penicillium chrysogenum	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	0.00 ± 0.000

Table 4.16: Percentage growth inhibition of moulds by methanol and hot water extract of uziza leaf.

Isolates	Concentration of extracts (mg/ml)			
	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000
Methanol				
Aspergillus flavus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 0.577
Aspergillus tamarii	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	7.00 ± 1.527
Aspergillus niger	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.00 ± 0.000
Aspergillus brunneoviolaceus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 1.154
Penicillium chrysogenum	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.577
Hot water				
Aspergillus flavus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.577
Aspergillus tamarii	0.00± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.00 ± 0.577
Aspergillus niger	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000
Aspergillus brunneoviolaceus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.00 ± 0.577
Penicillium chrysogenum	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

4.2 DISCUSSION.

A total of nine isolates were identified using their macroscopic and microscopic characteristics. They are as follows: *Aspergillus* sp. *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Penicillium chrysogenum*, *Fusarium* sp, *Rhizopus stolonifer*, *Rhizopus nigricans*, *Mucor* sp. When subjected to molecular identification, the moulds recovered were *Aspergillus flavus, Aspergillus tamarii, Aspergillus niger, Aspergillus brunneoviolaceus* and *Penicillium chrysogenum.* The colony forming units of isolates for unstored grains and legumes and the colony forming units of isolates for stored grains and legumes were determined. The numbers of colony forming units were more in Potato dextrose agar, sabouround dextrose agar and the least was in malt extract agar.

Results obtained showed that unstored Rice samples had *Aspergillus* sp as the highest occurring isolate followed by *Penicillium chrysogenum* and *Mucor* sp. Maize had *Aspergillus* sp followed by *Penicillium chrysogenum, Aspergillus niger, Mucor* sp, *Aspergillus flavus, Aspergillus ochraceus* and *Rhizopus stolonifer*. Wheat had *Aspergillus* sp, *Rhizopus stolonifer and Aspergillus flavus.* Groundnut had *Rhizopus stolonifer*, *Mucor* sp, *Aspergillu fl aus*, *Aspergillus sp.* and *Penicillium chrysogenum*. This was also reported by (Ikechi –Nwogu and Edith, 2012). Beans had *Aspergillus* sp. followed by *Penicillium chrysogenum*, *Aspergillus flavus*, *Rhizopus stolonifer* and *Mucor* sp.

Stored grains and legumes analysis showed that Rice had *Aspergillus* sp as the highest occurring isolate followed by *Penicillium chrysogenum, Rhizopus stolonifer*, *Aspergillus flavus and Rhizopus nigricans.* Maize had *Rhizopus stolonifer* as the highest occurring isolate followed by *Penicillium chrysogenum Aspergillus* sp, *Aspergillus flavus*, *Mucor* sp. and *Fusarium* sp. Wheat had *Penicillium chrysogenum* as the highest occurring isolate followed by *Rhizopus solonifer,*

Aspergillus sp, *Aspergillus niger, Aspergillus ochraceus, Aspergillus flavus* and *Rhizopus nigricans*. Groundnut had *Penicillium chrysogenum,* as the highest occurring isolate followed by *Aspergillus* sp, *Rhizopus stolonifer, Aspergillus flavus, Aspergillus ochraceus* and *Rhizopus nigricans* Beans had *Aspergillus* sp as the highest occurring isolate followed by *Penicillium chrysogenum, Aspergillus flavus, Aspergillus niger* and *Rhizopus nigricans*.

The overall best organism was from the genus *Aspergillus* followed by *Penicillium chrysogenum* this was also reported by Ikechi –Nwogu and Edith, (2012). All the moulds reported in this research have already been isolated in foods as reported by Hocking and Pitts, (1980). The genus *Aspergillus* was the most prevalent genus. The majority of *Aspergillus* species in this study are potential producer of toxic metabolites. However the minimum water activity (Aw) required to synthesize toxins is superior to the minimum required to fungal multiplication this was reported by Pitts and Hocking, (1997).

Evaluation of mycological media.

Potato dextrose agar recovered the highest number of isolates from both unstored and stored grains and legumes followed by sabouraud dextrose agar then malt extract agar recovered the least number of isolates. This agreed with the work of Steven, (1981) and Chalupova *et al*., (2013) that potato dextrose agar has a very high recovery rate of fungi compared to other mycological media.

Evaluation of samples.

Groundnut had the highest number of isolates followed by maize, beans, rice and wheat had the least number of isolates. This result agreed with the work of Bueno*et al*., (2001) saying that the

control of fungal development during the storage of food is mainly reach through the low A_w in the final product.

The work of Sohaib *et al*., (2019) observed that there were two dominate specie of *Aspergillus* namely; *Aspergillus flavus* and *Aspergillus fumigatus* form isolation and molecular characterization of rice samples. It suggested that there may be possible contamination of the rice products with aflatoxins since *Aspergillus* species were mainly responsible for the production of aflatoxins. It study encourage proper drying and storage devises for rice product to reduce *Aspergillus* contamination and subsequent contamination with aflatoxin. The study of Sohaib *et al*., (2019) went further to suggest the use of potato dextrose agar for isolation of moulds. These findings were similar to this research work.

The research done by Wagara, *et al*., (2014) observed that both the maize and groundnut samples were contaminated with at least one types of mycotoxigenic moulds like *Aspergillus*, *Penicillium* and *Fusarium*. That research also suggested that the highest occurring mould genera were *Aspergillus, Penicillium* and *Fusarum* in that order. It also stated that the maize and groundnut samples were contaminated with more than two types of moulds. *Aspergillus flavus* was the highest contaminating mould. The high prevalence of mycotoxigenic moulds remains a threat to healthy life. These finding were all similar to this research work.

Ryan and Ray, 2004 observed that the highest frequency of mould were in this order *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus* and *Mucor* which agreed with this research work.

Hussaini *et al*., (2009) observed that moulds contamination requires high moisture content. According to Varga *et al*., (2011). It found out that *Aspergillus* species have great affinity for cereals. Seven *Aspergillus* species namely *Aspergillus flavus, Aspergillus parasiticus,*

Aspergillus ochraceus, Aspergillus niger, Aspergillus fumigatus, Aspergillus wentii and *Aspergillus ruber*. *Aspergillus* genera are known to produce aflatoxins, ochratoxins, fumonisins, gliotoxin, aspertoxins as well as other harmful mycotoxins was observed by Schmale and Mmunkvoid, (2009). Okello, *et al*., (2010) suggested that traditional drying of cereals on bare floor may account for its high fungal contamination. Also poor storage practices may lead to the production of mycotoxins this was observed by Bhat and Vasanthi, (2003) and Wagacha and muthoni, (2008). According to Fandohan, *et al*., (2003) suggested that mechanical damage may have a contributed to mould invasion of cereals.

The study showed that all the stored grains and legumes (rice, maize, wheat. Groundnut and beans) analyzed had different types of moulds. The moulds identified are *Aspergillus flavus, Aspergillus tamarii, Aspergillu sniger, Aspergillus brunneoviolaceus,* and *Penicillium chrysogenum.* These results were similar to the results of Ranjana and Ananta, (2016). The frequent genus isolated was *Aspergillus* with four different species namely *Aspergillus flavus, Aspergillus tamarii, Aspergillus niger, Aspergillus brunneoviolaceus*. Results showed that the predominant mould species were in the order *Aspergillus flavus* (46%), *Aspergillus tamarii* (23%), *Aspergillus niger* (18%), and *Penicillium chrysogenum* (9%) while the least was (4%) *Aspergillus brunneoviolaceus*. *Aspergillus flavus* produce Aflatoxins and *Aspergillus* produce Ochratoxin A and their presence in stored and unstored grains and legumes can be detrimental to human health (Sambrook, & Russell 2001).

Results further showed that the frequency and percentage occurrence of mould from different grains and legumes with *A. flavus* (60%) from maize being high and *A. niger* from wheat predominating. This finding was similar to those reported by Jedidi *et al*., (2017). The occurrence of *Aspergillus flavus* is seen as important because they are believed to produce

aflatoxins which are among the most dangerous carcinogens to human and animals (Shalini and Amutha 2014). The moulds with the highest frequency of occurrence were *Aspergillus flavus, Aspergillus tamarii, Aspergillus niger, Aspergillus brunneoviolaceus,* and *Penicillium chrysogenum.* This has been reported by Omaima, *et al.*, (2018) and Ryan & Ray (2004).

It is likely that post-harvest infections and the storage structures greatly influence the mycoflora in storage (Wagacha & Muthomi, 2008). The two genera *Aspergillus* and *Penicillium* encountered are storage fungi while *Fusarium* is a field fungus (Bullerman & Bianchini 2011), (Andrew & Beatrice 2017). The high moisture contents could account for the variations in the frequency and percentage occurrence in the stored grains and legumes (Pitt & Hocking 1997).

The limits for Aflatoxins B_1, B_2, G_1, and G_2 varies from (0-40 µg/kg in food), for Ochratoxin A (0-50 µg/kg in food) , for Citrinin and Dihydrocitrinone (0-100 µg/kg in foods), for Fumonisins (0-1000 µg/kg in food), Zearalenone (0-1000 µg/kg in food), for Deoxynivaleno and Nivalenol (500-2000 µg/kg in food European) (Commission, 2006).

There is worldwide occurrence of mycotoxins especially aflatoxins in rice where the climatic environment favours the growth of moulds on rice (Aydin *et al*., 2011). The cultivation of rice is normally done on irrigated and flooded conditions that favour the growth of moulds and the subsequent production of mycotoxins, like aflatoxins (Majeed, *et al*., 2018). Aflatoxins are the most potent carcinogenic mycotoxins and are produced by *Aspergillus flavus, Aspergillus parasiticus* and rarely *Aspaergillus nomius* (Creppy, 2002).

Moulds grow on rice when the environment is favourable like during heavy rain fall, floods and storage. Insufficient sun drying and improper storage make rice prone to mould attack (Majeed, *et al*., 2018).

The four stored grains and legumes exceeded the maximum limit for Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1, Aflatoxin G_2, Ochratoxin A, Citrinin and Dihydrocitrinone. They did not exceed the limits for Fumonisin B_1, Fumonisin B_2, Fumonisin B_3, Fumonisin B_4, Zearalenone, Deoxynivalenol and Nivalenol.

The largest concentration of mycotoxins detected from all stored grains and legumes was the fumonisin 1350 ± 10.000 µg/kg followed by aflatoxins 1265.3 ± 1.327 µg/kg, then Citrinin (Dihydrocitrrinone) 709.8 ± 1.039 µg/kg, Trichothecenes: (Nivalenone Deoxynivalenone) 642.2 ± 1.900 µg/kg , Ochratoxin A 371.8 ± 1.616 µg/kg, and the least being Zearalenone 358.5 ± 2.500 µg/kg.

From this study, it was found that the predominant mycotoxin found was Aflatoxin, which was seen relatively on all the grains and legumes (rice, maize, wheat and groundnut). This was similar to the Research done over the years by Nigerian Stored Products Research Institute (NSPRI) that detected the presence of aflatoxin in Nigerian products like groundnut and livestock feed, maize, millet and sorghum (Oyeniran, 1978; Opadokun & Ikeorah 1979). Aflatoxin had 100% occurrence in all the 6 samples namely rice, corn, millet, sorghum, cassava flakes (garri) and yam flour in North-Central Nigeria that were analysed. Other mycotoxins detected were fumonisins and ochratoxin A. Rice had the highest levels of aflatoxin, fumonisins and ochratoxin A Onyedum *et al.,* (2020). Aflatoxin contamination is mainly reported in maize, peanuts and their products, fumonisin contamination in maize and maize products Misihairabgwi *et al*., (2019).

From the work of Atanda *et al*., (2013), it showed that aflatoxins in groundnuts and groundnut based products from Kaduna and Port Harcourt cities in the northern and southern part of Nigeria

exceeded the United States of America and Nigeria Minimum permissible limit. This is common in west and central Africa.

According to Ekhuemelo & Abu, (2018), groundnuts from Benue state in Nigeria were contaminated by aflatoxin B_1. According to Ojuri *et al*., (2018) various mycotoxins like aflatotins (AFs), fumonisins (FBs), ochratoxin A (OTA), citrinin (CIT), trichothecenes (deoxynivalenol (DON) and nivalenol (NIV)) and zearalenone (ZEN) were found in cereal, legume and their products in Nigeria. They used LC-MS/MS Liquid chromatography–tandem mass spectrometry method to analyze for the presence of mycotoxins. This agreed with this research work that cereals contain the above mentioned mycotoxins when analyzed with the LC-MS/MS Liquid chromatography tandem mass spectrometry method.

The antifungal susceptibility tests showed that all the isolates were susceptible to ketoconazole and voriconazole though at different millimeters. For Amphotericin B, *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus brunneoviolaceus*, were sensitive and *Aspergillus niger and Penicillium chrysogenum* were resistant. For fluconazole, *Aspergillus brunneoviolaceus*was sensitive while the other isolates were resistant.Susceptibility profile using only tetrazolium test (TTC) all the isolates (*Aspergillus flavus, Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum*) were resistant.

The antifungal susceptibility tsets using tetrazolium chloride showed that all the isolates were sensitive to ketoconazole and TTC, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus* were sensitive to voriconazole and TTC while *Aspergillus flavus and Penicillium chrysogenum w*ere intermediate. For Amphotericin B and TTC *and Aspergillus niger*, *Aspergillus brunneoviolaceus* were sensitive while *Aspergillus flavus*, *Aspergillus tamarii*

were intermediate, *Penicillium chrysogenum* was resistant. *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, and *Penicillium chrysogenum* were resistant to fluconazole and TTC while *Aspergillus brunneoviolaceus* was was intermediate.

Ketoconazole had the highest minimum inhibitory concentration among different anti fungi agents, followed by Voriconazole, Amphotericin B and then Fluconazole.

Statistical analysis done using the analysis of variance showed that there was no significant difeerence ($P > 0.05$) between ketoconazole, voriconazole and Amphotericin B. There was a significant difference ($P < 0.05$) between, ketoconazole, voriconazole Amphotericin B and fluconazole.

According to Lopez *et al*., (2006), the standard disk diffusion method of analysis was a good method for testing the susceptibility of various moulds to different oxoid antifungal agents.

This study had similarity with the work of Agbulu *et al*., (2015) where ketoconazole had the highest minimum inhibitory concentration among different antifungal agents. Another study by Kamal *et al*., (2018) had it that all isolates in the study were sensitive to Amphotericin-B and Ketoconazole and a high frequency of Fluconazole resistance was observed.

The work of Kulkarni *et al*., (2018) also observed that fluconazole showed the lowest antifungal efficacy when used with other antifungal agents like itraconazole, amphotericin B, terbinafine.

The study by Khan, *et al*., (2018) observed that Amphotericin was known as the main antifungal agent against invasive fungal infections which did not agree with this work. The study by Khan, *et al*., (2018) also observed that *Aspergillus* species were not susceptible to fluconazole but were susceptible to other azoles like ketoconazole, voriconazole and itraconazole which was similar to this research work. According to Samuel *et al*., (2014), moulds that may be resistant to

amphotericin B will usually be susceptible to voricinazole or posaconazole. This observation was also seen in this research work.

The results of the qualitative phytochemical screening of different solvent extracts of the four selected green leafy vegetables showed the presence of phytochemicals. It was reported that the health benefits of vegetables and other plants lie on their phytochemical compositions (Anderson, 2004).

The plant Percentage (%) growth inhibition of moulds by methanol and hot water extract in scent leaf gave the highest inhibition followed by bitter leaf, utazi and uziza gave the least growth inhibition. For scent leaf, *Aspergillus brunneoviolaceus* (19%) gave the highest growth inhibition at 100 mg/ml, followed by *Aspergillus tamarii* (15%), *Aspergillus flavus* (14%), *Aspergillus niger* (7%) then *Penicillium chrysogenum* (3%). For Bitter leaf extract, *Aspergillus tamarii* (15%) gave the highest growth inhibition at 100 mg/ml followed by *Aspergillus brunneoviolaceus* (12%), *Aspergillus flavus* (11%), *Aspergillus niger* (5%) then *Penicillium chrysogenum* (3%). For utazi leaf, *Aspergillus brunneoviolaceus* (10%) gave the highest growth inhibition at 100 mg/ml followed by *Aspergillus tamarii* (9%), *Aspergillus flavus* (7%), *Aspergillus niger* (3%) then *Penicillium chrysogenum* (1%). Uziza, leaf, *Aspergillus tamarii* (7%) gave the highest growth inhibition at 100 mg/ml followed by *Aspergillus brunneoviolaceus* (5%), *Aspergillus flavus* (4%), *Aspergillus niger* (3%) then *Penicillium chrysogenum* (1%), while the control gave no growth inhibition.

Minimum inhibitory concentration of moulds by methanol and hot water extract of Scent leaf was at 50 mg/ml for both extract. Minimum inhibitory concentration of moulds by methanol and hot water extract of Bitter leaf was 50 mg/ml for methanol and 50 mg/ml and 100 mg/ml for hot

water. Minimum inhibitory concentration of moulds by methanol and hot water extract of utazi leaf was at 50 mg/ml and 100 mg/ml for methanol extract and 100 mg/ml for hot water extract. Minimum inhibitory concentration of moulds by methanol and hot water extract of Uziza leaf was at 100 mg/ml for methanol and 100 mg/ml for some isolates for hot water extract.

The study of Nnam *et al*., (2013) observed that that scent leave (*Ocimum gratissimum*) and other local herbs contain both nutritive (fiber, potassium, phosphorus, ascorbate) and phytochemical (alkaloids, flavonoids, phenol, steroid) composition. These constitutes promotes good health and have antimicrobial activity. According to Nduche *et al*., (2018), uziza leaves (*Piper guineense*) and utazi leaves (*Gongronema latifolium*) and other medicinal plants have some level of antimicrobial activities because of the various phytochemicals they contain. This agreed with this research where there were some levels of susceptibility of moulds to the various medicinal plants. The work of Oladosu-Ajayi *et al*., (2017) observed that hot water extract of (*Vemonia amygdalina*) and (*Ocimum gratissimum*) showed the presence of different phytochemicals like alkaloid, tannin, flavonoid and steroid. They both contain similar antimicrobial properties though the former contains more quantity. These accounts for their medicinal use. According to the research of Onwuka, (2006), four local vegetable leaves which includes bitter leaf (*Vemonia amygdalina*) scent leave (*Ocimum gratissimum*), utazi leaves (*Gongronema latifolium*) and uziza leaves (*Piper guineense*) on fungal isolates especially *Aspergillus* species showed that the lowest microbial content was found on bitter leaves (*Vemonia amygdalina*), scent leaves (*Ocimum gratissimum*),, utazi leaves (*Gongronema latifolium*) and uziza leaves (*Piper guineense*). Uziza leaves (*Piper guineense*) has the least fungal efficacy which agreed with this research, but disagreed on bitter leaves (*Vemonia amygdalina*) having greater fungal efficacy

Aqueous extract of (*Ocimum gratissimum*) inhibited the growth of *Aspergillus*, *Fusarium* and *Mucor* as was observed by Ebimieowei, & Emiri, (2016). This has some similarity with this research in the sense that *Ocimum gratissimum* inhibited the growth of *Aspergilllus* species.

The study of Nwankwo, *et al*., (2014) suggested regular consumption of both uziza leaves (*Piper guineense*) and scent leaves (*Ocimum gratissimum*) as they both contain proximate and phytochemical components. The study observed that scent leaves (*Ocimum gratissimum*) had a higher components than uziza leaves (*Piper guineense*) which may account for its greater fungal activities as seen in this work.

CHAPTER FIVE.

5.0 CONCLUSION AND RECOMMENDATIONS.

5.1.1 CONCLUSION.

The grains and legumes studied were contaminated with various species of moulds. The moulds isolated and identified culturally were *Aspergillus* sp. *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Penicillium chrysogenum*, *Fusarium* sp, *Rhizopus stolonifer*, *Rhizopus nigricans* and *Mucor* sp. while those identified molecularly were *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum.* Evaluating the recovery rate of the various media, it was observed that potato dextrose agar had the highest rate of mould recovery and should be used in the culture of moulds.

The following mycotoxins were found ; Aflatoxin B_1, Aflatoxin B_2 , Aflatoxin G_1, Aflatoxin G_2, Ochratoxin A, Citrinin, Dihydrocitrinone, Fumonisin B_1, Fumonisin B_2, Fumonisin B_3, Fumonisin B_4, Zearalenone, Deoxynivalenol and Nivalenol. The presence of these mycotoxins in stored grains and legumes has serious implications for human health. Antifungal susceptibility test showed that ketoconazole gave the highest zone of inhibition and should be used in research purposes. Tetrazolium chloride did not have much inhibition effect even when combined with oxoid antifungal agents. Medicinal plants like scent leaves, bitter leaves, utazi leaves and uziza leaves gave varying levels of growth inhibition against varying isolates and should be used frequently at home.The grains and legumes studied were contaminated with various species of moulds and contained many mycotoxins of public health importance.

5.1.2 RECOMMENDATIONS.

1. There is urgent need to preserve and store these grains and legumes quickly and properly to avoid contamination with moulds.
2. Molecular characterization method should be used to identify moulds to the specie level.
3. There is urgent need to enlighten the public to avoid mycotoxicosis by ensuring that stored grains and legumes infested by moulds are not consumed.
4. Mycotoxins study should be embraced by more researchers to determine the mycotoxin level in some other stored grains and legumes.
5. Antifungal susceptibility test should be done using different types of antifungal agents to assay different moulds.
6. Local medicinal plants within our reach should be frequently studied.

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APPENDICES

1: CONCENTRATIONS OF MYCOTOXINS IN STORED GRAINS AND LEGUMES.

LOQ	0.722389402	0.211326816	0.54253691	1.69	1.52	2.45	3.76	7.9842379	7.08101209	19.23567874	7.0810120	0.633360953	9	2.565522952
LOD	0.2167168	0.063398045	0.16276107	0.51	0.46	0.73	1.13	2.395271379	2.124303627	5.770703622	2.124303627	0.19000828	3	0.769656885

	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Ochratoxin A	Citrinin	Dihydrocitrinone	Fumonisin B_1	Fumonisin B_2	Fumonisin B_3	Fumonisin B_4	Zearalenone	Deoxynivalenol	Nivalenol
Maize	58.2	60.8	84.7	67.8	87.8	27.1	147.3	85	85	85	85	85	80	72.9
Wheat	84.3	86.1	79.5	80.5	96.9	43.9	115.5	85	85	85	85	100.1	80	60
Rice	97.4	96.3	97.5	94.2	100.5	80	100.1	85	85	85	85	99.2	80	101.1
Groundnut	81.5.	69	65.3	62.2	86.6	98.8	97.1	85	85	85	85	74.2	80	88.2

STATISTICAL ANALYSIS.

2: One-way analysis of variance (anova) for occurrence of mycotoxins in grains and legume (maize, wheat, rice and groundnut).

Statistics descriptives /posthoc=lsd alpha (0.05).

Oneway

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
MAIZE	AFLATOXIN B1	2	58.2000	.00000	.00000	58.2000	58.2000	58.20	58.20
	AFLATOXIN B2	2	60.8500	.07071	.05000	60.2147	61.4853	60.80	60.90
	AFLATOXIN G1	2	84.7000	.28284	.20000	82.1588	87.2412	84.50	84.90
	AFLATOXIN G2	2	67.8000	.14142	.10000	66.5294	69.0706	67.70	67.90
	Ochratoxin A	2	87.8000	.00000	.00000	87.8000	87.8000	87.80	87.80
	Citrinin	2	27.1000	.14142	.10000	25.8294	28.3706	27.00	27.20
	Dihydrocitrinone	2	147.3000	.42426	.30000	143.4881	151.1119	147.00	147.60
	Fumonisin B1	2	85.0000	7.07107	5.00000	21.4690	148.5310	80.00	90.00
	Fumonisin B2	2	82.5000	10.60660	7.50000	-12.7965	177.7965	75.00	90.00
	Fumonisin B3	2	85.0000	.00000	.00000	85.0000	85.0000	85.00	85.00
	Fumonisin B4	2	85.0000	1.41421	1.00000	72.2938	97.7062	84.00	86.00

	Zearalenone	2	85.0000	.00000	.00000	85.0000	85.0000	85.00	85.00
	Deoxynivalenol	2	80.0000	14.14214	10.00000	-47.0620	207.0620	70.00	90.00
	Nivalenol	2	72.9000	.00000	.00000	72.9000	72.9000	72.90	72.90
	Total	28	79.2250	25.43268	4.80632	69.3632	89.0868	27.00	147.60
WHEAT	AFLATOXIN B1	2	84.3000	.14142	.10000	83.0294	85.5706	84.20	84.40
	AFLATOXIN B2	2	86.1000	.14142	.10000	84.8294	87.3706	86.00	86.20
	AFLATOXIN G1	2	79.5000	.70711	.50000	73.1469	85.8531	79.00	80.00
	AFLATOXIN G2	2	80.5000	.70711	.50000	74.1469	86.8531	80.00	81.00
	Ochratoxin A	2	96.9000	.14142	.10000	95.6294	98.1706	96.80	97.00
	Citrinin	2	43.9000	.00000	.00000	43.9000	43.9000	43.90	43.90
	Dihydrocitrinone	2	115.5000	.70711	.50000	109.1469	121.8531	115.00	116.00
	Fumonisin B1	2	85.0000	.00000	.00000	85.0000	85.0000	85.00	85.00
	Fumonisin B2	2	85.0000	.00000	.00000	85.0000	85.0000	85.00	85.00
	Fumonisin B3	2	85.0000	1.41421	1.00000	72.2938	97.7062	84.00	86.00
	Fumonisin B4	2	85.0000	7.07107	5.00000	21.4690	148.5310	80.00	90.00
	Zearalenone	2	100.1000	.14142	.10000	98.8294	101.3706	100.00	100.20
	Deoxynivalenol	2	80.0000	14.14214	10.00000	-47.0620	207.0620	70.00	90.00
	Nivalenol	2	60.0000	14.14214	10.00000	-67.0620	187.0620	50.00	70.00
	Total	28	83.3429	16.95227	3.20368	76.7695	89.9163	43.90	116.00
RICE	AFLATOXIN B1	2	97.4000	.56569	.40000	92.3175	102.4825	97.00	97.80
	AFLATOXIN B2	2	96.3000	.42426	.30000	92.4881	100.1119	96.00	96.60

	AFLATOXIN G1	2	97.5000	.70711	.50000	91.1469	103.8531	97.00	98.00
	AFLATOXIN G2	2	94.2000	.28284	.20000	91.6588	96.7412	94.00	94.40
	Ochratoxin A	2	100.5000	.70711	.50000	94.1469	106.8531	100.00	101.00
	Citrinin	2	80.0000	14.14214	10.00000	-47.0620	207.0620	70.00	90.00
	Dihydrocitrinone	2	100.1000	.14142	.10000	98.8294	101.3706	100.00	100.20
	Fumonisin B1	2	85.0000	7.07107	5.00000	21.4690	148.5310	80.00	90.00
	Fumonisin B2	2	85.0000	1.41421	1.00000	72.2938	97.7062	84.00	86.00
	Fumonisin B3	2	85.0000	5.65685	4.00000	34.1752	135.8248	81.00	89.00
	Fumonisin B4	2	85.0000	2.82843	2.00000	59.5876	110.4124	83.00	87.00
	Zearalenone	2	99.2000	.28284	.20000	96.6588	101.7412	99.00	99.40
	Deoxynivalenol	2	80.0000	7.07107	5.00000	16.4690	143.5310	75.00	85.00
	Nivalenol	2	101.1000	.14142	.10000	99.8294	102.3706	101.00	101.20
	Total	28	91.8786	8.65338	1.63534	88.5231	95.2340	70.00	101.20
GROUNDNUT	AFLATOXIN B1	2	81.5000	.70711	.50000	75.1469	87.8531	81.00	82.00
	AFLATOXIN B2	2	69.0000	1.41421	1.00000	56.2938	81.7062	68.00	70.00
	AFLATOXIN G1	2	65.3000	.42426	.30000	61.4881	69.1119	65.00	65.60
	AFLATOXIN G2	2	62.2000	.28284	.20000	59.6588	64.7412	62.00	62.40
	Ochratoxin A	2	86.6000	.14142	.10000	85.3294	87.8706	86.50	86.70
	Citrinin	2	98.8000	.14142	.10000	97.5294	100.0706	98.70	98.90
	Dihydrocitrinone	2	97.1000	.14142	.10000	95.8294	98.3706	97.00	97.20
	Fumonisin B1	2	85.0000	.00000	.00000	85.0000	85.0000	85.00	85.00

	Fumonisin B2	2	85.0000	7.07107	5.00000	21.4690	148.5310	80.00	90.00
	Fumonisin B3	2	85.0000	1.41421	1.00000	72.2938	97.7062	84.00	86.00
	Fumonisin B4	2	85.0000	2.82843	2.00000	59.5876	110.4124	83.00	87.00
	Zearalenone	2	74.2000	.28284	.20000	71.6588	76.7412	74.00	74.40
	Deoxynivalenol	2	80.0000	14.14214	10.00000	-47.0620	207.0620	70.00	90.00
	Nivalenol	2	88.2000	.28284	.20000	85.6588	90.7412	88.00	88.40
	Total	28	81.6357	11.02355	2.08326	77.3612	85.9102	62.00	98.90

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
MAIZE	Between Groups	17099.367	13	1315.336	50.478	.000
	Within Groups	364.805	14	26.058		
	Total	17464.172	27			
WHEAT	Between Groups	7305.669	13	561.975	17.346	.000
	Within Groups	453.580	14	32.399		
	Total	7759.249	27			
RICE	Between Groups	1678.087	13	129.084	5.258	.002
	Within Groups	343.700	14	24.550		
	Total	2021.787	27			
GROUNDNUT	Between Groups	3018.024	13	232.156	12.359	.000
	Within Groups	262.980	14	18.784		
	Total	3281.004	27			

Post Hoc Tests

Multiple Comparisons

LSD

Dependent Variable	(I) SAMPLE	(J) SAMPLE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
MAIZE	AFLATOXIN B1	AFLATOXIN B2	-2.65000	5.10465	.612	-13.5984	8.2984
		AFLATOXIN G1	-26.50000*	5.10465	.000	-37.4484	-15.5516
		AFLATOXIN G2	-9.60000	5.10465	.081	-20.5484	1.3484
		Ochratoxin A	-29.60000*	5.10465	.000	-40.5484	-18.6516
		Citrinin	31.10000*	5.10465	.000	20.1516	42.0484
		Dihydrocitrinone	-89.10000*	5.10465	.000	-100.0484	-78.1516
		Fumonisin B1	-26.80000*	5.10465	.000	-37.7484	-15.8516
		Fumonisin B2	-24.30000*	5.10465	.000	-35.2484	-13.3516
		Fumonisin B3	-26.80000*	5.10465	.000	-37.7484	-15.8516
		Fumonisin B4	-26.80000*	5.10465	.000	-37.7484	-15.8516
		Zearalenone	-26.80000*	5.10465	.000	-37.7484	-15.8516
		Deoxynivalenol	-21.80000*	5.10465	.001	-32.7484	-10.8516
		Nivalenol	-14.70000*	5.10465	.012	-25.6484	-3.7516

	AFLATOXIN B2	AFLATOXIN B1	2.65000	5.10465	.612	-8.2984	13.5984
		AFLATOXIN G1	-23.85000*	5.10465	.000	-34.7984	-12.9016
		AFLATOXIN G2	-6.95000	5.10465	.195	-17.8984	3.9984
		Ochratoxin A	-26.95000*	5.10465	.000	-37.8984	-16.0016
		Citrinin	33.75000*	5.10465	.000	22.8016	44.6984
		Dihydrocitrinone	-86.45000*	5.10465	.000	-97.3984	-75.5016
		Fumonisin B1	-24.15000*	5.10465	.000	-35.0984	-13.2016
		Fumonisin B2	-21.65000*	5.10465	.001	-32.5984	-10.7016
		Fumonisin B3	-24.15000*	5.10465	.000	-35.0984	-13.2016
		Fumonisin B4	-24.15000*	5.10465	.000	-35.0984	-13.2016
		Zearalenone	-24.15000*	5.10465	.000	-35.0984	-13.2016
		Deoxynivalenol	-19.15000*	5.10465	.002	-30.0984	-8.2016
		Nivalenol	-12.05000*	5.10465	.033	-22.9984	-1.1016
	AFLATOXIN G1	AFLATOXIN B1	26.50000*	5.10465	.000	15.5516	37.4484
		AFLATOXIN B2	23.85000*	5.10465	.000	12.9016	34.7984
		AFLATOXIN G2	16.90000*	5.10465	.005	5.9516	27.8484
		Ochratoxin A	-3.10000	5.10465	.553	-14.0484	7.8484
		Citrinin	57.60000*	5.10465	.000	46.6516	68.5484
		Dihydrocitrinone	-62.60000*	5.10465	.000	-73.5484	-51.6516
		Fumonisin B1	-.30000	5.10465	.954	-11.2484	10.6484

	Fumonisin B2	2.20000	5.10465	.673	-8.7484	13.1484
	Fumonisin B3	-.30000	5.10465	.954	-11.2484	10.6484
	Fumonisin B4	-.30000	5.10465	.954	-11.2484	10.6484
	Zearalenone	-.30000	5.10465	.954	-11.2484	10.6484
	Deoxynivalenol	4.70000	5.10465	.373	-6.2484	15.6484
	Nivalenol	11.80000*	5.10465	.037	.8516	22.7484
AFLATOXIN G2	AFLATOXIN B1	9.60000	5.10465	.081	-1.3484	20.5484
	AFLATOXIN B2	6.95000	5.10465	.195	-3.9984	17.8984
	AFLATOXIN G1	-16.90000*	5.10465	.005	-27.8484	-5.9516
	Ochratoxin A	-20.00000*	5.10465	.002	-30.9484	-9.0516
	Citrinin	40.70000*	5.10465	.000	29.7516	51.6484
	Dihydrocitrinone	-79.50000*	5.10465	.000	-90.4484	-68.5516
	Fumonisin B1	-17.20000*	5.10465	.005	-28.1484	-6.2516
	Fumonisin B2	-14.70000*	5.10465	.012	-25.6484	-3.7516
	Fumonisin B3	-17.20000*	5.10465	.005	-28.1484	-6.2516
	Fumonisin B4	-17.20000*	5.10465	.005	-28.1484	-6.2516
	Zearalenone	-17.20000*	5.10465	.005	-28.1484	-6.2516
	Deoxynivalenol	-12.20000*	5.10465	.031	-23.1484	-1.2516
	Nivalenol	-5.10000	5.10465	.335	-16.0484	5.8484
Ochratoxin A	AFLATOXIN B1	29.60000*	5.10465	.000	18.6516	40.5484
	AFLATOXIN B2	26.95000*	5.10465	.000	16.0016	37.8984

		AFLATOXIN G1	3.10000	5.10465	.553	-7.8484	14.0484
		AFLATOXIN G2	20.00000*	5.10465	.002	9.0516	30.9484
		Citrinin	60.70000*	5.10465	.000	49.7516	71.6484
		Dihydrocitrinone	-59.50000*	5.10465	.000	-70.4484	-48.5516
		Fumonisin B1	2.80000	5.10465	.592	-8.1484	13.7484
		Fumonisin B2	5.30000	5.10465	.317	-5.6484	16.2484
		Fumonisin B3	2.80000	5.10465	.592	-8.1484	13.7484
		Fumonisin B4	2.80000	5.10465	.592	-8.1484	13.7484
		Zearalenone	2.80000	5.10465	.592	-8.1484	13.7484
		Deoxynivalenol	7.80000	5.10465	.149	-3.1484	18.7484
		Nivalenol	14.90000*	5.10465	.011	3.9516	25.8484
	Citrinin	AFLATOXIN B1	-31.10000*	5.10465	.000	-42.0484	-20.1516
		AFLATOXIN B2	-33.75000*	5.10465	.000	-44.6984	-22.8016
		AFLATOXIN G1	-57.60000*	5.10465	.000	-68.5484	-46.6516
		AFLATOXIN G2	-40.70000*	5.10465	.000	-51.6484	-29.7516
		Ochratoxin A	-60.70000*	5.10465	.000	-71.6484	-49.7516
		Dihydrocitrinone	-120.20000*	5.10465	.000	-131.1484	-109.2516
		Fumonisin B1	-57.90000*	5.10465	.000	-68.8484	-46.9516
		Fumonisin B2	-55.40000*	5.10465	.000	-66.3484	-44.4516
		Fumonisin B3	-57.90000*	5.10465	.000	-68.8484	-46.9516

	Fumonisin B4	-57.90000*	5.10465	.000	-68.8484	-46.9516
	Zearalenone	-57.90000*	5.10465	.000	-68.8484	-46.9516
	Deoxynivalenol	-52.90000*	5.10465	.000	-63.8484	-41.9516
	Nivalenol	-45.80000*	5.10465	.000	-56.7484	-34.8516
Dihydrocitrinone	AFLATOXIN B1	89.10000*	5.10465	.000	78.1516	100.0484
	AFLATOXIN B2	86.45000*	5.10465	.000	75.5016	97.3984
	AFLATOXIN G1	62.60000*	5.10465	.000	51.6516	73.5484
	AFLATOXIN G2	79.50000*	5.10465	.000	68.5516	90.4484
	Ochratoxin A	59.50000*	5.10465	.000	48.5516	70.4484
	Citrinin	120.20000*	5.10465	.000	109.2516	131.1484
	Fumonisin B1	62.30000*	5.10465	.000	51.3516	73.2484
	Fumonisin B2	64.80000*	5.10465	.000	53.8516	75.7484
	Fumonisin B3	62.30000*	5.10465	.000	51.3516	73.2484
	Fumonisin B4	62.30000*	5.10465	.000	51.3516	73.2484
	Zearalenone	62.30000*	5.10465	.000	51.3516	73.2484
	Deoxynivalenol	67.30000*	5.10465	.000	56.3516	78.2484
	Nivalenol	74.40000*	5.10465	.000	63.4516	85.3484
Fumonisin B1	AFLATOXIN B1	26.80000*	5.10465	.000	15.8516	37.7484
	AFLATOXIN B2	24.15000*	5.10465	.000	13.2016	35.0984

		AFLATOXIN G1	.30000	5.10465	.954	-10.6484	11.2484
		AFLATOXIN G2	17.20000*	5.10465	.005	6.2516	28.1484
		Ochratoxin A	-2.80000	5.10465	.592	-13.7484	8.1484
		Citrinin	57.90000*	5.10465	.000	46.9516	68.8484
		Dihydrocitrinone	-62.30000*	5.10465	.000	-73.2484	-51.3516
		Fumonisin B2	2.50000	5.10465	.632	-8.4484	13.4484
		Fumonisin B3	.00000	5.10465	1.000	-10.9484	10.9484
		Fumonisin B4	.00000	5.10465	1.000	-10.9484	10.9484
		Zearalenone	.00000	5.10465	1.000	-10.9484	10.9484
		Deoxynivalenol	5.00000	5.10465	.344	-5.9484	15.9484
		Nivalenol	12.10000*	5.10465	.033	1.1516	23.0484
	Fumonisin B2	AFLATOXIN B1	24.30000*	5.10465	.000	13.3516	35.2484
		AFLATOXIN B2	21.65000*	5.10465	.001	10.7016	32.5984
		AFLATOXIN G1	-2.20000	5.10465	.673	-13.1484	8.7484
		AFLATOXIN G2	14.70000*	5.10465	.012	3.7516	25.6484
		Ochratoxin A	-5.30000	5.10465	.317	-16.2484	5.6484
		Citrinin	55.40000*	5.10465	.000	44.4516	66.3484
		Dihydrocitrinone	-64.80000*	5.10465	.000	-75.7484	-53.8516
		Fumonisin B1	-2.50000	5.10465	.632	-13.4484	8.4484
		Fumonisin B3	-2.50000	5.10465	.632	-13.4484	8.4484

	Fumonisin B4	-2.50000	5.10465	.632	-13.4484	8.4484
	Zearalenone	-2.50000	5.10465	.632	-13.4484	8.4484
	Deoxynivalenol	2.50000	5.10465	.632	-8.4484	13.4484
	Nivalenol	9.60000	5.10465	.081	-1.3484	20.5484
Fumonisin B3	AFLATOXIN B1	26.80000*	5.10465	.000	15.8516	37.7484
	AFLATOXIN B2	24.15000*	5.10465	.000	13.2016	35.0984
	AFLATOXIN G1	.30000	5.10465	.954	-10.6484	11.2484
	AFLATOXIN G2	17.20000*	5.10465	.005	6.2516	28.1484
	Ochratoxin A	-2.80000	5.10465	.592	-13.7484	8.1484
	Citrinin	57.90000*	5.10465	.000	46.9516	68.8484
	Dihydrocitrinone	-62.30000*	5.10465	.000	-73.2484	-51.3516
	Fumonisin B1	.00000	5.10465	1.000	-10.9484	10.9484
	Fumonisin B2	2.50000	5.10465	.632	-8.4484	13.4484
	Fumonisin B4	.00000	5.10465	1.000	-10.9484	10.9484
	Zearalenone	.00000	5.10465	1.000	-10.9484	10.9484
	Deoxynivalenol	5.00000	5.10465	.344	-5.9484	15.9484
	Nivalenol	12.10000*	5.10465	.033	1.1516	23.0484
Fumonisin B4	AFLATOXIN B1	26.80000*	5.10465	.000	15.8516	37.7484
	AFLATOXIN B2	24.15000*	5.10465	.000	13.2016	35.0984

		AFLATOXIN G1	.30000	5.10465	.954	-10.6484	11.2484
		AFLATOXIN G2	17.20000*	5.10465	.005	6.2516	28.1484
		Ochratoxin A	-2.80000	5.10465	.592	-13.7484	8.1484
		Citrinin	57.90000*	5.10465	.000	46.9516	68.8484
		Dihydrocitrinone	-62.30000*	5.10465	.000	-73.2484	-51.3516
		Fumonisin B1	.00000	5.10465	1.000	-10.9484	10.9484
		Fumonisin B2	2.50000	5.10465	.632	-8.4484	13.4484
		Fumonisin B3	.00000	5.10465	1.000	-10.9484	10.9484
		Zearalenone	.00000	5.10465	1.000	-10.9484	10.9484
		Deoxynivalenol	5.00000	5.10465	.344	-5.9484	15.9484
		Nivalenol	12.10000*	5.10465	.033	1.1516	23.0484
	Zearalenone	AFLATOXIN B1	26.80000*	5.10465	.000	15.8516	37.7484
		AFLATOXIN B2	24.15000*	5.10465	.000	13.2016	35.0984
		AFLATOXIN G1	.30000	5.10465	.954	-10.6484	11.2484
		AFLATOXIN G2	17.20000*	5.10465	.005	6.2516	28.1484
		Ochratoxin A	-2.80000	5.10465	.592	-13.7484	8.1484
		Citrinin	57.90000*	5.10465	.000	46.9516	68.8484
		Dihydrocitrinone	-62.30000*	5.10465	.000	-73.2484	-51.3516
		Fumonisin B1	.00000	5.10465	1.000	-10.9484	10.9484
		Fumonisin B2	2.50000	5.10465	.632	-8.4484	13.4484

	Fumonisin B3	.00000	5.10465	1.000	-10.9484	10.9484
	Fumonisin B4	.00000	5.10465	1.000	-10.9484	10.9484
	Deoxynivalenol	5.00000	5.10465	.344	-5.9484	15.9484
	Nivalenol	12.10000*	5.10465	.033	1.1516	23.0484
Deoxynivalenol	AFLATOXIN B1	21.80000*	5.10465	.001	10.8516	32.7484
	AFLATOXIN B2	19.15000*	5.10465	.002	8.2016	30.0984
	AFLATOXIN G1	-4.70000	5.10465	.373	-15.6484	6.2484
	AFLATOXIN G2	12.20000*	5.10465	.031	1.2516	23.1484
	Ochratoxin A	-7.80000	5.10465	.149	-18.7484	3.1484
	Citrinin	52.90000*	5.10465	.000	41.9516	63.8484
	Dihydrocitrinone	-67.30000*	5.10465	.000	-78.2484	-56.3516
	Fumonisin B1	-5.00000	5.10465	.344	-15.9484	5.9484
	Fumonisin B2	-2.50000	5.10465	.632	-13.4484	8.4484
	Fumonisin B3	-5.00000	5.10465	.344	-15.9484	5.9484
	Fumonisin B4	-5.00000	5.10465	.344	-15.9484	5.9484
	Zearalenone	-5.00000	5.10465	.344	-15.9484	5.9484
	Nivalenol	7.10000	5.10465	.186	-3.8484	18.0484
Nivalenol	AFLATOXIN B1	14.70000*	5.10465	.012	3.7516	25.6484
	AFLATOXIN B2	12.05000*	5.10465	.033	1.1016	22.9984

		AFLATOXIN G1	-11.80000*	5.10465	.037	-22.7484	-.8516
		AFLATOXIN G2	5.10000	5.10465	.335	-5.8484	16.0484
		Ochratoxin A	-14.90000*	5.10465	.011	-25.8484	-3.9516
		Citrinin	45.80000*	5.10465	.000	34.8516	56.7484
		Dihydrocitrinone	-74.40000*	5.10465	.000	-85.3484	-63.4516
		Fumonisin B1	-12.10000*	5.10465	.033	-23.0484	-1.1516
		Fumonisin B2	-9.60000	5.10465	.081	-20.5484	1.3484
		Fumonisin B3	-12.10000*	5.10465	.033	-23.0484	-1.1516
		Fumonisin B4	-12.10000*	5.10465	.033	-23.0484	-1.1516
		Zearalenone	-12.10000*	5.10465	.033	-23.0484	-1.1516
		Deoxynivalenol	-7.10000	5.10465	.186	-18.0484	3.8484
WHEAT	AFLATOXIN B1	AFLATOXIN B2	-1.80000	5.69197	.756	-14.0081	10.4081
		AFLATOXIN G1	4.80000	5.69197	.413	-7.4081	17.0081
		AFLATOXIN G2	3.80000	5.69197	.515	-8.4081	16.0081
		Ochratoxin A	-12.60000*	5.69197	.044	-24.8081	-.3919
		Citrinin	40.40000*	5.69197	.000	28.1919	52.6081
		Dihydrocitrinone	-31.20000*	5.69197	.000	-43.4081	-18.9919
		Fumonisin B1	-.70000	5.69197	.904	-12.9081	11.5081
		Fumonisin B2	-.70000	5.69197	.904	-12.9081	11.5081
		Fumonisin B3	-.70000	5.69197	.904	-12.9081	11.5081
		Fumonisin B4	-.70000	5.69197	.904	-12.9081	11.5081

	Zearalenone	-15.80000*	5.69197	.015	-28.0081	-3.5919
	Deoxynivalenol	4.30000	5.69197	.463	-7.9081	16.5081
	Nivalenol	24.30000*	5.69197	.001	12.0919	36.5081
AFLATOXIN B2	AFLATOXIN B1	1.80000	5.69197	.756	-10.4081	14.0081
	AFLATOXIN G1	6.60000	5.69197	.266	-5.6081	18.8081
	AFLATOXIN G2	5.60000	5.69197	.342	-6.6081	17.8081
	Ochratoxin A	-10.80000	5.69197	.079	-23.0081	1.4081
	Citrinin	42.20000*	5.69197	.000	29.9919	54.4081
	Dihydrocitrinone	-29.40000*	5.69197	.000	-41.6081	-17.1919
	Fumonisin B1	1.10000	5.69197	.850	-11.1081	13.3081
	Fumonisin B2	1.10000	5.69197	.850	-11.1081	13.3081
	Fumonisin B3	1.10000	5.69197	.850	-11.1081	13.3081
	Fumonisin B4	1.10000	5.69197	.850	-11.1081	13.3081
	Zearalenone	-14.00000*	5.69197	.028	-26.2081	-1.7919
	Deoxynivalenol	6.10000	5.69197	.302	-6.1081	18.3081
	Nivalenol	26.10000*	5.69197	.000	13.8919	38.3081
AFLATOXIN G1	AFLATOXIN B1	-4.80000	5.69197	.413	-17.0081	7.4081
	AFLATOXIN B2	-6.60000	5.69197	.266	-18.8081	5.6081
	AFLATOXIN G2	-1.00000	5.69197	.863	-13.2081	11.2081
	Ochratoxin A	-17.40000*	5.69197	.009	-29.6081	-5.1919
	Citrinin	35.60000*	5.69197	.000	23.3919	47.8081

		Dihydrocitrinone	-36.00000*	5.69197	.000	-48.2081	-23.7919
		Fumonisin B1	-5.50000	5.69197	.350	-17.7081	6.7081
		Fumonisin B2	-5.50000	5.69197	.350	-17.7081	6.7081
		Fumonisin B3	-5.50000	5.69197	.350	-17.7081	6.7081
		Fumonisin B4	-5.50000	5.69197	.350	-17.7081	6.7081
		Zearalenone	-20.60000*	5.69197	.003	-32.8081	-8.3919
		Deoxynivalenol	-.50000	5.69197	.931	-12.7081	11.7081
		Nivalenol	19.50000*	5.69197	.004	7.2919	31.7081
	AFLATOXIN G2	AFLATOXIN B1	-3.80000	5.69197	.515	-16.0081	8.4081
		AFLATOXIN B2	-5.60000	5.69197	.342	-17.8081	6.6081
		AFLATOXIN G1	1.00000	5.69197	.863	-11.2081	13.2081
		Ochratoxin A	-16.40000*	5.69197	.012	-28.6081	-4.1919
		Citrinin	36.60000*	5.69197	.000	24.3919	48.8081
		Dihydrocitrinone	-35.00000*	5.69197	.000	-47.2081	-22.7919
		Fumonisin B1	-4.50000	5.69197	.442	-16.7081	7.7081
		Fumonisin B2	-4.50000	5.69197	.442	-16.7081	7.7081
		Fumonisin B3	-4.50000	5.69197	.442	-16.7081	7.7081
		Fumonisin B4	-4.50000	5.69197	.442	-16.7081	7.7081
		Zearalenone	-19.60000*	5.69197	.004	-31.8081	-7.3919
		Deoxynivalenol	.50000	5.69197	.931	-11.7081	12.7081
		Nivalenol	20.50000*	5.69197	.003	8.2919	32.7081
	Ochratoxin A	AFLATOXIN B1	12.60000*	5.69197	.044	.3919	24.8081

	AFLATOXIN B2	10.80000	5.69197	.079	-1.4081	23.0081
	AFLATOXIN G1	17.40000*	5.69197	.009	5.1919	29.6081
	AFLATOXIN G2	16.40000*	5.69197	.012	4.1919	28.6081
	Citrinin	53.00000*	5.69197	.000	40.7919	65.2081
	Dihydrocitrinone	-18.60000*	5.69197	.006	-30.8081	-6.3919
	Fumonisin B1	11.90000	5.69197	.055	-.3081	24.1081
	Fumonisin B2	11.90000	5.69197	.055	-.3081	24.1081
	Fumonisin B3	11.90000	5.69197	.055	-.3081	24.1081
	Fumonisin B4	11.90000	5.69197	.055	-.3081	24.1081
	Zearalenone	-3.20000	5.69197	.583	-15.4081	9.0081
	Deoxynivalenol	16.90000*	5.69197	.010	4.6919	29.1081
	Nivalenol	36.90000*	5.69197	.000	24.6919	49.1081
Citrinin	AFLATOXIN B1	-40.40000*	5.69197	.000	-52.6081	-28.1919
	AFLATOXIN B2	-42.20000*	5.69197	.000	-54.4081	-29.9919
	AFLATOXIN G1	-35.60000*	5.69197	.000	-47.8081	-23.3919
	AFLATOXIN G2	-36.60000*	5.69197	.000	-48.8081	-24.3919
	Ochratoxin A	-53.00000*	5.69197	.000	-65.2081	-40.7919
	Dihydrocitrinone	-71.60000*	5.69197	.000	-83.8081	-59.3919
	Fumonisin B1	-41.10000*	5.69197	.000	-53.3081	-28.8919
	Fumonisin B2	-41.10000*	5.69197	.000	-53.3081	-28.8919

	Fumonisin B3	-41.10000*	5.69197	.000	-53.3081	-28.8919
	Fumonisin B4	-41.10000*	5.69197	.000	-53.3081	-28.8919
	Zearalenone	-56.20000*	5.69197	.000	-68.4081	-43.9919
	Deoxynivalenol	-36.10000*	5.69197	.000	-48.3081	-23.8919
	Nivalenol	-16.10000*	5.69197	.013	-28.3081	-3.8919
Dihydrocitrinone	AFLATOXIN B1	31.20000*	5.69197	.000	18.9919	43.4081
	AFLATOXIN B2	29.40000*	5.69197	.000	17.1919	41.6081
	AFLATOXIN G1	36.00000*	5.69197	.000	23.7919	48.2081
	AFLATOXIN G2	35.00000*	5.69197	.000	22.7919	47.2081
	Ochratoxin A	18.60000*	5.69197	.006	6.3919	30.8081
	Citrinin	71.60000*	5.69197	.000	59.3919	83.8081
	Fumonisin B1	30.50000*	5.69197	.000	18.2919	42.7081
	Fumonisin B2	30.50000*	5.69197	.000	18.2919	42.7081
	Fumonisin B3	30.50000*	5.69197	.000	18.2919	42.7081
	Fumonisin B4	30.50000*	5.69197	.000	18.2919	42.7081
	Zearalenone	15.40000*	5.69197	.017	3.1919	27.6081
	Deoxynivalenol	35.50000*	5.69197	.000	23.2919	47.7081
	Nivalenol	55.50000*	5.69197	.000	43.2919	67.7081
Fumonisin B1	AFLATOXIN B1	.70000	5.69197	.904	-11.5081	12.9081
	AFLATOXIN B2	-1.10000	5.69197	.850	-13.3081	11.1081

	AFLATOXIN G1	5.50000	5.69197	.350	-6.7081	17.7081
	AFLATOXIN G2	4.50000	5.69197	.442	-7.7081	16.7081
	Ochratoxin A	-11.90000	5.69197	.055	-24.1081	.3081
	Citrinin	41.10000*	5.69197	.000	28.8919	53.3081
	Dihydrocitrinone	-30.50000*	5.69197	.000	-42.7081	-18.2919
	Fumonisin B2	.00000	5.69197	1.000	-12.2081	12.2081
	Fumonisin B3	.00000	5.69197	1.000	-12.2081	12.2081
	Fumonisin B4	.00000	5.69197	1.000	-12.2081	12.2081
	Zearalenone	-15.10000*	5.69197	.019	-27.3081	-2.8919
	Deoxynivalenol	5.00000	5.69197	.395	-7.2081	17.2081
	Nivalenol	25.00000*	5.69197	.001	12.7919	37.2081
Fumonisin B2	AFLATOXIN B1	.70000	5.69197	.904	-11.5081	12.9081
	AFLATOXIN B2	-1.10000	5.69197	.850	-13.3081	11.1081
	AFLATOXIN G1	5.50000	5.69197	.350	-6.7081	17.7081
	AFLATOXIN G2	4.50000	5.69197	.442	-7.7081	16.7081
	Ochratoxin A	-11.90000	5.69197	.055	-24.1081	.3081
	Citrinin	41.10000*	5.69197	.000	28.8919	53.3081
	Dihydrocitrinone	-30.50000*	5.69197	.000	-42.7081	-18.2919
	Fumonisin B1	.00000	5.69197	1.000	-12.2081	12.2081
	Fumonisin B3	.00000	5.69197	1.000	-12.2081	12.2081
	Fumonisin B4	.00000	5.69197	1.000	-12.2081	12.2081

	Zearalenone	-15.10000*	5.69197	.019	-27.3081	-2.8919
	Deoxynivalenol	5.00000	5.69197	.395	-7.2081	17.2081
	Nivalenol	25.00000*	5.69197	.001	12.7919	37.2081
Fumonisin B3	AFLATOXIN B1	.70000	5.69197	.904	-11.5081	12.9081
	AFLATOXIN B2	-1.10000	5.69197	.850	-13.3081	11.1081
	AFLATOXIN G1	5.50000	5.69197	.350	-6.7081	17.7081
	AFLATOXIN G2	4.50000	5.69197	.442	-7.7081	16.7081
	Ochratoxin A	-11.90000	5.69197	.055	-24.1081	.3081
	Citrinin	41.10000*	5.69197	.000	28.8919	53.3081
	Dihydrocitrinone	-30.50000*	5.69197	.000	-42.7081	-18.2919
	Fumonisin B1	.00000	5.69197	1.000	-12.2081	12.2081
	Fumonisin B2	.00000	5.69197	1.000	-12.2081	12.2081
	Fumonisin B4	.00000	5.69197	1.000	-12.2081	12.2081
	Zearalenone	-15.10000*	5.69197	.019	-27.3081	-2.8919
	Deoxynivalenol	5.00000	5.69197	.395	-7.2081	17.2081
	Nivalenol	25.00000*	5.69197	.001	12.7919	37.2081
Fumonisin B4	AFLATOXIN B1	.70000	5.69197	.904	-11.5081	12.9081
	AFLATOXIN B2	-1.10000	5.69197	.850	-13.3081	11.1081
	AFLATOXIN G1	5.50000	5.69197	.350	-6.7081	17.7081
	AFLATOXIN G2	4.50000	5.69197	.442	-7.7081	16.7081

		Ochratoxin A	-11.90000	5.69197	.055	-24.1081	.3081
		Citrinin	41.10000*	5.69197	.000	28.8919	53.3081
		Dihydrocitrinone	-30.50000*	5.69197	.000	-42.7081	-18.2919
		Fumonisin B1	.00000	5.69197	1.000	-12.2081	12.2081
		Fumonisin B2	.00000	5.69197	1.000	-12.2081	12.2081
		Fumonisin B3	.00000	5.69197	1.000	-12.2081	12.2081
		Zearalenone	-15.10000*	5.69197	.019	-27.3081	-2.8919
		Deoxynivalenol	5.00000	5.69197	.395	-7.2081	17.2081
		Nivalenol	25.00000*	5.69197	.001	12.7919	37.2081
	Zearalenone	AFLATOXIN B1	15.80000*	5.69197	.015	3.5919	28.0081
		AFLATOXIN B2	14.00000*	5.69197	.028	1.7919	26.2081
		AFLATOXIN G1	20.60000*	5.69197	.003	8.3919	32.8081
		AFLATOXIN G2	19.60000*	5.69197	.004	7.3919	31.8081
		Ochratoxin A	3.20000	5.69197	.583	-9.0081	15.4081
		Citrinin	56.20000*	5.69197	.000	43.9919	68.4081
		Dihydrocitrinone	-15.40000*	5.69197	.017	-27.6081	-3.1919
		Fumonisin B1	15.10000*	5.69197	.019	2.8919	27.3081
		Fumonisin B2	15.10000*	5.69197	.019	2.8919	27.3081
		Fumonisin B3	15.10000*	5.69197	.019	2.8919	27.3081
		Fumonisin B4	15.10000*	5.69197	.019	2.8919	27.3081
		Deoxynivalenol	20.10000*	5.69197	.003	7.8919	32.3081
		Nivalenol	40.10000*	5.69197	.000	27.8919	52.3081

	Deoxynivalenol	AFLATOXIN B1	-4.30000	5.69197	.463	-16.5081	7.9081
		AFLATOXIN B2	-6.10000	5.69197	.302	-18.3081	6.1081
		AFLATOXIN G1	.50000	5.69197	.931	-11.7081	12.7081
		AFLATOXIN G2	-.50000	5.69197	.931	-12.7081	11.7081
		Ochratoxin A	-16.90000*	5.69197	.010	-29.1081	-4.6919
		Citrinin	36.10000*	5.69197	.000	23.8919	48.3081
		Dihydrocitrinone	-35.50000*	5.69197	.000	-47.7081	-23.2919
		Fumonisin B1	-5.00000	5.69197	.395	-17.2081	7.2081
		Fumonisin B2	-5.00000	5.69197	.395	-17.2081	7.2081
		Fumonisin B3	-5.00000	5.69197	.395	-17.2081	7.2081
		Fumonisin B4	-5.00000	5.69197	.395	-17.2081	7.2081
		Zearalenone	-20.10000*	5.69197	.003	-32.3081	-7.8919
		Nivalenol	20.00000*	5.69197	.003	7.7919	32.2081
	Nivalenol	AFLATOXIN B1	-24.30000*	5.69197	.001	-36.5081	-12.0919
		AFLATOXIN B2	-26.10000*	5.69197	.000	-38.3081	-13.8919
		AFLATOXIN G1	-19.50000*	5.69197	.004	-31.7081	-7.2919
		AFLATOXIN G2	-20.50000*	5.69197	.003	-32.7081	-8.2919
		Ochratoxin A	-36.90000*	5.69197	.000	-49.1081	-24.6919
		Citrinin	16.10000*	5.69197	.013	3.8919	28.3081
		Dihydrocitrinone	-55.50000*	5.69197	.000	-67.7081	-43.2919

		Fumonisin B1	-25.00000*	5.69197	.001	-37.2081	-12.7919
		Fumonisin B2	-25.00000*	5.69197	.001	-37.2081	-12.7919
		Fumonisin B3	-25.00000*	5.69197	.001	-37.2081	-12.7919
		Fumonisin B4	-25.00000*	5.69197	.001	-37.2081	-12.7919
		Zearalenone	-40.10000*	5.69197	.000	-52.3081	-27.8919
		Deoxynivalenol	-20.00000*	5.69197	.003	-32.2081	-7.7919
RICE	AFLATOXIN B1	AFLATOXIN B2	1.10000	4.95480	.828	-9.5270	11.7270
		AFLATOXIN G1	-.10000	4.95480	.984	-10.7270	10.5270
		AFLATOXIN G2	3.20000	4.95480	.529	-7.4270	13.8270
		Ochratoxin A	-3.10000	4.95480	.542	-13.7270	7.5270
		Citrinin	17.40000*	4.95480	.003	6.7730	28.0270
		Dihydrocitrinone	-2.70000	4.95480	.594	-13.3270	7.9270
		Fumonisin B1	12.40000*	4.95480	.025	1.7730	23.0270
		Fumonisin B2	12.40000*	4.95480	.025	1.7730	23.0270
		Fumonisin B3	12.40000*	4.95480	.025	1.7730	23.0270
		Fumonisin B4	12.40000*	4.95480	.025	1.7730	23.0270
		Zearalenone	-1.80000	4.95480	.722	-12.4270	8.8270
		Deoxynivalenol	17.40000*	4.95480	.003	6.7730	28.0270
		Nivalenol	-3.70000	4.95480	.468	-14.3270	6.9270
	AFLATOXIN B2	AFLATOXIN B1	-1.10000	4.95480	.828	-11.7270	9.5270
		AFLATOXIN G1	-1.20000	4.95480	.812	-11.8270	9.4270

		AFLATOXIN G2	2.10000	4.95480	.678	-8.5270	12.7270
		Ochratoxin A	-4.20000	4.95480	.411	-14.8270	6.4270
		Citrinin	16.30000*	4.95480	.005	5.6730	26.9270
		Dihydrocitrinone	-3.80000	4.95480	.456	-14.4270	6.8270
		Fumonisin B1	11.30000*	4.95480	.039	.6730	21.9270
		Fumonisin B2	11.30000*	4.95480	.039	.6730	21.9270
		Fumonisin B3	11.30000*	4.95480	.039	.6730	21.9270
		Fumonisin B4	11.30000*	4.95480	.039	.6730	21.9270
		Zearalenone	-2.90000	4.95480	.568	-13.5270	7.7270
		Deoxynivalenol	16.30000*	4.95480	.005	5.6730	26.9270
		Nivalenol	-4.80000	4.95480	.349	-15.4270	5.8270
	AFLATOXIN G1	AFLATOXIN B1	.10000	4.95480	.984	-10.5270	10.7270
		AFLATOXIN B2	1.20000	4.95480	.812	-9.4270	11.8270
		AFLATOXIN G2	3.30000	4.95480	.516	-7.3270	13.9270
		Ochratoxin A	-3.00000	4.95480	.555	-13.6270	7.6270
		Citrinin	17.50000*	4.95480	.003	6.8730	28.1270
		Dihydrocitrinone	-2.60000	4.95480	.608	-13.2270	8.0270
		Fumonisin B1	12.50000*	4.95480	.024	1.8730	23.1270
		Fumonisin B2	12.50000*	4.95480	.024	1.8730	23.1270
		Fumonisin B3	12.50000*	4.95480	.024	1.8730	23.1270
		Fumonisin B4	12.50000*	4.95480	.024	1.8730	23.1270
		Zearalenone	-1.70000	4.95480	.737	-12.3270	8.9270
		Deoxynivalenol	17.50000*	4.95480	.003	6.8730	28.1270

	Nivalenol	-3.60000	4.95480	.479	-14.2270	7.0270
AFLATOXIN G2	AFLATOXIN B1	-3.20000	4.95480	.529	-13.8270	7.4270
	AFLATOXIN B2	-2.10000	4.95480	.678	-12.7270	8.5270
	AFLATOXIN G1	-3.30000	4.95480	.516	-13.9270	7.3270
	Ochratoxin A	-6.30000	4.95480	.224	-16.9270	4.3270
	Citrinin	14.20000*	4.95480	.012	3.5730	24.8270
	Dihydrocitrinone	-5.90000	4.95480	.254	-16.5270	4.7270
	Fumonisin B1	9.20000	4.95480	.085	-1.4270	19.8270
	Fumonisin B2	9.20000	4.95480	.085	-1.4270	19.8270
	Fumonisin B3	9.20000	4.95480	.085	-1.4270	19.8270
	Fumonisin B4	9.20000	4.95480	.085	-1.4270	19.8270
	Zearalenone	-5.00000	4.95480	.330	-15.6270	5.6270
	Deoxynivalenol	14.20000*	4.95480	.012	3.5730	24.8270
	Nivalenol	-6.90000	4.95480	.185	-17.5270	3.7270
Ochratoxin A	AFLATOXIN B1	3.10000	4.95480	.542	-7.5270	13.7270
	AFLATOXIN B2	4.20000	4.95480	.411	-6.4270	14.8270
	AFLATOXIN G1	3.00000	4.95480	.555	-7.6270	13.6270
	AFLATOXIN G2	6.30000	4.95480	.224	-4.3270	16.9270
	Citrinin	20.50000*	4.95480	.001	9.8730	31.1270
	Dihydrocitrinone	.40000	4.95480	.937	-10.2270	11.0270

		Fumonisin B1	15.50000*	4.95480	.007	4.8730	26.1270
		Fumonisin B2	15.50000*	4.95480	.007	4.8730	26.1270
		Fumonisin B3	15.50000*	4.95480	.007	4.8730	26.1270
		Fumonisin B4	15.50000*	4.95480	.007	4.8730	26.1270
		Zearalenone	1.30000	4.95480	.797	-9.3270	11.9270
		Deoxynivalenol	20.50000*	4.95480	.001	9.8730	31.1270
		Nivalenol	-.60000	4.95480	.905	-11.2270	10.0270
	Citrinin	AFLATOXIN B1	-17.40000*	4.95480	.003	-28.0270	-6.7730
		AFLATOXIN B2	-16.30000*	4.95480	.005	-26.9270	-5.6730
		AFLATOXIN G1	-17.50000*	4.95480	.003	-28.1270	-6.8730
		AFLATOXIN G2	-14.20000*	4.95480	.012	-24.8270	-3.5730
		Ochratoxin A	-20.50000*	4.95480	.001	-31.1270	-9.8730
		Dihydrocitrinone	-20.10000*	4.95480	.001	-30.7270	-9.4730
		Fumonisin B1	-5.00000	4.95480	.330	-15.6270	5.6270
		Fumonisin B2	-5.00000	4.95480	.330	-15.6270	5.6270
		Fumonisin B3	-5.00000	4.95480	.330	-15.6270	5.6270
		Fumonisin B4	-5.00000	4.95480	.330	-15.6270	5.6270
		Zearalenone	-19.20000*	4.95480	.002	-29.8270	-8.5730
		Deoxynivalenol	.00000	4.95480	1.000	-10.6270	10.6270
		Nivalenol	-21.10000*	4.95480	.001	-31.7270	-10.4730
	Dihydrocitrinone	AFLATOXIN B1	2.70000	4.95480	.594	-7.9270	13.3270

	AFLATOXIN B2	3.80000	4.95480	.456	-6.8270	14.4270
	AFLATOXIN G1	2.60000	4.95480	.608	-8.0270	13.2270
	AFLATOXIN G2	5.90000	4.95480	.254	-4.7270	16.5270
	Ochratoxin A	-.40000	4.95480	.937	-11.0270	10.2270
	Citrinin	20.10000*	4.95480	.001	9.4730	30.7270
	Fumonisin B1	15.10000*	4.95480	.009	4.4730	25.7270
	Fumonisin B2	15.10000*	4.95480	.009	4.4730	25.7270
	Fumonisin B3	15.10000*	4.95480	.009	4.4730	25.7270
	Fumonisin B4	15.10000*	4.95480	.009	4.4730	25.7270
	Zearalenone	.90000	4.95480	.858	-9.7270	11.5270
	Deoxynivalenol	20.10000*	4.95480	.001	9.4730	30.7270
	Nivalenol	-1.00000	4.95480	.843	-11.6270	9.6270
Fumonisin B1	AFLATOXIN B1	-12.40000*	4.95480	.025	-23.0270	-1.7730
	AFLATOXIN B2	-11.30000*	4.95480	.039	-21.9270	-.6730
	AFLATOXIN G1	-12.50000*	4.95480	.024	-23.1270	-1.8730
	AFLATOXIN G2	-9.20000	4.95480	.085	-19.8270	1.4270
	Ochratoxin A	-15.50000*	4.95480	.007	-26.1270	-4.8730
	Citrinin	5.00000	4.95480	.330	-5.6270	15.6270
	Dihydrocitrinone	-15.10000*	4.95480	.009	-25.7270	-4.4730
	Fumonisin B2	.00000	4.95480	1.000	-10.6270	10.6270

	Fumonisin B3	.00000	4.95480	1.000	-10.6270	10.6270
	Fumonisin B4	.00000	4.95480	1.000	-10.6270	10.6270
	Zearalenone	-14.20000*	4.95480	.012	-24.8270	-3.5730
	Deoxynivalenol	5.00000	4.95480	.330	-5.6270	15.6270
	Nivalenol	-16.10000*	4.95480	.006	-26.7270	-5.4730
Fumonisin B2	AFLATOXIN B1	-12.40000*	4.95480	.025	-23.0270	-1.7730
	AFLATOXIN B2	-11.30000*	4.95480	.039	-21.9270	-.6730
	AFLATOXIN G1	-12.50000*	4.95480	.024	-23.1270	-1.8730
	AFLATOXIN G2	-9.20000	4.95480	.085	-19.8270	1.4270
	Ochratoxin A	-15.50000*	4.95480	.007	-26.1270	-4.8730
	Citrinin	5.00000	4.95480	.330	-5.6270	15.6270
	Dihydrocitrinone	-15.10000*	4.95480	.009	-25.7270	-4.4730
	Fumonisin B1	.00000	4.95480	1.000	-10.6270	10.6270
	Fumonisin B3	.00000	4.95480	1.000	-10.6270	10.6270
	Fumonisin B4	.00000	4.95480	1.000	-10.6270	10.6270
	Zearalenone	-14.20000*	4.95480	.012	-24.8270	-3.5730
	Deoxynivalenol	5.00000	4.95480	.330	-5.6270	15.6270
	Nivalenol	-16.10000*	4.95480	.006	-26.7270	-5.4730
Fumonisin B3	AFLATOXIN B1	-12.40000*	4.95480	.025	-23.0270	-1.7730
	AFLATOXIN B2	-11.30000*	4.95480	.039	-21.9270	-.6730

		AFLATOXIN G1	-12.50000*	4.95480	.024	-23.1270	-1.8730
		AFLATOXIN G2	-9.20000	4.95480	.085	-19.8270	1.4270
		Ochratoxin A	-15.50000*	4.95480	.007	-26.1270	-4.8730
		Citrinin	5.00000	4.95480	.330	-5.6270	15.6270
		Dihydrocitrinone	-15.10000*	4.95480	.009	-25.7270	-4.4730
		Fumonisin B1	.00000	4.95480	1.000	-10.6270	10.6270
		Fumonisin B2	.00000	4.95480	1.000	-10.6270	10.6270
		Fumonisin B4	.00000	4.95480	1.000	-10.6270	10.6270
		Zearalenone	-14.20000*	4.95480	.012	-24.8270	-3.5730
		Deoxynivalenol	5.00000	4.95480	.330	-5.6270	15.6270
		Nivalenol	-16.10000*	4.95480	.006	-26.7270	-5.4730
	Fumonisin B4	AFLATOXIN B1	-12.40000*	4.95480	.025	-23.0270	-1.7730
		AFLATOXIN B2	-11.30000*	4.95480	.039	-21.9270	-.6730
		AFLATOXIN G1	-12.50000*	4.95480	.024	-23.1270	-1.8730
		AFLATOXIN G2	-9.20000	4.95480	.085	-19.8270	1.4270
		Ochratoxin A	-15.50000*	4.95480	.007	-26.1270	-4.8730
		Citrinin	5.00000	4.95480	.330	-5.6270	15.6270
		Dihydrocitrinone	-15.10000*	4.95480	.009	-25.7270	-4.4730
		Fumonisin B1	.00000	4.95480	1.000	-10.6270	10.6270
		Fumonisin B2	.00000	4.95480	1.000	-10.6270	10.6270
		Fumonisin B3	.00000	4.95480	1.000	-10.6270	10.6270

	Zearalenone	-14.20000*	4.95480	.012	-24.8270	-3.5730
	Deoxynivalenol	5.00000	4.95480	.330	-5.6270	15.6270
	Nivalenol	-16.10000*	4.95480	.006	-26.7270	-5.4730
Zearalenone	AFLATOXIN B1	1.80000	4.95480	.722	-8.8270	12.4270
	AFLATOXIN B2	2.90000	4.95480	.568	-7.7270	13.5270
	AFLATOXIN G1	1.70000	4.95480	.737	-8.9270	12.3270
	AFLATOXIN G2	5.00000	4.95480	.330	-5.6270	15.6270
	Ochratoxin A	-1.30000	4.95480	.797	-11.9270	9.3270
	Citrinin	19.20000*	4.95480	.002	8.5730	29.8270
	Dihydrocitrinone	-.90000	4.95480	.858	-11.5270	9.7270
	Fumonisin B1	14.20000*	4.95480	.012	3.5730	24.8270
	Fumonisin B2	14.20000*	4.95480	.012	3.5730	24.8270
	Fumonisin B3	14.20000*	4.95480	.012	3.5730	24.8270
	Fumonisin B4	14.20000*	4.95480	.012	3.5730	24.8270
	Deoxynivalenol	19.20000*	4.95480	.002	8.5730	29.8270
	Nivalenol	-1.90000	4.95480	.707	-12.5270	8.7270
Deoxynivalenol	AFLATOXIN B1	-17.40000*	4.95480	.003	-28.0270	-6.7730
	AFLATOXIN B2	-16.30000*	4.95480	.005	-26.9270	-5.6730
	AFLATOXIN G1	-17.50000*	4.95480	.003	-28.1270	-6.8730
	AFLATOXIN G2	-14.20000*	4.95480	.012	-24.8270	-3.5730

		Ochratoxin A	-20.50000*	4.95480	.001	-31.1270	-9.8730
		Citrinin	.00000	4.95480	1.000	-10.6270	10.6270
		Dihydrocitrinone	-20.10000*	4.95480	.001	-30.7270	-9.4730
		Fumonisin B1	-5.00000	4.95480	.330	-15.6270	5.6270
		Fumonisin B2	-5.00000	4.95480	.330	-15.6270	5.6270
		Fumonisin B3	-5.00000	4.95480	.330	-15.6270	5.6270
		Fumonisin B4	-5.00000	4.95480	.330	-15.6270	5.6270
		Zearalenone	-19.20000*	4.95480	.002	-29.8270	-8.5730
		Nivalenol	-21.10000*	4.95480	.001	-31.7270	-10.4730
	Nivalenol	AFLATOXIN B1	3.70000	4.95480	.468	-6.9270	14.3270
		AFLATOXIN B2	4.80000	4.95480	.349	-5.8270	15.4270
		AFLATOXIN G1	3.60000	4.95480	.479	-7.0270	14.2270
		AFLATOXIN G2	6.90000	4.95480	.185	-3.7270	17.5270
		Ochratoxin A	.60000	4.95480	.905	-10.0270	11.2270
		Citrinin	21.10000*	4.95480	.001	10.4730	31.7270
		Dihydrocitrinone	1.00000	4.95480	.843	-9.6270	11.6270
		Fumonisin B1	16.10000*	4.95480	.006	5.4730	26.7270
		Fumonisin B2	16.10000*	4.95480	.006	5.4730	26.7270
		Fumonisin B3	16.10000*	4.95480	.006	5.4730	26.7270
		Fumonisin B4	16.10000*	4.95480	.006	5.4730	26.7270
		Zearalenone	1.90000	4.95480	.707	-8.7270	12.5270
		Deoxynivalenol	21.10000*	4.95480	.001	10.4730	31.7270

GROUNDNUT	AFLATOXIN B1	AFLATOXIN B2	12.50000*	4.33408	.012	3.2043	21.7957
		AFLATOXIN G1	16.20000*	4.33408	.002	6.9043	25.4957
		AFLATOXIN G2	19.30000*	4.33408	.001	10.0043	28.5957
		Ochratoxin A	-5.10000	4.33408	.259	-14.3957	4.1957
		Citrinin	-17.30000*	4.33408	.001	-26.5957	-8.0043
		Dihydrocitrinone	-15.60000*	4.33408	.003	-24.8957	-6.3043
		Fumonisin B1	-3.50000	4.33408	.433	-12.7957	5.7957
		Fumonisin B2	-3.50000	4.33408	.433	-12.7957	5.7957
		Fumonisin B3	-3.50000	4.33408	.433	-12.7957	5.7957
		Fumonisin B4	-3.50000	4.33408	.433	-12.7957	5.7957
		Zearalenone	7.30000	4.33408	.114	-1.9957	16.5957
		Deoxynivalenol	1.50000	4.33408	.734	-7.7957	10.7957
		Nivalenol	-6.70000	4.33408	.144	-15.9957	2.5957
	AFLATOXIN B2	AFLATOXIN B1	-12.50000*	4.33408	.012	-21.7957	-3.2043
		AFLATOXIN G1	3.70000	4.33408	.408	-5.5957	12.9957
		AFLATOXIN G2	6.80000	4.33408	.139	-2.4957	16.0957
		Ochratoxin A	-17.60000*	4.33408	.001	-26.8957	-8.3043
		Citrinin	-29.80000*	4.33408	.000	-39.0957	-20.5043
		Dihydrocitrinone	-28.10000*	4.33408	.000	-37.3957	-18.8043
		Fumonisin B1	-16.00000*	4.33408	.002	-25.2957	-6.7043

	Fumonisin B2	-16.00000*	4.33408	.002	-25.2957	-6.7043
	Fumonisin B3	-16.00000*	4.33408	.002	-25.2957	-6.7043
	Fumonisin B4	-16.00000*	4.33408	.002	-25.2957	-6.7043
	Zearalenone	-5.20000	4.33408	.250	-14.4957	4.0957
	Deoxynivalenol	-11.00000*	4.33408	.024	-20.2957	-1.7043
	Nivalenol	-19.20000*	4.33408	.001	-28.4957	-9.9043
AFLATOXIN G1	AFLATOXIN B1	-16.20000*	4.33408	.002	-25.4957	-6.9043
	AFLATOXIN B2	-3.70000	4.33408	.408	-12.9957	5.5957
	AFLATOXIN G2	3.10000	4.33408	.486	-6.1957	12.3957
	Ochratoxin A	-21.30000*	4.33408	.000	-30.5957	-12.0043
	Citrinin	-33.50000*	4.33408	.000	-42.7957	-24.2043
	Dihydrocitrinone	-31.80000*	4.33408	.000	-41.0957	-22.5043
	Fumonisin B1	-19.70000*	4.33408	.000	-28.9957	-10.4043
	Fumonisin B2	-19.70000*	4.33408	.000	-28.9957	-10.4043
	Fumonisin B3	-19.70000*	4.33408	.000	-28.9957	-10.4043
	Fumonisin B4	-19.70000*	4.33408	.000	-28.9957	-10.4043
	Zearalenone	-8.90000	4.33408	.059	-18.1957	.3957
	Deoxynivalenol	-14.70000*	4.33408	.004	-23.9957	-5.4043
	Nivalenol	-22.90000*	4.33408	.000	-32.1957	-13.6043
AFLATOXIN G2	AFLATOXIN B1	-19.30000*	4.33408	.001	-28.5957	-10.0043
	AFLATOXIN B2	-6.80000	4.33408	.139	-16.0957	2.4957

	AFLATOXIN G1	-3.10000	4.33408	.486	-12.3957	6.1957
	Ochratoxin A	-24.40000*	4.33408	.000	-33.6957	-15.1043
	Citrinin	-36.60000*	4.33408	.000	-45.8957	-27.3043
	Dihydrocitrinone	-34.90000*	4.33408	.000	-44.1957	-25.6043
	Fumonisin B1	-22.80000*	4.33408	.000	-32.0957	-13.5043
	Fumonisin B2	-22.80000*	4.33408	.000	-32.0957	-13.5043
	Fumonisin B3	-22.80000*	4.33408	.000	-32.0957	-13.5043
	Fumonisin B4	-22.80000*	4.33408	.000	-32.0957	-13.5043
	Zearalenone	-12.00000*	4.33408	.015	-21.2957	-2.7043
	Deoxynivalenol	-17.80000*	4.33408	.001	-27.0957	-8.5043
	Nivalenol	-26.00000*	4.33408	.000	-35.2957	-16.7043
Ochratoxin A	AFLATOXIN B1	5.10000	4.33408	.259	-4.1957	14.3957
	AFLATOXIN B2	17.60000*	4.33408	.001	8.3043	26.8957
	AFLATOXIN G1	21.30000*	4.33408	.000	12.0043	30.5957
	AFLATOXIN G2	24.40000*	4.33408	.000	15.1043	33.6957
	Citrinin	-12.20000*	4.33408	.014	-21.4957	-2.9043
	Dihydrocitrinone	-10.50000*	4.33408	.030	-19.7957	-1.2043
	Fumonisin B1	1.60000	4.33408	.718	-7.6957	10.8957
	Fumonisin B2	1.60000	4.33408	.718	-7.6957	10.8957
	Fumonisin B3	1.60000	4.33408	.718	-7.6957	10.8957
	Fumonisin B4	1.60000	4.33408	.718	-7.6957	10.8957

	Zearalenone	12.40000*	4.33408	.013	3.1043	21.6957
	Deoxynivalenol	6.60000	4.33408	.150	-2.6957	15.8957
	Nivalenol	-1.60000	4.33408	.718	-10.8957	7.6957
Citrinin	AFLATOXIN B1	17.30000*	4.33408	.001	8.0043	26.5957
	AFLATOXIN B2	29.80000*	4.33408	.000	20.5043	39.0957
	AFLATOXIN G1	33.50000*	4.33408	.000	24.2043	42.7957
	AFLATOXIN G2	36.60000*	4.33408	.000	27.3043	45.8957
	Ochratoxin A	12.20000*	4.33408	.014	2.9043	21.4957
	Dihydrocitrinone	1.70000	4.33408	.701	-7.5957	10.9957
	Fumonisin B1	13.80000*	4.33408	.007	4.5043	23.0957
	Fumonisin B2	13.80000*	4.33408	.007	4.5043	23.0957
	Fumonisin B3	13.80000*	4.33408	.007	4.5043	23.0957
	Fumonisin B4	13.80000*	4.33408	.007	4.5043	23.0957
	Zearalenone	24.60000*	4.33408	.000	15.3043	33.8957
	Deoxynivalenol	18.80000*	4.33408	.001	9.5043	28.0957
	Nivalenol	10.60000*	4.33408	.028	1.3043	19.8957
Dihydrocitrinone	AFLATOXIN B1	15.60000*	4.33408	.003	6.3043	24.8957
	AFLATOXIN B2	28.10000*	4.33408	.000	18.8043	37.3957
	AFLATOXIN G1	31.80000*	4.33408	.000	22.5043	41.0957

		AFLATOXIN G2	34.90000*	4.33408	.000	25.6043	44.1957
		Ochratoxin A	10.50000*	4.33408	.030	1.2043	19.7957
		Citrinin	-1.70000	4.33408	.701	-10.9957	7.5957
		Fumonisin B1	12.10000*	4.33408	.014	2.8043	21.3957
		Fumonisin B2	12.10000*	4.33408	.014	2.8043	21.3957
		Fumonisin B3	12.10000*	4.33408	.014	2.8043	21.3957
		Fumonisin B4	12.10000*	4.33408	.014	2.8043	21.3957
		Zearalenone	22.90000*	4.33408	.000	13.6043	32.1957
		Deoxynivalenol	17.10000*	4.33408	.001	7.8043	26.3957
		Nivalenol	8.90000	4.33408	.059	-.3957	18.1957
Fumonisin B1		AFLATOXIN B1	3.50000	4.33408	.433	-5.7957	12.7957
		AFLATOXIN B2	16.00000*	4.33408	.002	6.7043	25.2957
		AFLATOXIN G1	19.70000*	4.33408	.000	10.4043	28.9957
		AFLATOXIN G2	22.80000*	4.33408	.000	13.5043	32.0957
		Ochratoxin A	-1.60000	4.33408	.718	-10.8957	7.6957
		Citrinin	-13.80000*	4.33408	.007	-23.0957	-4.5043
		Dihydrocitrinone	-12.10000*	4.33408	.014	-21.3957	-2.8043
		Fumonisin B2	.00000	4.33408	1.000	-9.2957	9.2957
		Fumonisin B3	.00000	4.33408	1.000	-9.2957	9.2957
		Fumonisin B4	.00000	4.33408	1.000	-9.2957	9.2957
		Zearalenone	10.80000*	4.33408	.026	1.5043	20.0957

	Deoxynivalenol	5.00000	4.33408	.268	-4.2957	14.2957
	Nivalenol	-3.20000	4.33408	.473	-12.4957	6.0957
Fumonisin B2	AFLATOXIN B1	3.50000	4.33408	.433	-5.7957	12.7957
	AFLATOXIN B2	16.00000*	4.33408	.002	6.7043	25.2957
	AFLATOXIN G1	19.70000*	4.33408	.000	10.4043	28.9957
	AFLATOXIN G2	22.80000*	4.33408	.000	13.5043	32.0957
	Ochratoxin A	-1.60000	4.33408	.718	-10.8957	7.6957
	Citrinin	-13.80000*	4.33408	.007	-23.0957	-4.5043
	Dihydrocitrinone	-12.10000*	4.33408	.014	-21.3957	-2.8043
	Fumonisin B1	.00000	4.33408	1.000	-9.2957	9.2957
	Fumonisin B3	.00000	4.33408	1.000	-9.2957	9.2957
	Fumonisin B4	.00000	4.33408	1.000	-9.2957	9.2957
	Zearalenone	10.80000*	4.33408	.026	1.5043	20.0957
	Deoxynivalenol	5.00000	4.33408	.268	-4.2957	14.2957
	Nivalenol	-3.20000	4.33408	.473	-12.4957	6.0957
Fumonisin B3	AFLATOXIN B1	3.50000	4.33408	.433	-5.7957	12.7957
	AFLATOXIN B2	16.00000*	4.33408	.002	6.7043	25.2957
	AFLATOXIN G1	19.70000*	4.33408	.000	10.4043	28.9957

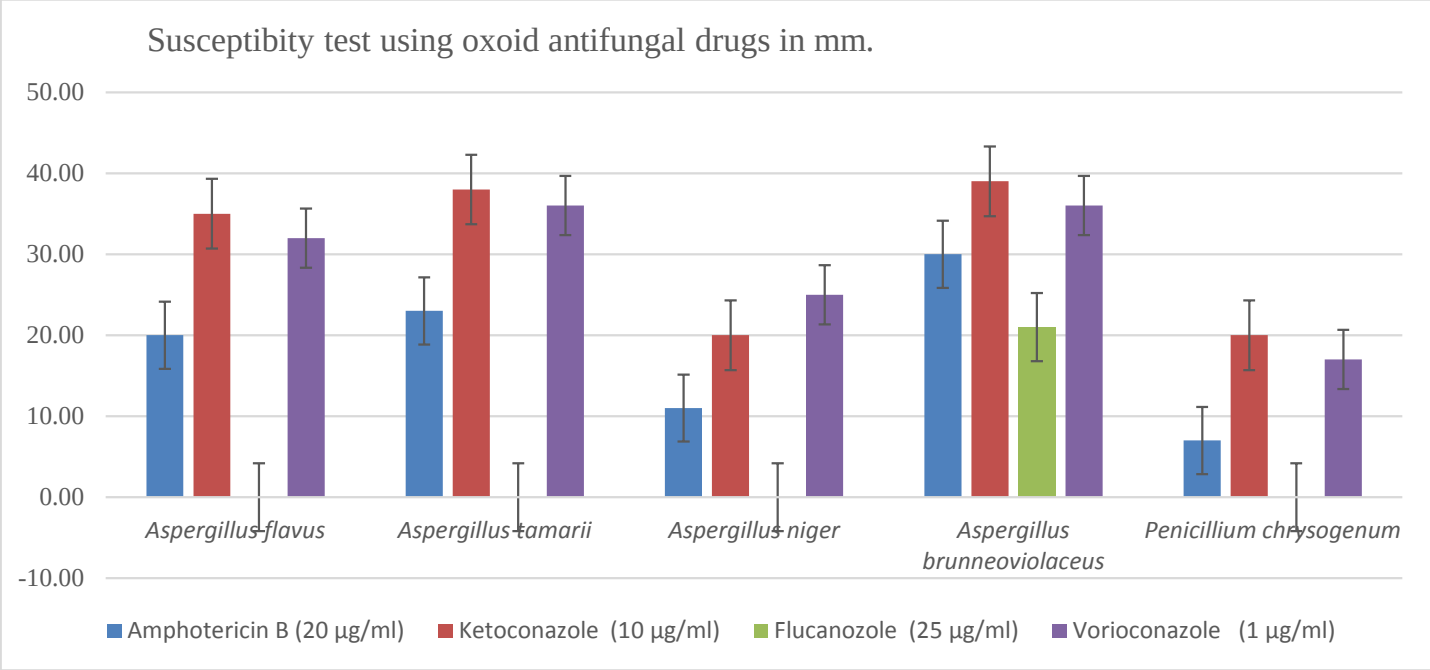
		AFLATOXIN G2	22.80000*	4.33408	.000	13.5043	32.0957
		Ochratoxin A	-1.60000	4.33408	.718	-10.8957	7.6957
		Citrinin	-13.80000*	4.33408	.007	-23.0957	-4.5043
		Dihydrocitrinone	-12.10000*	4.33408	.014	-21.3957	-2.8043
		Fumonisin B1	.00000	4.33408	1.000	-9.2957	9.2957
		Fumonisin B2	.00000	4.33408	1.000	-9.2957	9.2957
		Fumonisin B4	.00000	4.33408	1.000	-9.2957	9.2957
		Zearalenone	10.80000*	4.33408	.026	1.5043	20.0957
		Deoxynivalenol	5.00000	4.33408	.268	-4.2957	14.2957
		Nivalenol	-3.20000	4.33408	.473	-12.4957	6.0957
	Fumonisin B4	AFLATOXIN B1	3.50000	4.33408	.433	-5.7957	12.7957
		AFLATOXIN B2	16.00000*	4.33408	.002	6.7043	25.2957
		AFLATOXIN G1	19.70000*	4.33408	.000	10.4043	28.9957
		AFLATOXIN G2	22.80000*	4.33408	.000	13.5043	32.0957
		Ochratoxin A	-1.60000	4.33408	.718	-10.8957	7.6957
		Citrinin	-13.80000*	4.33408	.007	-23.0957	-4.5043
		Dihydrocitrinone	-12.10000*	4.33408	.014	-21.3957	-2.8043
		Fumonisin B1	.00000	4.33408	1.000	-9.2957	9.2957
		Fumonisin B2	.00000	4.33408	1.000	-9.2957	9.2957
		Fumonisin B3	.00000	4.33408	1.000	-9.2957	9.2957
		Zearalenone	10.80000*	4.33408	.026	1.5043	20.0957

	Deoxynivalenol	5.00000	4.33408	.268	-4.2957	14.2957
	Nivalenol	-3.20000	4.33408	.473	-12.4957	6.0957
Zearalenone	AFLATOXIN B1	-7.30000	4.33408	.114	-16.5957	1.9957
	AFLATOXIN B2	5.20000	4.33408	.250	-4.0957	14.4957
	AFLATOXIN G1	8.90000	4.33408	.059	-.3957	18.1957
	AFLATOXIN G2	12.00000*	4.33408	.015	2.7043	21.2957
	Ochratoxin A	-12.40000*	4.33408	.013	-21.6957	-3.1043
	Citrinin	-24.60000*	4.33408	.000	-33.8957	-15.3043
	Dihydrocitrinone	-22.90000*	4.33408	.000	-32.1957	-13.6043
	Fumonisin B1	-10.80000*	4.33408	.026	-20.0957	-1.5043
	Fumonisin B2	-10.80000*	4.33408	.026	-20.0957	-1.5043
	Fumonisin B3	-10.80000*	4.33408	.026	-20.0957	-1.5043
	Fumonisin B4	-10.80000*	4.33408	.026	-20.0957	-1.5043
	Deoxynivalenol	-5.80000	4.33408	.202	-15.0957	3.4957
	Nivalenol	-14.00000*	4.33408	.006	-23.2957	-4.7043
Deoxynivalenol	AFLATOXIN B1	-1.50000	4.33408	.734	-10.7957	7.7957
	AFLATOXIN B2	11.00000*	4.33408	.024	1.7043	20.2957
	AFLATOXIN G1	14.70000*	4.33408	.004	5.4043	23.9957

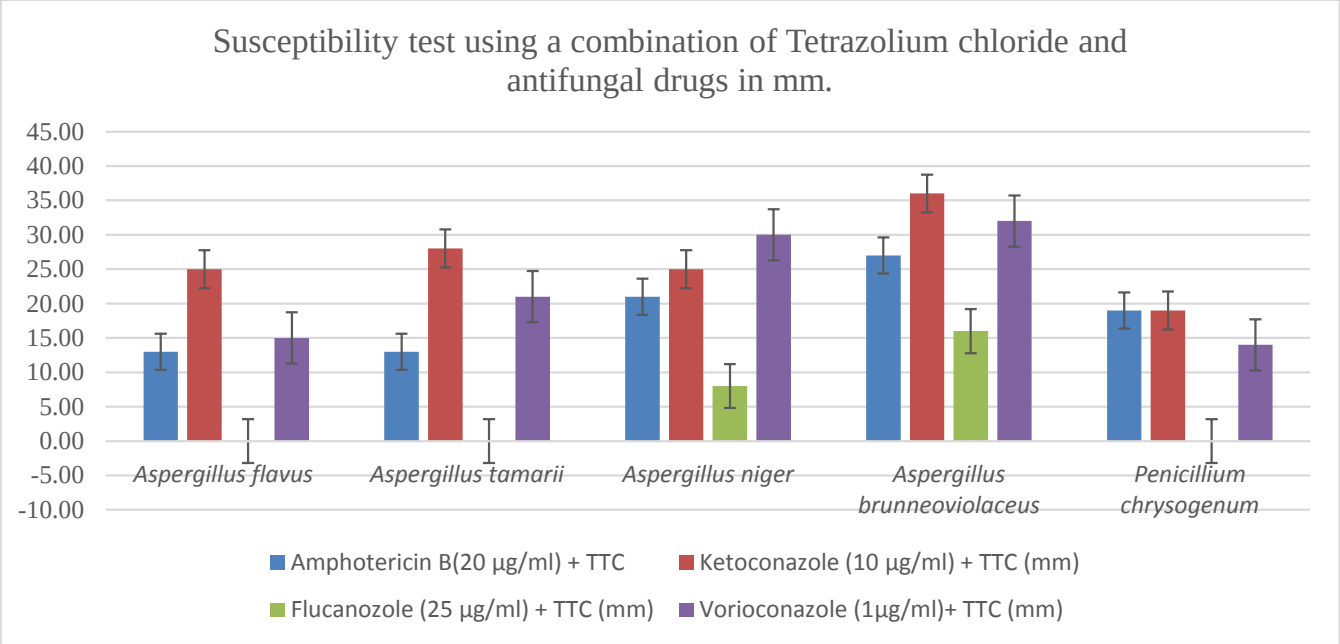
		AFLATOXIN G2	17.80000*	4.33408	.001	8.5043	27.0957
		Ochratoxin A	-6.60000	4.33408	.150	-15.8957	2.6957
		Citrinin	-18.80000*	4.33408	.001	-28.0957	-9.5043
		Dihydrocitrinone	-17.10000*	4.33408	.001	-26.3957	-7.8043
		Fumonisin B1	-5.00000	4.33408	.268	-14.2957	4.2957
		Fumonisin B2	-5.00000	4.33408	.268	-14.2957	4.2957
		Fumonisin B3	-5.00000	4.33408	.268	-14.2957	4.2957
		Fumonisin B4	-5.00000	4.33408	.268	-14.2957	4.2957
		Zearalenone	5.80000	4.33408	.202	-3.4957	15.0957
		Nivalenol	-8.20000	4.33408	.079	-17.4957	1.0957
Nivalenol		AFLATOXIN B1	6.70000	4.33408	.144	-2.5957	15.9957
		AFLATOXIN B2	19.20000*	4.33408	.001	9.9043	28.4957
		AFLATOXIN G1	22.90000*	4.33408	.000	13.6043	32.1957
		AFLATOXIN G2	26.00000*	4.33408	.000	16.7043	35.2957
		Ochratoxin A	1.60000	4.33408	.718	-7.6957	10.8957
		Citrinin	-10.60000*	4.33408	.028	-19.8957	-1.3043
		Dihydrocitrinone	-8.90000	4.33408	.059	-18.1957	.3957
		Fumonisin B1	3.20000	4.33408	.473	-6.0957	12.4957
		Fumonisin B2	3.20000	4.33408	.473	-6.0957	12.4957
		Fumonisin B3	3.20000	4.33408	.473	-6.0957	12.4957
		Fumonisin B4	3.20000	4.33408	.473	-6.0957	12.4957

	Zearalenone	14.00000*	4.33408	.006	4.7043	23.2957
	Deoxynivalenol	8.20000	4.33408	.079	-1.0957	17.4957

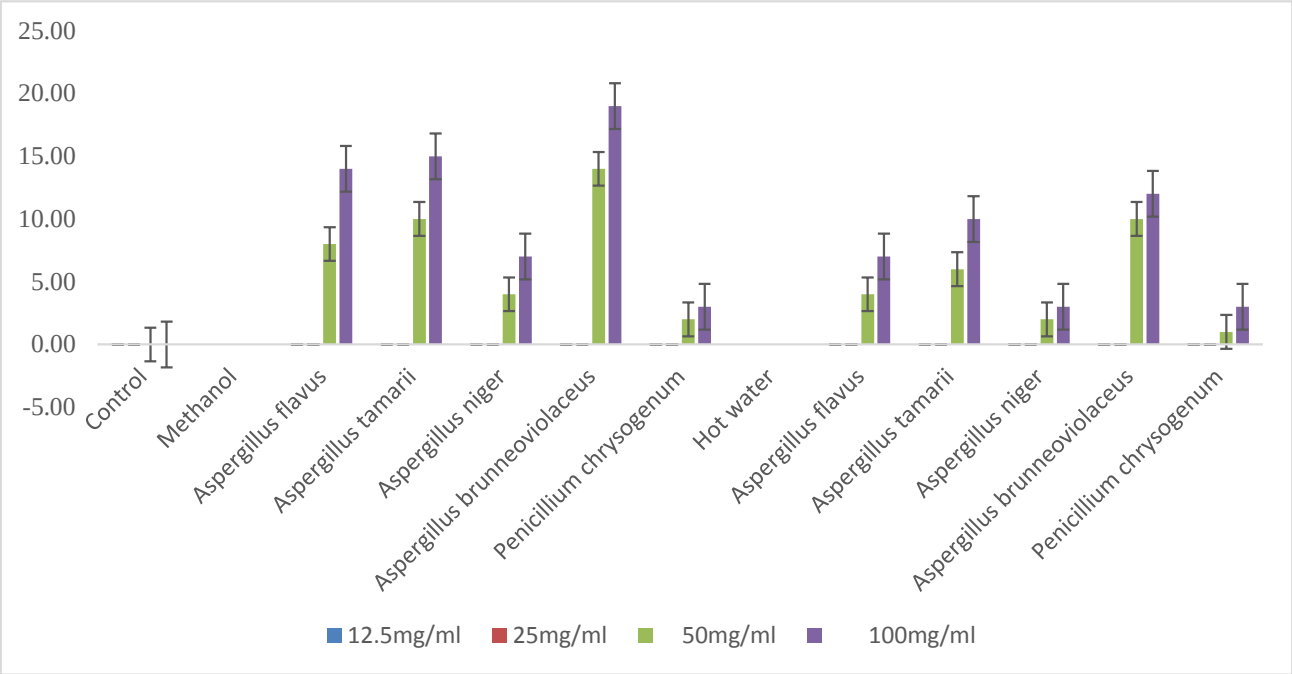
*. The mean difference is significant at the 0.05 level.



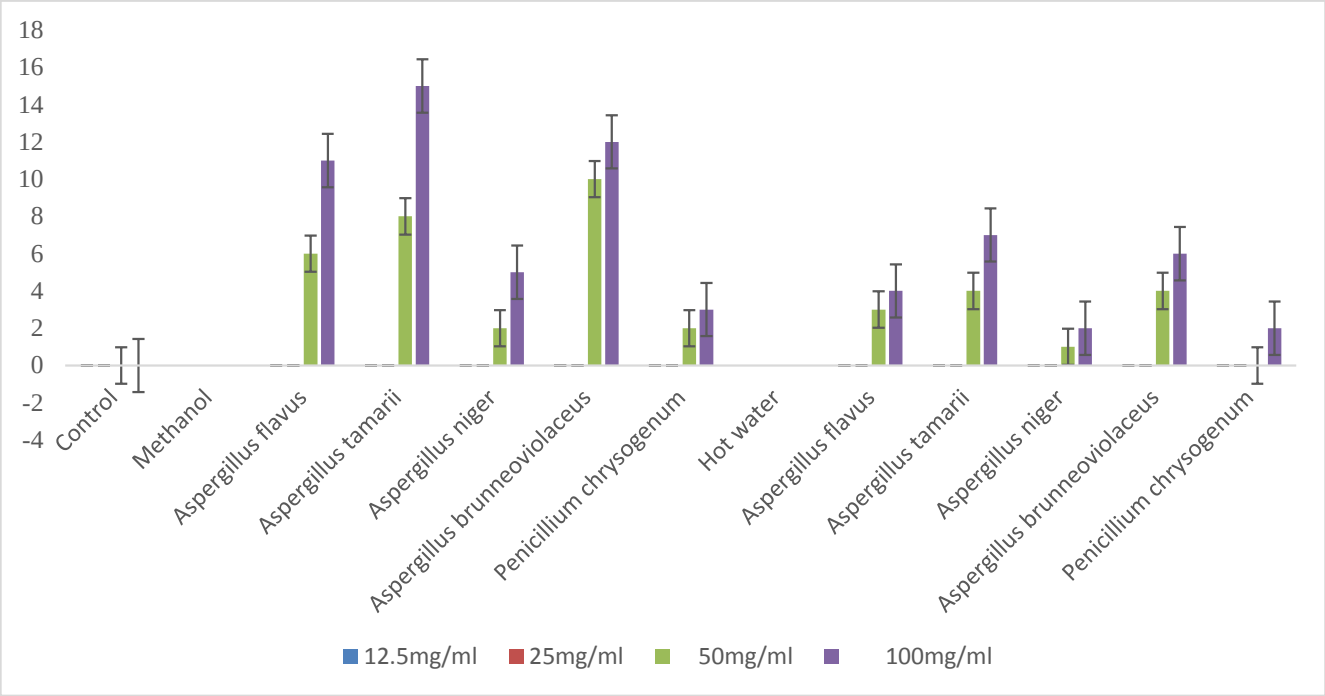
3: Susceptibity profile using oxoid antifungal drugs in mm.



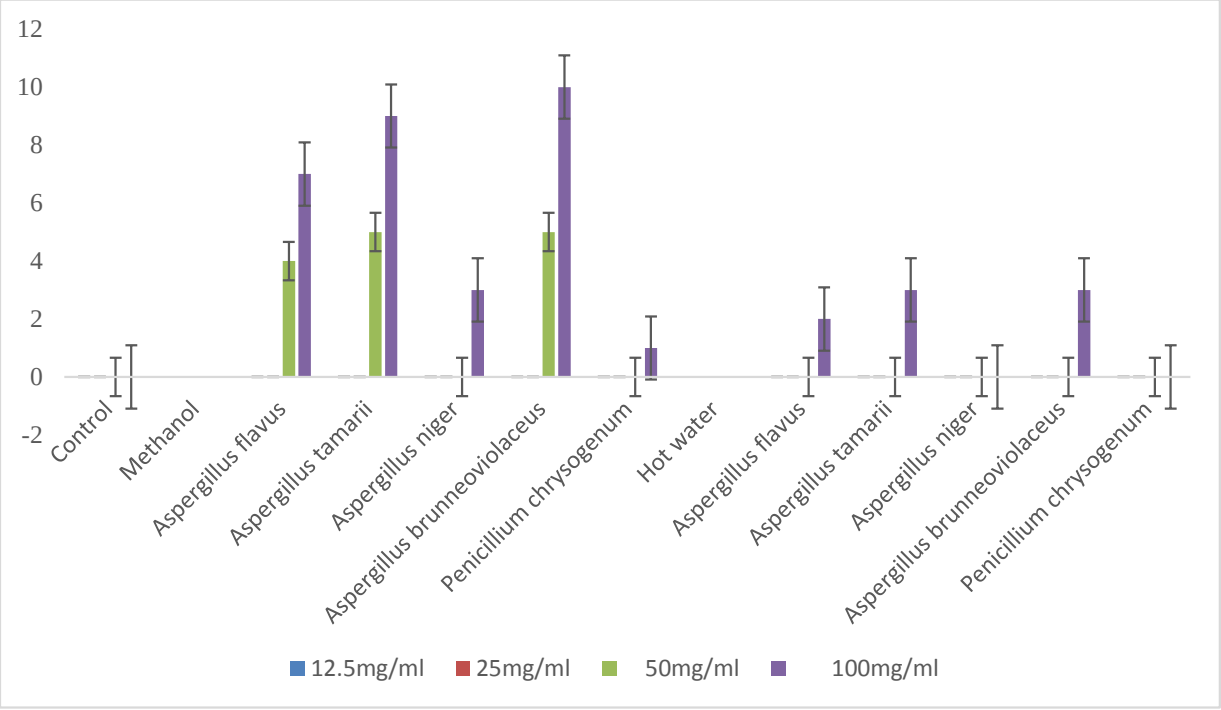
4: Susceptibility profile using a combination of tetrazolium chloride and antifungal drugs in mm.



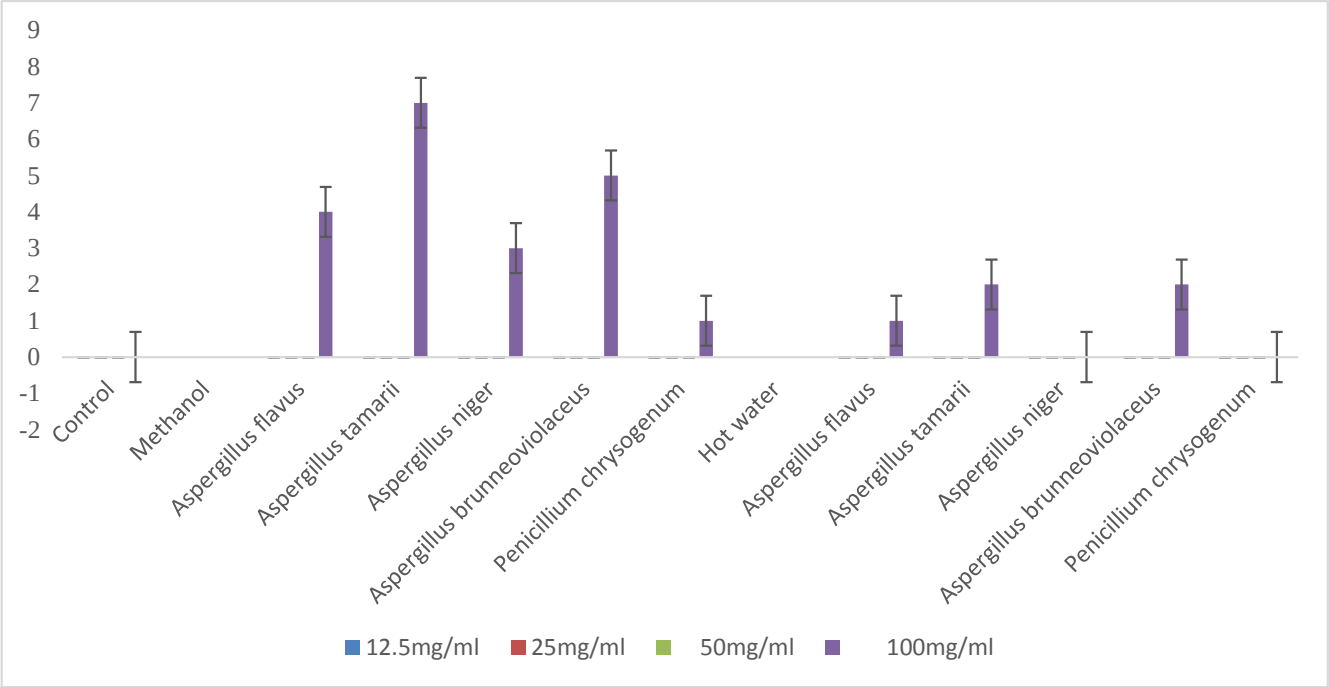
5: Percentage growth inhibition of moulds by methanol and hot water extract of scent leaf.



6: Percentage growth inhibition of moulds by methanol and hot water extract of bitter leaf.



7: Percentage growth inhibition of moulds by methanol and hot water extract of utazi leaf.



8: Percentage growth inhibition of moulds by methanol and hot water extract of uziza leaf.