Sudan University of Science and Technology

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Contamination of some Poultry Feed with *Aspergillud* and Aflatoxin In Khartoum State

تلوث بعض علف الدواجن بالرشاشيات والافلاتوكسين في ولاية الخرطوم

A Thesis submitted to The Sudan University of Science and Technology College of Graduate Studies in the fulfilment of the requirement for the degree of Doctor of philosophy in Veterinary Medicine (Preventive Medicine)

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DEDICATION

To the soul of my father

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Abstract

Aspergillus flavus is an important fungal species which may occur in foods and feed, producing a number of toxins including aflatoxins. There are several diseases that affect poultry and cause severe losses; among which is mycotoxicosis caused by strains of *Aspergillus-flavus*; consequently the research objective concerned the isolation and identify of *Aspergillus flavus*in poultry feed. Detection of Aflatoxin-producing strains of *Aspergillus flavus* and these toxins that affect poultry, animal and human. Poultry farms are suitable environments for growth of fungi. Most poultry feeds are prone to fungal growth during different stages of the manufacturing process, production, transportation and storage. This study was carried in Khartoum State, from different localities to identify *Aspergillus-flavus* from food poultry Peanuts (cakes) Identificationofthe*Aspergillus-flavus* was based on the morphological and characteristics of the colonies and microscopic examinations. Thirty samples (Cake) were collected from different places in Khartoum State(Omdurman, Bahri and Sharg-elnile). They were preparing samples and culture media. Samples cultured in Sabouraud's Dextrose Agar (SDA) for isolation of the fungal sub culture in potato dextrose agar (PDA)**.**The morphological characteristics under microscopic with wet smear slide of lactophinol cotton blue.Thin Layer Chromotography used to detection the aflatoxin in samples which are positive *Aspergillus-flavus*.**.**Polymerase chain reaction (PCR) was used to identify *Aspergillus-flavus.* Out of 30 samples 7 were found positive for *Aspergillus-flavus* . Aflatoxin showed that all the positive sample of *Aspergillus flavus* were found positive for aflatoxin B1 (2.5 – 10mg/kg) with different concentrations. One sample is positive for aflatoxin B2 (2.5mg/kg).But PCR tested all recovered samples were not amplified by PCR. Poultry food was found contaminated with *Aspergillus flavus* and aflatoxins B1and B2 but no G1and G2. These results obtained support the idea that of the used of peanuts was the main factor of aflatoxin contamination. *Aspergillus flavus* contaminated food is a serious risk for public health having long-term health effects in Human, Animals and poultry because it produces aflatoxin.

Abstract (Arabic)

ملخص

أجريت هذه الدراسة بهدف تحديد مدى تلوث الفول السوداني المأخوذ من ثلاثة مناطق بولاية الخرطوم بفطر الاسبيروقلس والسم الفطري المعروف بالأفلاتوكسين.جُمعت ثلاثون عينة من الفول السوداني المخزون من مناطق مختلفة بولاية الخرطوم العينات أختبرت عن طريق التحليل الميكروبي للتعرف على فطر الاسبيروقلس وعُزله وذلك بواسطة الزراعة على وسط أجار السابرود ديكستروز وبتيتو دكستروز اجار وكذلك سموم الأفلاتوكسين عن طريق التحليل الكيميائي بواسطة تقنية التصويرالملون ذات الطبقة الرقيقة لمعرفة وجود السم ومقداره (ب1 .ب 2 وج1 وج2). إحتوت سبعة عينات من مجموع عينات الفول السوداني المخزون على سم الأفلاتوكسين ب 1عن طريق تقنية التصوير الملون ذات الطبقة الرقيقة. وعينة واحدة فقط من سم الأفلاتوكسين ب2 تتراوح تراكيز سم الافلاتوكسين ب 1في العينات المختبرة مابين (2.5 – 10 g/kg) وب2(2.5g/kg) ،مما يودي من خطورة محتملة للإنسان والحيوان.وقد اثبت البحث تلوث اغذية الدواجن بفطر الاسبيروقلس وسم الأفلاتوكسين ب1 وب2 هذه النتائج تدعم ان استخدام الفول السوداني كان العامل الاساسي للتلوث بالافلاتوكسن. . يعتبر الغذاء الملوث **ب**الاسبيروقلس مهدد خطير للصحة العامة وله اثار صحية بعيدة المدى على الانسان والحيوان والدواجن لانه ينتج سم الافلاتوكسن.

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LIST OF ABBREVIATIONS

AF	Aflatoxin
AFs	*Aspergillus flavus*
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AOAC	Association of Official Analytical Chemists
CAC	Codex Alimentarius Commission
CPA	Cyclopiozonic acid
DAD	Diode Array Detector
ELISAs	Enzyme-Linked Immunosorbent Assays
EU	European Union
FAO	Food and Agriculture Organization
FD	fluorescence detector
FDA	Food and Drug Administration
FPIA	Fluorescence Polarization Immunoassay
HPLC	High Performance Liquid Chromatography
IAC	Immunoaffinity Column
IARC	International Agency for Research on Cancer
ITS	Internal transcribed spacer
LC/MS	Liquid Chromatography/Mass Spectrometry
MRC	Medical Research Council
ppb	Parts Per Billion
PCR	polymerase chain reaction
RT-PCR	reverse transcription-polymerase chain reaction
TLC	Thin-Layer Chromatography
USA	United States of America
USDA	United States Department of Agriculture
USAID	United States Agency for International Development
WHO	World Health Organization

Introduction

Aspergillus flavus is a kind of fungus which the known to inhabit in soil and other substrates (Powell *et al*., 1994). *Aspergillus flavus* an important fungal species which may occur in food and feeds producing number of toxins including aflatoxins being the most relevant with food safety. The aflatoxinogenic fungi can contaminate several food commodities including cereals (Sultana *et al*., 2013), peanuts (Jelinek *et al*., 1989), spices (Bartine and Tantaoui, 1997) and figs. *Aspergillus flavus* is reported to be associated with many diseases of human, with severe invasive aspergillosis. It can also cause diseases in insects (Campbell, 1994) as well as in crops (such as maize, rice, peanuts etc). Agricultural products including cereals e.g. maize, wheat, sorghum and by products of variety of oilseeds are major constituents of poultry feed (Okoli *et al*., 2006). *Aspergillus flavus* occurs in warm temperate and subtropical climates all over the world. Although the fungus is not a very aggressive pathogen, under weather conditions conducive for its growth *A. flavus* can colonize seeds in the field and contaminate them with aflatoxin. Because of its ability to grow at low water activity, *A. flavus* is a thermotolerant fungus, so can survive at temperatures that other fungi cannot. It can contribute to the storage rots, especially when the plant material is stored at high moisture levels. *A. flavus* grows and thrives in hot and humid climates *(Hedayati, 2007).*

A. flavus producing mycotoxin can be injurious for animals and human health. Aflatoxins are family of myctoxin that contaminate peanuts, cereals, cotton seed, corn, rice and others commodities with wide spread contamination in hot and humid region in the world. (Murphy *et al*., 2006)

Out of all different types of mycotoxins, aflatoxins are the most potent natural carcinogens known possessing hepatoxic and immunosuppressive properties which can cause acute liver damage, liver cirrhosis, tumor induction and teratogenesis (JECFA, FAO *et al*., 1996). They have been recognized as a possible human carcinogen by International Agency of Research on Cancer (IARC,1993).

Poultry feeding is one of the most important branches of poultry farming. Nutritionally balanced diets are provided during phases of productive life in eggers, chicks, grower and layer stages, in broilers starter and finishing stages (Gopalakrishana and Lal, 1985). Poultry feed are prepared basically with plants materials such as maize, soybean and sorghum and these materials are known to have fungi as the commonest contaminations (Oyeka and Onochie, 1992). A mycotoxin contaminated diet may lead to substantial economic losses in livestock due to feed refusal, poor feed conversion, diminished body weight gain, immune-suppression, interference with reproductive capabilities and residues in animal products (Varga and Toth, 2005). Low yields of animals and crops can also be occurred due to aflatoxin contamination (Phillips *et al*., 1996)

Different mycotoxins have been reported as contaminant of poultry feed, most important of which are aflatoxins (B1, B2, G1, and G2) and Ochratoxin A (OTA) (Gentles *et al*., 1999).
Control of aflatoxin contamination can be achieved by either controlling the fungus or controlling aflatoxin production. Detoxification by many control measures, such as chemical, biological, and physical means, have been tried to reduce or eliminate aflatoxin contamination in maize, but none appears to be economically feasible (Lillehoj and Wall, 1987).
This study was carried out in Khartoum State, in different localities to identify *Aspergillus flavus* from food poultry Peanuts (cakes) and detection of Aflatoxins. Identification of the *Aspergillus flavus* based on the morphological characteristics of the colonies .This review focuses on resources, detection and identification of *Aspergillus flavus* and production of aflatoxins in Peanuts (cakes).To provid safety food for humans and poultry.

Consequently the research problem concerned the prevalence of Aflatoxin-producing strains of *Aspergillus flavus*in poultry feed. Research Problem Limits
a. spatial limit: Khartoum State
b. Temporal limit during two years.

Rationale of the study.
a. Aflatoxin-producing strains of *Aspergillus flavus* and Aflatoxin affect poultry and human.
b. Poultry farms are suitable environments for growth of fungi
c. Most poultry feeds are prone to fungal growth during different stages of the manufacturing process, production, transportation and storage.
d. Poultry are source of meat and eggs for human consumption
e. scarce information about prevalence of Aflatoxin-producing strains of *Aspergillus flavus* and Aflatoxin in poultry feed in Khartoum State.
f. Ignorance about prevalence of Aflatoxin-producing strains of *Aspergillus flavus* and Aflatoxin in poultry feed leads to ignorance about control and prevention of Aspergillosis and Aflatoxicosis.

g. consequently spread of the fungi and its toxin in poultry farms may lead to reduction in poultry population and production, and infection of human consumers

h**.**hazard occurring in these food products is likely to affect a large population and consequently contribute to increasing poverty and food insecurity. So the research Significance

a. knowing the prevalence of Aflatoxin-producing strains of *Aspergillus flavus* and Aflatoxin in poultry feed will help in implementing the proper strategic plans for control and prevention.

b. Control and prevention of Aspergillosis and Aflatoxicosis reduce poultry losses, increase poultry population and production, encourage poultry industry, and protect human consumers

Research Objective

a. **General objective:**

Identification of Aflatoxin-producing fungi in poultry feeds

b. **Specific objectives**

1) Isolation and identification of Aflatoxin-producing strains of *Aspergillus flavus* from poultry feed Peanuts (cakes) in Khartoum state.

2) Identification of aflatoxin.

Theresearch hypothesis content

a. There is high prevalence of Aflatoxin-producing strains of *Aspergillus flavus*in poultry feed Peanuts (cakes) in Khartoum state.

b**.** The aflatoxins levels in stored peanuts lead to an effect on contamination of peanuts and it product.

The basic research postulates.

a. Poultry feed are prone to fungal contamination in the presence of suitable environment during production, storage and poultry feeding.

b. Antibiotics in poultry feeds increase the probability of fungal contamination.

CHAPTER ONE

Literature review

1. Historic Background

The first report of Aflatoxins(AF) goes back to the early 1960s, when they were initially isolated and identified as the causative toxins for the deaths of more than 100,000 turkeys in the United Kingdom (Filazi and Sireli, 2013). Since then, several cases have been reported worldwide. Currently, it has been estimated that more than 4.5 billion people in developing countries worldwide are at risk of chronic exposure to AFs through contaminated foods (USAID, 2012).While in developed countries the problem of AF seems to be under control, it remains a big challenge to food safety in several developing countries (DANYA and USAID, 2012).Indeed, AF poisoning in Eastern Africa has almost become epidemic, especially in arid and semi-arid areas. The most obvious case is that of Kenya where several outbreaks of aflatoxicosis were reported (Strosnider *et al*.,2006).

Moreover, contamination of products by AF is also encountered in Western and Central Africa.Ghana, Nigeria, Senegal, Togo, Burkina Faso and Cameroon have recorded AF contamination in sorghum, maize, cottonseed, groundnuts and groundnut products, yam and cassava at different levels with contamination levels generally exceeding the European Union (EU) and the United States Department of Agriculture (USDA) standards (Bankole and Adebanjo, 2003)(Kpodo *etal.*,200*)*).Contamination of food products by AFs was also reported in some Southern African countries. In Botswana, AFs were found in maize meal (DANYA and USAID, 2012). Furthermore, a study in Malawi revealed a high concentration of AF of up to 1,020 ppb in grain consumption (Glaston.,*et al* 2000).Despite recent initiatives from WHO and the CDC (The central of disease control) advocating for reduced antibiotic prescription rates in an effort to curb antibiotic resistance, these plans focus on bacterial infections while ignoring considerations for fungal infections. Not yet recognized as a public health threat, the emergence of multidrug-resistant fungal infections has the potential to become a grave national issue (Vincent and Bruno,2016).Aflatoxins are mycotoxins produced by fungi of the genus *Aspergillus* that essentially belongsto grains storage flora and soil. *Aspergillus* grows optimally at 25 °C with a minimum necessary water activity of 0.75. It starts to produce secondary metabolites at 10-12 ºC, but the most toxic ones are produced at 25°C with a water activity of 0.95 (Gimeno and Martins,2003).

There are about twenty types of Aflatoxins, but the naturally occurring and well-known ones are aflatoxinB1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1)

and aflatoxin G2 (AFG2) (Wu *et al*., 2013). These names were given due to their blue (B) or green (G) fluorescence properties under ultraviolet light and their migration patterns during chromatography (Saleemullah *et al*., 2006).
There are also aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) that are hydroxylated derivatives of AFB1 and AFB2, respectively. These may be found in milk, milk products or meat (hence the designation M1 and M2). They are formed by the metabolism of B1 and B2 in the body of the animals following absorption of contaminated feeds (Wild and Gong, 2010).
With regard to *Aspergillus flavus*(AF) contamination in foods imported into Japan, relatively low incidences and low levels of AFs have been found in various commodities. Aflatoxin inspection of imported peanuts in Japan (1999–2000) indicated that 355 (6.9%) of 5108 samples were contaminated with AFB1 at levels ranging from 0.2 to 760 ppb, and 145 samples (2.8%) contained over 10ppb, the maximum permissible limits (MPLs) (IARC, 2002). In commercial nuts and nut products in markets, AFB1 was found in 23 (3.4%) of 673 samples at levels of 0.3–128 ppb in the same country. Imported spices (white and red pepper, paprika and nutmeg) contained AFB1in 106 (19.4%) of 546 samples at levels of 0.2–27.7 ppb (IARC, 2002).

1.1. Aspergillus-flavus

The conidial states of *Aspergillus* and *Penicillium* are generally arranged in phialides, and their arrangements are generally used as diagnostic state. In *Aspergillus*, the conidiophore tip is swollen, forming the vesicle, and phialides start directly on its surface (uniseriate) or present a palisade of sterile cells, metulae, followed by phialides (biseriate) (Raper and Fennell, 1965). *Penicillium* lacks vesicles, and the conidiophore tip has directly a monoverticillate arrangement or series of metulae followed by philiades, the levels of ramification could be from one to several series of metulae (Raper and Thon, 1968). The cell walls are composed mainly by chitin and glucans and in general the septum is incomplete, forming a central pore that result in coenocytic mycelia (Webster and Weber, 2007).

Aspergillus and *Penicillium* genera are important to humankind not only because of their detrimental effects, but also because of their use in biotechnology, enzymes and other compounds synthesized as part of their primary and secondary metabolisms are used, as well as a direct inoculation of fungi on foodstuff. *Aspergillus* and *Penicillium* have been used in food production for several centuries in fermentation processes to produce beverages, sauces and in the cheese industry. Likewise, proteases, amylases, lipases and pectinases are

important in the manufacture of dairy, bakery, distillery and brewery products, juices and leather, and in the starch industry. Furthermore, they have been used to synthesize antibiotics, such as penicillins and cephalosporins that comprise around the 50% of antibiotics production worldwide (Kavanagh, 2017); or griseofulvin used as antitumoral and in dermatology (Banani *et al*., 2016).

Few fungi have as broad an economic impact as *Aspergillusflavu*. It is a pathogen of plants, animals human and insects, causes storage rots in numerous crops, and it produces the highly regulated mycotoxin, aflatoxin B1.as human pathogens, Aspergillus species have become increasingly important because immune suppressed people are very susceptible to infection by these fungi. Of the aspergilli causing mycoses in humans, only A. fumigates is more important than *A. flavus* (Stevens *et al*., 2000).

The genus *Aspergillus* was first described by Florentine priest and mycologist (Ross *et al*., 1951). During 20th century. Named based on the structural similarity of its conidiophore structure to the aspergillum, a liturgical implement used to sprinkle holy water (Bennett, 1992). This common genus has been classified based on morphology many times (Samson, 1992) and currently contains over 200species. One of the most important ubiquitous fungal species in tropical environments *Aspergillus flavus* that can be found in soil and other substrates (Powell *et al*., 1994). Recently the sexual stage of *A. flavus* has been reported and classified as *Petromyces flavus*(Horn , 2009).

Invasive aspergillosis (IA) is a chronic fungus caused disease by aspergillus. It affects the respiratory, blood, digestive, and other systems. It is the most common respiratory system fungal infection (Arvanitis andAnagnostou, 2015).*A. flavus* is also an allergen causing allergic broncho pulmonary aspergillosis.Children who have organ transplants such as lung or stem are highly susceptible to Aspergillus infections (AI). Mortality can be as high as 66 % and 76 % depending on risk factors (Geltner and Lass, 2016).

Aspergillus flavus in Aspergillus section Flavi, is most often associated with food spoilage and toxicity to animals and humans due to its ability to produce the potent toxins and carcinogens called aflatoxins (Cleveland *et al*., 2009) (Abbas *et al*., 2009).

The residue of AF in edible eggs may induce health hazards to human (Cast, 2003). However, residue of AF in poultry meat has been reviewed (Milicevic and Skrinjar, 2010).

The over wintering reservoir of *A. flavus* propagules in soils and plant debris can serve as a primary inoculum for infestation of below-ground plant parts, especially in peanuts. Although soil can serve as the primary habitat for *A. flavus*, *A. parasiticus*, and *A. nominus*, little is known about the life cycle of these fungi in soil. *A. flavus* is capable of surviving and overwintering in plant residues as mycelium (hypha) or sclerotia (Abbas *et al*., 2008) (Horn, 2007) (Payne, 1998)(Wicklow *et al*.,1993) that in turn serve as the source of new conidia

There are few methodologies, all of which are quite tedious, available to ascertain if propagules of*Aspergillus* are actively growing in soil or present in quiescent propagules, e.g., conidia or sclerotia. The majority of studies that evaluate *A. flavus* soil ecology have traditionally used selective plating techniques (Abbas *et al*., 2004) (Horn and Dorner, 1998) (Zablotowicz *et al*., 2007). When using cultural techniques for enumeration such as serial dilution technique and plating, it is difficult to ascertain whether propagules enumerated are present as hyphal fragments or as spores. Sieving techniques can be usedfor the recovery of sclerotia from soil or plant tissue homogenates; however, the actual identification of species can be difficult as sclerotia of a given species can vary tremendously in size and shape (Klich and Pitt, 1988) (Abbas *et al*., 2005) (Horn, 2003). When suitable environmental conditions arise, sclerotia and conidia germinate into mycelia that produce numerous conidiophores and release conidia into the air that can be available for colonizing plants

The *A. flavus* life cycle can be divided into two major phases the colonization of plant residues in soil, and the infection of crop tissues, including grain and seeds of actively growing plant tissues. At the beginning of the growing season, usually in spring and sometimes at the end of winter, when sclerotia are exposed to the soil surface, they quickly germinate and form new conidial inoculum. This new inoculums will be vectored by insects or carried by the wind to begin the colonization and infection of the freshly planted crops (Horn, 2007) (Payne, 1992). During the growing season, infected plant tissues can serve as sources of secondary conidial inoculum, which colonize new non infected plant tissues

Despite our understanding of how the initialand secondary inocula occur for plant infection, little information is available about the saprotrophic activities of these fungi in soil. Recently (Accinelli *et al*., 2008) confirmed the presence of *A. flavus* in the soil actively synthesizing aflatoxins.

Aspergillusflavus is a soil fungus found in temperate regions worldwide. In the United States it is found as a preharvest contaminant of corn, peanuts, cottonseed, and tree nuts. *Aspergillusflavus* was thought to lack a sexual stage, but recent studies have proven that it not only propagates vegetatively through asexual spores (conidiospores or conidia) but also can form ascospores. The sexual teleomorph is called Petromyces flavus (Horn *et al*., 2009). Recent genomic studies have shed light on *A. flavus's* ability to produce aflatoxins and also have revealed its population diversity and evolution (Chang *et al*., 2006).

Individual strains of *A. flavus* are not specialized to any particular host plant or insect (Leger *et al*., 2000). Aflatoxin B1 is the most potent naturally formed carcinogen. It is one of the few mycotoxins that have been developed for use as a biological weapon (Bennett and Klich, 2003). The risk of hepato cellular carcinoma is particularly elevated in individuals with chronic hepatitis B virus infection who also are exposed to AFs (IARC, 2002). Further, the International Agency for Researcher on Cancer (IARC) classifies the aflatoxin M as Group 2B(possibly carcinogenic to humans).

flavus may be isolated in all climatic zones, it is isolated relatively more frequently in warm temperate zones (latitudes 26–35°) than in tropical or cooler temperate zones, and is quite uncommon in latitudes above 45° (Klich, 2002)(Manabe and Tsuruta, 1978). It is therefore not surprising that chronic aflatoxin problems are associated with crops in latitudes below 35°.

flavus, as a species, encompasses two morphotypes and various chemotypes. Some strains produce large sclerotia (L-strain) while others produce numerous small sclerotia (S-strain) (Cotty, 1989). *A. flavus* L-strains can exhibit either a non aflatoxigenic phenotype or they will produce only one type (B) of aflatoxins, and the S-strain morphotype will produce only B aflatoxins (SB), or they can produce B and G aflatoxins (SBG) (Cotty and Cardwell, 1999). Production of G aflatoxins is rarely observed in *A. flavus* L-strains (Ehrlich *et al*., 2004).

1.1.1. Characteristics of Aspergillus

The genus Aspergillus contains more than 100 recognized species and belongs to the phylum Ascomycota.The basic morphological structure of Aspergillus. include conidial head which consists of a swollen vesicle bearing either one or two layers of synchronously formed specialized cells. The specialized cells bearing the asexual spores (conidia) are called phialides. A conidial head with only phialides is referred to as uniseriate. When a second layer of specialized cells (metulae) is present between the vesicle and phialides, the conidial head is referred to as biseriate.
The conidial head is borne on a long stipe, the basal part of which forms the 'foot cell' characteristic of Aspergillus. The structures that support the formation of conidia (foot cell, stipe, vesicle, metulae, and phialides) are collectively called the conidiophore. In addition to the typical conidial state (anamorph) characteristic of the genus, some species also reproduce sexually and have an ascosporic state (teleomorph). (Perng, Chang *et al*., 2014).

1.1.2. Culture Media

A substance on which a mould is grown on in the laboratory is called a medium and the mould growing on it is culture. Culture media can be solid or liquid. For the purposes of identification, solid culture media are usually more useful, as they allow the mould to sporulate more easily (Encyclopedia, 2009)

1.1.3. Characteristics of *Aspergillus flavus* in culture Media

In culture, *Aspergillus flavus* is characterized by fast-growing yellow–green colonies, after 7 days growth in the dark at 25 °C on Czapek yeast extract (CYA), CYA with 200 g sucrose or malt extract agars. It grows well at 37 °C. The stipe of the conidiophore is usually long and rough-walled. Seriation is variable, but usually at least 20% of the aspergilla produce both metulae and phialides on CYA. Conidia are globose to ellipsoidal, mostly with smooth to finely roughened walls Figure (1.1) (1.2) (1.3) (1.4). Black globose to elongate firm-walled structures called sclerotia are produced by some strains. (Klich, 2002) (Raper and Fennell, 1965).

Several species are morphologically similar to *A.flavus*, *Aspergillus oryzae* differs from *A.flavus* in producing colonies that are more floccose and turn brown with age on CYA as well as conidia that are larger. *Aspergillus*

parasiticus colonies are generally darker green, and the conidial walls are very rough; *Aspergillus bombycis* grows more slowly on CYA at 37 °C and has smooth-walled stipes; *A. nomius* is most easily distinguished from *A. flavus* by its mycotoxin profile *A. flavus* produces only B aflatoxins (see below) whereas A. nomius produces both B and G aflatoxins (Klich, 2002). There are also molecular means to differentiate these fungi (Peterson *et al*., 2001)

1.2.4. Classification

Aspergillus is an anamorphic genus consisting of about 250 recognized species. It is characterized by a distinctive spore-bearing structure, the aspergillum.Some members of the genus produce teleomorphs (sexual states). The genus has been divided into a number of sections. *Aspergillus flavus* belongs to section Flavi. This section contains the major economically important aflatoxin-producing fungi *A. flavus and A. parasiticus*. Less common aflatoxin-producing species in this section are *A. nomius, A. pseudotamarii, A. bombysis and A. parvisclerotigenus*. Section Flavi includes a number of other economically important species, including the food fermentation industrial species *A. oryzae and A. sojae*. At the molecular level these two fungi are closely related to *A. flavus and A. parasiticus*, respectively, but they are morphologically distinct and do not produce aflatoxin (Klich, 2002) (Raper and Fennell, 1965).

1.2.5. Global distribution of *Aspergillusflavus*

Surveys of *A. flavus* isolates from various geographic regions have revealed differences in the proportions of isolates that produce low, medium, and high amounts of aflatoxins (Cotty, 1999).

This mold is common and widespread in nature.AFs are most prevalent in latitudes between 40° N and 40° S of the equator, and the greatest health risk lies within developing countries in tropical regions Figure (1.5), which relyon commodities susceptible to contamination by these toxins as their staple food source (IITA,2011). The Food and Agriculture Organization (FAO) estimates that the fungus affects 25% of the world's crops (USAID, 2012). The Centers for Diseases Control (CDC) estimate that more than4.5 billion people are chronically exposed to AF through consumption of contaminated foods such as maize and groundnuts (USAID, 2012).

1.2.6. Presence of *Aspergillus flavus* in Food

Aspergillus flavus is a common soil fungus and is predominately saprotrophic, growing on dead plant tissue in the soil. The species is also a facultative parasite on a broad range of plants and often colonizes oil-rich seeds, such as corn, peanuts, cottonseed, and tree nuts (almond and pistachio), as well as other crops such as barley, wheat, and rice. *Aspergillus flavus* is an opportunistic pathogen of animals and humans, particularly in individuals who are immune compromised. Infection by *A. flavus* has become the second leading cause of human as Apergillosis next to *Aspergillus fumigatus*. Because of its ubiquitous nature, *A. flavus* has been isolated from a wide variety of food items, including dried vine berries, sour lime, cocoa beans, smoked dried meat products, cured ham, dried salted fish, and spices (Perng and Kuang, 1999).

Aspergillus flavus is widely distributed in nature and is largely found at cereal and grains.Before harvest or during storage, *A. flavus* grows on agricultural crops (Saini, and Kaur, 2012). Its growth is affected by the environmental condition such as temperature and relative humidity (Giorni, Leggrieri *et al*., 2012).

Fungi produce aflatoxin in the presence of higher moisture, temperature, and adequate substratum. Before harvest, the risk for the development of aflatoxin is greatest during major droughts, where soil moisture is below normal and temperatures are high, the number of *Aspergillus* spores in the air increases. These spores infect crops through areas of damage caused by insects, and inclement weather. Once injected, plant stress occurs; the production of aflatoxin is favored. During postharvest stage, proliferation of aflatoxin can be exacerbated in susceptible environment (Risk Assessment Studies, 2001)

1.2.7. Preharvest Contamination

Temperature and moisture have a significant effect on the host–pathogen interaction because of their combined effect on both the host plant and the fungus (Schmidt, Heydt *et al*., 2009). Under conditions optimum for these fungi (i.e., high temperature and low moisture), they thrive and outcompete other soil and plant microflora. Under such conditions, the fungi are able to produce abundant conidia that are easily dispersed in the air. These conditions may allow *A. flavus* to outcompete other microflora on the seed surface, placing them in an ideal position to colonize both insect-injured or otherwise susceptible seeds.

Under drought conditions, many of the physiological defense systems of the host plant are compromised due to high temperatures and water stress. Further, these conditions often lead to cracks in the seed, which allow the fungi to breach the seed's structural barriers. An example of an ear of corn contaminated by *A. flavus* is injury especially that caused by insects, is very important in the epidemiology of *Aspergillus flavus* infection (Abbas *et al*., 2009). Abundant sporulation of *A. flavus and A. parasiticus* is often observed on developing seeds damaged by insects. Injury not only allows an easy means of entry to the fungus, but it also provides increased access to plant nutrients and a more favorable aerobic environment for sporulation and aflatoxin production. Also causes dehydration of the kernels, thus creating a more favorable environment for growth and aflatoxin production. Studies found that only minor injury to the seed is needed to increase aflatoxin contamination.

1.2.8. Postharvest Contamination

Aspergillus flavus can also rot improperly stored grain and contaminate the grain with aflatoxins (Magan and Aldred, 2007). The two major environmental conditions for contamination, like those for preharvest contamination, are temperature and moisture. Properly dried grain does not support growth of the fungus. Insect activity in stored products sometimes creates favorable microclimates for fungal growth, and once fungal growth starts, the water from metabolism by the fungus provides sufficient additional water for further growth and mycotoxin development. The contamination of aflatoxins in various foodstuffs and agricultural commodities is amajor problem and may vary with geographical conditions, production and storage practices, and also with the type of food. The pre-harvest practices employed in crop production such as tillage practices, fertilizer application practices, crop rotation, plant population, planting date and irrigation if not effectively managed, has a significant association of high aflatoxin levels (Campos *et al*., 2008).

1.2.9. Biology and Habitat of *Aspergillus flavus*

Aspergillus flavus is found in temperate regions of the world as well as in subtropical regions (Abbas *et al*., 2009). From an agronomic perspective, *A. flavus* is a plant pathogen, but living tissue is only a minor substrate for these soil-borne filamentous fungi. From an ecological perspective, *A. flavus* grows mainly on dead matter (saprophytically) and can grow on a wide variety of substrates including decaying plant and animal debris found in the soil, where it

must compete with the other soil microflora. The two major factors that influence soil populations of *A. flavus* are soil temperature and soil moisture. *Aspergillus flavus* can grow at temperatures of 12–48° C and at water activity (aw) as low as 0.80. The optimum temperature for growth is 25–42 °C. Fungal growth and conidial germination are ideal at water activity greater than 0.90 and are completely inhibited at aw <0.75. Thus, these organisms are semi thermophilic and semixerophytic. When *A. flavus* interacts with plants, the primary source of inoculum (predominantly conidia) appears to be from the soil. Existing data suggest that fungal mycelia in debris are most likely the primary soil propagule (Probst*et al*., 2010) (Horn and Dorner, 2009) (Dorner, 2009). The presence of sclerotia (highly melanized, compacted mycelial bodies) in infected tissue and in the soil in the southern United States suggests that these structures play an important role in fungal survival when conditions are unfavorable for growth and propagation. For peanuts, populations of the fungi in the soil are important in contaminating the pods, whereas for other plant hosts, airborne conidia appear to be most important (Horn and Dorner, 2009).
Climate change can result in fragmentations and bottlenecks, eliminating or reducing the population sizes of endemic species, and selecting for more fit taxa (Ali and Roossinck, 2008), (Opdam and Wascher, 2004). Climate has been reported to influence the aflatoxin producing ability of *A. flavus*, alter the numbers of aflatoxigenic fungi in the environment, and change fungal population structure (Cotty and Jaime, 2007). Soil type (e.g., clay, sand) and quality factors such as pH and mineral content can impact the presence and distribution of *Aspergillus* in a field (Ahmad and Singh, 1994) (Wassila *et al*., 2015).

1.2.10.Impacts of *Aspergillus flavus*

- Deterioration of food and nutritional quality of agricultural products with an accompanying reduction in sensory characteristics, e.g., taste, odor, texture and color
- Health-related productivity losses due tomutagenic and carcinogenic effects on humans who consume aflatoxin-contaminated food over an extended period of time.
- Loss of income from livestock resulting from feeding aflatoxin contaminated feedstuffs, e.g., higher mortality rates and lower feed to weight conversion ratios for chickens, ducks, egg-layers, and pigs
- Loss of export market and related economic losses due to regulations that restrict international trade of aflatoxin-contaminated grain (Lubulwa and Davis, 1994).

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1.2.11. Methods for Detection of *A. flavus* in Foods

Generally, detection of *A. flavus* in foods and feeds is carried out by using traditional microbiological plating methods, by either surface spread or direct plating of kernels and seeds (Klich, 2006). Media used for detection include potato dextrose agar (PDA), acidified PDA, and PDA with antibiotics such as chlortetracycline, chloramphenicol, oxytetracycline, gentamycin, and streptomycin. Because these fungi are semixerophytic, a selective medium containing up to 7% sodium chloride has been used to isolate *A. flavus*. A differential medium, called Aspergillus differential medium (ADM), contains ferric citrate (0.05%) as the differential ingredient. This compound reacts with *A. flavus* metabolites such as kojic acid and aspergillic acid to produce a bright orange–yellow pigment on the reverse side of the colony. Dichloran and chloramphenicol have been added to ADM to make a new medium called *A. flavus* and *A. parasiticus* agar (AFPA). This medium contains peptone 10 g, yeast extract 20 g, ferric ammonium citrate 0.5 g, chloramphenicol 100 mg, agar 15 g, distilled water 1 l and dichloran 2 mg (the final pH of 6.2). Cultures on AFPA are routinely incubated at 30° C for 42–48 h. Dichloran inhibits spreading of fungi, and chloramphenicol inhibits bacterial contamination. *Aspergillus flavus* and *A. parasiticus* are identified on this medium by production of typical yellow to olive green spores and a bright orange reverse. This medium permits rapid identification of *A. flavus and A. parasiticus* (within 3 days) because these fungi grow rapidly at 30° C. Another advantage of the use of this medium is the isolation and identification of potentially aflatoxigenic fungi from other aspergilli. For example, *Aspergillus niger* produces a yellow but not orange reverse color, and after 48 h of incubation *A. niger* starts to develop its dark brown to black conidia, which easily distinguish it from *A. flavus*. *Aspergillus ochraceus* grows relatively slowly at 30° C, and the yellow color appears after 48 h. (Perng and Kuang, 2014).

1.2.12. Entry of *Aspergillus Flavus* to the Plant

Once conditions of temperature, drought stress and inoculums levels are met, *A. flavus* is capable of entering the plant through a number of portals. This variability has made development of control measures more difficult. Insect damage is associated with increased aflatoxin in all affected crops. The insects damage plant tissue, thereby creating entry portals for the fungus. Bright

greenish yellow (BGY) fluorescence from *Aspergillus flavus* is frequently associated with insect damage, indicating that insects provide entry or act as vectors (Lee *et al*., 1977) (Marsh *et al*., 1969). Aflatoxin does occur, however, in the absence of apparent injury. In corn, *A. flavus* may be brought to the surface of developing seeds by insects or by colonizing the silks and growing down to the seed area. *Aspergillus flavus and A. parasiticus* do invade young peanut plants systemically as seedlings from seed and soil and disseminate throughout the plant, but stems and roots are more commonly infected than leaves and petioles (Pitt *et al*., 1991). Recovery of these fungi was shown to be higher in root and pod tissue than other plant parts in more mature peanuts (Kisyombe *et al*., 1985)

1.2.13. Pathogenicity *Aspergillus flavus* in plants and Humans

Aspergillus flavus is a minor pathogen of corn, peanuts and cotton. In corn, *A. flavus* causes an ear rot (Taubenhaus, 1920). In peanuts, it causes a seedling disease known as yellow mould of seedlings or aflaroot. Aspergillus species cause several allergic and infective conditions of humans and certain other vertebrates. These include allergic broncho pulmonary aspergillosis and invasive pulmonary aspergillosis. The most common cause of most of these conditions is *Aspergillus fumigatus*. However, other aspergilli, including members of the *A. flavus* group, are sometimes implicated (Hedayati.,*et al* 2007).

1.2.14.Molecular biology applications to *Aspergillus* ecology and aflatoxin contamination

To achievea more complete picture of *Aspergillus* spp. ecology,rapid methods of identification are needed so that a broader array of conditions can be investigated.Unfortunately *Aspergillus* species are not suitablefor molecular techniques such as fluorescent in situ hybridization (FISH). The molecular ecology of several bacteria and fungi has been advanced by (FISH) coupled with autoradiography; however, the nucleotide probes are unable to penetrate through the cellwalls of *Aspergillus* (Teertsta *et al*., 2004). Thus, the majority of ecological population studies rely up one numeration and vegetative compatibility groups to characterize *A. flavus* (Papa, 1986).

These traditional methods are based upon morphological and genetic characteristics and are boththime consuming and complex (Klich and Pitt, 1998) (Klich *et al*., 1992). Recently, additional molecular techniques have been

developed to help identify and classify these fungi (Wilkinson and Abbas, 2008). One of the more reliable molecular methods for distinguishing *Aspergilli* is ribosomal DNA (rDNA) comparisons. Comparisons of variations within 16S rRNA gene sequences of bacterial species and 18S rRNA gene sequences for eukaryotes are used extensively for ecological investigations. In particular, the internal transcribed spacer (ITS) regions are useful for identifications of fungal species and strains within a species (Hugenholtz and Pace, 1996) (White *et al*., 1990). PCR amplification and sequencing has also been used for resolving filamentous fungal species, including *A. flavus* (Accinelli.,*et al* 2008)(Brun*et al*., 1991)(Peterson, 2008)(Scully and Bidochka, 2006) (White *et al*., 1990), using comparisons based upon the aflatoxin biosynthetic pathway, which is unique to the*Aspergilli*.The genes involved in the aflatoxin biosynthesis pathway have been identified and sequenced(Bhatnagar *et al*., 2003)(Scheidegger and Payne, 2003) (Yu *et al*., 2004). These genes are highly conserved in multiple *Aspergilli*, most notably *A. parasiticus* and*A. flavus.* PCR based on selected genes from the biosynthetic pathway are slowly being adapted for detection, identification, or examination of ecological aspects of *A. flavus*. Amplification of these specific genes is extremely sensitive and has the potential to be used to detect the presence of *A. flavus* in agricultural commodities and environmental samples, including soil and insects (Accinelli *et al*., 2008)(Criseo *et al*., 2001)(Geisen, 1996)(Manonmani *et al*., 2005).
Recently completed and ongoing studies using molecular techniques are beginning to greatly increase our knowledge of *A. flavus* population structures in soil. PCR has been employed to identify the presence of *Aspergilli* in the soil by (Accinelli *et al*., 2008), whileRT-PCR (reverse transcription-polymerase chain reaction) has also been employed to distinguish toxigenic and nontoxigenic *A. flavus* (Degola *et al*., 2006) (Scherm *et al*., 2005), as well as to track the presenceof aflatoxin biosynthetic genes (Accinelli *et al*., 2008). The novel technique of pyrosequencing may prove useful in quantifying the presence of introduced *A. flavus* strains in mixed communities of *A. flavus* (Das *et al*., 2008).

1.2. Mycotoxin

Mycotoxins are toxic substances produced by fungi that contaminate various food and feedstuffs. There are about a hundred different types of mycotoxins which are produced by awide range of fungal species *(*Bennett and Klich, 2003).

In tropical and subtropical climates, such as seen in Brazil, the fungal development finds favorableconditions of humidity and temperature (Dilein, 2002). However, the occurrence of mycotoxins is not only aproblem from developing countries. Agribusiness in many countries is affected, and this may interfere with even prohibit exportation, reducing animal and agricultural production (Leung *et al*., 2006).

Mycotoxicosis symptoms depend on several variables that interacts synergistically, includingthe mycotoxin chemical nature, the exposure time (duration and dose), the organism that intakes themycotoxin (species, sex, age, health, diet), and the mixed effects of mycotoxin with otherxenobiotics. The effects exerted on vertebrates could be chronic (low doses, long periods of time) or acute toxic (high doses, short periods of time), mutagenic, teratogenic, carcinogenic, nephrotoxic,hepatotoxic, immunotoxic and estrogenic. The main target organs depend on the mycotoxin and the organisms that ingest it, include liver, kidney, lungs, nervous, digestive, endocrine and immune systems (Bhatnagar *et al*., 2002). In general, more than one mycotoxin is found instaples, and a mix of them is thus usually ingested. The interaction between mycotoxins can producedifferent effects in the organism: antagonists, additive or synergic, which are linked to the mycotoxin nature, the decontamination pathway, of the host species, the time of exposure, and the doses andratio of mycotoxins (Peraica *et al*., 1999)(Alassane ,Kpembi *et al*., 2017).

Livestock production can be threatened when feeds are contaminated by fungi and their toxic metabolites. Several mycotoxins, including aflatoxins (AFs), cyclopiazonic acid(CPA), fumonisins (FUMs), nivalenol (NIV) and zearalenone (ZEN) have been reported to contaminate poultry feed and their ingredients (Labuda *et al*., 2005)(De Boevreet *et al*., 2012). Multiple mycotoxins can be produced by one fungi or can be combined from different sources of contamination (Devegowda and Murthy, 2005).

Levels of mycotoxins in the feed largely depend on the mycotoxins in the individual feed ingredients, the mix proportion of feed ingredients, feed processing techniques, and storage practices (Warth *et al*., 2012)(Ezekiel *et al*., 2014).

In animals, ruminants have a gastric system with a rich microbiota that facilitates the degradation of mycotoxins, whereas monogastric species, like pork and poultry, are especially sensible to mycotoxicosis because intestinal microbiota is

less diverse. Poultry is less prone to biotransform toxins to less toxic compounds before the intestine absorbs them. For ruminants it has been documented that rumen function is nevertheless affected negatively by the presence of mycotoxins, as well some biotrasformation can produce toxic products, which can be excreted thus making it available, like the case of AFM1 (Hussein and Brasel, 2001). The major problem for livestock and poultry associated with ingestion of mycotoxins is the poor animal performance. Which can be difficult to diagnose and quantify because of the diversity of life histories, physiological status, biotransformation pathways, detoxification mechanisms and the intra- and inter variability of species that ingested them Similarly, the type and level of mycotoxin in feed, the time of the exposure, and the interaction between mycotoxins are also a problem (Bryden, 2012)(Alassane , Kpembi *et al*., 2015)

At the end of 1959, peanuts from Brazil were imported in England as protein supplements in farming feeds. Soon afterwards, young turkeys began to die and other animals such as pigs fell ill. 100,000 turkey poult were killed by the so-called “turkey X disease”, “X” referring to its supposedly viral origin (Cole, 1986). Shortly after, aflatoxins were identified as the source of this intoxication (Nesbitt *et al*., 1962).

The reason for the production of mycotoxins is not yet known; they are not necessary for the growth or the development of the fungi(Fox and Howlett, 2008)Because mycotoxins weaken the receiving host, the fungus may use them as a strategy to better the environment for further fungal proliferation.The production of toxins depends on the surrounding intrinsic and extrinsic environments and these substances vary greatly in their toxicity, depending on the organism infected and its susceptibility, metabolism, and defense mechanisms(Hussein and Brasel, 2001)

Before 1900, in Italy, researchers believed consumption of moldy corn by children led to the development of illness (Christensen, 1975). Some experiments, done at that time, included the isolation, and growth of the suspected fungus in pure culture, and isolation of toxic compounds from the fungus that the researchers believed to be the cause of the illness. However, since the compound was not identified and was not actually isolated from the moldy corn, it could not be concluded that this compound was the cause of the illness or that the compound in question was even present on the moldy corn (

Burnside *et al*., 1957) studied an extensive outbreak of moldy corn disease in the southeastern United States in the early 1950's where hundreds of wild pigs for aging in cultivated corn fields became ill, and many died. Teams of veterinarians and mycologists collaborated to determine the cause of the deaths of these pigs. They isolated a number of different fungi from the moldy corn and inoculated each fungus on moist corn that had been sterilized and then fed them to pigs. The consumption of corn inoculated with *Aspergillus flavus* caused outward signs and inward lesions found in other cases of the so-called moldy corn disease. However, since there was no toxin(s) isolated, there was little attention paid to this paper since it still seemed like old news until 1960, when approximately 100,000 turkeys and a lesser number of other domestic birds died in England, causing losses of approximately several hundred thousand dollars, before the first mycotoxin would be isolated and identified. Initially, the disease was thought to be caused by a virus and the syndrome was named "turkey-X disease". The "X" here indicated that the cause of the disease was unknown. However, with a great deal of detective work, on the part of the researchers, soon the cause of the disease was traced to feed that was produced by Oil Cake Mills, Ltd. (research always seems to get done more quickly and receive more priority when loss of large sums of money is involved). The oil cake feed was composed mostly of peanuts. However, it seemed unlikely that the peanut meal itself was toxic, since peanut meal had long been used as a feed ingredient and was known to be an excellent source of protein. Thus, it was reasoned that something must have been added to the peanut meal to make it toxic, and one possibility that was investigated was that peanuts had been made toxic by fungi growing in them. From their isolations, the investigators identified *Aspergillus flavus*, the same fungus that was isolated by.(Burnside *et al*., 1957) and his research teams.(Jay, 1970) and(Banwart, 1981) in their study, observedthere are certain conditions needed for the contamination of food with mycotoxins. First mycotoxin producing fungus must be present. These fungi are widely spread and have been found in many foods or feeds. Secondly, the food must be suitable as a substrate and environmental factors must be acceptable for growth of the fungus and elaboration of the mycotoxin. Growth is not synonymous with the presence of mycotoxin, as some substrates are suitable for growth but not suitable for production of the secondary metabolite. These mycotoxins areproduced primarily by *Aspergillus parasiticus*and *A. flavus* which may invade agriculturalproducts during plant growth, during harvest and finally in storage, resulting in significanteconomic losses (Bennett *et al*., 2009). The three main

mycotoxins producers are *Aspergillus, Penicillium and Fusarium genera*. Currently, 300 to 400 mycotoxins are known, among which 30 have been studied for their toxic and or disturbing impacts for human and animal (Bennett and Klich, 2003) (Boudergue *et al*., 2009).

(Geoffrey, 1979) reported that mycotic abortions due to *Aspergillus* have been found in cattle. In survey of 1828 abortions conducted in Britain, mycotic infection was found in 5.8 percent of fetal stomachs and 19.2% of 177 aborted after brith. In NewYork State (Hillman ,1969) found mycotic placentitis to be the most frequently identifiable cause of cattle abortions.

In fact, all countries with mycotoxin regulations in 2003 have at least regulatory limits for aflatoxin B1 or the sum of aflatoxins B1, B2, G1 and G2 in foods or feeds, a situation that was also observed in 1995. The number of countries regulating mycotoxins has significantly increased over the years. Comparing the situation in 1995 and 2003, it appears that in 2003 more mycotoxins are regulated in more commodities and products, whereas tolerance limits generally remain the same or tend to decrease.

The majority of the African countries, specific mycotoxin regulations (probably) do not exist. The fact that countries have no specific regulatory limit for mycotoxins does not mean that the problem is ignored. Several of these countries recognize that they have problems due to mycotoxins and that regulations should be developed, and they indicated this in their responses to the inquiry.The mycotoxin issue in Africa needs to be viewed, however, in the overall context of local food safety, health and agricultural issues (Shephard, 2004).

Figures (1.6) and (1.7) illustrate the occurrences of the regulatory limits for various mycotoxins in Africa in food and feed respectively.

1.3.1. Interaction between mycotoxins and Poultry immunity

With the consumption of contaminated food, cells of the intestinal mucosa, which possesses both components of innate as specific immunity may be exposed to large concentrations of these toxins (Prelusk *et al*., 1996). As described by (Bouhetand Oswald, 2005), the function performed by the physical barrier of the intestinal epithelium is achieved by trans-epithelial electrical resistance that exists in the cell monolayer. Some toxins may affect this trans-

epithelial electrical resistance in the intestinal mucosa (Mclaughlin *et al*., 2004) explained this can happen due to the decrease in the amount of proteins found in

the cell junctions.On the other hand, cells of the intestinal mucosa which make this innate physical protection are comprised of a constantly renewed tissue to maintain the integrity of the epithelium, which occurs from the proliferation of differentiated cells from the crypt, which differentiate and move along, being eliminated by extrusion at the height of intestinal villi. It is also known that mucus production has an important function as a lubricant and protective barrier of this epithelium, and when the intestinal mucosa is challenged, there is an increase in the number of these cells in the intestine, with increased mucus production. The influence of toxic fungal metabolites in mucosal immunity can greatly affect the animal performance, as the induction of immunity is very important to ensure protection against various pathogens which typically invade

these surfaces (Strea and tfield ,2006).
The administration of oral vaccines is a very practical and economical route of animal immunization; however, when mycotoxins interfere with the immune response, the result of vaccination may be affected. Several changes observed in the scientific literature show that mycotoxins alter the immune response of animals, may interfere with the vaccine response and make the animals susceptible to nonspecific infections, which animals have not been vaccinated against (Gertner *et al*., 2008).

There are six known types of mycotoxins that affect animals (Binder and Eva, 2007)

Table (1.1): Significant mycotoxins produced by the *Aspergillus flavus* group and their toxic effect

Mycotoxin(s)	Toxicity	Species producing
Aflatoxins B1 and B2	Acute liver damage, cirrhosis, carcinogenic (liver), teratogenic, immunosuppressive	*A. flavus, A. parasiticus, A. nomius*
Aflatoxins G1 and G2	Effects similar to those of B aflatoxins: G1 toxicity less than that of B1 but	*A. parasiticus, A. nomius*

	greater than that of B2	
Cyclopiozonic acid (CPA)	Degeneration and necrosis of various organs, tremorgenic, low oral toxicity	*A. flavus*

1.3.2. Mycotoxin prevention

Mycotoxins show that there are three ways to preventing them from contaminating feed

- Firstoccurs before there is a possibility of fungal infection.
- When the fungi are starting to produce thetoxins.
- When the material is known to be heavily infected.*(*Jouany and Jean, 2007)

After contamination of grains and feed, different methods of decontamination can be used, including biological, physical and or chemical procedures. According to

(Eman, 2000),the ideal decontamination process must be easy to apply, economical, should not lead to the formation of compounds which retain toxicity, or change the nutritional properties and palatability of grains and feed. The degree ofdecontamination depends on the used method and the toxicity that remains in the sample to be treated(SorianoandDragacci, 2004).

1.3.2.1 .Biological agents

The decontamination by biological agents, which began in the 1960s, has been using microorganisms, as fermentation processes, started in the 1980s, employ the use of yeast (Bata and Lasztity, 1999).

In recent years, compounds present in plant extracts have also been used, since the biological agentcompete with the fungus by the ecological niche in the plant or food (Bacon *et al*., 2001). Some extractsinhibit fungal growth and transgenics increase the plants' resistance to fungi and insects (Duvick, 2001).

1.3.2.2. Physical agents

The effectiveness of the method depends on the physical milling and manufacturing to which grainsare subjected, since many mycotoxins are inside the grain, and removal of the germ and pericarpeliminates a large amount of toxin. This, however, is a preliminary process of decontamination. The dry orwet milling may reduce the mycotoxin content in food (Soriano and Dragacci, 2004).

In dry milling, anincrease in the concentration of mycotoxins occurs in the pericarp. In wet milling, toxins migrate to the aqueous solution, reducing its concentration in the grain (Eman, 2000). The use of elevated temperatures is also an alternative for the removal of toxins, but the time and temperature used in the process should be considered (Seefelder *et al*., 2003). Processes such as grain flocculation and extrusion can also reduce the content of toxins (Kim *et al*., 2003).Adsorbent materials, with capacity to bind mycotoxins and immobilize them in the gastrointestinal tract of animals, reducing the bioavailability of the toxin, are widely used.

Anotherphysical method is the use of radiation.

1.3.2.3. Chemical agent

For the chemical form of partial decontamination, water and sodium bisulfite solution, sodium chloride and ammonium hydroxide washing is used, although, in this process, toxins must be not totallyeliminated (Sorianoand Dragacci, 2004).The physical and chemical methods have the disadvantage of not being totally effective and may result in loss of nutrients and generate high costs, making many studiesreport the best solution in the future will be decontamination by biodegradation.

1.3.3. Other methods

Other method of prevention of mycotoxins include

- planting species that are able to defend naturally against mycotoxins, proper fertilization, weed control, and proper crop rotation
- The way the crops are storedafter harvesting alsoplays an important role in staying mycotoxin free. If there is too much moisture then fungi has a better chance of growing and producing mycotoxins. Along with moisture levels, factors such as temperature, grain condition.

- Chemical or biological can determine whether or not mycotoxin producing fungi will grow(Binder and Eva, 2007).

1.3.4. Impact of mycotoxins

The presence of mycotoxins in staples is a major concern, not only for public health, but alsofor their economic impact. Food commodities losses due to mycotoxin contamination representabove 25% of spoiled food (FAO, 2003). For instance, only in the United States the Food and DrugAdministration (FDA) has estimated that the losses exceed $900 million per year (Cast, 2003). Due to its impacts, food security associated to mycotoxin contamination is a major issue worldwide;public health commissions all over the world try to ensure safe and healthy feed and food for animalsand humans (Stoev, 2013). In developed countries, food security is carried out better than indeveloping countries, in which food quality monitoring and the infrastructure to avoid mycotoxincontamination are more difficult to settle. Nevertheless, regulation of mycotoxins reduced the intakein those countries that have proper regulation and monitoring, and increase exportation standardsaround the world. On the other hand, it could result in a higher risk of consumption of mycotoxins by human and animal populations of developing countries, as the best quality staples are exported,whereas the poor quality ones remain for domestic consumption (Wild and Gong, 2009)(Stoev, 2013).As aforementioned, the global distribution of mycotoxins is not homogenous, the conditions ineach region will favor the development of certain fungi over others, thus favor some mycotoxins overothers.

In addition, climate change is shifting distribution and prevalence of some fungi, and thereby,mycotoxin distribution.(Streit *et al*., 2013) determined the presence of the main mycotoxins(aflatoxins, zearalenone, deoxynivalenol, fumonisins and ochratoxins) around the world for a periodof eight years. Their results showed that most of the samples (72%) were mycotoxin positive, and 38% showed a multicontamination (more than one mycotoxin). In addition, they determined that thepercentages of each mycotoxin were more or less stable during the years, with the exception ofaflatoxins, their level increased between 2005 and 2009 in tropical regions.

1.3.5. What is a secondary metabolite andmycotoxin

In order to cope with their environment, fungi have developed the ability to produce severalextracellular chemicals, called secondary metabolites, which are not essential in the primarymetabolism of fungi (*i.e.* growth, reproduction, respiration), and not required for their survival whengrowth in laboratory conditions. These compounds are low-weight molecules (< 1000 Daltons) produced by their secondary metabolism, which encloses the molecular pathways that are notessential for the survival of the organism (Bennet, 1987) (Bennet and Klich, 2003). These molecules arediverse in their chemical nature, including polyketides (Aflatoxin-Lovastatin- Fusarin- Patulin- Fumonisin and Zearalenone), non-ribosomal peptides, terpene, indoleterpenes and hybrids. These organisms are capable to produce a large number of thesecompounds, and their secondary metabolic profile will vary depending on the genetic information (presence of secondary metabolic gene clusters), environmental conditions (mainly nutrients andwater availability), and community composition (Brakhage, 2013), (Bills and Gloer, 2016)

1.3.6.Climate and mycotoxin prevalence

It is common knowledge that different mycotoxin producing fungal species favor different climate conditions making mycotoxin prevalence highly variable across the world. Mycotoxins, such as trichothecenes, are typically regarded as toxins of the regions with temperate climate, while the presence of others, such as aflatoxins, are associated with tropical and subtropical climate areas. A large amount of information is available in the literature on the environmental factors impacting the presence of mycotoxins in food and feed crops e.g (Van and Kler *et al*., 2012)(Battilani *et al*., 2012)(Paterson and Lima, 2011). A year-to-year variation occurs. While on the other years high mycotoxin contamination rates are observed, on the other years the prevalence of mycotoxins is low (Edwards and Jennings, 2018) (Kos *et al*., 2013) (Meyer *et al*., 2019). Climate change is further believed to affect fungal growth and agricultural practices and, subsequently to the mycotoxin incidences and levels on the agricultural crops (Battilani *et al*., 2013) (Paterson and Lima, 2011) (Van and FelsKlerx *et al*., 2016). It is also projected that changes in climate will shift the geographic distributions of mycotoxin-producing fungi and hence the patterns of mycotoxin occurrence in the world. The amount of mycotoxin contaminated crops are generally anticipated to increase with the global warming, but at the same time

major variations in mycotoxin contamination are also expected as well as decreased occurrences of mycotoxins (Battilani *et al*., 2013) (Paterson and Lima, 2011) (Van and Klerx *et al*., 2016). Furthermore, most of the mycotoxins are known to be chemically stable, with pre- and post-harvest mitigation strategies, such as with food and feed processing; only eliminating them to a certain extent (Leslie and Logrieco, 2014).

1.4. Aflatoxins

Aflatoxins were first discovered and characterized in the early 1960s after more than 100 000 turkey poult in England died of apparent poisoning from mould-contaminated peanut meal (Blout, 1961)(Goldblatt, 1969). There are two general forms of the disease caused by exposure to aflatoxin, aflatoxicosis. Acute aflatoxicosis results in death. Chronic aflatoxicosis causes cancer, with the liver as the primary target organ, immune suppression, teratogenicity and other symptoms (Bennett , Klich, 2003). There is also some evidence that respiratory exposure to aflatoxin increases the occurrence of respiratory and other cancers (Dvorackova, 1990).These toxins are named after the fungus producing them, e.g. "A"from the genus name *Aspergillus*, "fla" from the species name *flavus* added to toxin to givethe name aflatoxin. There are 18 different types of toxins in the aflatoxin group identified.Among these aflatoxin toxins, the major members are aflatoxins B1, B2, G1, G2, M1 and M2(Wrather, 2008). Predominant species with aflatoxin production ability include *A. flavus and A. parasiticus* (Yu.,*et al* 2004). Which are known to affect plants including cereal grains, figs, nuts, and tobacco(Binder, Eva , 2007)) Cereal grains are one of the main ingredient in animal feed. The animals most at risk of having serious problems with aflatoxins are trout, ducklings, and pigs, while cattle are less at risk. Another animal feed product is genetically altered grass and animals including cattle, sheep, and horses eat tons of it. Some of the conditions that result from ergot ingestion in animals include gangrene, abortion, convulsions, hypersensitivity, and ataxia(Binder, Eva, 2007).

Aflatoxins are prevalent in food crops, particularly maize, groundnuts, oilseeds and tree nuts, in tropical and subtropical regions worldwide. Host crops are particularly susceptible to infection by Aspergillus following exposure to high humidity and temperature, or damage from stressful conditions such as drought and insects and the average aflatoxin-producing potential of the fungal community associated with the crops (Cotty , Jaime.,*et al*2007). Crops can

become contaminated in the field, during harvesting and after harvest during food storage, transportation and processing (Probst *et al*., 2010) (Wu *et al*., 2011). While the presence of Aspergillus in food products does not always indicate harmful levels of aflatoxin are present, it does imply a significant risk in consumption, particularly in food products with frequent and high consumption in developing world populations.Maize and groundnuts are the major source of aflatoxin exposure in humans because of the frequent and high consumption rates of these foods worldwide and their susceptibility to Aspergillus infection (Strosnide *et al*., 2006).

Of at least 16 structurally close aflatoxin has been detected to date, aflatoxins B1, B2, G1 and G2 are the major four (Goldblatt, 1969). Most studied aflatoxin is aflatoxin B1 as it is identified to be most toxic and potentially hepato carcino genic (Bennett and Klich, 2003). Considerable amount of crop and livestock losses occur due to contamination of toxigenic fungi and mycotoxin. Economic loss increased also due to conducting regulatory programs to prevent such contamination. Feeding livestock and poultry with aflatoxin contaminated feeds can cause death and immune suppression as well as growth reduction. Lowe yields of animals and crops can also be occurred due to aflatoxin contamination (Phillips *et al*., 1996).

Producing strains of *A. flavus* can produce AFB1 and AFB2 (Horn and Corner,1999).Contamination by AFB1 can occur in several agricultural products and commonly found in maize. AFB1 production on maize feed appeared to be greatly influenced by environment under hot and humid weather conditions and it may cause serious consequences to animal health (Hussein and Brasel, 2001). Several reports have shown the impact of AFB1 on animal health including pigs (Harvey *et al*., 1989) (Lindemann *et al*., 1993), poultry birds (Espada *et al*., 1992) and dairy cows (Fink and gremmels,2008)

The highest levels of aflatoxins are usually found in warmer regions of the world where there is a great deal of climatic variation (Paterson and Lima , 2011).It is important to recognize that, although it is primary food commodities that usually become contaminated with aflatoxins by mould growth, these toxins are very stable and may pass through quite severe processes. For this reason they can be a problem in processed foods, such as peanut butter (Volkel ,2011)

At high enough exposure levels, aflatoxins can cause acute toxicity, and potentially death, in mammals, birds and fish, as well as in humans

(Sahoo,2001). The liver is the principal organ affected, but high levels of aflatoxin have also been found in the lungs, kidneys, brains and hearts of individuals. Chronic toxicity is probably more important from a food safety point of view, certainly in more developed regions of the world.

Aflatoxin B1 is a very potent carcinogen and a mutagen in many animals, and therefore potentially in humans, and the liver is again the main target organ. Ingestion of low levels over a long period has been implicated in primary liver cancer, chronic hepatitis, jaundice, cirrhosis and impaired nutrient conversion (Wild,2009).
Aflatoxins may also play a role in other conditions, such as Reye's syndrome and acute liver failure (a childhood condition linked to malnutrition) (Kocabas,2003) (Rogan,1985). Less is known about the chronic toxicity of aflatoxin G1 and M1, but these are also thought to be carcinogens.

1.4.1. Threatening of acute poisoning

Large doses of aflatoxins lead to acute poisoning (aflatoxicosis) that can be life threatening, usually through damage to the liver. Outbreaks of acute liver failure (jaundice, lethargy, nausea, death), identified as aflatoxicosis, have been observed in human populations since the 1960s. Most recently deaths attribute to aflatoxins were reported during the summer of 2016 in the United Republic of Tanzania. Adults are more tolerant to acute exposure than children. The consumption of food containing aflatoxin concentrations of 1 mg/kg or higher has been suspected to cause aflatoxicosis. Based on past outbreaks it has been estimated that, when consumed over a period of 1–3 weeks, an AFB1 dose of 20–120 μg/kg bw per day (μgram is one billionth [1×10^{-9}] of a kilogram) is acutely toxic and potentially lethal.(WHO, 2018).
Aflatoxin is associated with large scale deaths of livestock, such as chicken, trout (Fish) and other species of animals around the world, which ultimately proves that the fungus poses a threat to food supplies and overshadows its ability to decompose plant materials (Goldblatt, 1969). In a recent study, in some countries, such as Gulf countries, India and Sudan, *A. flavus* is the predominant etiological agent among patients with fungal rhinos inusitis and endohthalmitis (Rudramurthy *et al*., 2011).

1.4.2. Infection of Aspergillus flavus in Humans

Most human exposure comes from nuts and grains Long-term or chronic exposure to aflatoxins has several health consequences including:

- aflatoxins are potent carcinogens and may affect all organ systems, especially the liver and kidneys; they cause liver cancer, and have been linked to other types of cancer AFB1 is known to be carcinogenic in humans; the potency of aflatoxin to cause liver cancer is significantly enhanced in the presence of infection with hepatitis.
- aflatoxins are mutagenic in bacteria (affect the DNA), genotoxic, and have the potential to cause birth defects in children
- children may become stunted, although these data have yet to be confirmed because other factors also contribute to growth faltering e.g. low socioeconomic status, chronic diarrhoea, infectious diseases, malnutrition
- aflatoxins cause immunosuppression, therefore may decrease resistance to infectious agents
- Aflatoxins ingested by the birds accumulate in different tissue organs and eggs and entered into human food chain which posses a major risk to human health, as these aflatoxins are not inactivated by dry heating at160 °C for one hour or by steam heating (Carnaghan, 1964) and are unlikely to be affected by cooking. In poultry the severity of clinical disease and lesions of aflatoxicosis may vary with varying levels of dietary aflatoxins (Espada.,*et al*1992).

- Allergies and asthma are known to be triggered by active host immune responses to the presence of fungal spores or hyphae (Bennett.,*et al* 2010). *Aspergillus* can cause allergic responses and colonising and invasive diseases as well as becoming an immune competent host (Widstrom.,*et al* 2003). Atopy is a type of genetic predisposition that causes a person to develop certain hypersensitive reactions, such as asthma, hay fever (allergic rhinitis) and food allergies. An allergic reaction to *Aspergillus* in atopic individuals can be triggered by fungal spores in the air and from fungi ingested in food. Airborne spores are readily inhaled when we breathe or when they come into contact with our eyes and other exposed body parts. Molds are involved in the initiation and exacerbation of the lower airway diseases, such as asthma, although the specific aetiology is poorly understood (Bush *et al*., 2006) (Bennett *et al*., 2010). The inhalation of spores varies enormously according to the local environmental conditions. Some forms of these ill-defined human diseases have been particularly associated with exposure to high concentrations of *Aspergillus* spores. Predominantly it is for the lungs of farmers, malt workers, compost workers and bird fanciers (Bennett *et al*., 2010).

Epidemiological studies of human populations exposed to diets naturally contaminated with aflatoxins reveal an association between the high incidence of liver cancer in Africa and elsewhere and dietary intake of aflatoxins (MRC, 2006). Hepatitis B virus infection and chronic aflatoxin exposure places a person at a risk 30 times greater of developing liver cancer than people who are

hepatitis B virus negative. Sub-Saharan African and Asian populations that have endemically high rates of infection of hepatitis B virus and hepatitis C virus are, therefore, likely to have a significantly increased disease burden from liver cancer (USAID, 2003).

Exposure to aflatoxin leads to, or is associated with, several health-related conditions including acute and chronic aflatoxicosis, aflatoxin-related immunosuppression, liver cancer and liver cirrhosis, and nutritional-related problems such as stunted growth in children.

1.4.2.1. Acute aflatoxicosis

Acute aflatoxicosis is associated with sporadic outbreaks caused by the consumption of highly contaminated foods. Early symptoms of acute aflatoxicosis include diminished appetite, malaise and low fever. Later symptoms, which include vomiting, abdominal pain and hepatitis, can signal potentially fatal liver failure (USAID, 2003). Severe acute liver injury with high morbidity and mortality has been associated with high-dose exposures to aflatoxins (Chao *et al*., 1991). Ingestion of 2–6 mg of aflatoxin per day by adults for a month can cause acute hepatitis and death (Patten ,1981).

Kenya has experienced several recurrences of acute aflatoxicosis in humans and has recorded hundreds of deaths in the last four decades. The largest reported outbreak of aflatoxicosis to date occurred in Kenya in 2004 where 317 cases and 125 deaths were reported with significant mortality among domesticated livestock and widespread socio-economic impact (Azziz,Baumgartner *et al*., 2005) (Lewis *et al*., 2005) (Wagacha and Muthomi ,2008).

In the 2004 outbreak in Kenya, concentrations of aflatoxin B1 in maize were found to be as high as 4400 parts per billion (ppb), which is 220 times greater than the 20 ppb limit for food suggested by Kenyan authorities (Azziz,Baumgartner *et al*., 2005). During this outbreak, children younger than 14 years (51% of the child population) had a greater predisposition to aflatoxicosis risk (Obura, 2013).

1.4.2.2.Chronic aflatoxicosis

Chronic aflatoxicosis is caused by long-term exposure to low to moderate levels of aflatoxins in the food supply.Chronic exposure to moderate or even low levels of aflatoxin has been linked to development of liver cancer. In a study of Gambian liver cirrhosis patients, those that had eaten groundnuts at least once daily over the previous two months were classified in the high aflatoxin intake group (Kuniholm *et al*., 2008).

In addition to the links to liver cancer, chronic aflatoxin exposure has been associated with impaired growth and immunosuppression in young West African children (Turne *et al*., 2003), (Gong *et al*., 2004). Immunosuppression predisposes humans and animals to many secondary infections by fungi, bacteria

and viruses (McLean, 1995). Thus, chronic aflatoxin exposure could exacerbate the burden of disease in already vulnerable populations.

Chronic aflatoxin exposure is evident from the presence of aflatoxin M1 in human breast milk in Ghana, Kenya, Nigeria, Sierra Leone, Sudan, Thailand and the United Arab Emirates, and in umbilical cord blood samples in Ghana, Kenya, Nigeria and Sierra Leone (Bhat and Vasanthi, 2003).

1.4.2.3. Liver cancer

A large body of experimental, clinical and epidemiological evidence has defined aflatoxin as one of the most potent naturally occurring liver cancer-causing agents. Globally, it is estimated that aflatoxin exposure contributes to 4.6–28.2% of all liver cancer cases, most of which occur in subSaharan Africa, southwest Asia and China, the regions with the highest aflatoxin exposure. Each year 550,000–600,000 new cases of liver cancer are recorded worldwide, and of these, approximately 25,200–155,000 is attributable to aflatoxin exposure (Liu and Wu, 2010).According to the World Health Organization (WHO), liver cancer is the third leading cause of cancer deaths globally. Approximately 83% of liver-related deaths in East Asia and sub-Saharan African are due to liver cancer (USAID, 2003).Figure(1.7)

1.4.2.4. Links to Aflatoxin and tuberculosis

New research has linked high aflatoxin levels to an increased risk of developing tuberculosis in human immune deficiency virus (HIV)-positive individuals (Keenan *et al*., 2011).

In a study done on HIV-positive Ghanaians, hazard ratios for developing symptomatic tuberculosis were significantly higher for those in the highest aflatoxin-albumin (hazard ratio 3.30; 95%) compared to those in the lowest. Those with the highest levels of aflatoxinalbumin from dietary intake have an increased hazard of symptomatic tuberculosis but not malaria, hepatitis B virus or pneumonia (Keenan *et al*., 2011).

1.4.3. Detecting aflatoxicosis in humans and animals

Detection aflatoxicosis in humans and animals is difficult due to variations in clinical signs and the presence of other factors such as suppression of the immune system caused by an infectious disease. Of the two techniques most often used to detect levels of aflatoxins in humans, one measures a breakdown product in urine (which however is only present for 24 hours after exposure), and the other measures the level of an AFB–albumin compound in the blood serum, providing information on exposure over weeks or months. These biomarker measurements are important in investigating outbreaks where aflatoxin contamination is suspected (WHO, 2018)

1.4.4. Economic Impact of Aflatoxins

Aflatoxins have a considerable negative impact on the national economies particularly indeveloping countries. Indeed, for most developing countries, the export of agricultural productsincluding cereals, oilseeds, tubers, dried fruits and coffee grains to Europe and Americaconstitute the basis of the economy in the agricultural sector. However, stringent measuresimposed either by importing countries or by international guidelines relating to thecontaminated products by AFs are a real barrier to the cross-border or transcontinental trade(Otsuki *et al*., 2001). Also the economic impact of aflatoxins is derived directly from crop and livestock losses due to flatoxins and directly from the cost of regulatory programs designed to reduce risks to human and animal health. Nevertheless, aflatoxins reputation as a potentpoison may explain why it has been adopted for use in bioterrorism (Bennett and Klich,2004).

1.4.5.Human Exposure of aflatoxins

Humans are primarily exposed to AF through dietary intake (IARC, 2002). Two pathways ofthe dietary exposure have been identified:

(a) Direct ingestion of AFs (mainly B1) in contaminated foods of plant origin such asmaize, groundnuts and their products. Food crops can become contaminated both before and after harvesting. Pre-harvest contamination with aflatoxins is mainly limited to maize, cotton seed, peanuts and tree nuts. Post-harvest contamination can be found in a variety of other crops such as coffee, rice and spices. Improper storage under conditions that favour mould growth (warm and humid storage environments) can typically lead to levels of contamination much higher than those found in the field.

(b) Ingestion of AFs carried over from feed into milk and milk products including cheeseand powdered milk, where they appear mainly as AFM1. In addition to the carry overinto milk, residues of AFs may be present in the tissues of animals that consumecontaminated feed.

However, exposure to AF is a result of both the level of contamination in a given commodityand the quantity of the commodity that is consumed. Thus, in some areas of the world AFlevels in foods might be relatively high but with modest exposures because of a varied diet.While, in Sub-Saharan Africa, similar levels of food contamination will translate to a muchhigher exposure because, dietary staples in this region (peanuts and maize) are

highlysusceptible to AFs contamination due to poor grain storage conditions. In many countries andregions, these staples are consumed daily for the majority of the year and may constitute morethan 50% of the diet (Wild and Turner, 2002). In addition, these toxins present an elevatedthermal stability enabling them to remain in some cooked foods as well as freezing has verylittle effect on their presence in foods (Sáez *et al*., 2011).

Several incidences of aflatoxicosis in humans have been reported in many countries including South East Asia and Africa. Furthermore, it is estimated that 4.5 billion people in the developing nations are chronically exposed to aflatoxins in their food, hence putting them at a risk of cancer related diseases (Li *et al*., 2001).

1.4.6. Effects of aflatoxins in animals and poultry

- Poultry

  Is primarily a disease of the liver and it showed the typical lesions on it, which ultimately cause production problems and mortality. The main clinical signs in affected birds are decreased feed intake, decreased body weight, poor skin, decreased egg production and decreased immunity. The disease may be fatal and resulted in heavy mortality. The main lesions of aflatoxicosis in birds are also appeared on liver and kidneys which are, Jaundice, generalized oedema and hemorrhages, tan or yellow discoloration of the liver, periportal necrosis with bile duct.proliferation and fibrosis and depletion of lymphoid organs. (Charlton *et al*., 2006).

- Dogs

  More than 100 dogs were reported to be killed from aflatoxin-contaminated commercial dog food in 2005–2006 in the United States (Dereszynski *et al*., 2008) (Newman, Smith *et al*., 2007) (Stenske, Smith *et al*., 2006).

- Pigs

  Pigs also highly affected by aflatoxins,with the chronic effects largely apparent as liver damage.

- Cattles

  The primary symptoms are reduced weight gain as well as liver and kidney damage; where milk production is also reduced. Different forms of the enzymes that metabolize aflatoxins (e.g. cytochrome P450s, glutathione S-transferases) are considered responsible for the different susceptibilities of

different animals to the toxic effects of aflatoxins(WHO, 2018).

In dairy cattlein ruminants, aflatoxin B1 is metabolized to aflatoxin M1 in the liver and excreted in the milk ofdairy cows. Aflatoxin intoxication in dairy cattle is characterized by liver cell injury, fatty liversyndrome, poor feed conversion and a significant reduction in milk yield. High-yielding dairy cowsare considered to be more sensitive to aflatoxins than fattening cattle. Even low levels of aflatoxinsare able to affect the cellular and humoral immune system, resulting in increased susceptibility toinfectious diseases in exposed animals (Fink and Gremmels, 2008). Aflatoxicosis is usually considered aherd rather than an individual cow problem (Feddern *et al*., 2013).Because aflatoxins are degraded by flora in the cow's rumen, the amount of aflatoxin M1 excreted in milk is only around 1–7% of the total amount of aflatoxin B1 ingested (Fink and Gremmels, 2008).Cows in early lactation can excrete 3.8–6.2% of dietary aflatoxin B1 as aflatoxin M1 and cows in late lactation can excrete 1.8–2.5% of dietary aflatoxin B1 as aflatoxin M1 (Coppock *et al*., 2012). Higher yielding animals consuming large amounts of concentrates typically have higher levels of aflatoxin in their milk. The dietary threshold for aflatoxin excretion in cows' milk appears to be 15 ppb or 230 µg aflatoxin B1 per cow per day (Coppock *et al*., 2012). The presence of mastitis may increase the secretion of aflatoxins.

Aflatoxin levels are around three times higher in soft cheese and five times higher in hard cheesethan in the milk of origin. Since cheese is more concentrated, using aflatoxin-contaminated milkfor cheese production is risk mitigating (for example, if 10 litres of milk makes 1 kg of cheese and aflatoxins are five times higher in hard cheese than in milk, then the exposure to aflatoxin byconsuming 1 kg of cheese is half as much as that from consuming 10 litres of milk). Aflatoxins mayalso be present in yoghurt and other dairy products. Recent studies have suggested that a relatedtoxin called aflatoxicol may also be excreted in significant amounts in milk, a subject that requires further research (Grace, 2013)

1.4.7. Factors affecting production of aflatoxin

1.4.7.1 The fungus

The *Aspergullus flavus* species is a normal constituent of the air microflora, soil, and in living or dead plants and animals throughout the world(Raper and

Funnel, 1965). It has been found associated with peanut soils and peanut wherever they are grown (Diener, 1973).Aflatoxin arise naturally when toxin producing strains *A.flavus*or *A. parasitic* were grown on a substrate where environmental conditions are suitable for the development of the mould (Gold and Blatt, 1969).

1.4.7.2 Pod condition

Rapid invasion of pod by *A.flavus*in the soil has been associated primary with physical and biological damage of the shell and kernels.(McDonald and Harkness, 1964)(Dickens and Pattee, 1966) reported that pre-harvest development of aflatoxin occurred only in kernels of broken pods. Damage pods contain more aflatoxin than undamaged pods.

1.4.7.3 Temperature

*A.flavus*was classified as a mesophilic, having cardinal growth temperature, minimum temp 6º - 8ºc, optimum temp 36º – 38ºc and maximum temp 44º–46ºc. The minimum temperature for growth is affected by moisture content, oxygen content, availability of nutrient, and other factors (Semeniuk, 1954). Several studies were conducted to determine the effect of temperature on aflatoxin production. (Diener *et al*., 1965) found that optimum temperature for aflatoxin production by *A.flavus*was 35ºCincase of peanuts and liquid media. Those authors reported that *A. parsiticus* produced maximum amount of aflatoxin at 25ºc – 30ºc. (Ahmed, 1981) showed that no aflatoxin was produced by *A.flavus*on groundnut at 20ºc and 42ºc under Sudan conditions.

1.4.7.4. Relative humidity and substrate moisture content

It is widely recognized that the most important factor in growth of *A.flavus*and aflatoxin production by the fungus is the moisture content of the natural substrate and the relative humidity (RH) surrounding it(Austwick and Ayerst, 1963). Those authors found that the optimum moisture content of the substrate for *A.flavus*growth 15 - 25% where aflatoxin formation could occur at much higher moisture content about 39%. Safe storage moisture content for seeds and other natural substrates has generally been established at substrate moisture in equilibrium with 70% RH, at which very few fungi will grow. (Dickens and Pattee, 1966) stated that 15 – 30% moisture content in groundnut seeds were more conductive to aflatoxin production than higher or lower moisture content

1.4.7.5. Harvest, drying and storage environments

Post-harvest conditions are also important. Poor harvesting and storage conditions can lead to rapid development of the fungi and thus high production of the toxin. Studies in Africa have shown that storage conditions are usually inadequate. Groundnut (peanuts) is often stored in the pods and insects can easily damage the pods and facilitate penetration by fungi. Groundnut is also stored as shelled seed in poorly ventilated areas, which favor fungal development net 2,(2005).

1.4.8. Aflatoxin limits

The number of countries regulating aflatoxins is significantly increasing every year. The aflatoxin regulations are often detailed and specific for various foodstuffs, and dairy products. Some countries have regulations specifying different tolerated levels for foods and feeds, while others have set only one tolerated level for instance for all feeds, net 8 (2005).

1.4.8.1. Aflatoxin B1 in food

Compared to the situation in 1995, the maximum tolerated levels for aflatoxin B1 in food have not changed dramatically since 2003, although the range of limits has narrowed a little (1-20 ppb), and 2 ppb is now a limit in force in at least 29 countries. Another major limit is visible at 5 ppb, followed by 21 countries, spread over Africa, Asia, Latin America and Europe, 5 ppb followed by 21 countries, 10 ppb followed by 5 countries net 8, (2005).

1.4.8.2. Total aflatoxins in food

As in 1995, in 2003 many countries regulated the aflatoxins with limits for the total of aflatoxin B1, B2, G1 and G2. The range of limits (0 – 35 ppb) has narrowed a little compared to 1995, whereas the median limit (10 ppb) is slightly higher.Also the United States, one of the first countries that established an aflatoxin action limit, follows the 20 ppb limit. Aflatoxin B1 is the most important of the aflatoxins, considered from both the viewpoints of toxicology

and occurrence. It is most unlikely that commodities will contain aflatoxin B2, G1 and G2 not aflatoxin B1, and the concentration of the sum of the aflatoxins B2, G1, G2 is generally less than the concentration ofaflatoxin B1 alone net 8, (2005)

1.4.8.3. Sudan limits for Aflatoxin

Peanut is an important cash crop in the Sudan, providing about 5% of the gross national income. Two varieties of Peanut are grown in Sudan; one is grown in the western part of the county (Darfur and Kurdofan) 80% of the total production whilst the other variety grows in Gazeria and East Sudan. Figure (1.9)

For exported groundnut, aflatoxin limits was 15 ppb, while aflatoxin in groundnut butter for local consumption must not exceed 10 ppbas proposed by (Sudanese Standard, 2004).

1.4.8.4. Aflatoxin in Sudan

The importance of aflatoxins in peanut in Sudan stems from the huge production (>million tons/annum), its high consumption (as food and feed) and as an important export (Mehan *et al*., 1989). Peanut is very susceptible to aflatoxin contamination, and this fact is manifested by a lot of literature of research. That is, in Sudan 100% contamination by aflatoxins was reported in 43 samples of peanut butter from Khartoum state (Elzubair *et al*., 2011). In addition, a lot of work preceded this experiment includethe following references (Habish *et al*., 1971).

1.4.9. Methods for Detection of Aflatoxin in Foods and Feeds

Aflatoxins are of major importance and techniques for their detection and analysis have been extensively investigated to develop those that are highly specific, useful and practical. A lot of methods are available for different needs, ranging from techniques or methods such as

1.4.9.1. Thin-Layer Chromatography (TLC)

Thin-layer chromatography is widely used in laboratories throughout the world for food analysis and quality control. Applications of TLC have been reported in areas of food composition, intentional additives, adulterants, contaminants, etc. TLC has been used to analyze agricultural products and plants. It has advantages as: simplicity of operation; availability of many sensitive and selective reagents for detection and confirmation without interference of the mobile phase; ability to repeat detection and quantification; and cost effectiveness analysis, because many samples can be analyzed on a single plate with low solvent usage, and the time that TLC employs to analyze the sample is less than LC (Liquid

chromatography) method(Sherma, 2000) (Fuch *et al*., 2010) The most important differences between TLC and HPTLC arethe different particular size of stationary phase; the care used to apply the samples; and the way to process the obtained data (Fuch *et al*., 2010).

1.4.9.2.High performance liquid chromatography (HPLC)

HPLC coupled with ultra-violet, a diode array detector (DAD) or a fluorescence detector (FD)currently is the most widely used technique for the identification of the major mycotoxins infood commodities (Giniani *et al*., 2011). HPLC/FD is highly sensitive, selective, accurate and repeatable.These methods have been adopted as official or standard methods by the InternationalAssociation of Official Analytical Chemists (AOAC) or the European StandardizationCommittee. In particular, methods for measuring AFs in maize, raw peanuts and peanut butter;AFB1 and total aflatoxins in pistachios, figs and paprika; AFM1 in milk; AFB1 in baby food(Fu *et al*., 2008).

1.4.9.3. Enzyme-linked immunosorbent assays (ELISA)

Since the late 1970s, AF-specific antibodies have been developed. The antibody developmenthas led to the development of enzyme-linked immunosorbent assays (ELISAs) for AFs (Li *et al*., 2009). The ELISAs are mainly used for qualitative, semi-quantitative and quantitativeanalysis of AF and other mycotoxins in a number of food matrices. In general,(ELISAs) do notrequire clean-up procedures and the extract containing the mycotoxin is analyzed directly (Li *et al*., 2009). It provides fast and inexpensive screening assays; but lack accuracy at very lowconcentrations (competitive assays) and is limited in the range of matrices examined.This assay was bothaccurate and reliable giving no false compliant and only a few false noncompliant results (Li *et al*., 2009).

1.4.9.4.Biosensors

A biosensor is an analytical device that incorporates a specific biological element, *e.g.*, anantibody, which creates a recognition event and a physical element that transduces therecognition event into acoustic, electrical or optical signal. Immunochemical biosensors thatuse surface plasmon resonance, quartz crystal microbalance and screen-printed carbonelectrodes have been described for the detection of mycotoxins.Competitive surface plasmon resonance-based immunoassays have been used for rapidscreening of AFB1 and other

mycotoxins in naturally contaminated matrices (Van *et al*., 2003). These methods are able to detect AFs at very low levels with good accuracy andprecision.

1.4.9.5. Polymerase chain reaction (PCR)

In recent years, polymerase chain reaction (PCR)-based methods have emerged as major tools for detection of*Aspergillus flavus* andaflatoxin-producing fungi in foods (Shapira *et al*., 1996) (Degola *et al*., 2007) (Cruz and Buttner, 2008) (Passone *et al*., 2010) (Levin, 2012) (Rodriguez *et al*., 2012). In general, these methods are highly sensitive, specific and accurate and can be performed and interpreted by personnel with no specialized taxonomical expertise. Furthermore, these techniques are rapid and less laborious than conventional methods because isolation of the fungus from the infected tissue is not required (Lievens and Thomma, 2005).

1.4.10. Prevention and Control

The AF contamination is a serious health concern rooted in the entire food chain, hence theneed for a multidisciplinary approach in the search for solutions. Interventions to reduce aflatoxin-induced illness can be roughly grouped into three categories, agricultural, dietary, andclinical.

1.4.10.1.Agricultural

Agricultural interventions are methods or technologies that can be applied either in the field(pre-harvest) or in drying, storage and transportation (post-harvest) to reduce AF levels in food. Agricultural interventions can thus be considered "primary" interventions, because theydirectly reduce AF levels in food.

1.4.10.1.1. Pre-harvest handling methods

These include the use of bio-control agents that establish a process of competitive displacement using non-toxigenic strains by different agricultural techniques, and developing breeds stronger or more resistant crop strains. These methods are

- **Biological control**

  Biological strategies employed instead of traditional chemical pesticides, are environmentallyfriendly and come from natural resources. These strategies

include beneficial insects, plantextracts (Reddy *et al*., 2009), or the introduction of other natural organisms such asMethyleugenol (Sudhakar *et al*., 2009).In the prevention of AF contamination, biocontrol methods can be applied before harvest or inthe fields when the plants grow and become mature. However, these methods are not aseffective as those using chemical methods.

- **Competitive displacement**

The application of non-toxigenic strains of *A. flavus* on crops promotes competition with thosethat are toxic, resulting in a significant reduction or elimination of toxigenic strains thatproduce AF (Probst *et al*., 2011). This method has proven to be very effective in the fight against AF contamination in variouscultures (Probst *et al*., 2011).The application of this method can be done in different ways and this influences theeffectiveness of results. It may involve:

(1) Inoculating the soil with a non-toxigenic strain

(2) Spraying of crops with conidia or spores of non-toxigenic strain

(3) Spraying the plants witha product which comprises hydrosoluble non-toxigenic strain(Lyn *et al*., 2009).

- **Agricultural techniques**

The use of agricultural techniques, such as crop rotation (Jaime and Cotty, 2010) andinterventions to reduce exposure to environmental stress (Cotty and Jaime, 2007) mayalso reduce AF contamination.

1.4.10.1.2. Post-harvest handling methods

These methods provide a set of tools that eliminate or limit the spread of AFs in agriculturalproducts through out the harvest. These include food processing, storage strategies such asdrying and improved conditions and measures that are appropriate and well adapted to the agroecological zone.

- **Food processing**

Food processing does not completely eliminate AFs contained in harvested crops but itsignificantly reduces AF levels in the final product (Scudamore, 2008). This procedure can be done using various techniques including those that involve removing theshell of certain foods such as oats and pistachios (Scudamore, 2008)

➢ Storage strategies

These include

(a) Drying

The heat does not eliminate exposure to AFs, but the gradual elimination of humidity in thecrop is an effective method to prevent fungal growth and mold. The method often used is theheated drying. It is effective in limiting the spread of A. flavus but also AFs (Magan andAldred, 2007).

(b) Storage conditions

Measures should be taken to minimize fluctuations in temperature and humidity in silos andother storage buildings to prevent the increased risk of AF contamination of harvested crops.Furthermore, the presence of insects in warehouses must be avoided since their activityincreases the temperature and level of humidity in the cultures. This can lead to fungal growth,which in turn may result in the production of mycotoxins (Magan and Aldred, 2007).The useof short-term silo bags can help guard against infiltration of insects and moisture. However, forlong-term storage, the impermeability may result in fungal growth (Udoh *et al*., 2000).

➢ Handling contaminated products

TheAF contaminated foods can be detoxified by the use of inorganic salts and organic acidssuch as sodium carbonate, sodium bicarbonate, potassium carbonate, ammonium carbonate,acetic acid, the preopinante sodium and ammonia (Shekha *et al*., 2009); and AFB1 bindingagents (Oluwafemi and DaSilva, 2009).

1.4.10.2.Dietary and Clinical

Dietary and clinical interventions can be considered as secondary interventions. They cannotreduce actual AF levels in food, but they can reduce aflatoxin-related illness.

❖ Vaccination against Hepatitis B

Vaccination against hepatitis B is not officially considered as a control intervention againstAFs, because the vaccine has no effect on AF levels in diets. However, knowing that AF isinvolved in the occurrence of hepato cellular carcinoma, together with hepatitis B and hepatitisC as major risk factors, vaccination therefore reduces the synergistic impact of hepatitis B

andthe AF in inducing liver cancer (Wild and Hall, 2000), (Wu and Khlangwiset, 2010).

❖ Awareness campaigns

Awareness campaigns aimed at improving the knowledge in order to change attitudes and practices of both agricultural and food production methods in the community are a major pillarin the fight and prevention against AFs. This method proved to be effective during aflatoxicosis outbreak in Kenya in 2005 (Strosnider *et al*., 2006).

CHAPTER TWO

Materials and Methods

2.1. Materials

2.1.1. Study area

The study was conducted in Khartoum State which is the capital of Sudan located at 15.30° N and 32.33° E.Khartoum state is one of the eighteen states of Sudan. Although it is smallest state by area (22.142km), it is the most population (7,687.547 in 2017 census). Density 347/km² 900/sqmj).Khartoum is capital of states as well as the national capital of Sudan. (Map 2.1).It located in the heart of Sudan at the confluence of the White Nile and Blue Nile where the two river unite to form the River Nile.The confluence of the two river creates unique effect as they join each river retain it own color.The White Nile with bright white and blue Nile with it's alluvial brown color. These colors are more visible in the flood season.

2.1.2. Sample collection

Thirty Samples of Peanuts from solid grain (Cakes) collected from different loclities in Khartoum State such as Omdurman (Seed markets, peanut oil factory), Bahri(peanut oil factory)and Sharg-elnile(seed market – hillatkoko). Each sample contained 5-10 grams, samples from the oil factory are freshly squeezed while other had been saved for long time in the open markets.

The samples were placed in sampling plastic bags and labeled. In the laboratory, they stored in a dry and airy place until processing. The laboratory handbook is an important laboratory document used to assign information about samples collected.

2.1.3. Transport of Samples

Samples transportedto the laboratory on plastic bages and then put in another plastic bag, to avoid any contamination through transportation.

2.1.4. Storage of Samples upon arrival to the lab.

Samples do not need to be kept refrigerated or frozen during transportation. They stored in a cool place till used.

2.1.5. Media used for conventional isolation and identification of *Aspergillus*

- Potato Dextrose Agar(PDA) media for subculture
- Czapex yeast extract (CYA) media to confirmation
- Microscopic and lactophenol cotton blue for identification
- Molecular techniques (detection of *Aspergillus flavus* gene).

2.1.5.1. Culture media for isolation of *Aspergillus*

- Sabouraud Dextrose Agar (SDA) is a selective medium primarily used for the isolation of dermatophytes, other fungi and yeasts but can also grow filamentous bacteria such as Nocardia.

2.1.5.2. Biochemical and biological tests

2.1.5.2.1. Microbiological test

Samples were first cultured on Sabouraud Dextrose Agar (SDA) which is a selective medium used for the isolation of*Aspergillus Flavus*.

Then sub cultured on other media, such as potato dextrose agar and later on Czapek yeast agar (CYA) media in appendix(1,2). Finally, lacto phenol cotton blue was used for microscopic examination of cultures.

2.1.5.2.2. Biochemical test

PCR test was used.

2.1.6. Identification of aflatoxin

Thin layer chromatography (TLC) was used to quantitatively detect aflatoxins that recovered from collected samples.Samples tested by Natinal Public Health Laboratory.

Reagentandchemicals

- Methanol
- Chloroform
- Acetone
- Benzene

- N-hexane
- Acetonitrile
- Petroleum ether
- Distilled water
- Sodium sulfate anhydrous as analytical reagent.
- Silica gel.

2.1.7. Molecular identification of isolates

2.1.7.1. Genomic DNA extraction and purification from the isolates

The genomic DNA was extracted and purified from 5 days-old mycelia following (Mölle r*et al*., 1992) and (Suleiman *et al* ., 2015) with some modifications.

2.1.7.1.1. Tris-EDTA-SDS solution (TES)

A solution was made containing100 mMTris at pH 8.0, 10 mM EDTA (Disodiumethylenediaminetetraacetate-2H_2O**)** and 2% SDS (Sodium dodecyl sulfate solution).

2.1.7.1.2. Liquid nitrogen

Liquid nitrogen was brought in a container and used for grindin culture.

2.1.7.1.3. Proteinase K (PK)

An ampoule containing 100 mg of lyophilised PK (Promega) was dissolved in 5 ml of ddH_2O to make a final concentration of 20 mg/ml (w/v), aliquoted and then stored at -20◦C.

2.1.7.1.4. 5 M NaCl

A mass of 29.2 g of NaCl was added to a graduated bottle, supplemented with 80 ml of ddH2O, stirred to completely dissolve NaCl, final volume adjusted to 100 ml with ddH2O, autoclaved and finally stored at room temperature.

2.1.7.1.5. 0.7 M NaCl

2.1.7.1.6. 10% CTAB (Cetyltrimethyl-ammonium bromide)

A volume of 50 ml of 0.7 M NaCl and 2.5 g of CTAB (Fishier Scientific) were combined into a 50 ml polypropylene tube (Fisher Scientific), rotated slowly at 60 ℃ for several hours to dissolve the powder completely and finally stored at 4°C.

2.1.7.1.7. Chloroform:Isoamylalcohol (Sigma Aldrich)

The ready to use premix concentration of the different ingredients is 24:1 (v/v).

2.1.7.1.8. 5 M ammonium acetate

A mass of 385 g of ammonium acetate was dissolvedin 800 ml of ddH2O, volume adjust to 1 litre by ddH2O and finally filter sterilized.

2.1.7.1.9. TE (Tris-HCl, EDTA) buffer

A volume of 1 ml from the 1M Tris-HCl (10mM) was mixed with, 2 ml from the 0.5M EDTA (10mM) and 97 ml of ddH_2O.

2.1.7.1.10. Other solutions

a. Isopropanol
b. Absolute ethanol.
c. 70% ethanol in ddH_2O.

2.1.7.2. Analysis of DNA by agarose gel

2.1.7.2.1. Blue/Orange 6X Loading Dye (Promega)

A dye for loading DNA samples into the agarose gel wells and tracking migration during electrophoresis.

2.1.7.2.2. Ethidium bromide solution (Promega)

A fluorescent dye for staining DNA and its visualization with UV light. The concentration of the solution is 10 mg/ml.

2.1.7.2.3. DNA size makers (Vivantis)

A100 bp Plus DNA Ladder.

2.1.7.2.4. TBE (Tris-borate-EDTA) running buffer

A 5x TBE was prepared by mixing 54 g Tris base, 27.5 g boric acid, and 20 ml 0.5M EDTA. This was followed by adjusting the pH to 8.0 before bringing the final volume to 1L with ddH_2O.

2.1.7.2.5. Agarose Gel

A 1.5% (w/v) agarose gel was prepared by combining 1.5 g SeaKem LE Agarose (Cambrex) and 1xTBE buffer to 100 ml in a 250 ml bottle. The cover of the bottle was left loose then the agarose was melt by heating in a microwave. The gel was left to cool down to about 60◦C before the addition of 5 µl ethidium bromide.

2.1.7.2.6. Electrophoresis equipment

A minigel electrophoresis apparatus (8-cm-long) was used.

2.1.7.3. Polymerase chain reaction (PCR) for amplification of the*ß*-tubulin gene (*ben*A)

2.1.7.3.1 Oligonucleotide Primers

The primers Bt2a and Bt2b (Muilenberg.,*et al* 1990) (Glassand Donaldson, 1995) were synthesised by Invitrogen. The Bt2a sequence is 5'-GGTAACCAAATCGGTGCTGCTTTC-3' and the Bt2b sequence is 5'-ACCCTCAGTGTAGTGACCCTTGGC-3'.

2.1.7.3.2. Master mixes

A ready to use DreamTaq Green PCR Master Mix (2x) (Fermentas, Cat. # K1072).

2.1.7.3.3. Reaction mixes

The volume of a reaction mix was 50 µl containing 25 µl of DreamTaq Green PCR Master Mix (2x) (Fermentas, Cat. # K1072), 0.3 µl (0.6 uM), of each primer, 5 µl of the respective template DNA and 19.4 µl nuclease ddH_2O. The reaction mixes were kept on ice.

2.2. Methods

2.2.1. Conventional isolation and identification of *Aspergillus*

Preparing samples by Grain the solid samples, one gram from each sample were suspended in 10 ml normal saline, after half an hour diluted2-3 times of each samples using 5m normal saline.

Sabouraud Dextrose Agar (SDA).The acidic pH of this medium (pH about 5.0) inhibits the growth of bacteria but permits the growth of yeasts and most filamentous fungi.

6.5 gram from Sabouraud Dextrose Agar powder was dissolved in 100ml distill water and autoclaved for half an hour.

Adding (0.02mg/ml) chloramphenicol antibiotics (vet services) to inhabit the growth of bacteria.Pour the plates and let to solidify in Refrigerator.

Using a loop few amount from each sample culture in plates. The plates were incubated at25- 30ºC for 6-7 days, then isolates and Identification according to method described by (Baily and Scott ,1998).Then sub cultured on potato dextrose agar to get pure culture to confirm the purity of *Aspergillus-flavus.*

Identification of *Aspergillus* specis was made as described by Raper and Fennell(1965).

2.2.2. Biochemical and biological tests

2.2.2.1. Microbiological test

Slide from the pure culture of the colony was mounted in lacto phenol cotton blue (wet smear) and examined under the light microscope . The morphological characteristics of the isolates were described.The morphological characteristics of isolates were described microscopically according to(Domsch and Gams, 1980) and(Klich, 2002).

2.2.2.2. Biochemical test

Aflatxins producing by the fungus.

2.2.3. Detection of aflatoxin

Thin Layer Chromatographic (TLC) was used to identify types of Aflatoxins.

❖ Objective

This method was used for determining different aflatoxin classes.

Aflatoxin B1

Aflatoxin B2

Aflatoxin G1

Aflatoxin G2

2.2.3.1.Principle

A test aflatoxin extracted with an aqueous solvent mixture (methanol:water) in addition of an non-polar solvent such as hexane or petroleum ether, the sample extracted was filtered and evaporated to specific solvent concentration Safety and quality.

2.2.3.2.Procedure

Extraction of aflatoxin was done as per AOAC method with screening by TLC andquantification using reference standards B1, B2, G1, and G2.

Fifty grams of cake powder sample from each sample added to 250ml. methanol water, ratio (55:45) and 100ml hexane. The bottle was shaken for 3 minutes then centrifuged 300 round for 5 minutes for separation of aflatoxin extraction. then 50ml of extraction was transfered to the extract chloroform. (50ml) was added. The mixture was steaming until evaporated and dry of forming lower phase.

Dissolve extract with 0.5ml (Benzen – a acetonitrile)(98:2) for spotting on TLC plate use methanol : water : diethyl ether (3 : 1 :96) mobile phase up to the mark after saturation of twin trough chamber.

The AOAC method (970:40/990)

2.2.4. Molecular identification of isolates

2.2.4.1.GenomicDNA extraction and purification from the isolates

The genomic DNA was extracted and purified from the isolates following(Möller .,*et al* 1992) and (Suleiman .,*et al* 2015) with some modifications. Briefly, 5 days-old mycelia were harvested from the surface of SDA Petri dishes, transferred to a mortar, supplemented with liquid nitrogen and ground with a pestle. About 45 mg of the mycelia powder were transferred to a 2.0 ml eppendorf tube, supplemented with 500 µl TES (100 mMTris, pH 8.0, 10 mM EDTA, 2% SDS) followed by addition of 3.75 µl of Proteinase K (stock 20 mg/ml; w/v; Promega), then the tube was incubated for 1 hour at 60°C with occasional gentle mixing of the tube. The reaction mix was then supplemented with 140 µl of 5 M NaCl, 65 µl of 10% CTAB (Cetyltrimethyl-ammonium bromide), and was incubated for 10 min at 65°C. After adding 700 µl of chloroform: isoamylalcohol (24:1, v/v; Sigma Aldrich), the tube was mixed and incubated for 30 min at 0°C before centrifugation for 10 minutes at 4°C and 12000 rpm. The supernatant was then transferred to a 1.5 ml micro-tube, supplemented with 225 µl of 5 M ammonium acetate, mixed, placed on ice for one hour, before centrifugation again. The supernatant was transferred to a fresh 1.5 ml micro-tube, supplemented with 510 µl of isopropanol, placed on ice for 30 minutes, centrifuged for 5 min at the same conditions mentioned above. Finally the supernatant was decanted, the pellet was washed twice with cold ethanol (70%, v/v), dried at room temperature, dissolved in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) and stored at -20°C for later experiments.

2.2.4.2. Thermo cycling conditions for amplification of *β*-tubulin gene by PCR

The PCR was performed with a TC-512 (Techne) thermo cycler. The heat lid temperature was adjusted to 104°C. The thermo cycling conditions were one cycle of 94°C for 1 min; 32 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min; and one cycle of 72°C for 5 min.

2.2.4.3. Agarose gel electrophoresis

A horizontal mini-gel electrophoresis system (MSMINI, Cleaver Scientific) was used. To the agarose gel, a well was loaded with 5 µl of 100 bp DNA size marker (Vivantis), and the other wells were loaded with 8 µl of the respective PCR amplicons mixed with 2 µl of loading dye 6x (Vivantis). The power supply was adjusted to 80 V and the DNA was left to separate for 45 min. The gel was inspected and photographed by a gel documentation system (Ingenius, Syngene Bio Imaging).

CHAPTER THREE

3. 3. Results

3.1.Fungual Growth

Different species were grown on Sabouraud Dextrose Agar with different colour (Figure 3.1,3.2, 3.3). Out of 30 samples 7 (23.30) were found positive for *Aspergillus-flavus*with green color.The fungus was rapidly grown on Sabouraud Dextrose Agar. The colonies began to develop after 3 days as granular flat, with radial grooves, yellow at first but quickly turned to bright and dark yellow green. After 7 days the mature colonies covered the whole plate with green color. Sub culture on potato dextrose agar revealed pure growth of *Aspergillus-flavus.(* Figure 3.4, 3.5, 3.6)

3.2. Morphological examination

3.2. 1.Visual examination

The fungal isolates were identified according to (Klich, 2002). Primary identification gross green colour colonies revealed grown on Sabouraud Dextrose Agar (SDA) and sub culture in selective media Potato Dextrose Agar (PDA). Slide culture from pure colony was carried out.

3.2.2. Physiological characterization of *A. flavus* isolates

Sub culture on Czapek media in appendix 1showed growth of *Aspergillus flavus*that changed the colour of the media to reddish (Figure 3.7).

3.2.3. Microscopic characteristics.

Small amont of the growth from the plate culture put in slide with loob, added drop from lacto phenol cotton blue and covered with slide cover and examine under microscopic.

The morphological characteristics of the isolates described microscopically according to (Domsch and Gams, 1980) and(Klich, 2002) revealed characteristics conidiophore, septated hyphae, vesicle and conidia of *Aspergillus flavus*(Figure 3.8,3.9).

3.3. Aflatoxins

Detection of aflatoxin tested by thin layer Chromotography in showed that all the positive sample of *Aspergillus flavus*were found positive for aflatoxin B1 with different concentrations. One sample is positive for aflatoxin B2. While G1,G2 are found negative in all samples tested (table 3).the which is positive for B1 and B2 from oil factory.The proportion and concentration of Aflatoxin in (Figure3.10, 3.11)

Cake samples

Table (3) Concentration of Aflatoxin (mg\kg) in samles.

No	Samples	B1	B2	G1	G2
1	Hi-Alarab	2,5	-	-	-
2	Hi-Alarab (Factory)	10	2,5	-	-
3	Suage shapey. omdu	2,5	-	-	-
4	Suage shapey. omdu	2,5	-	-	-
5	Alhalfay	2,5	-	-	-
6	Kopar	2,5	-	-	-
7	Kopar	2,5	-	-	-

3.4. Amplification of β-tubulin gene by PCR

All recovered samples were not amplified by Polymerase chain reaction (PCR). Were shown in Figure (3.12, 3.13) Molecular identification of isolate

CHAPTER FOUR

4.1. Discussions

This study focused on isolation and identification of *Aspergillus flavus* and aflatoxin from peanuts (cake) of poultry feed in Khartoum State. with positive samples of *Aspergillus flavus* which is agree with study in Khartoum state done by (Muhammed, 2010) who isolated *A. falvus* from poultry feed.

Aspergillus flavus strains can cause disease in plants, animals and poultry, during the contamination of food.This study considers the isolation and identification of *Aspergillus flavus* and identification of aflatoxin presence in peanuts (cake) in Khartoum State.In culture, *Aspergillus flavus* is characterized by fast-growing yellow–green colonies this is agreement with(Klich, 2002) (Raper and Fennell, 1965),in studies.

Other similar study in isolation of Aspergillus *flavus* from drinking water from poultry farm, by (Wisal Abdalla, 2017). The isolation of fungi from water has demonstrated a common presence of fungi in water distribution systems of poultry farms.

Isolation of *Aspergillus flavus* from tanks in poultry farms was in accordance with(Paterson.,*et al* 1997)who detected aflatoxins, produced by *A. flavus* in water from a cold water storage tank.

Aresent study in water Identified *Aspergillus* species from Dal Lake, Kashmir from water samples .(Azra and Kamili,2012).

Aspergillus flavus could contaminate eggs this approve the study of fungal contamination of table eggs sold in Khartoum State, Sudan by(Elham and Naglaa, 2018) who isolated *Aspergillus flavus and* other species from Egg samples (shell and egg contents). Contamination of eggshell and content might be due to the use of contaminated poultry feeds or poultry feeds raw materials or general low hygienic measures in such farms. However, contamination with *A. flavus* is a threat to health due to the production of aflatoxins that have been found to be carcinogenic, teratogenic and mutagenic in humans and birds.

Contamination of egg contents might be due to occurrence of fungi in the oviduct of the hen that contaminate chicken droppings or poultry feeds (Obi and Igbokwe, 2007).

The most important factor in growth of *A.flavus*and aflatoxin production by the fungus is the temperature. Which is suitable for growthof *A.flavus*in poultry farm and places when cake store.This was in agreement with that reported by (Elbeeli, 1989).

Aflatoxin identified in the present study was produced by tested of poultry peanuts (cake) which contamened with *Aspergillus flavus* strain was known to produce both aflatoxin B1 and aflatoxin B2. AFB1 is the most toxic fraction of all aflatoxins . finding of present study are in line with (Shotwell.,*et al*1966.).

In my study the concentration of aflatoxin B1 range (2.5 -10) B2(2.5)ppb.

Tthere are several surveys that showed a relatively lower level of contamination of aflatoxin B1 in peanuts and their products (Siame.,*et al* 1998) who did a study in Botswana, Africa, reported that the levels of aflatoxin B1 in a range of (0.8 – 16.00 µg/ Kg) for the raw shelled peanut samples and for peanut butter were (3.2 – 16.00 µg/ Kg). In Tokyo, Japan (Tabata.,*et al*1993) found that several peanut products were contaminated by aflatoxin B1 in a range of (0.4 – 21.7 µg/ Kg).

Beside studies for *Aspergillus Species* in water there are more studies for it in Soil.Identification of *A.flavus* species from the soil of Larkana District (Sindh, Pakistan)in that study two differential media, Czapek Solution Agar (CZA) and Malt Exract Agar (MEA) were used for the identification of *Aspergillus* species using microscopic characteristics such as colony growth, conidial color, colony reverse, and microscopic characteristics including conidiophore, vesicle, matulae, phialides and conidia. All the Aspergillus species(*Aspergillus ficcum*, *Aspergillus flavus, Aspergillus terreus* . , *Aspergillus fumigates*)appeared to be a new recordsfrom Pakistan.(Saleem and Shahzad, 2015).

Several species are morphologically similar to *A. flavus* such as *A. nomius* which is most easily distinguished from *A. flavus* by its mycotoxin profile *A. flavus* produces only B aflatoxins whereas *A. nomius* produces both B and G aflatoxins (Klich, 2002). In this study only aflatoxin B1 and B2 producing by *Aspergillus flavus*

The contamination range of aflatoxin B1 in my study is (2.5 – 10 µg/ Kg) (23.3%).Different studies conducted in Sudan from different places revaled aflatoxin in peanuts those results are similar to that obtained by (Lund.,*et al* 2000) who reported that the 27 samples (23.5%) of peanut and peanut products of one hundred and fifteen (27/115) showed positive aflatoxin B1 with a range

of (1.6 – 26.0 µg/ Kg). This range is similar to the twenty two samples (18.33%) of the one hundred and twenty samples of peanut and peanut butter studied. Also(Suliman.,*et al* 2007) reported that the 73/145 (50%) stored peanut kernels showed positive to aflatoxin B1 with ranges of (0.8 – 547.5 µg/ Kg).Peanuts is the bone marrow for poultry feed.

A survey done in Philippines on peanut-based products revealed that 60% of the samples were positive for aflatoxin B1 in range of 1.00 - 244 µg/ Kg (Ali.,*et al* 1999).

Studies had done in Khartoum showed positive aflatoxin B1 contamination.(Hanafi, 1987) reported, there were some causes of certain Sudanese groundnuts consignments which had been rejected as exported goods because of aflatoxin contamination resulting in loss of millions of foreign exchange in 1982.

(Habish and Elshafie, 1974) whom surveyed the incidence of aflatoxin ongroundnut cakes in the local Sudanese market. They found that freshly pressed cake contained less aflatoxin than cakes stored in poultry farms. They concluded that freshly pressed cake are more acceptable for both local use and exportation.

Poultry feed are suitable environment for fungal contamination during production, storage and poultry feeding, also high dose of antibiotics in poultry feeds increase the probability of fungal contamination. This study is agreement with (Nazmul andHussain ,2014) who isolated and identified *Aspergillus flavus* from poultry feed.

In Kenya a study by (Azziz,Baumgartner.,*et al*2005) reported that males were more likely to die from aflatoxicosis, in spite of eating similar quantities of maize as females. One hypothesis for males being at higher risk of dying from aflatoxicosis .

One study examined the possible association between aflatoxins and male fertility. Semen from 40% of infertile men had aflatoxins compared to semen from 8% of fertile men. The concentrations of aflatoxins detected in the semen were consistently higher among infertile men compared to the fertile men. Fifty percent of infertile men with high aflatoxin semen levels also showed abnormalities (sperm count, morphology and motility) of their spermatozoa on semen analysis. In comparison, only 10–15% of the fertile men showed comparative abnormalities of spermatozoa (Ibeh.,*et al* 1994).

A study carried out in the Gambia by (Turner.,*et al*2003) found that elevated aflatoxin-albumin levels were associated with stunting and underweight among children aged 6 to 9 years. The study detected aflatoxin-albumin adducts in 93% of sampled children and provided evidence that immunoglobulin A in saliva may be reduced because of aflatoxin exposure.

(Magid and Magid, 1995) recorded the incidence of *A. flavus*to be in groundnut seeds in Sudan. They detected many aflatoxinsfrom their isolates.

(Mohammed and ElRasheed, 1995) collected 6 samples ofpeanut butter "Dakwa" and 6 samples of peanut kernel from 6different locations in Khartoum and investigated the occurrence ofaflatoxin in the samples. In peanut butter samples, all the sampleshave aflatoxins but in varying ratios. In the peanut kernel samples the amount of aflatoxin was much less than in the peanut butter where only trace amount was detected.

4.2. Conclusion

Poultry food was found contaminated with *Aspergillus flavus* and aflatoxins B1and B2 but no G1and G2. These results obtained support the idea that of the used of peanuts was the main factor of aflatoxin contamination.

Aspergillus flavus contaminated food is a serious risk for public health having long-term health effects in Human, Animals and poultry because it produce aflatoxin.There are high percentage of Apergillus sp, *A.flavus* isolated from poultry feed product in different studies.
The summaries of all using of damage groundnut pods by moisture content, relative humidity, temperature, soil pests, poor harvesting practices and bad storage conditionsfor oil and cause contamination of *Aspergillus flavus* and aflatoxin.My study confirmed the presence of *Aspergillus flavus* in peanuts which is the present of the poultry feed.

4.3. Recommendations

To reduce the critical problems of the aflatoxin contamination of peanuts (cake) the following practices are suggested:

1. Proper field drying to avoid the development of contamination that may generate aflatoxins during storage period.
2. Careful handling to avoid physical damage of the groundnut kerneks to reduce the contamination.
3. Proper storage conditions to avoid insects damage, moisture and humidity.

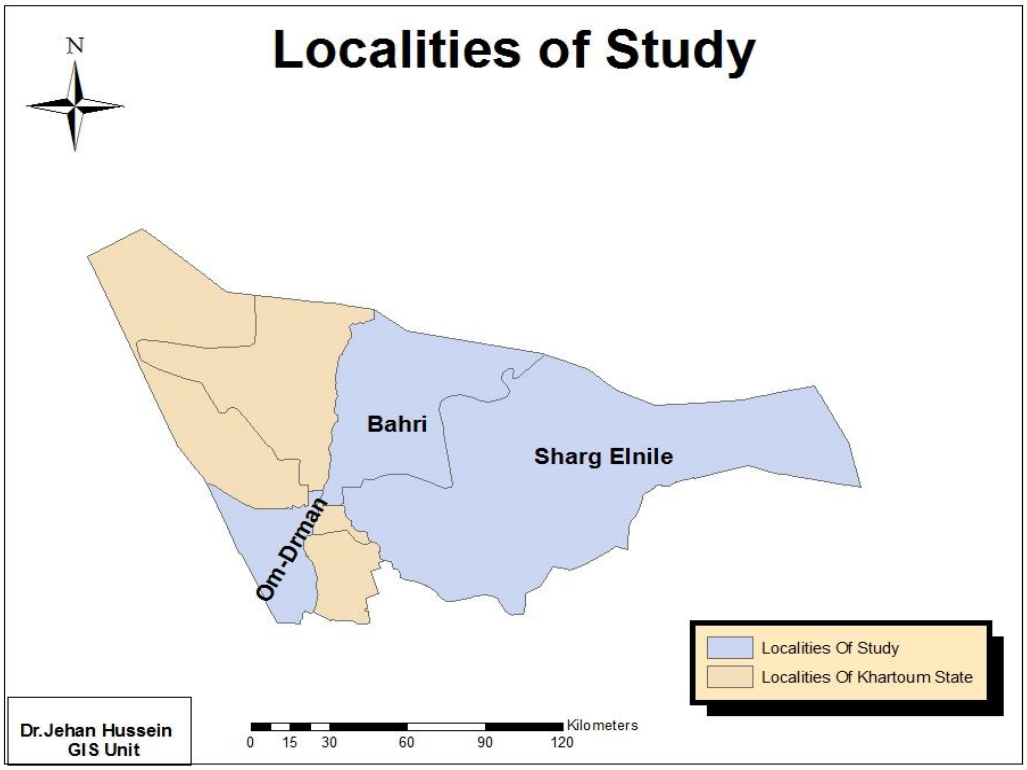
4. Health authorities should activate the regulations that stated in the standards concerning the maximum limits of aflatoxin levels of human consumption in Sudan.
5. Consumers should be aware of the harmful effects of aflatoxin on human health and economy

6. Eliminate these toxins from food and guarantee the food safety and health concerns of consumers. There are chemical and biological methods for their control and management in food must be studies and discuseed.

4.4.Research restrains

a. High cost of reagents.
b. Limited resources

C. High cost of the kits for PCR tested.



Map (2.1) showing the Khartoum State Localities include in study

Aspergillus Spp were grown in Sabouraud DextroseAgar with different colour

Before isolation *Aspergillus flavus*

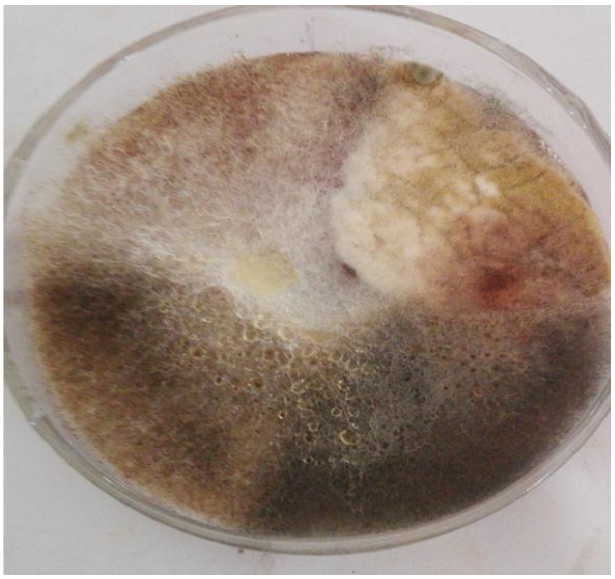


Figure (3.1) *Aspergillus flavus* growthing in Sabouraud Dextrose Agar

Figure (3.2) *Aspergillus flavus* growthing in Sabouraud Dextrose Agar

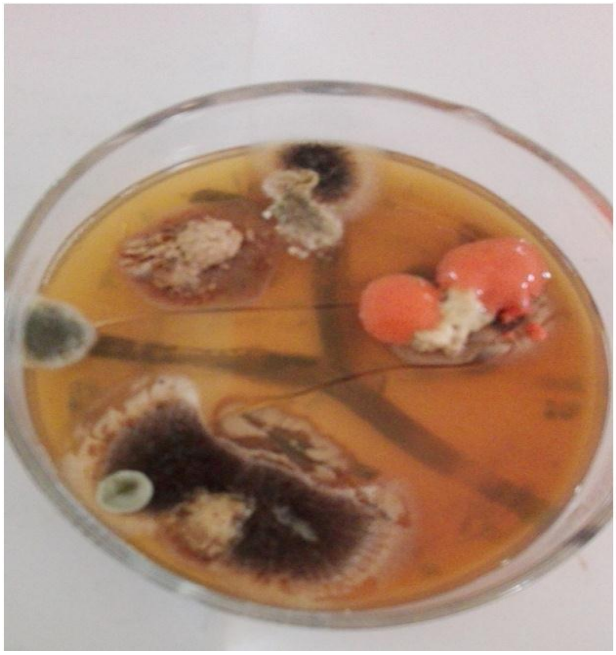


Figure (3.3) *Aspergillus flavus* growthing in Sabouraud Dextrose Agar

Figure (3.4) *Aspergillus flavus* growthing in potato dextrose agar

Colony after 5 days

Figure (3.5) *Aspergillus flavus* growthing in potato dextrose agar

Colony after 3 days

Figure (3.6) *Aspergillus flavus* growthing in potato dextrose agar

Colony after 5 days

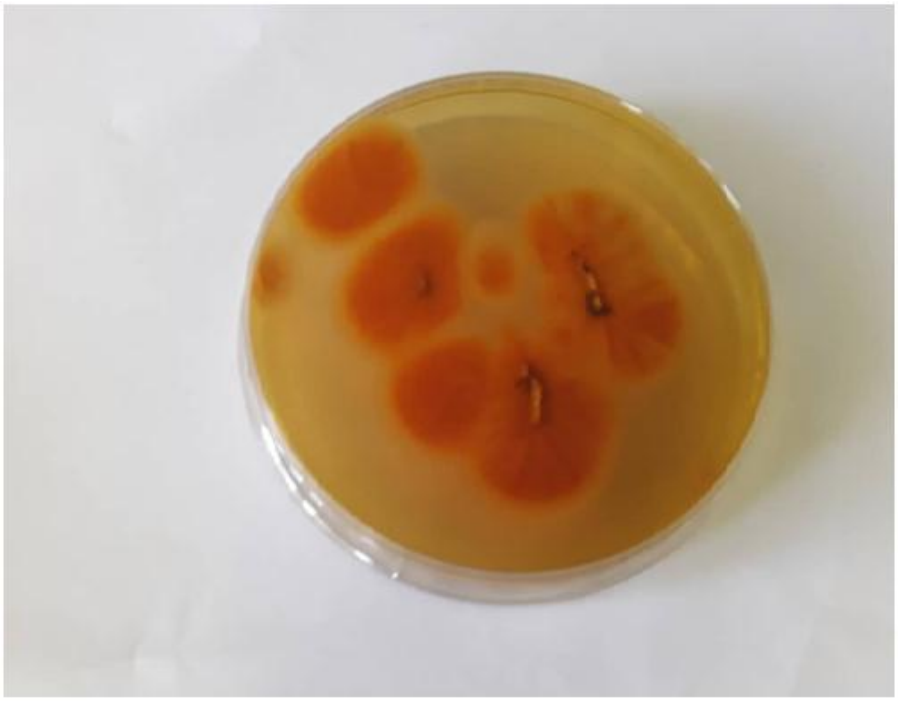


Figure (3.7) An orange reverse colony of *A. flavus* on Aspergillus differentiation base media **(**Czapeck)

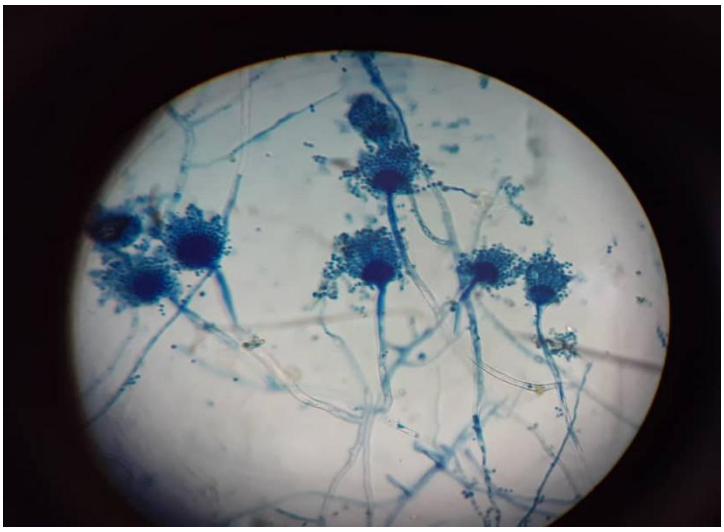


Figure (3.8) *Aspergillus Flavus* under microscopic Hyphae

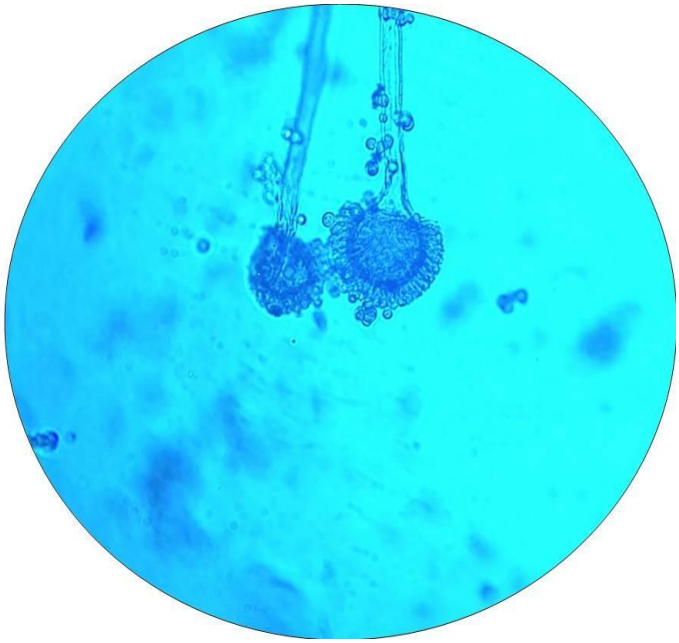


Figure (3.9) *Aspergillus Flavus* under microscopic septate hyphae

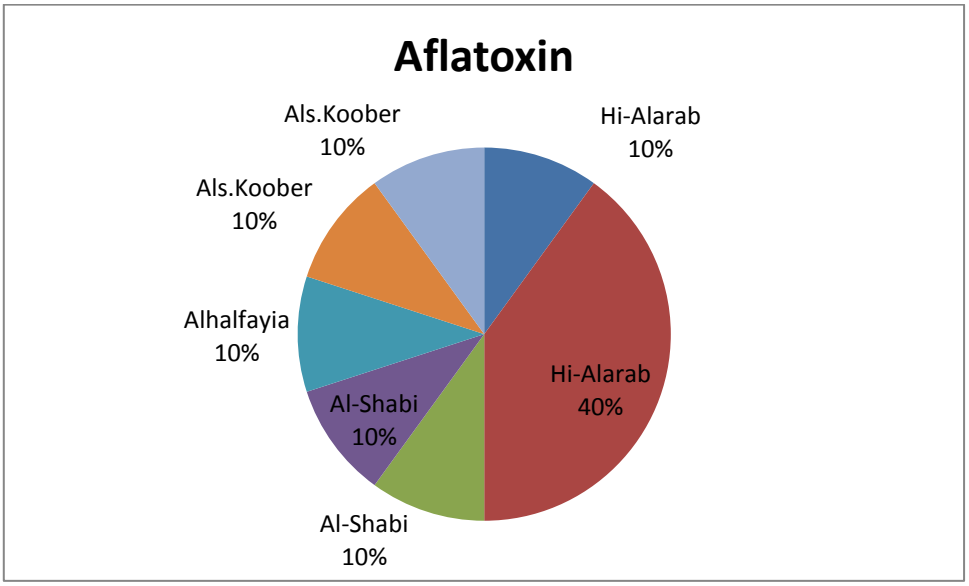


Figure (3.10) Proportion of Aflatoxin contamination

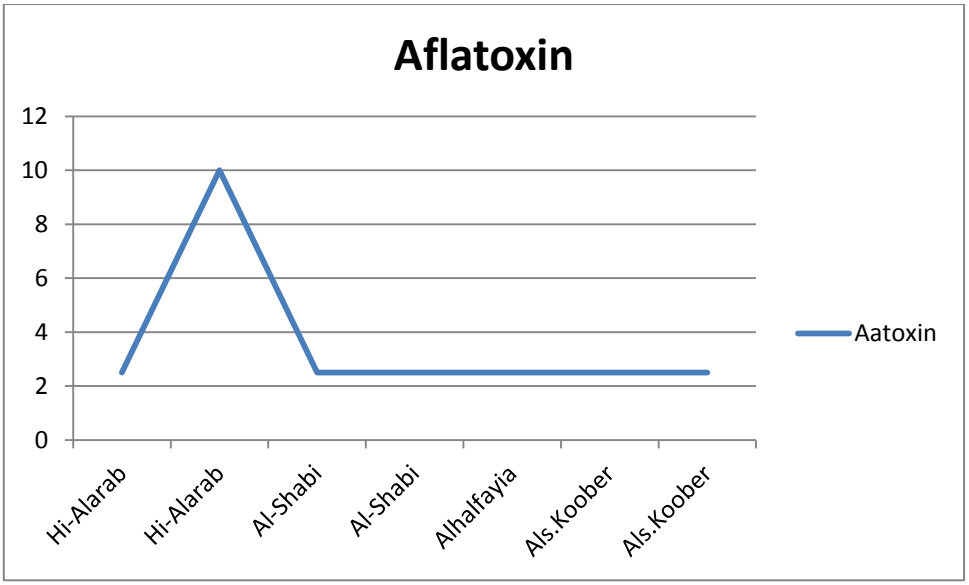


Figure (3.11) Histogram of Aflatoxin contamination

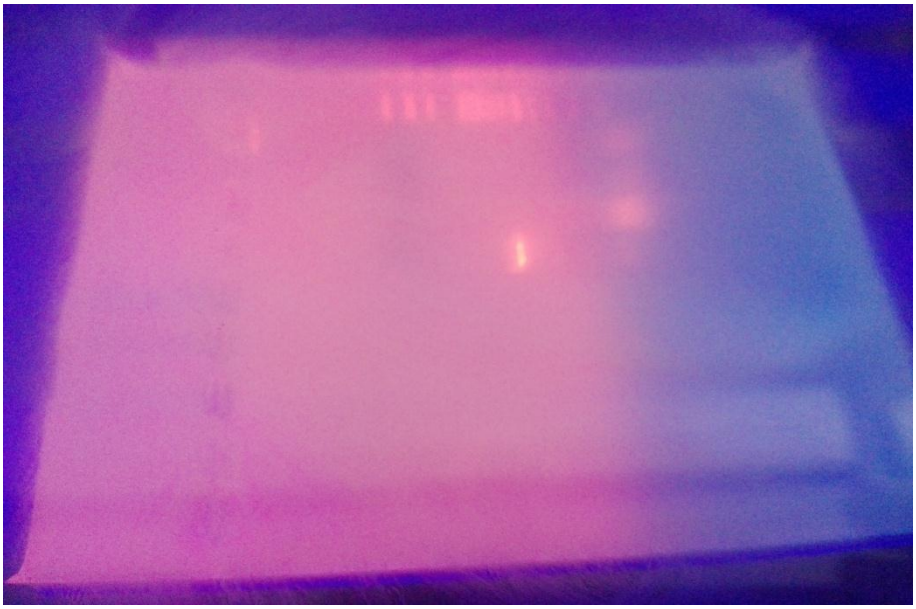
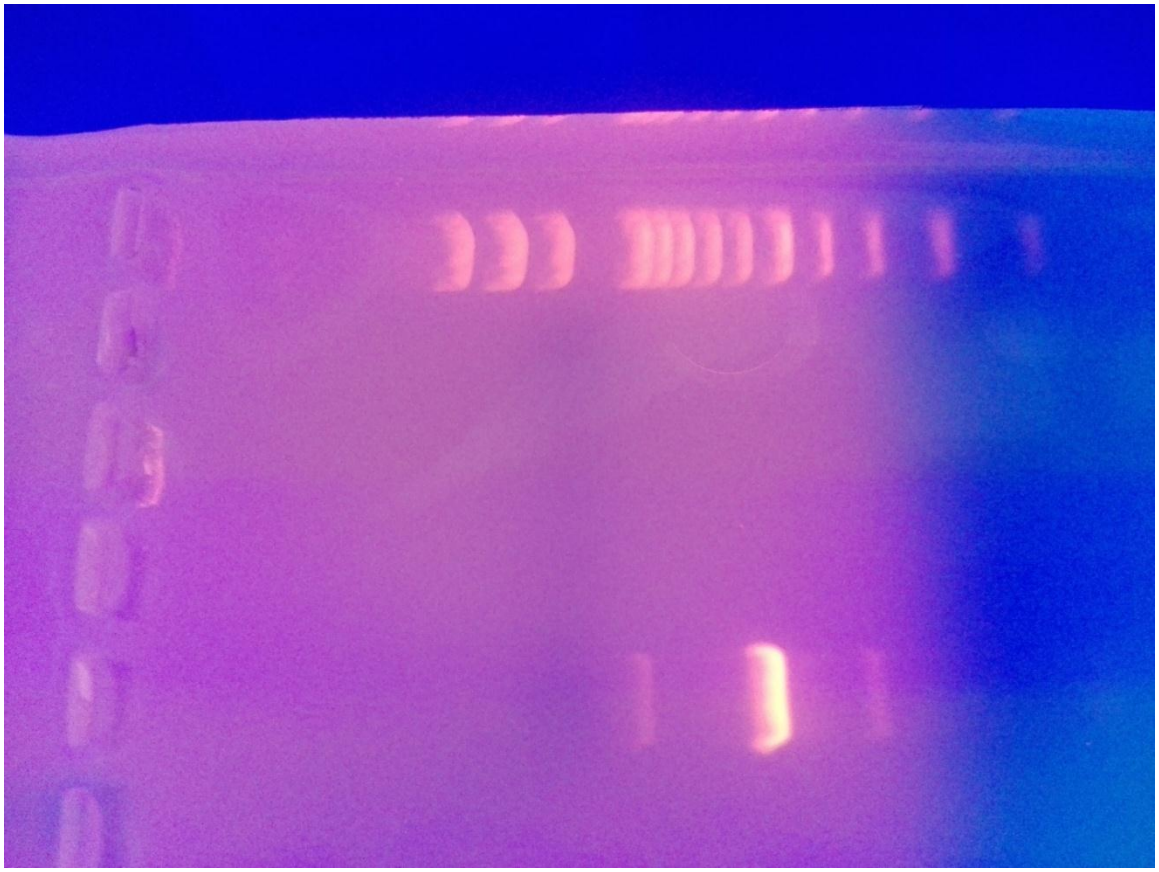


Figure (3.12): PCR were not amplified for fungal isolate



PCR Result

Figure (3.13): All recovered samples were not amplified genefor fungal isolate by PCR

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APPENDIX

Appendix 1

Czapixl yeast agar (CYA)

9.4g of commercialized powder. Dissolved in100ml distilled water

Boil white mixing to dissolve completely. Sterilized media by autoclaveing at 121ºc for 15 minutes. Adding chloroamphincol (0.02mg/ml)

Asptically dispense into sterile petri dishes.

Appendix 2

Potato Dextrose Agar (PDA)

5.4g of commercialized powder. Dissolved in100ml distilled water

Boil white mixing to dissolve completely. Sterilized media by autoclaveing at 121ºc for 15 minutes. Adding chloroamphincol (0.02mg/ml)

Asptically dispense into sterile petri dishes

Appendix 3

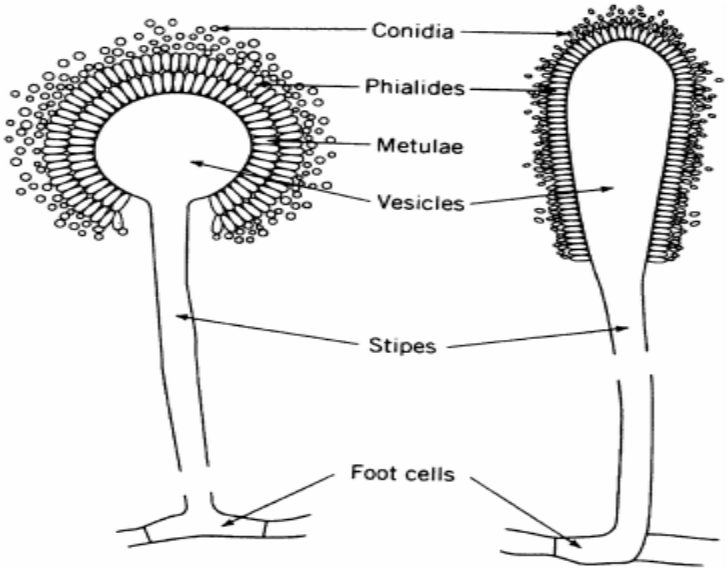


Figure1.1 Characterstic condiophores of Apegillus.

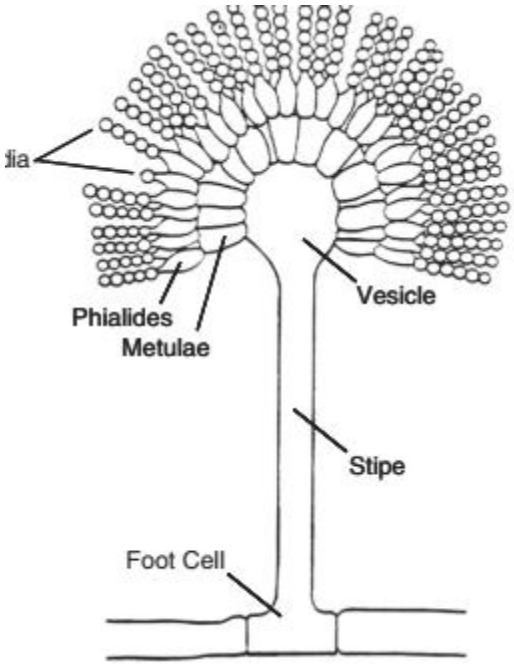
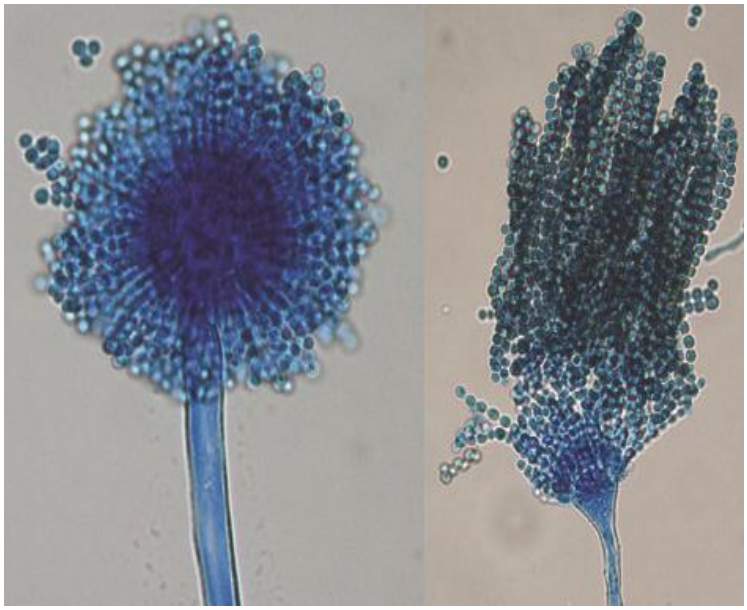


Figure (1.2): Basic morphological structure of *Apegillus*



Under microscope

Figure (1.3) *Aspergillus flavus* hyphae on slide culture

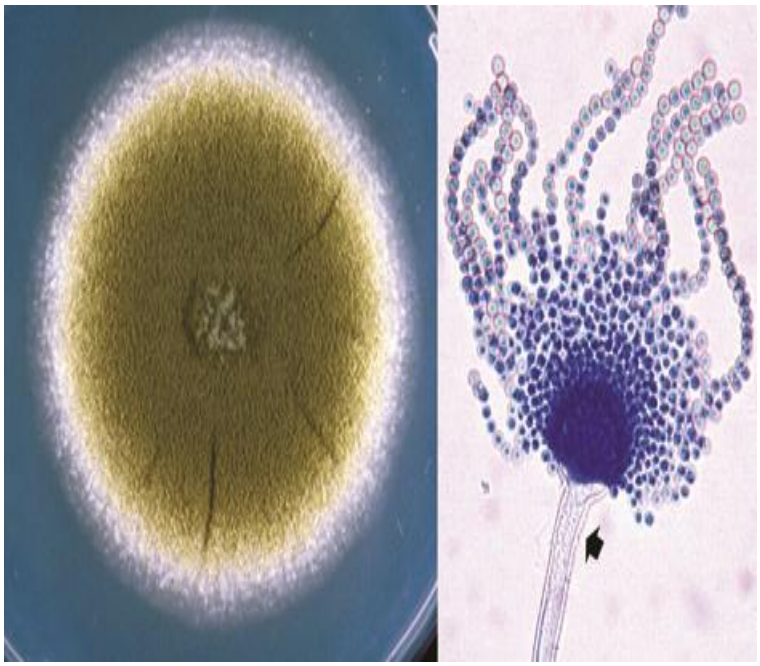


Figure (1.4) : Culutre and Condia head of Aspergillus

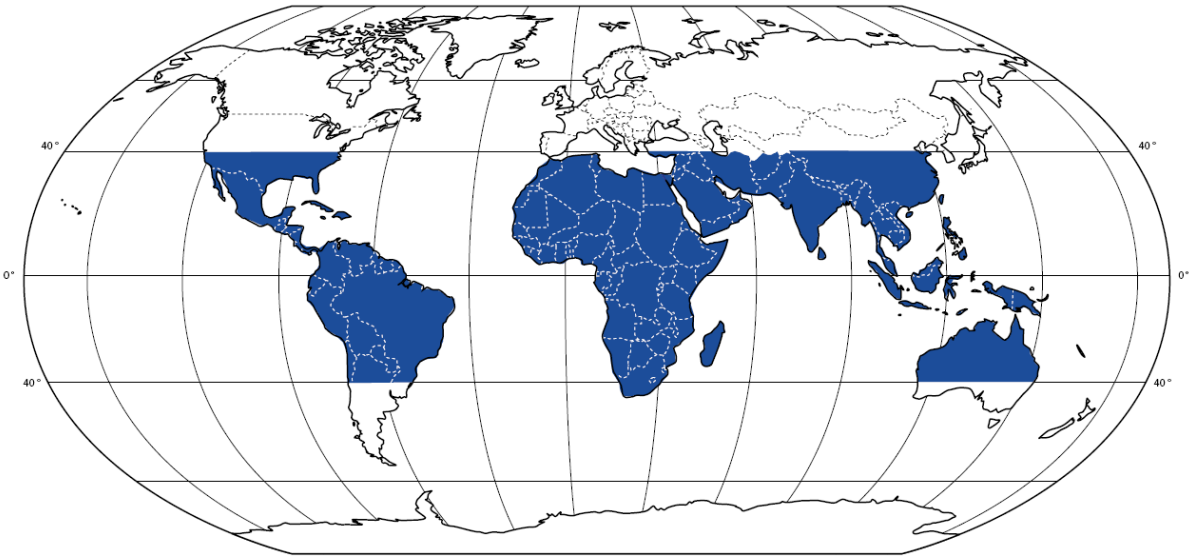


Figure (1.5) Map of regional and populations at risk of chronic exposure to uncontrolled contaminationof AF (Source: Williams.,*et al*2004)

Figure (1.6)

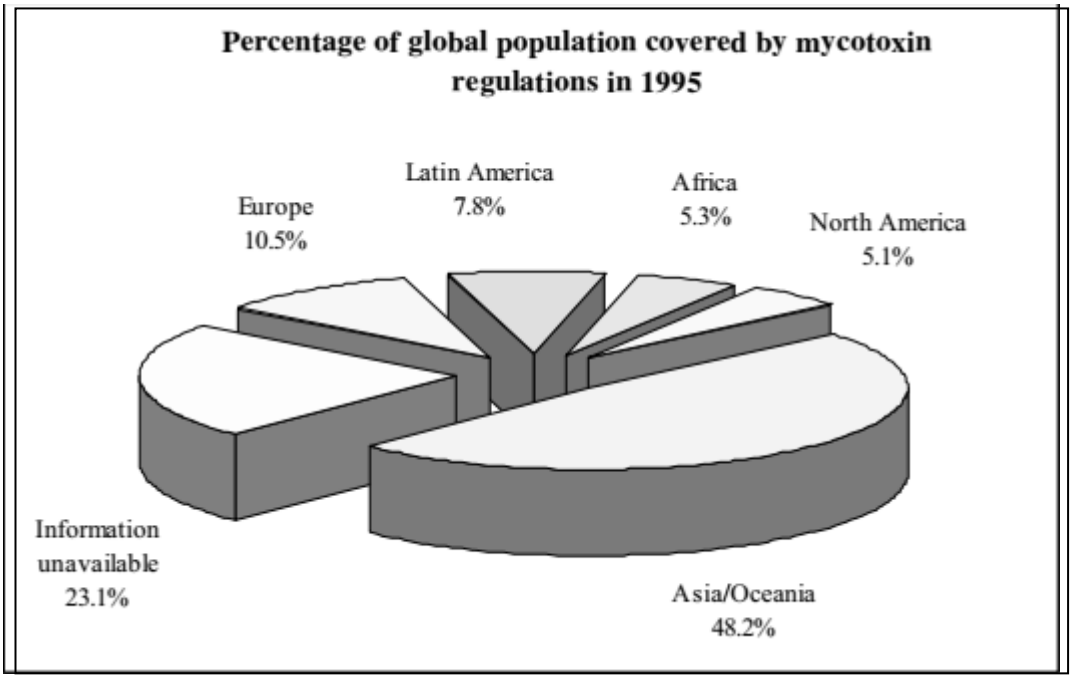
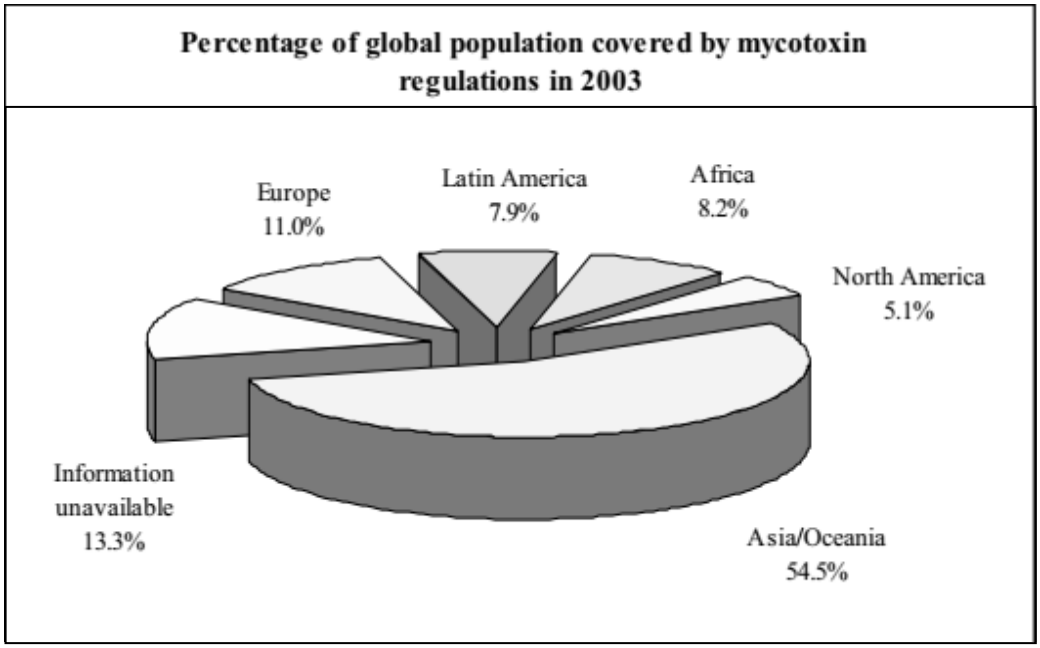
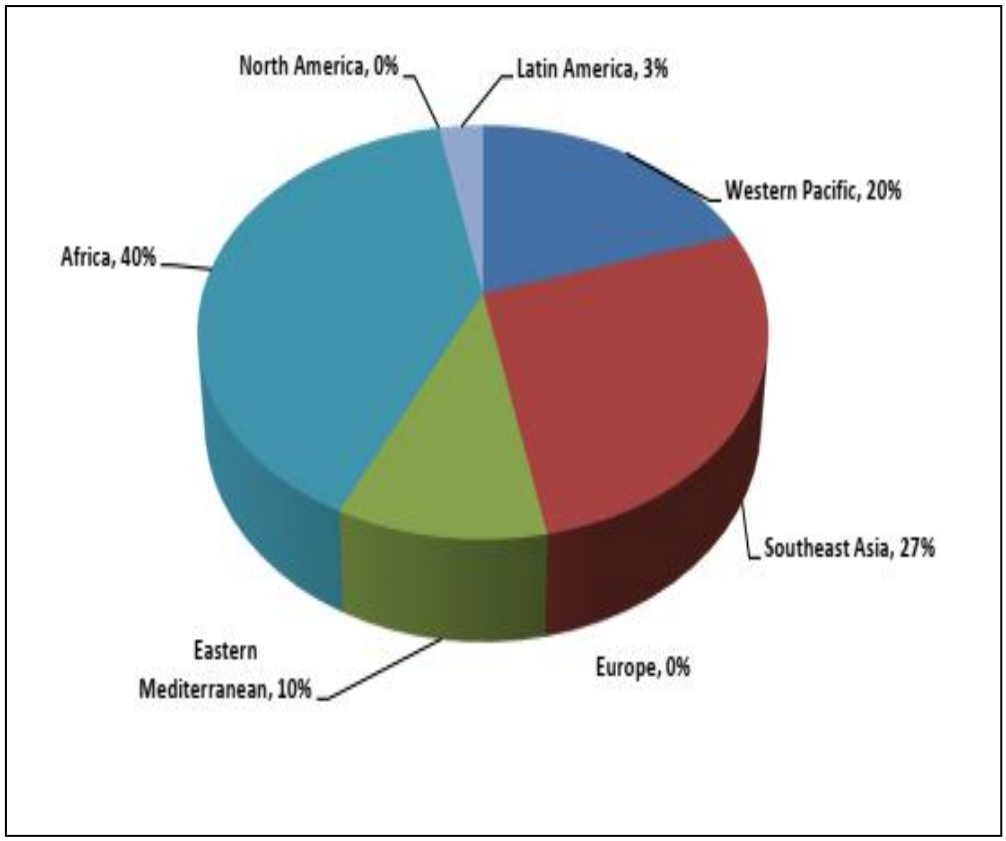


Figure (1.7)





Source: USAID (2003).

Figure(1.8): Distribution of liver cancer attributable to aflatoxin

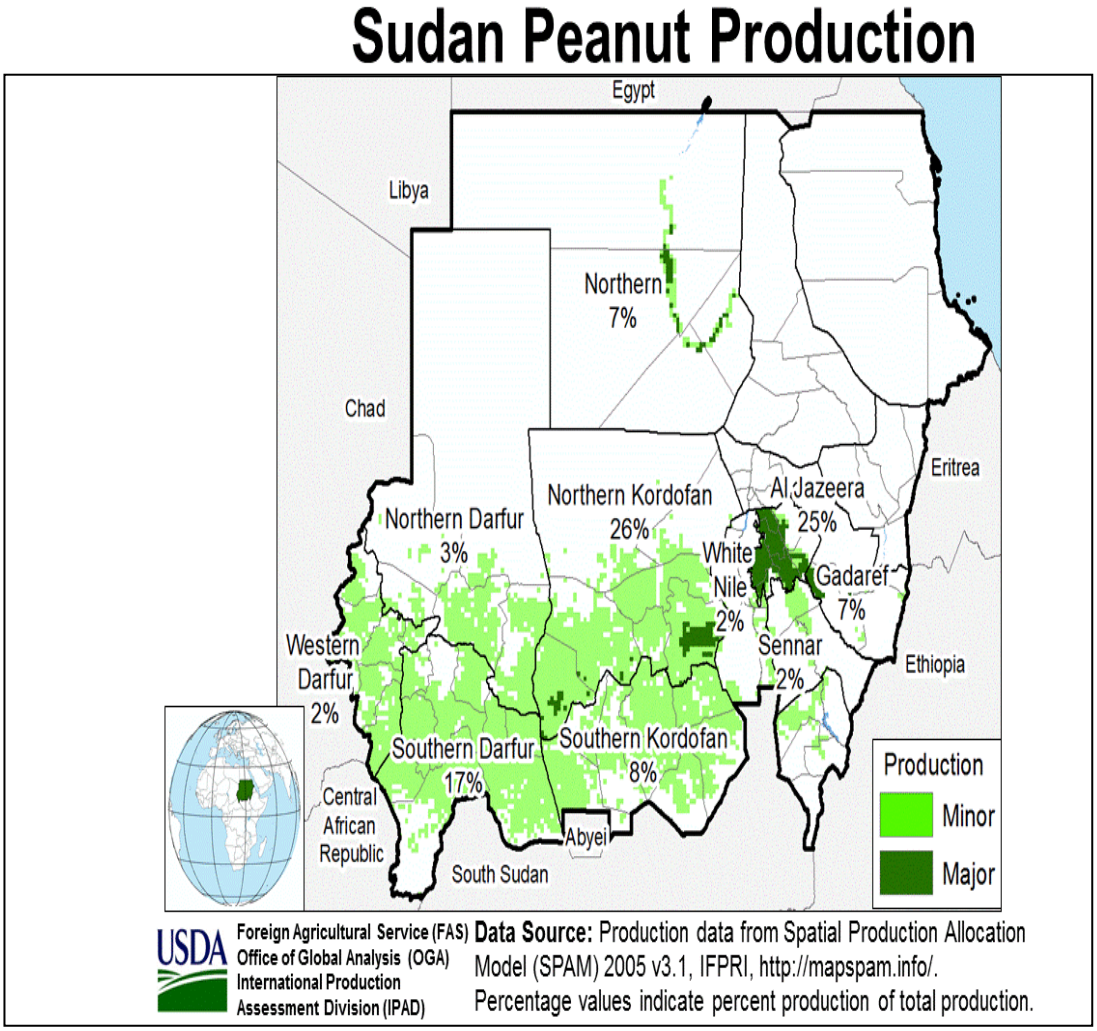


Figure (1.9) Map of Sudan States Peanuts Production